The study of the deubiquitinase USP8 in Parkinson's disease Pathogenesis

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ABSTRACT

Parkinson's disease is the second commonest neurodegenerative disease currently treated symptomatically. It is a multifactorial disease involving mechanisms ranging from protein aggregation to mitochondrial dysfunction, oxidative stress and dopamine dysregulation. The levels of α-synuclein have been causatively linked to the development and progression of Parkinson's disease. Therefore α-synuclein lowering strategies are valid approaches in Parkinson's disease. Neuropathologically, Lewy Bodies in the vulnerable substantia nigra of Parkinson's disease patients are less ubiquitinated and specifically less K-63 ubiquitinated than Lewy bodies in the cortex, suggesting differential activation or regulation of ubiquitin interactors. A targeted screen for such interactors revealed that the Deubiquitinating enzyme Usp8 is upregulated in the substantia nigra of Parkinson's disease brains and is inversely correlated with the degree of total and K-63 ubiquitination. Using genetic knockdown and overexpression techniques, Usp8 was found to colocalize and directly interact with α-synuclein. It was found to de-ubiquitinate α-synuclein and increase its half-life. Its knockdown increased the total and K-63 α-synuclein ubiquitination and decreased its levels by 35% at least partly by increasing its degradation via the lysosome. *In vivo* in the *Drosophila melanogaster*, Usp8 knockdown demonstrated protection against α-synuclein toxicity. It rescued in a specific manner the rough eye phenotype, the age-dependent locomotive defect and the loss of dopaminergic neurons caused by the expression of α-synuclein. Specific and effective pharmacological Usp8 inhibition also has the potential to lower α-synuclein levels. Collectively, the evidence produced in my thesis suggests that Usp8 could be a potential target for the future disease-modifying therapies in Parkinson's disease.

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GLOSSARY OF ABBREVIATIONS

120: α-synuclein 1-120 amino acids

130: α-synuclein 1-130 amino acids

17-AAG: geldanamycin derivative

2F12: α-synuclein antibody

3D: 3 Dimensions

A20: a deubiquitinating enzyme

A50P: mutant form of α-synuclein with Alanine to Proline change at position 50

A53T: mutant form of α-synuclein with Alanine to Threonine change at position 53

aa: amino acids

AC: Anterior cingulate

Ala: Alanine

ALDH: Aldehyde Dehydrogenase

ALS: Amyotrophic Lateral Sclerosis

AMPA receptors: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

AMSH= STAMBP: STAM-binding protein

ANOVA: Analysis of Variance

AP2: Adaptor protein driving macroautophagy which possesses a LIR-ATG8 binding domain

ASOs: Antisense oligonucleotides

ATG5/12/16L: Autophagy protein complex

ATG8, 7, 5, 9: Autophagy proteins

ATP: adenosine triphosphate

ATP13A2: gene encoding for cation-transporter ATPase

AU: Arbitrary Units

BACE1: beta (β) secretase 1 enzyme

BCA: bicinchoninic acid assay

BiFC: Bifluorescence complementation assay

BMP: Bone morphogenic Protein

BS: Baseline

C20: α-synuclein antibody

CA3: Cornu Ammonis region 3, one of the hippocampal subfields

C-CBL: E3 Ubiquitin ligase

CHIP: Carboxyl-terminus of HSC70-Interacting Protein

CHMP: Charged multivesicular body protein

CLOCK proteins: Circadian Locomotor Output Cycles Kaput gene involved in circadian rhythms

CMA: Chaperone-mediated autophagy

CO_2: Carbon dioxide

COMT: Catechol-O-methyl transferase

COX-2: Cyclooxygenase 2

CQ: Chloroquine

C-Terminus: Carboxyl-terminus

CyO: *Drosophila* balancer on the 2nd chromosome

Cys: Cysteine

DAPI: 4',6-diamidino-2-phenylindole: Fluorescent stain staining DNA

DAT: Dopamine Active Transporter

Ddc: Dopamine decarboxylase

DIV: Day *in vitro*

DJ-1: Protein deglycase DJ-1=PARK7

DLB: Dementia with Lewy Bodies

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNAse I: Deoxyribonuclease I

DOPAC: 3,4-Dihydroxyphenylacetic acid

DOPAL: 3,4-Dihydroxyphenylacetaldehyde

DUBs: Deubiquitinases

dVMAT: *Drosophila* ortholog of VMAT2

E1: Ubiquitin-activating enzymes

E18: Embryonic day 18

E2: E2 ubiquitin-conjugating enzyme

E3 ligase: E3 ubiquitin ligase

E46K: mutant form of α-synuclein with Glutamate to Lysine change at position 46

EC: Entorhinal cortex

EC_{50}: Half maximal effective concentration

EEA1: Early Endosome Antigen 1

EGFR: Epidermal growth factor receptor

elav: *Drosophila* neuron-specific driver

ELISA: Enzyme-Linked Immunosorbent Assay

e-mi: endosomal microautophagy

ER: endoplasmic reticulum

ErbB3: human epidermal growth factor receptor 3

ESCRT: endosomal sorting complexes required for transport

EV: Empty Vector

FCS: Foetal calf serum

FL: Full length

FLAG: tag containing the sequence motif DYKDDDDK

FTD: Frontotemporal Dementia

GABA: gamma-Aminobutyric acid

GABARAPL1: Gamma-aminobutyric acid receptor-associated protein-like1

GAL4: yeast transcription activator protein GAL4

GBA: β-glucocerebrosidase

GFAP: Glial fibrillary acidic protein

GFP: Green Fluorescent Protein

GMR: glass multiple reporter

GWAS: Genome-wide Association Studies

h: hours

H^+: hydrogen ions=protons

H_20_2: hydrogen peroxide

HA: Human influenza hemagglutinin tag

HBSS: Hanks' Balanced Salt Solution

HC: Healthy controls

HCl: Hydrochloric acid

HDAC: Histone deacetylase

HEK293-T: Human Embryonic Kidney 293 cell line with expression of T antigen from SV40 virus

HeLa cells: Immortalized cell line derived from cervical cancer cells from Henrietta Lacks

Hh: Hedgehog

HRP: Horseradish peroxidase

HRS: component of ESCRT-0

HSF1: Heat shock factor protein 1

HSP70, 73, 40, 90: Heat Shock proteins

HSR: Heat shock response

HSV: herpes simplex virus

HTT: Huntingtin

IC_{50}: half maximal inhibitory concentration

If: *Drosophila* marker on the 2nd chromosome

IgG: Immunoglobulin G

IP: Immunoprecipitation

iPSc: Induced- pluripotent stem cells

JAMMs: Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) protease

JOSD2: Josephin Domain Containing 2, DUB

K0: Ubiquitin with no lysines

K48, 63, 33, 11, 6: Lysine at position X of ubiquitin

kDa: kiloDaltons

L2-L5: Longitudinal veins of *Drosophila* wings

Lact: Lactacystin

LAG3: Lymphocyte activation gene 3

LAMP2/LAMP1/LAMP2A: Lysosome-associated membrane protein 2/1/2A

LB: Lewy bodies

$LC3_{II}$: light chain 3 cleaved isoform II

LC-MS/MS: Liquid chromatography–mass spectrometry- mass spectrometry

LDH: Lactate Dehydrogenase

L-Dopa: levodopa= L-3,4-dihydroxyphenylalanine

LIR domain: LC3-binding domain

LPS: lipopolysaccharide

LRIG1: Leucine-rich repeats and immunoglobulin-like domains protein 1

LRRK2: Leucine-rich repeat kinase 2

MALDI-TOF: Matrix-assisted laser desorption/ionization Time of Flight Mass Spectrometer

MAO/MAOB: Monoamine oxidase/ Monoamine oxidase B

MEM: Minimum Essential Medium

MET: gene for c-Met (proto-oncogene protein)

Mins: minutes

MIT domain: Microtubule Interacting and Transport domain

MJD: Machado–Joseph disease

mL: mili litre

mM: mili molar

MPP^+: 1-methyl-4-phenylpyridinium

MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA: messenger ribonucleic acid

MS1096: wing driver in *Drosophila*

mTOR: Mammalian target of Rapamycin

mTORC1/2: mammalian target of rapamycin complex 1/2

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MVBs: multi-vesicular bodies

n: number

NAC: non-αβ component

NaCl: Sodium Chloride

NADH: dihydronicotinamide adenine dinucleotide dehydrogenase = reduced diphosphopyridine nucleotide diaphorase

NADPH: nicotinamide adenine dinucleotide phosphate-oxidase

$NaHCO_3$: Sodium Bicarbonate

NB: neurobasal

NBR1: Neighbour of BRCA1 gene 1 protein

NDP52: nuclear dot protein 52 kDa, Ubiquitin adaptor

Ndufs4: NADH dehydrogenase iron-sulphur protein 4 = NADH-ubiquinone oxidoreductase 18 kDa subunit

Nedd4: neural precursor cell expressed developmentally down-regulated protein 4, E3 ligase

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NHS: National Health System

NK: not known

NP40: Tergitol-type NP-40 = nonyl phenoxypolyethoxylethanol, detergent

Nrdp1: E3 ligase

N-terminus: Amino terminus

OTUB1: Ubiquitin thioesterase otubain-1

p value: The probability of obtaining a result equal to or more extreme than was actually observed when the null hypothesis is true

p53: Tumour protein (suppressor) p53

p62: Sequestosome-1= SQSTM1

p70S6K: Ribosomal protein S6 kinase beta-1 =S6K1

PBS: phosphate buffered solution

PBS-T: PBS containing 0.05 Triton X-100

pC12 cell line: cell line derived from pheochromocytoma of the rat adrenal medulla

PD: Parkinson's disease

PFA: Performaldehyde

pH: power of hydrogen: numeric scale used to specify acidity

PHGH: Peptidyl-glutamyl peptide hydrolysing activity

PINK1: PTEN-induced putative kinase 1

PMAT transporter: plasma membrane monoamine transporter

PMSF: phenylmethane sulfonyl fluoride = phenylmethylsulfonyl fluoride

Poly-Q: Poly-glutamine

PPM1/2: cluster of dopaminergic neurons in *Drosophila melanogaster*

pre-mRNA: pre-messenger Ribonucleic acid

qPCR: real-time PCR= Quantitative PCR

RAB1a, 7, 11: Trafficking GTPases

Rad23: UV excision repair protein

RNA: Ribonucleic Acid

RNAi: RNA interference

RNS: Reactive nitrogen species

ROS: Reactive Oxygen species

rp49: ribosomal protein 49

Rpn10: subunit of the 26S proteasome recognising polyubiquitinated proteins

S6, pS6: ribosomal protein S6, phosphorylated ribosomal protein S6

SAHA: histone deacetylase inhibitor

SAIT301: Antibody for MET

SCAs: Spinocerebellar ataxia

SCR: Scramble

SDS: Sodium dodecyl sulphate, a surfactant

SEM: Scanning electron microscopy

Ser-129: serine at position 129

shRNA: Short hairpin RNA

SH-SY5Y: neuroblastoma-like cell line

SIAH: seven in absentia homologs E3 ligase

SN: Substantia nigra

SNARE: SNAP REceptor (Soluble NSF Attachment Protein)

SNCA: Gene encoding α-synuclein

SNPs: Single nucleotide polymorphisms

STAM: Signal transducing adaptor molecule

SYBR Green: asymmetrical cyanine dye that stains nucleic acids

Syn 120: α-synuclein 1-120 amino acids

Syn1: monoclonal antibody against α-synuclein

TARDBP gene: gene encoding TAR DNA-binding protein 43 =TDP-43, transactive response DNA binding protein 43 kDa

TAX1BP1: Tax1-binding protein 1

TBS-T: Tris-buffered saline containing Polysorbate 20 (Tween 20)

TD_{50}: median toxic dose

TDP-43: Transactivation element DNA-binding protein of 43kDa

TE Buffer: Buffer containing Tris and EDTA

TH: Tyrosine Hydroxylase

TM3: *Drosophila* balancer on the 3rd chromosome

TM6: *Drosophila* balancer on the 3rd chromosome

TRIS: tris(hydroxymethyl)aminomethane

TSC1: Tuberous sclerosis 1=Hamartin

TUBE: Tagged concentrated ubiquitin binding domain constructs

UAS: Upstream activating sequence

Ub AMC: Ubiquitin-AMC= fluorogenic substrate for a wide range of deubiquitinating enzymes

Ub: Ubiquitin

UBA: Ubiquitin-binding domain

UBPY: *Drosophila* orthologue of Usp8

UCH: ubiquitin C-terminal hydrolase

UCHL1: Ubiquitin carboxyl-terminal hydrolase L1

UPR: Unfolded protein response

UPS: Ubiquitin-proteasome system

Usp1,2,16,27x,28,36,45,20,25, 7/HAUSP, 9x, 30, 24, 40, 14, 47,1: Ubiquitin specific proteases

USP8^{CA}: Catalytically inactive Usp8 (cysteine-> alanine)

USP8^{WT}: Wild type form of Usp8

USPs: Ubiquitin specific proteases

VFP: Venous fluorescent protein

VKKDQ: amino acid sequence valine-lysine-lysine-aspartate-glutamine

VMAT2: vesicular monoamine transporter 2

vOTU: Viral Ovarian Tumour Domain Protease

VPS35: Vacuolar protein sorting-associated protein 35

VPS54: Vacuolar protein sorting-associated protein 54

W^{1118}: wild-type *Drosophila melanogaster*

WB: Western blot

Wg/Wnt signalling: Wingless-related integration site

WNK: with no lysine

α-syn: α-synuclein

Aβ: alpha-beta

Δ: deletion

μL: micro litre

μM: micro molar

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1. Introduction

1.1. Parkinson's Disease

1.1.1. Clinical Features and Epidemiology of Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder affecting 1% of people over 60 years (de Lau, Breteler 2006). The disease was first described by James Parkinson in 1817 in his work "*An essay on the Shaking Palsy*" (Parkinson 1817). Clinically, it is characterised by bradykinesia, rigidity, tremor and postural instability (**Fig 1.1.**) (Gowers 1888). These are classified as extrapyramidal motor symptoms. There are also non-motor symptoms which affect olfaction, behaviour, cognition, mood, and the autonomic nervous system such as constipation and sialorrhoea (Jankovic 2008). For example, patients with Parkinson's disease can develop depression, anxiety and sleep disorders, apathy and inattention (Caballol, Martí et al. 2007, Parker, Lamichhane et al. 2013), hypomimia, hypophonia and micrographia (Jankovic 2008). In addition, eye movement disorders can occur such as decreased blinking rate, dry eyes, saccadic movement and defective pursuit (Bronstein, Kennard 1985, Husain, Kennard 1995, Hodgson, Dittrich et al. 1999, Armstrong 2008). Importantly, up to 80% of patients with PD who survive 20 years post-diagnosis were found to have dementia (Hely, Reid et al. 2008). All these are part of the syndrome, but are not part of the extrapyramidal symptoms. Thus, it is evident that Parkinson's is a disease affecting many systems.

Fig. 1.1. Well-documented illustration of Parkinson's disease demonstrating some of the motor symptoms. Illustrated by Paul de Saint-Leger in 1879 in his doctoral thesis and included in the William Gowers's book "Manual of Diseases of the Nervous System" in 1888 (Gowers 1888).

The incidence of Parkinson's disease is increasing with the ageing population, as it is a disease affecting the elderly (1% of the population older than 60 years old and 5% older than 80 years old (de Lau, Breteler 2006, Wirdefeldt et al. 2011). In our increasingly ageing population, the prevalence of PD is predicted to double over the next 20 years with significant impact on funding of health care and social services. For example, PD care costs the NHS between 449 million and 3.3 billion pounds annually (Findley 2007). The majority of these costs are spent in inpatient care and nursing homes. In addition, PD reduces significantly the quality of life of both patients and caregivers (Findley 2007). Thus, it is crucial to identify the pathophysiology of the disease in order to be able to design disease-modifying treatments to slow or arrest the progression of pathology.

1.1.2. Genetics of Parkinson's Disease

Approximately 90 % of Parkinson's disease cases are sporadic (de Lau, Breteler 2006). However, several monogenic variants that cause PD have been identified (**Table 1**). Importantly, GWAS studies showed that common variants in the same genes (e.g. *SNCA* or *LRRK2*) increase the risk of sporadic PD suggesting that there are shared pathomechanisms between sporadic and certain familial forms of the disease (Simón-Sánchez, Schulte et al. 2009, Hamza, Zabetian et al. 2010).

Gene	Clinical Features	Pathology
α-Synuclein mutations or multiplications	Typical PD but can sometimes have a dementia presentation	Lewy bodies
LRRK2	Typical PD	Usually Lewy bodies: sometime tangles, sometimes neither
VPS35	Late-onset Parkinsonism	No Lewy bodies
EIF4G1	Parkinsonism with Dementia	Lewy bodies
PINK1	Early onset Parkinsonism	Classically non-Lewy bodies associated but Lewy bodies have been identified in certain cases
Parkin	Early onset Parkinsonism	Classically non-Lewy bodies associated but Lewy bodies have been identified in certain cases
DJ1	Early onset Parkinsonism	Unknown
ATP13A2	Early onset Parkinsonism with dementia, oculomotor dysfunction, spasticity, pyramidal syndrome and psychiatric symptoms	Unknown
VPS13C	Early-onset rapidly progressing severe Parkinsonism associated with dementia	Diffuse Lewy bodies
GBA	Typical PD	Lewy bodies
SNCA	Typical PD	Lewy bodies
MAPT		Lewy bodies (although tau pathology not systematically assessed)
LRRK2	Typical PD	Lewy bodies
HLA	Typical PD	Lewy bodies

Table 1: Mendelian Genes that Lead to Parkinsonism and Their Pathology. Text in **Red font**: dominant genes. Text in **Blue font**: recessive genes. Text in **Green font**: High risk loci. Test in **Purple/plum font**: Low-risk loci. Figure adapted from (Hardy 2010).

Cell-based and animal studies of the genetic mutations and variants as identified by both genetic and GWAS studies have shed important new insights into mechanisms

of Parkinson's disease pathogenesis. Some of the pathways affected by PD mutations involve mitophagy, protein trafficking and the lysosomal pathway (Tofaris 2012). Most of the loci identified by GWAS alter gene expression rather than causing protein coding changes. This can explain their small but significant increase in the risk of developing Parkinson's disease. The PDGene project identified and validated the following loci to be involved in increasing the risk of PD: MAPT, SNCA, GBA, LRRK2, PM20D1, GAK, MCCC1, STK39, BST1, GPNMB (Lill, Roehr et al. 2012). Nall et al, by performing a metanalysis of GWAS studies identified a few more loci: SIPA1L2, INPP5F, MIR4697, GCH1, VPS13C and DDRGK1, GAK, Rab7L1 (Nalls, Pankratz et al. 2014). All these genes/loci identified must be extensively studied using pathway analyses to determine their exact interaction. Several pathways have been implicated in Parkinson's disease, both stemming from Mendelian mutations and by loci increasing the risk for developing Parkinson's disease.

Mitophagy is the autophagic clearance of defective mitochondria. Mutations in Pink1 and Parkin cause Parkinson's disease by preventing effective mitophagy. Pink1 is a mitochondrial kinase, which upon mitochondrial damage it is activated and recruits the E3 ligase Parkin to the mitochondrial membrane promoting mitophagy (further discussed in **Chapter 3**) (Clark et al. 2006, Park et al. 2006, Ibáñez et al. 2006, Gegg et al. 2009, Schmidt et al. 2011). In addition, DJ-1 translocates to mitochondria upon oxidative stress and has protective effects for the cells (Canet-Avilés, Wilson et al. 2004).

The second pathway most evidently involved in PD is the autophagic-lysosomal pathway. Examples of lysosomal enzymes that cause PD or PD-like conditions include heterozygous mutations in GBA and mutations in ATP13A2. Homozygous

GBA mutations cause Gaucher's disease which is a lysosomal storage disorder, whereas heterozygous GBA mutations increase the risk of developing PD by 5-fold (Sidransky, Lopez 2012). The function of ATP13A2 and its involvement in PD is less well understood although we know that it impairs lysosomal function (Tsunemi, Krainc 2014) . An example of a mutation of a trafficking enzyme causing PD is Vps35. Vps35 forms part of the retromer complex and is involved in protein sorting, rescue and recycling (Vilariño-Güell, Wider et al. 2011). The Vps35 D620N mutation causes autosomal dominant PD by impairing protein sorting at the maturing endosome and autophagy defects and/or mitochondrial defects (Zavodszky, Seaman et al. 2014, Tang, Liu et al. 2015, Wang, Wang et al. 2016). In addition, ATP6AP2 mutations cause acidification defects of the lysosomes and result in neurodegeneration and loss of Rab7LA impairs the endosomal/lysosomal trafficking of proteins reviewed in (Perrett, Alexopoulou et al. 2015).

The first mutation to be identified in PD was the A53T point mutation in SNCA coding for α-synuclein (Polymeropoulos, Higgins et al. 1996). This was followed by the discovery of A30P, E46K, H50Q, G51D, duplications and triplications (Krüger, Kuhn et al. 1998, Conway, Lee et al. 2000, Choi, Zibaee et al. 2004, Greenbaum, Graves et al. 2005, Zarranz, Alegre et al. 2004). α-synuclein amongst other functions, is involved in synaptic vesicle release (Cabin, Shimazu et al. 2002) and relates to SNARE proteins (Burré, Sharma et al. 2010). Mutations or overexpression of α-synuclein cause Parkinson's disease, but this will be discussed further in **Chapter 1.2**. LRRK2 is a cytoplasmic kinase and sometimes recruited to the outer mitochondrial membrane and is involved in signalling (Dauer, Ho 2010), especially in cytoskeletal dynamics (Hardy 2010, MacLeod et al. 2006, Meixner et al. 2011). LRRK2 mutations cause neuritic defects (MacLeod, Dowman et al. 2006) due to

defective autophagy (Plowey, Cherra III et al. 2008, Alegre-Abarrategui, Christian et al. 2009, Ramonet, Daher et al. 2011, Friedman, Lachenmayer et al. 2012). This defect can be abolished by regulation of LC3 by protein Kinase A (Cherra III, Kulich et al. 2010). Mutant forms of LRRK have been recently found to increase the phosphorylation of certain Rab proteins and further link trafficking defects with Parkinson's disease (Steger, Tonelli et al. 2016).

1.1.3. Pathophysiology

The majority of PD is sporadic (around 90%) (de Lau, Breteler 2006). PD is a multifactorial disease and can be caused by a multitude of pathological changes. Dopaminergic neurons have very long axons with extensive arborisation, so they require an excellent trafficking system across microtubules both for maintaining their function but also for housekeeping functions. Thus, it is not surprising that mutations involving trafficking mechanisms predispose to Parkinson's disease (Sulzer 2007). However, other neurons too are long and have complex synapses, so although this predisposes dopaminergic neurons to death, more attributes are needed to understand why these neurons die. Thus, the theory of "multiple hits" has been suggested, like in cancer. In most cases, more than one risk factor/pathologic event should take place in order to initiate the disease (Sulzer 2007).

There are known risk factors for developing the disease, such as exposure to herbicides and pesticides (e.g. rotenone, paraquat) (de Lau, Breteler 2006, Tanner et al. 2011, Van Maele-Fabry et al. 2012, Moretto, Colosio 2013) and head injury. In addition, exposure to certain chemicals causes parkinsonism (e.g. MPTP) (Meredith, Rademacher 2011). MPTP is not toxic itself, but being lipophilic, it crosses the blood-brain barrier and is transformed to MPP+ by MAO-B in astrocytes. It then concentrates in the mitochondria of dopaminergic neurons

inhibiting the respiratory Complex I of the oxidative phosphorylation pathway, reducing the production of ATP, forming free radicals and activating the mitochondrial transition pore dissipating the potential. These cells then undergo apoptosis (Meredith, Rademacher 2011). The accumulation of heavy metals in the SN has also be implicated, although debatable in the field (de Lau, Breteler 2006). Rotenone being lipophilic, like MTPT, crosses the blood-brain barrier and enters the dopaminergic neurons. It impairs oxidative phosphorylation by its binding to the complex I of the electron transport chain. (Schuler, Casida 2001). It also inhibits microtubule formation from tubulin (Brinkley, Barham et al. 1974, Marshall, Himes 1978). Unlike MPTP and rotenone, paraquat does not cross the BBB spontaneously (Shimizu, Ohtaki et al. 2001), but it has striking structural similarity to MPP+ (Bové, Prou et al. 2005). Its toxicity stems from redox species formation in conjunction with nitric oxide synthase (Day, Patel et al. 1999).

As dopaminergic neurons die in Parkinson's disease, dopamine levels are reduced as the disease develops. However, at the earlier stages, dopamine dysregulation is believed to be involved in the pathophysiology, with the overproduction of toxic intermediate dopamine species, such as DOPA-L and possibly dopamine storage defects. These toxic species and other defective processes lead to the formation of reactive oxygen and nitrogen species, which create oxidative stress that are very harmful for the cell (Nakamura, Bindokas et al. 2001). The cells use various mechanisms under physiological conditions to overcome this toxicity (e.g. by dopamine sequestration by VMAT2, its metabolism by MAO (monoamine oxidase), feedback inhibition of Tyrosine Hydroxylase (TH) and the antioxidant properties of glutathione (Nakamura, Bindokas et al. 2001). Since dopamine is stored in vesicles using a hydrogen electrochemical force, the ATP production from mitochondria is

essential for its vesicle storage (Sulzer, Bogulavsky et al. 2000). If mitochondrial oxidative phosphorylation is impaired for any reason (e.g. rotenone, MPP+), dopamine uptake could be conceivably impaired causing accumulation in the cytosol and oxidation into toxic intermediates (Sulzer, Bogulavsky et al. 2000). The toxic metabolites (e.g. DOPA-L) and oxidized species interfere with and delay the breakdown mechanisms of α-synuclein and enhance its aggregation. This is because toxic dopamine metabolites create reactive oxygen and nitrogen species, which cause lipid peroxidation that in turn promote α-synuclein aggregation. Dopamine quinones form an ionic bond with residues 125-129 of α-synuclein (Norris, Giasson et al. 2005). This keeps α-synuclein in protofibrils, which are more toxic and inhibits the bigger aggregate formation. These protofibrils can cause H+ leakage in small chromaffin vesicles (Mosharov, Staal et al. 2006), and if this also happens in dopamine-vesicles, they could be releasing more dopamine in the cytosol, thus creating a vicious cycle. Dopamine-modified α-synuclein also inhibits CMA in a way similar to pathogenic α-synuclein mutations (Martinez-Vicente, Talloczy et al. 2008). There is a suggested model for a biphasic dopamine function: at physiological concentrations, it decreases the oligomerization of α-synuclein, increases cell viability and promotes normal lysosomal function). At doses above 100μM (e.g. if VMAT2 is malfunctioning etc.), it impairs proteasomal function and promotes the accumulation of ubiquitinated proteins (Jiang, Gan et al. 2013).

Furthermore, inflammation is one aspect of PD (Fahn 2003) also shown in animal models of PD (Dauer, Przedborski 2003) where there is toxicity from T-cells or microglia. One possible reason could be that dopaminergic neurons that express the cyclooxygenase enzyme 2 (Cox-2) upon an infective trigger directly signal to T-

cells or microglia to attack them (Sulzer 2007). However, the mechanism behind neuroinflammation and Parkinson's disease remains elusive.

A major pathophysiological event in PD is protein aggregation. This results from protein misfolding and the inability of dopaminergic neurons to clear or re-fold/disaggregate the defective proteins. The protein which gets aggregated in PD is α-synuclein, discussed in **Chapters 1.1.4 and 1.2.** The protein systems responsible for protein breakdown, which if defective cause α-synuclein overload and aggregation are discussed in **Chapter 1.3** as they represent both a major component of Parkinson's disease but also of my thesis.

1.1.4. Neuropathology of Parkinson's disease

Neuropathologically, PD is characterized by loss of dopaminergic neurons and the presence of intraneuronal Lewy bodies and Lewy neurites containing aggregated misfolded α-synuclein and ubiquitin. There is also evidence of α-synuclein accumulation within the synaptic terminals (Muntané, Dalfó et al. 2008, Schulz-Schaeffer 2010). In addition, there is also dopaminergic cell loss in the nigrostriatal pathway (from the Substantia nigra pars compacta to the putamen) which is also accompanied by loss of the black neuromelanin pigment of the dopaminergic neurons in the substantia nigra and of noradrenergic neurons in the locus ceruleus (Dickson 2012). Neuromelanin is the product of oxidized toxic dopamine species. These are stored in endosomal autophagic vacuoles since they cannot be broken down in order to protect cells from toxicity and thus give the SN nigra its characteristic black colour (Sulzer, Bogulavsky et al. 2000). In PD, upon the death of dopaminergic neurons, the neuromelanin vacuoles are released extracellularly

and neuromelanin is broken down by microglia, thus the black colour of the SN is lost (Zecca, Zucca et al. 2006).

Lewy bodies are eosinophilic cytoplasmic inclusions of dense core, surrounded by a halo. They are 10-15nm in diameter and contain dense granular species and straight filaments (Dickson 2012, Forno 1969, Tiller-Borcich, Forno 1988, Galloway, Mulvihill et al. 1992). Amongst their contents, there is aggregated α-synuclein which is mislocalized from its normal location mainly at the synaptic terminals (Spillantini, Schmidt et al. 1997) but also at the nucleus and based on a limited number of reports in association with mitochondria (Yu, Li et al. 2007, Guardia-Laguarta, Area-Gomez et al. 2015). Factors that increase the aggregation of α-synuclein include its phosphorylation at position 129, truncation at the C-terminus and its oxidation/association with toxic oxidative species. (Dickson 2001). Lewy bodies are immunoreactive against neurofilament (Galvin, Lee et al. 1997), TH, heat shock proteins, mitochondria, lipid vesicles and components of the ubiquitin system such as ubiquitin (Kuzuhara, Mori et al. 1988), the ubiquitin binding protein p62 (Dickson 2012, Kuusisto, Parkkinen et al. 2003)and the ubiquitin ligases CHIP (Shin, Klucken et al. 2005)and Nedd4 (Tofaris et al. 2011). There are some theories proposing that LB have a protective role in PD, containing the toxic oligomeric aggregates. It is thus possible that in PD neurons start dying when the toxic α-synuclein oligomers can no longer be stored in a compact fibrillar form (Sulzer 2007). On the other hand, there are theories stating that LB are toxic and their presence increases cellular toxicity. It remains unclear which form of α-synuclein is the most toxic species in the human brain.

Lewy bodies are not only found in the substantia nigra, but also in other regions (Jellinger 1991). In 2004 Braak and colleagues proposed a staging system for α-synuclein pathology in PD (Braak, Ghebremedhin et al. 2004). They suggest that pathology starts from the dorsal motor nucleus of the vagus (medulla) and the anterior olfactory nucleus (olfactory bulb). In later stages, LBs are found in the dopaminergic neurons of the SN and in the locus ceruleus neurons (pons) and then pathology spreads to the forebrain, amygdala and temporal lobe before spreading to other cortical areas (Braak, Ghebremedhin et al. 2004). It should be mentioned here that Braak's classification is the staging of α-synuclein pathology and not of neuronal death and that it was somewhat biased towards early medullary pathology, selecting to study many patients with such pathology (Dickson 2012). Other facts that should be considered when using/interpreting the 2004 Braak staging should include pathologies in long prodromal phase of PD when some non-motor features appear, such as REM sleep behavioural disorders, loss of olfaction and autonomic dysfunction. In 2009, Hawkes et al (including Braak) proposed the "dual hit" hypothesis, according to which a neurotropic virus that can infect the nasal mucosa can wither enter the brain via the olfactory bulb, or can be swallowed, infect the enteric wall and transferred to the brain via the enteric nervous system and the vagal nerve causing α-synuclein accumulation in these sites (Braak, De Vos et al. 2006, Braak, Del Tredici 2009, Hawkes, Del Tredici et al. 2009). Holmqvist et al later showed that α-synuclein injected into the intestinal wall is transported to the brain via the vagus nerve by an active retrograde transport system through microtubules (Holmqvist, Chutna et al. 2014). Taking all the above evidence into consideration, it is evident that the progression of PD pathology is likely to involve multiple factors.

1.2. α-Synuclein biology

α-Synuclein is the protein that aggregates in PD and forms part of the Lewy Bodies. It is a natively unfolded protein (Weinreb, Zhen et al. 1996) of 14.46 kDa and 140 amino acids (Tofaris, Spillantini 2005) and undergoes a number of post-translational modifications, including truncation, phosphoprylation and ubiquitination (Tofaris, Garcia Reitböck et al. 2006). Its structure comprises of NAC, a central hydrophobic region (the non-amyloid component- amino acids 61-95) which is involved in the aggregation process, a negatively charged C-terminus and N-terminal region which contains imperfect KTKEGV repeats that mediate membrane binding (Tofaris, Spillantini 2005). The synuclein family has three members, α, β and γ-synucleins. They all consist of a highly conserved α-helical lipid-binding motif. Their exact function remains elusive, however the best studied of the three synucleins is α-synuclein that is involved in Parkinson's disease. γ-Synuclein is sometimes used as a tumour marker in breast cancer progression.

Although α-synuclein is natively unfolded in solution, it acquires a partial α-helical structure when incubated with lipid membranes (Davidson, Jonas et al. 1998), and misfolds into β-pleated sheet containing amyloid fibrils when shaking in solution (Conway, Lee et al. 2000). Some studies have suggested that when cross-linked, a fraction of α-synuclein may exist as a tetramer in cells (Dettmer, Newman et al. 2013).

Its normal physiological functions are not fully understood but are thought to involve a role in synaptic transmission, including vesicle release, acting as a chaperone in the formation of SNARE complexes and it also may have a less well-understood nuclear and mitochondrial function. In nucleus it is known to bind to histones and reducing the levels of acetylated histone H3 (Kontopoulos, Parvin et al.

2006). In fact, histone-deacetylase (HDAC) inhibitors have been shown to reduce α-synuclein toxicity *in vitro* and in the *Drosophila* (Kontopoulos, Parvin et al. 2006). α-Synuclein can also be found on the inner mitochondrial membrane where it inhibits the function of Complex I (Li, Yang et al. 2007, Devi, Raghavendran et al. 2008, Nakamura, Nemani et al. 2008, Liu, Zhang et al. 2009, Loeb, Yakunin et al. 2010). At the synapse, it interacts with phospholipids and proteins and can be either membrane-bound or unbound (cytosolic) (Burré, Sharma et al. 2010). It facilitates the maintenance of synaptic vesicle supply and release (Murphy, Rueter et al. 2000), including that of dopamine (Abeliovich, Schmitz et al. 2000). Other properties of α-synuclein include its interaction with tubulin and microtubules (like tau) (Alim, Hossain et al. 2002, Zhou, Huang et al. 2010), the facilitation of neuronal Golgi and vesicular trafficking and its functions in normal development regarding the cognitive functions of spatial learning and working memory (Kokhan, Afanasyeva et al. 2012).

Depletion of α-synuclein in cells has provided limited insights into its function or role in disease. There have been studies with either α-synuclein, double (α, β) or triple (α, β, γ) synuclein knockout mice. These rodents had less dopaminergic synapses (Al-Wandi, Ninkina et al. 2010) and decreased dopamine levels (due to increased dopamine release) as α-synuclein has an inhibitory effect in activity-dependent modulation of dopamine transmission (Abeliovich, Schmitz et al. 2000) and an important function in reserving vesicles in neurotransmission (Murphy, Rueter et al. 2000). In addition, there were effects in spatial and working memory (Kokhan, Afanasyeva et al. 2012). Interestingly though, these knockout mice showed resistance to MPTP toxicity, connecting mitochondrial defects and oxidative damage due to complex I inhibition to α-synuclein (Dauer, Kholodilov et

al. 2002). Finally, the knockout mice were reported to have slightly less dopaminergic neurons than controls (Robertson, Schmidt et al. 2004, Greten-Harrison, Polydoro et al. 2010). In fact, endogenous α-synuclein is shown to increase the number of dopaminergic neurons in the SN of mice (Garcia-Reitboeck, Anichtchik et al. 2013). However, these effects were observed when α-synuclein was knocked out from birth and not silenced in elderly animals (e. g. with a conditional knockout). There was a study where mice were injected with siRNA against α-synuclein unilaterally which caused dopaminergic neuron death in one month (Gorbatyuk, Li et al. 2010). In contrast, when siRNAs reduced α-synuclein levels by 50-60% in monkeys, no toxicity was seen (McCormack, Mak et al. 2010). Therefore, excess and too little α-synuclein can be harmful, suggesting that loss of function, e.g. by sequestration of alpha-synuclein into fibrils and complete depletion from the synapse may also contribute to the pathogenesis of PD.

Pathological Functions of α-synuclein

Despite the incomplete understanding of its function, there is a major interest in its role in disease, which is primarily to a toxic gain of function due to accumulation and/or misfolding. Certain modifications of α-synuclein increase its predisposition towards aggregation such as phosphorylation at serine 129 (Fujiwara, Hasegawa et al. 2002), dopamine-intermediates, oxidizing or nitrating agents associated with α-synuclein (Ostrerova-Golts, Petrucelli et al. 2000, Giasson, Duda et al. 2000, Hodara, Norris et al. 2004, Lee, Shin et al. 2002) and α-synuclein mutations or C-terminus truncations (Crowther et al. 1998, Serpell et al. 2000, Kim, Paik & Yang 2002, Murray et al. 2003, Tofaris et al. 2003). The initially identified mutations in humans are the A53T and E46K and make α-synuclein more prone to aggregation (Polymeropoulos, Higgins et al. 1996, Choi, Zibaee et al. 2004, Choi, Zibaee et al.

2004, Greenbaum, Graves et al. 2005, Zarranz, Alegre et al. 2004, Conway, Rochet et al. 2001) whereas A30P reduce its binding to lipid membranes ((Jensen, Nielsen et al. 1998) suggesting that these properties may contribute to PD pathomechanisms. Interestingly, rodents carry the A53T variant as part of their normal genome (Stefanis 2012). Under physiological conditions, only 4% of α-synuclein is phosphorylated in rat, whereas in LB over 90% is phosphorylated (Paleologou, Schmid et al. 2008).

The theory of prion-like cell-to-cell transmission has stemmed from the fact that foetal mesencephalic intrastriatal transplants that were offered as treatment to PD patients developed LB pathology (Kordower, Chu et al. 2008, Li, Englund et al. 2008), due to cell-to-cell α-synuclein transmission (Kordower, Dodiya et al. 2011). Cell-to-cell transmission of α-synuclein pathology has been replicated in animal models of Parkinson's disease. For example, a single intrastiatal inoculation of synthetic α-synuclein fibrils in wild type mice resulted in cell-to-cell transmission of the α-synuclein pathology and the formation of Lewy pathology. This in turn led to loss of dopaminergic neurons in the SN pars compacta but not the VTA (Ventral tegmental area) and subsequent decrease in dopamine levels with associated motor syndrome (Luk, Kehm et al. 2012, Masuda-Suzukake, Nonaka et al. 2013). Similar effects were observed in wild type mice with the injection of Parkinson's brain lysates (Masuda-Suzukake, Nonaka et al. 2013). Likewise, exogenous preformed fibrils of α-synuclein can enter neurons probably via adsorptive endocytosis and convert the endogenous α-synuclein into Lewy body and Lewy neurite aggregates (Volpicelli-Daley, Luk et al. 2011). There are many possible ways of interneural transmission of pathologic α-synuclein species. Such species can either be secreted freely in to the intercellular space or in exosomes. α-Synuclein monomers and

oligomers have been shown to be secreted from cells in exosomes via the multi-vesicular bodies (Emmanouilidou, Melachroinou et al. 2010, Danzer, Kranich et al. 2012). It remains to be seen if fibrillar α-synuclein species can also be secreted via exosomes. Free species could be taken up via free membranous penetration, via fluid-phase endocytosis (micropinocytosis) or via receptor-mediated endocytosis (Guo, Lee 2014). Indeed, very recently, Mao et al showed that pathogenic neuron-to-neuron transmission of aggregated α-synuclein is initiated at least partly by its binding to the membrane lymphocyte-activation gene 3 (LAG3) and its subsequent endocytosis. LAG3 knockout or its deactivation by antibodies reduces the transmission and pathology both in vitro and in vivo (Mao, Ou et al. 2016). Another possible way of transmission includes nanotubules that directly connect the cytoplasm between two adjacent cells (Guo, Lee 2014). **Figure 1.2** depicts the different possible ways of α-synuclein transmission.

Another important consideration is what forms α-synuclein can take, how these contribute to the aggregation process and how they confer toxicity. In this regard, it was found that oligomeric and fibrillar α-synuclein species exist in equilibrium (Conway, Harper & Lansbury 1998, Giasson et al. 1999, Hashimoto et al. 1998). Oligomeric forms can be of low or high molecular weight, spherical, chain-like or annular both found in post-mortem PD brains (Tsigelny et al. 2008, Kahle et al. 2001, Baba et al. 1998) and in brains of PD animal models (Tsigelny et al. 2008, Kahle et al. 2000). Oligomers precede fibril formation (Horvath et al. 2012). Many factors can cause monomeric α-synuclein to convert to oligomers, including interactions with lipids, small molecules, oxidative stress (Hashimoto et al. 1999, Souza et al. 2000), post-translational modifications (e.g. S129 phosphorylation) and truncation (Oueslati, Fournier & Lashuel 2010). In addition, toxic dopamine

metabolites slow the conversion of protofibrils of α-synuclein to fibrils (Conway, Rochet et al. 2001, Mazzulli, Mishizen et al. 2006, Tsika, Moysidou et al. 2010)

Evidence suggests that the oligomeric and protofibrillar species are the most toxic forms of α-synuclein configuration (Uversky 2010, Conway et al. 2000a, Conway et al. 2000b) whereas the fibrillar forms are more stable and less dynamic conferring less toxicity. According to some in vitro studies, protofibrils also form pores in phospholipid vesicles, permeabilizing them (Volles, Lansbury Jr. 2002, Lashuel, Hartley et al. 2002). Conceivably this mechanism could involve dopamine-storing vesicles causing a vicious cycle (Mosharov, Staal et al. 2006, Conway, Rochet et al. 2001, Mazzulli, Mishizen et al. 2006) and also in the outer mitochondrial membrane, releasing pro-apoptotic mitochondrial proteins in the cytosol (Luth, Stavrovskaya et al. 2014). This also leads to abnormal calcium currents across the plasma membrane and subsequent death (Danzer et al. 2007). Cellular damage from oligomeric species is also caused by triggering lysosomal leakage (Hashimoto et al. 2004), microtubule disruption (Alim et al. 2004), damage to mitochondria (Hsu et al. 2000), proteasomal impairment and by interfering with axonal transport of synaptic proteins (e.g. synapsin 1) resulting in synaptic dysfunction and neuronal death (Scott et al. 2010, Lashuel et al. 2013). Two criticisms in the literature on the toxicity of oligomeric and fibrillar species consist of the facts that recombinant α-synuclein was mostly used in the experiments and that detection of the oligomeric forms was indirect, via native and/or denaturing gel electrophoresis (Lashuel et al. 2013).

An interesting new concept is that neuronal toxicity not only stems from oligomers and protofibrils, but also from the process of fibrillization itself (Lashuel et al. 2013). This theory is based upon observations that fibrillization-promoting

mutations (e.g. S129A) confer increased toxicity in rat PD models (Gorbatyuk et al. 2008), whereas mutations blocking oligomerization and fibrillization (e.g. S87E) reduce both α-synuclein aggregation and neurodegeneration (Oueslati et al. 2012). **Figure 1.2** shows the different α-synuclein species and how each one is involved in the aggregation process or other pathologic processes (e.g. pore formation).

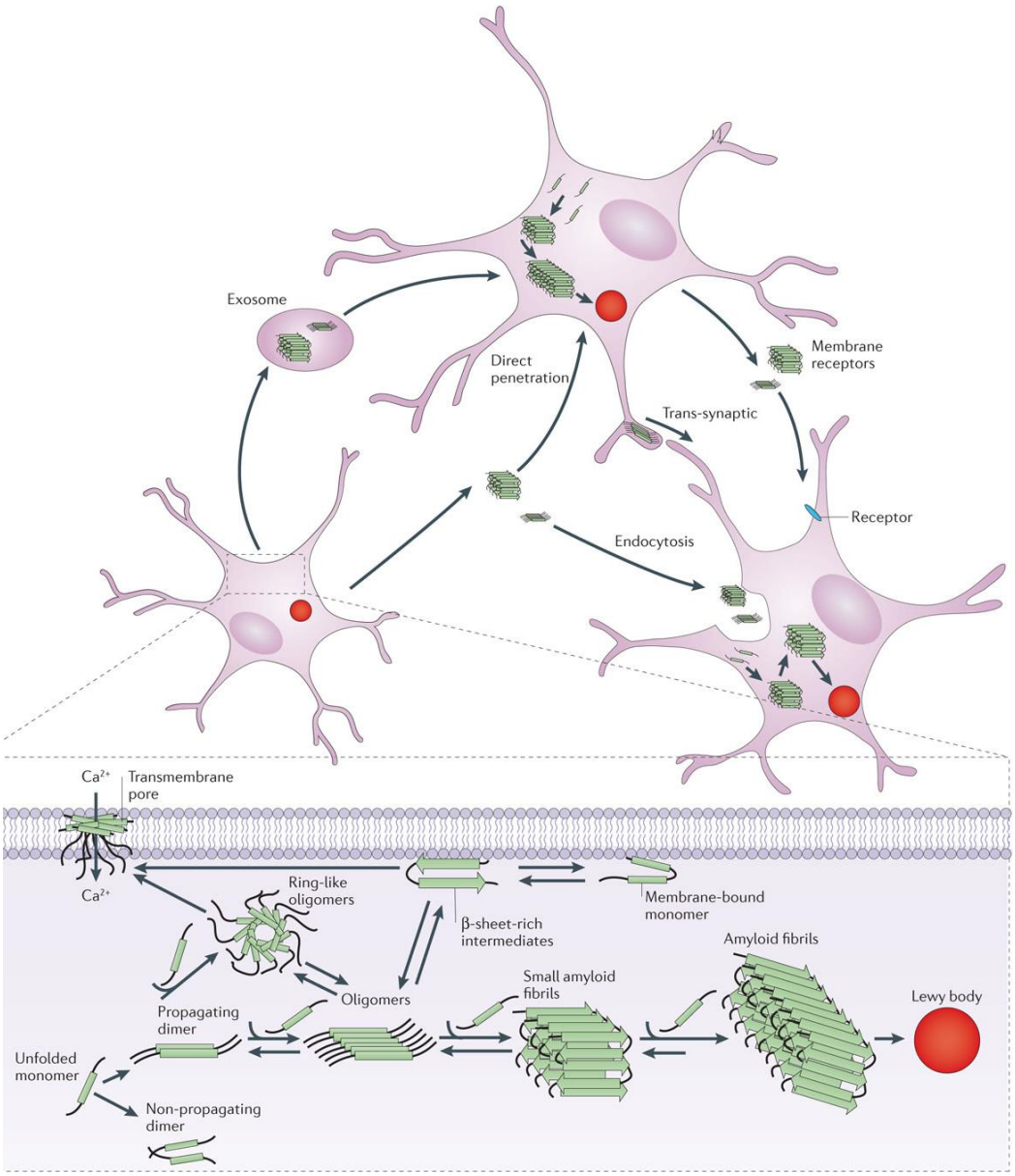


Figure 1.2. Possible ways of α-synuclein transmission/propagation and different stages of α-synuclein aggregation and formation of other species. Figure adapted from Lashuel et al 2013 (Lashuel et al. 2013).

α-Synuclein is expressed in many tissues (e.g. heart, muscles, erythrocytes and platelets) but its expression is very strong in many brain regions, consisting the 1% of proteins expressed in neurons. As the number of *α-synuclein* gene copy number increases, the age of onset of PD decreases and the severity of the disease gets worse (Fuchs, Nilsson et al. 2007, Ross, Braithwaite et al. 2008). Similarly, mutations causing expansions of the α-syn promoter (Rep1) increasing the expression levels of α-synuclein are a risk factor of PD (Maraganore, De Andrade et al. 2006, Nalls, Plagnol et al. 2011). These findings strongly suggest that intraneuronal accumulation of α-synuclein is sufficient to cause neurodegeneration with Lewy bodies. Therefore, understanding how α-synuclein is targeted for degradation is an important research question as identification of enzymes that mediate this process could be used for targeted therapies.

One prediction from the aforementioned studies is that the extent of α-synuclein aggregation may depend on its synthesis and breakdown (clearance) rates. The rate of production of α-synuclein has been debated with some studies showing decreased mRNA levels (Dächsel, Lincoln et al. 2007) and some increased (Gründemann, Schlaudraff et al. 2008) in post-mortem brains of Parkinson's disease patients depicting increased production. α-Synuclein is normally broken down via multiple pathways including the ubiquitin-proteasomal system, the autophagic-lysosomal system and chaperone-mediated autophagy (CMA), mechanisms which will be further discussed in **Chapter 1.3.**

When α-synuclein accumulates e.g. due to impaired clearance, it causes various defects in neurons. These include decreased synaptic vesicle release, formation of

enlarged vesicles and synaptic dysfunction (Chung, Koprich et al. 2009, Nemani, Lu et al. 2010, Scott, Tabarean et al. 2010), SNARE protein redistribution (Garcia-Reitboeck, Anichtchik et al. 2013), impaired energy production from oxidative phosphorylation in mitochondria (Li, Yang et al. 2007, Devi, Raghavendran et al. 2008, Nakamura, Nemani et al. 2008, Liu, Zhang et al. 2009, Loeb, Yakunin et al. 2010), accumulation of substrates of chaperone-mediated autophagy (Orenstein, Kuo et al. 2013), proteasome impairment and induction of apoptosis (Tanaka, Engelender et al. 2001). Another important effect is the impairment of protein trafficking in endosomes (e.g. by interfering with Rab proteins) (Gitler, Bevis et al. 2008, Chua, Tang 2011) and from the endoplasmic reticulum (ER) to Golgi (Cooper, Gitler et al. 2006) resulting in Golgi fragmentation (Gosavi, Lee et al. 2002), ER stress (Smith, Jiang et al. 2005) and defects in protein sorting (Soper, Kehm et al. 2011) and autophagy (Winslow, Chen et al. 2010).

One will observe that all these pathways affected by α-synuclein aggregation/dysfunction are similar to ones involved in genetic forms of Parkinson's disease. Therefore, there could be some unifying underlying pathways connecting sporadic to genetic Parkinson's disease. The lysosomal system is such a theme, which is a membrane trafficking system that processes membrane-bound proteins via endosomes or organelles via autophagy (e.g. from the plasma membrane or the Golgi). In the endosomal pathway, cargo is sorted through the ESCRT pathway (explained below) either to the lysosomes for degradation, or recycled back to the plasma membrane via the retromer complex ((Mellman 1996). Early endosomes are characterized by the presence of EEA1/Rab5 (Stoorvogel, Strous et al. 1991), late endosomes (or multivesicular bodies-MVB) by Rab7 (Russell 2006) and recycling endosomes by Rab11 (Ullrich, Reinsch et al. 1996).

This system is going to be explored in greater detail due to its involvement in Parkinson's disease.

1.3. Mechanisms of protein degradation in health and in α-synuclein pathology

From the data mentioned above, it is evident that α-synuclein homeostasis is perturbed in Parkinson's disease. It is thus important to understand how this protein is handled in cell homeostasis and the mechanisms that regulate its degradation. In general, protein homeostasis is regulated at the level of protein synthesis, correct folding, trafficking and correct localization and finally degradation (Narayan, Ehsani et al. 2014). If any of the above is defective, protein homeostasis (termed proteostasis) will be impaired and certain aggregation-prone proteins may accumulate in inclusion bodies. In the case of α-synuclein, we have discussed how synthesis and post-translational modifications (e.g. phosphorylation) can lead to α-synuclein aggregation. In relation to correct protein folding, the cells have two distinct but similar mechanisms to achieve proteostasis: the Heat Shock Response (HSR) and the Unfolded Protein Response (UPR). HSR involves several heat shock proteins, which act as molecular chaperones, identifying and refolding misfolded proteins. The UPR is similar to the HSR but ensures correct protein homeostasis in the ER (Narayan, Ehsani et al. 2014). In fact, the Heat Shock Protein 70 (HSP70) and its overexpression has been shown to be protective in preventing α-synuclein aggregation (Auluck, Chan et al. 2002). The last step in achieving proteostasis is protein breakdown. Identifying the mechanisms by which α-synuclein is broken down is very important in finding ways to prevent its aggregation and might

identify new targets for therapy of Parkinson's disease. Thus, the remainder of this section will focus on mechanisms of protein breakdown.

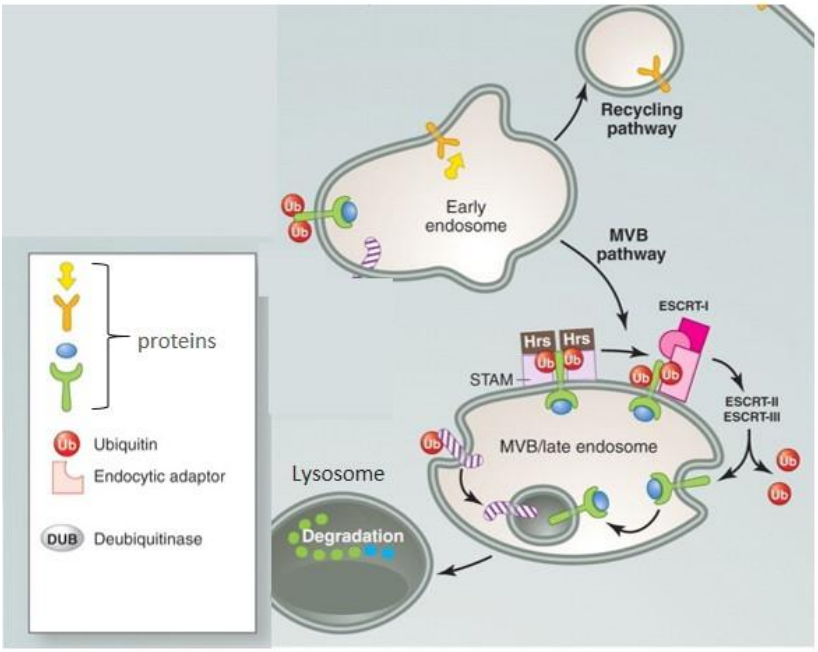


Fig 1.3. The ESCRT pathway involvement in the autophagic lysosomal and endosomal-lysosomal protein degradation (figure adapted from (Mukhopadhyay, Riezman 2007).

Most cellular proteins are selectively targeted for degradation after conjugation to a ubiquitin chain. This modification involves activation of ubiquitin by the enzyme E1, transfer of the reactive ubiquitin to a ubiquitin conjugating enzyme (E2), and then conjugation by a ubiquitin ligase (E3) to a protein substrate or a preceding ubiquitin to form a ubiquitin chain (Rubinsztein 2006) **(Fig. 1.4.).** Ubiquitin contains seven lysine residues, each of which can be linked to another ubiquitin molecule through an isopeptide bond **(Fig. 1.4.)**. There are more than 650 E3s and about 40 E2s in the mammalian genome, ensuring substrate selectivity in this pathway. The efficiency and fidelity of protein degradation is regulated in part, by

the recycling of the ubiquitin chains by deubiquitinating enzymes (DUBs) (Clague et al. 2013) **(Fig. 1.4.)**. The three main ways of protein breakdown are through the proteasome (Ubiquitin Proteasomal System-UPS), autophagic pathways and endosomal degradation. While formation of ubiquitin chains, in which the ubiquitins are covalently linked through their K48 or K11 residues, leads to the degradation of cytosolic proteins by 26S proteasomes, attachment of chains linked through K63 residues to membrane-associated proteins amongst other functions, targets them for lysosomal degradation (Nathan, Tae Kim et al. 2013).

The Endosomal Sorting Complex Required for Transport (ESCRT) components STAM1/2 and HRS deliver K63-linked chains to the endosomal pathway (Nathan, Tae Kim et al. 2013) **(Fig. 1.3.)** in a process regulated by two distinct DUBs, Usp8 and AMSH (Urbé, McCullough et al. 2006, Wollert, Hurley 2010). On the other hand, the Rad23 family binds to ubiquitinated proteins via their two UBA (ubiquitin-associated) domains and delivers them to the proteasome via docking of the Rad23 UBL (ubiquitin-like) domain (Goh, Walters et al. 2008).

CMA involves selective identification of cargo by heat shock proteins and co-factors (e.g. HSP70, more discussed below). The cargo is then transferred to the lysosome, where it is recognized and taken up by the lysosomal receptor LAMP2A in the lysosomal lumen where it is broken down. The importance of the process of autophagy and of the identification of the molecular machines that form the core of this process were recognized with the 2016 Nobel Prize in Medicine or Physiology to Yoshinori Oshumi. It should be mentioned that effective protein breakdown is especially essential in post-mitotic cells, such as neurons, which cannot perform protein dilution through mitosis (Narayan, Ehsani et al. 2014). In addition, normal

protein breakdown capacity decreases with ageing, rendering neurons more prone to proteotoxic stress.

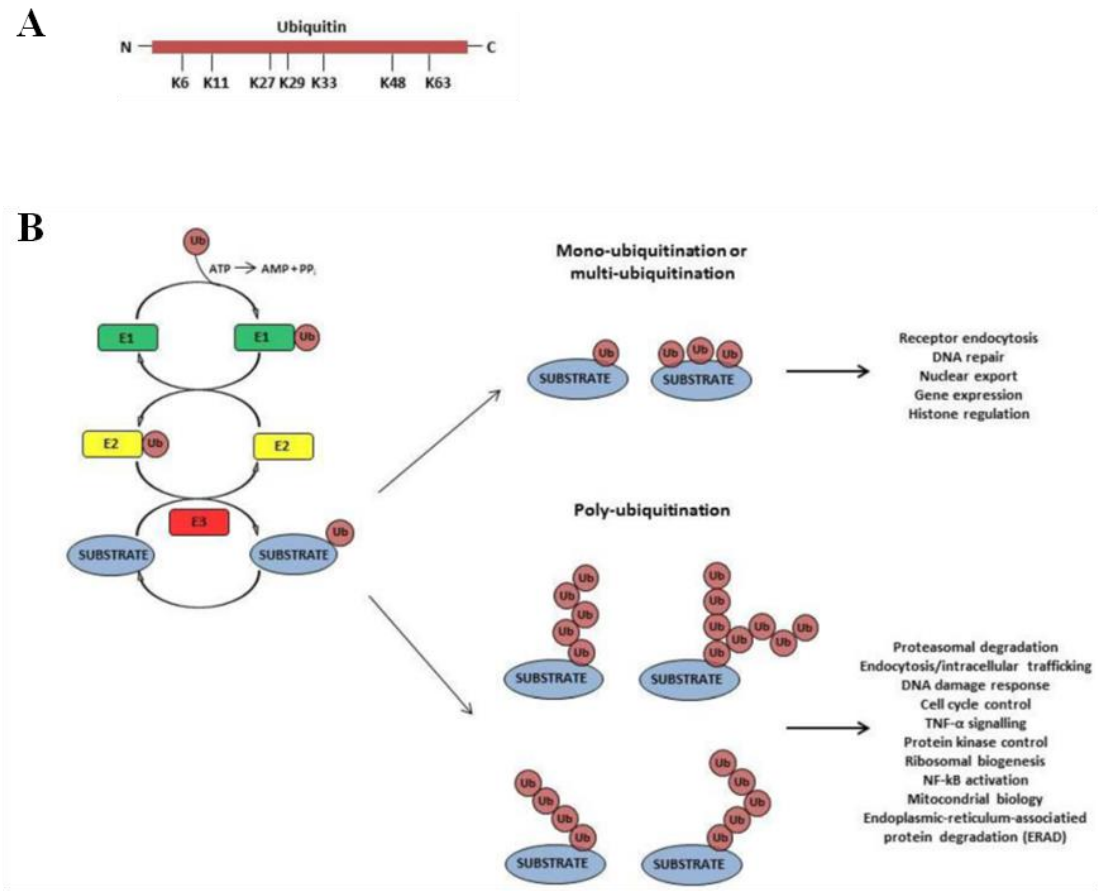


Fig. 1.4.: A: the positions of lysines in the ubiquitin protein. **B:** Ubiquitin metabolism and functions (adapted from (Calistri, Munegato et al. 2014)).

Protein breakdown mechanisms

The two major degradative systems in the cell are the proteasome and the lysosome. Lysosomal degradation can either be through the endosomal or autophagic pathways. Autophagy consists of macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy (**Fig 1.5**). Proteasomal degradation, macroautophagy, CMA and endosomal/lysosomal degradation will be discussed in further detail. Microautophagy involves a non-selective direct lysosomal engulfment of cytoplasmic cargo by membrane invagination (Li, Li et al. 2012).

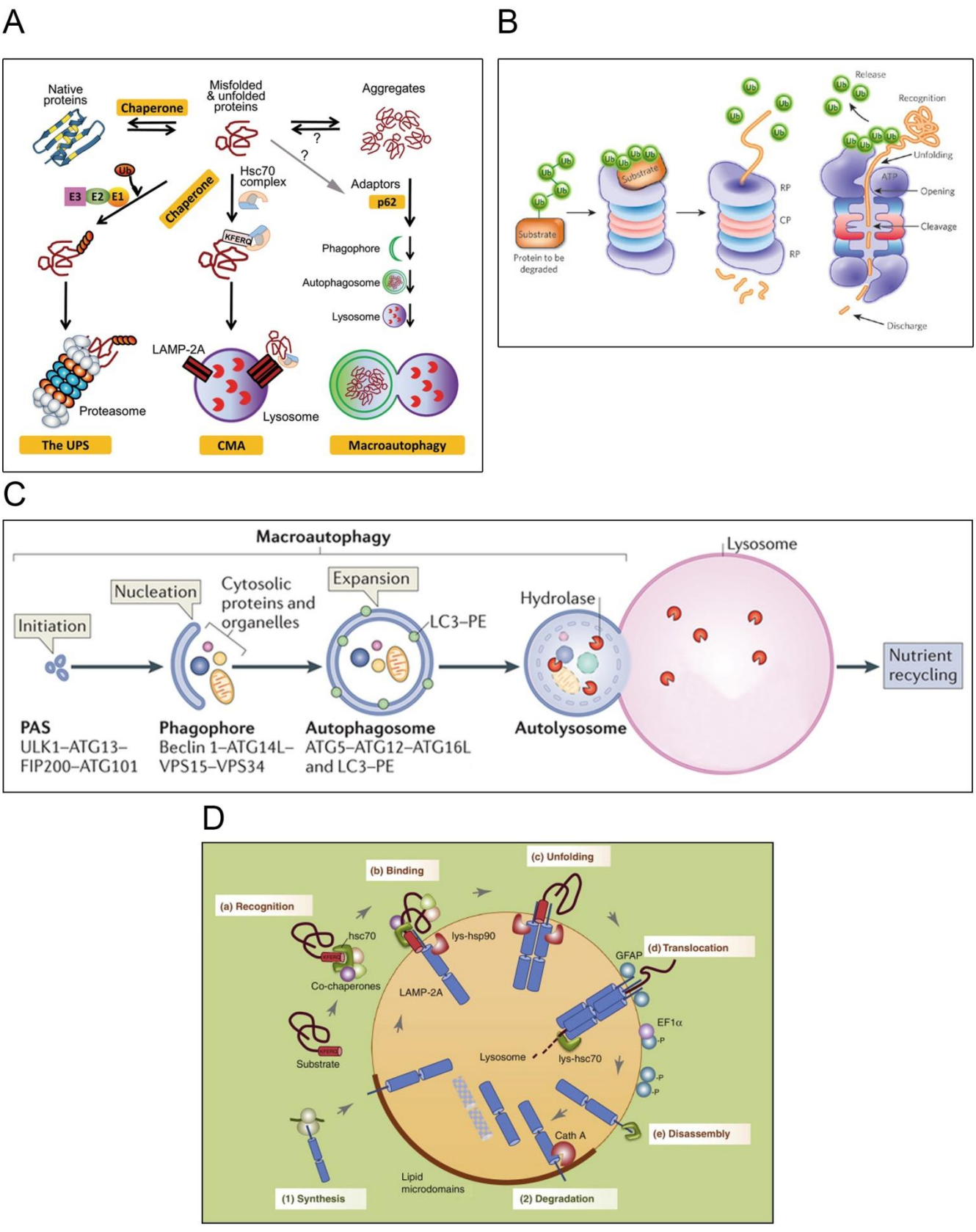


Fig. 1.5. Protein breakdown mechanisms: (A) Schematic depicting the 3 main protein breakdown mechansisms which are illustrated in more detail in: **(B)**

Ubiquitin-mediated Proteasomal degradation, **(C)** Macroautophagy, **(D)** Chaperone-Mediated Autophay. Figures adapted from (Wang et al. 2013, Hochstrasser 2009, Kaur, Debnath 2015, Kaushik, Cuervo 2012)

Proteasomal protein degradation

The Ubiquitin-proteasome System (UPS) of protein degradation is very selective in breaking down short-lived cytosolic, membranous, endoplasmic reticulum (ER)-associated and defective/misfolded proteins. The 26S proteasome is a multiprotein complex with an enclosed cavity and two openings at its two ends. Proteolysis occurs in the enclosed cavity. Professor Alfred Goldberg identified in 1977 the proteasome as a non-lysosomal proteolytic system in reticulocytes (which lack lysosomes) (Etlinger, Goldberg 1977). It was later identified as an ATP-dependent proteolytic complex which degraded ubiquitin-tagged proteins and was denoted as the 26S proteasome (Tanaka, Waxman et al. 1983, Hough, Pratt et al. 1987). Typically, ubiquitin chains of 4 or more ubiquitins linked via Lysines at position 48 are required for proteasomal degradation. The chains are recognized by ubiquitin adaptors such as Rad23 and Rpn10 which attach both to the proteasome and the ubiquitin chain (Verma, Oania et al. 2004, Kim, Mi et al. 2004, Elsasser, Chandler-Mitilello et al. 2004). For degradation to occur proteins are first unfolded by the AAA-ATPases of the cap, followed by non-energy dependent translocation of the polypeptide into the 20S core. The three catalytic subunits of the 20S complex perform the proteolysis through a threonine-dependent nucleophilic attack. The three subunits process different substrates according to their specificities, which are chymotrypsin-like, trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing (PHGH)-like. Previous studies showed that the 20S proteasome can degrade α-

synuclein monomers (Tofaris, Layfield et al. 2001, Liu, Corboy et al. 2003) but not α-synuclein aggregates (Weinhofer, Forss-Petter et al. 2002).

The role of UPS in Parkinson's disease:

The UPS is a major cellular defense mechanism against misfolded proteins whereas accumulation of misfolded proteins can in turn impair UPS function (Bence, Sampat et al. 2001). In fact, in sporadic PD post-mortem brains there is a 40% reduction in the α-subunit of the proteasome in the substantia nigra (Tofaris et al. 2003, McNaught et al. 2003, Grünblatt et al. 2004). On the other hand, in non-vulnerable brain areas, such as the cortex, there is compensatory upregulation of the α-subunit. The α-subunit provides structural support for the β-subunit to perform its catalytic activities (Olanow, Mcnaught 2011). In addition, chymotrypsin-like, trypsin-like, and PHGH-like activities are reduced by 45-55% in the SN irrespective of cell loss but no other brain areas in sporadic PD patients (Tofaris et al. 2003, McNaught et al. 2003). The above suggests that there is reduced proteasomal function in the SN of PD patients.

Early studies have provided a variety of conflicting and often controversial evidence as to whether proteasomal dysfunction is a cause or consequence of protein aggregation. However, evidence both form genetic and pharmacological studies suggest that under certain conditions impaired proteasomal degradation can cause parkinsonism. For example, pharmacological inhibition of the proteasome causes parkinsonian features in rats and mice with Lewy-body like aggregates (McNaught, Mytilin et al. 2002, McNaught, Perl et al. 2004, Petrucelli, O'Farrell et al. 2002, Fornai, Lenzi et al. 2003, McNaught, Björklund et al. 2002, Rideout, Lang-Rollin et

al. 2005, Miwa, Kubo et al. 2005, Schapira, Cleeter et al. 2006, Nair, McNaught et al. 2006, Bukhatwa, Zeng et al. 2010). However, a knockout mouse for 26S proteasome also developed nigrostriatal degeneration and pathological and behavioural features of PD but without abundant α--synuclein aggregates (Bedford, Hay et al. 2008). In cells the proteasome degrades α-synuclein in a non-ubiquitin dependent manner, suggesting that ubiquitination of at least soluble monomeric α-synuclein is not an absolute requirement for its proteasomal degradation (Tofaris, Layfield & Spillantini 2001, Liu et al. 2003). Additionally, chronic proteasomal inhibition in cells caused the accumulation of non-ubiquitinated α-synuclein (Tofaris, Layfield et al. 2001). In this regard, it has been suggested that ubiquitination may occur after α-synuclein aggregation in later stages of the disease (Gómez-Tortosa, Newell et al. 2000). In summary, these initial studies indicated that in cells α-synuclein is degraded by the proteasome and provided evidence that proteasomal dysfunction is detected preferentially in the SN in Parkinson's disease.

Macroautophagy/Autophagy

Macroautophagy is a process by which unwanted proteins or organelles are engulfed by double-membraned vesicles called autophagosomes. These then fuse with lysosomes, which break down their contents by proteolytic enzymes that are activated in acidic pH. Lysosomal enzymes include proteases, glycosidases, phosphatases and lipases and if deficient can cause lysosomal storage diseases, such as Gaucher's disease, Tay-Sachs disease and Pompe's disease. Autophagosome maturation also involves fusion with endosomes (forming amphisomes) en route to the lysosome. Macroautophagy is induced by various mechanisms in conditions of

cellular stress, such as oxidation, starvation, protein aggregation and proteasomal inhibition (Rubinsztein 2006). The most well-understood pathway that inhibits this type of autophagy is the mTOR pathway (discussed below).

Although autophagy was initially thought to be a bulk non-selective process, specific cargo is also identified by poly-ubiquitination via K63-linked chains. Certain E3 ligases such as CHIP, Nedd4 and TRAF6 (Linares, Duran et al. 2013) have been implicated in the ubiquitination of autophagy substrates. These ubiquitinated substrates are recognized by specific ubiquitin adaptors (such as p62, optineurin, NDP52, NBR1 and TAX1BP1), which in turn trigger LC3 binding via an LC3 interacting domain. LC3 and ATG5/12/16L complex is then recruited to form the autophagosome. In addition, some proteins (e.g. β-catenin and huntingtin) have been shown to interact directly with ATG8 family members such as LC3B or GABARAPL1 followed by macroautophagic breakdown (Petherick, Williams et al. 2013, Ochaba, Lukacsovich et al. 2014). Some other proteins interact with AP2 and c-CBL which both have a LIR-ATG8 binding domain but no Ubiquitin-binding domain again driving macroautophagy (Sandilands, Serrels et al. 2012, Tian, Chang et al. 2013).

The role of macroautophagy in α-synuclein breakdown:

There are multiple levels of evidence that macroautophagy contributes to the breakdown of α-synuclein, from neuropathological specimens, knockdown of autophagic machinery, PD genetics and pharmacological treatments (Olanow, Mcnaught 2011). In brain tissue, Lewy bodies co-localize to a large extent with autophagic proteins, whereas autophagy is increased and lysosomes tend to be depleted (Anglade 1997, Alvarez-Erviti, Rodriguez-Oroz et al. 2010, Dehay, Bové et al. 2010). This could suggest an unsuccessful attempt to increase autophagy to

clear the aggregates which could conceivably deplete the autophagic machinery inside affected cells. In the SN of PD patients and cellular models of PD there is decreased expression of certain lysosomal receptors and substrates such as LAMP2a, LAMP1, Cathepsin D, HSP70 and HSP73 (Alvarez-Erviti, Rodriguez-Oroz et al. 2010, Chu, Dodiya et al. 2009). Furthermore, GBA heterozygous mutations that decrease lysosomal function increase the risk of Parkinson's disease by 6-fold (Sidransky, Lopez 2012, Sidransky, Nalls et al. 2009). The above observations suggest an involvement of the lysosomal system in the breakdown of α-synuclein, which was further validated in knockdown experiments. Although mice with impaired autophagy develop ubiquitin-positive inclusions, the majority are not α-synuclein positive (Komatsu, Waguri et al. 2006). However, Atg7 knockout animals showed early degeneration with motor symptoms, reduced dopamine levels and presynaptic accumulation of α-synuclein and LRRK-2 (Friedman, Lachenmayer et al. 2012). In cellular studies, Webb et al. used an α-synuclein inducible model by doxycycline in the PC12 cell line. In this model, inhibition of autophagy decreased the breakdown of A53T mutants of α-synuclein which tend to aggregate more than WT or A30P mutants (Webb, Ravikumar et al. 2003). This suggests that autophagic pathways are especially important in clearing α-synuclein aggregates and A53T mutant α-synuclein. Another line of evidence comes from the fact that rapamycin, an autophagy activator, increases the clearance of both soluble and aggregated forms of α-synuclein (Webb, Ravikumar et al. 2003).

Winslow et al., showed that increased expression of α-synuclein in mammalian cells and mice impairs macroautophagy. They showed that the mechanism is that α-synuclein inhibits Rab1a and mislocalizes Atg9 and thus inhibits autophagy. In fact,

Rab1a overexpression rescues this defect (Winslow, Chen et al. 2010). In addition, it was shown that excessive cytoplasmic α-synuclein levels in SN are associated with a steady decline in lysosomal function markers, together with cytoplasmic retention of TFEB, a transcriptional regulator of the autophagic-lysosomal pathway. Overexpression of TFEB rescued the decrease in lysosomal function, by enhancing the lysosomal degradation of α-synuclein oligomers, whereas downregulation of TFEB produced the opposite effects (Decressac, Mattsson et al. 2013).

mTOR Pathway and relevance to neurodegeneration

The mTOR pathway is implicated in cellular growth, proliferation and metabolism, in transcription and protein synthesis, but also in the regulation of autophagy. The mTOR complex consists of mTORC1 and mTORC2 and is subject to multiple levels of regulation, such as phosphorylation, by hormones (e.g. insulin) and various proteins.

p70S6K is a substrate of mTORC1 and forms a complex with mTORC2. P70S6K's activity is regulated by its phosphorylation, which in turn phosphorylates the ribosomal protein S6. The phosphorylation of p70S6K (and thus phosphorylation of S6) signifies mTOR activation and is correlated with inhibition of autophagy (Datan, Shirazian et al. 2014).

Increased mTOR activity has been associated with neurodegeneration and its inhibition is neuroprotective. A notable example is the alteration of the pathway in Alzheimer's disease, where in neuropathological specimens of post-mortem brains from Alzheimer's disease patients, there is upregulation of mTOR (Chano, Okabe et al. 2007) and pS6 (An, Cowburn et al. 2003). Similarly, mTOR is upregulated in the

hippocampi of animal models of Alzheimer's disease (Caccamo, Majumder et al. 2010), which returns to normal levels once the Aβ-levels are normalized either genetically or pharmacologically (Caccamo, Maldonado et al. 2011). With the same rationale, blocking mTOR signaling by rapamycin (an mTOR inhibitor) helps clear huntingtin aggregates in models of Huntington's disease (Ravikumar, Vacher et al. 2004) probably due to increased autophagy (Sarkar, Ravikumar et al. 2009). In addition, one of the major problems with the mainstream symptomatic treatment in Parkinson's disease, L-Dopa, is that it can cause dyskinesias. L-Dopa administration in a mouse mode of Parkinson's disease activated the mTORC1 in the GABAergic medium spiny neurons projecting from the striatum to basal ganglia output structures and caused dyskinesia. This dyskinesia was abolished with simultaneous administration of rapamycin, which didn't abolish the therapeutic effects of L-Dopa (Santini, Heiman et al. 2009). Regarding α-synuclein pathology, metformin and rapamycin have shown to lower the phosphorylated from of the protein via mTOR signalling. More specifically, metformin activates phosphatase 2A (part of the mTOR pathway), which in turn dephosphorylates α-synuclein at position Ser-129, which renders it less pathogenic and aggregation-prone (Pérez-Revuelta, Hettich et al. 2014).

Finally, inhibition of the mTOR pathway has proven neuroprotective beyond neurodegenerative aggregation diseases. A notable example is that hippocampal CA3 cells are protected against ischaemia by overexpressing hamartin (TSC1), a constituent of mTORC1, by inducing autophagy. This neuroprotective effect is abolished by hamartin knockdown (Papadakis, Hadley et al. 2013). In Leigh's syndrome, a mitochondrial disorder due to respiratory chain deficiency, rapamycin delayed the onset of neurological symptoms and enhanced survival in a transgenic

mouse model (Johnson, Yanos et al. 2013). Zheng et al showed that rapamycin preserves ATP levels in neurons, especially during defective oxidative phosphorylation. The authors suggest that this might be due to rapamycin reducing protein synthesis, thus preventing energy waste (Zheng, Boyer et al. 2016). Similarly, it was found that rapamycin protects against neuronal death both in *in vitro* and *in vivo* models of Parkinson's disease (Malagelada, Jin et al. 2010). Therefore, it is evident that inhibition of the mTOR pathway has neuroprotective effects in a wide range of pathologic conditions and its effect is not limited to autophagy.

Chaperone-mediated autophagy and its role in Parkinson's disease

In various studies, it has been shown that α-synuclein is also degraded via chaperone-mediated autophagy (CMA). CMA involves degradation of client-proteins, which is mediated by the recognition of a pentapeptide (VKKDQ) on their sequence by heat shock proteins and co-factors. The cargo is recognized by heat shock protein 70 (HSP70/HSC70), transferred to the lysosome, where it is recognized by the lysosomal receptor LAMP2a. LAMP2a then multimerizes in a translocation complex in a GFAP and Ef1a-dependent manner. This lateral mobility allows lysosomal Hsp70 to internalize cargo in the lysosomal lumen where it is broken down by lysosomal enzymes. The levels of LAMP2a have been directly associated with the levels of Chaperone Mediated Autophagy, as the rate of internalization of the substrates in the lysosome depends on the levels of LAMP2a on the lysosomal membrane (Cuervo, Dice 2000b). Another interesting note is that chaperone-mediated autophagy is upregulated during oxidative stress and acts protectively for the cell (Kiffin, Christian et al. 2004).

α-Synuclein contains the CMA-recognition sequence (95VKKDQ99) consistent with HSP70 binding (Fred Dice 1990). Chaperone-mediated autophagy has been shown to degrade α-synuclein via HSP70 in isolated lysosomal preparations (Cuervo, Stafanis et al. 2004), in neurons (Alvarez-Erviti, Rodriguez-Oroz et al. 2010, Vogiatzi, Xilouri et al. 2008) and *in vivo* in α-synuclein transgenic and paraquat-induced Parkinson's disease mouse models (Mak, McCormack et al. 2010). The model proposes that HSP70 forms a complex with α-synuclein, which together with the co-chaperones hip, HSP40 and HSP90 transfer α-synuclein to the lysosome (Roodveldt, Bertoncini et al. 2009). There, it is taken up by the surface lysosomal receptor LAMP2 and degraded. Studies have shown that overexpression of HSP70 or any of its positive feedback systems is beneficial in Parkinson's disease models, whereas inhibiting HSP70's action or reinforcing its negative feedback worsens the phenotypes (Nagel, Falkenburger et al. 2008, Ebrahimi-Fakhari, Saidi et al. 2013). Currently, small molecule inhibitors and activators of various parts of Chaperone-mediated autophagy including targeting Hsp70 expression and developing chemical chaperones are under investigation (Ebrahimi-Fakhari, Saidi et al. 2013). For example, geldanamycin increases HSP70 intracellular levels and protects mice from MTPT toxicity (Shen, He et al. 2005). It also increases HSF1 binding to HSP70 promoter enhancing its transcription. Thus, pharmacological activation of HSP70 could be protective in *in vivo* models of PD. However, geldanamycin is a toxic antibiotic causing hepatotoxicity, therefore alternatives are sought. 17-AAG is a geldanamycin derivative which is less toxic and also has neuroprotective function in animal models of PD (Waza, Adachi et al. 2005, Fujikake, Nagai et al. 2008), but it requires parenteral administration (Pacey, Gore et al. 2012). An alternative approach is to activate HSF-1 without inhibiting

Hsp90 with agents such as arimoclomol, HSF1A and celastrol. Indeed, this approach decreased toxicity. Arimoclomol has been shown to be safe and tolerable in clinical trials for ALS (Cudkowicz, Shefner et al. 2008, Phukan 2010). HSF-1A upregulates Hsp70 and reduces polyQ toxicity in *Drosophila* (Neef, Turski et al. 2010). Another agent which upregulates HSP proteins is Celastrol, it appears to be superior having rapid kinetics and low EC50 values (Westerheide, Bosman et al. 2004) and has been shown to rescue toxicity *in vitro* and *in vivo* in several neurodegenerative models, including Parkinson's disease, Alzheimer's disease, ALS and polyglutamine expansion diseases. This compound is also an NFκB inhibitor and has been shown to lower Aβ levels in *in vivo* models of Alzheimer's disease (Paris, Ganey et al. 2010). The authors suggest that this effect is due to NFκB-dependent regulation of BACE-1 expression. Similarly, it has been shown to rescue neuronal death and extend the lifespan of transgenic mouse models of ALS (Kiaei, Kipiani et al. 2006). With respect to Parkinson's disease, celastrol decreased the loss of dopaminergic neurons and depletion of dopamine caused by MPP+ toxicity (Cleren, Calingasan et al. 2005). Finally, it also rescues the death of polyglutamine-expansion expressing cells (Zhang, Sarge 2007). The authors showed that this is an HSF1-dependent effect and is correlated with lower amounts of SDS-insoluble polyglutamine aggregates.

It is noteworthy that abnormal forms of α-synuclein block chaperone-mediated autophagy, by binding to and inhibiting LAMP2 multimerization in the translocation complex, ultimately inhibiting their internalization of themselves and other proteins, acting like toxic gain of function (Cuervo, Stefanis et al. 2004). Notable such examples are the pathogenic mutant forms of α-synuclein A53T and A30P (Cuervo, Stafanis et al. 2004), post-translational modifications of α-synuclein

which promote its aggregation and dopamine-modified α-synuclein (Martinez-Vicente, Talloczy et al. 2008). A similar effect is exerted on LAMP2a by mutant forms of the deubiquitinase UCHL-1 (Kabuta, Furuta et al. 2008). Other pathogenic proteins inhibit CMA by binding to LAMP2, however there are some important differences. For example, acetylated tau (in contrast to normal tau) binds to LAMP2A, successfully triggers its multimerization in the translocation complex, but is not internalized or dissociated from LAMP2A. This is because in acidic environments the affinity of the lysosomal hsp70 for acetlylated tau is greatly reduced in comparison to normal tau, thus does not associate with the protein (Caballero, Gan and Cuervo, unpublished data). This is also the case in post-mortem brains of Parkinson's disease patients where lysosomes from such preparations show CMA defects (Caballero, Cuervo, Korech, Goete, unpublished data).

Model based on current understanding

It has been shown that there is functional crosstalk between the UPS and autophagic mechanisms for the breakdown of α-syn. Depending on the cell type used, both proteasomes and lysosomes contribute to the basal breakdown of α-synuclein. However, when α-synuclein aggregates and can no longer be degraded by the proteasome, cellular mediators compensate by upregulating or promoting autophagy (Pan, Kondo et al. 2008, Shaid, Brandts et al. 2013). For example, HDAC6 (microtubule-associated deacetylase) interacts with poly-ubiquitinated proteins and acts at the intersection of USP and autophagy (Pandey, Nie et al. 2007). *In vivo* evidence for this HDAC6-dependent compensation came from a fly model of spinobulbar muscular atrophy with USP dysfunction, where expression of HDAC6 was sufficient to rescue the degeneration by upregulating autophagy (Pandey, Nie et

al. 2007). HDAC6 was also able to rescue toxicity caused by Aβ in a fly model of AD. This rescue was abolished by ATG12 knockdown, showing that HDAC6 rescue comes from upregulating autophagy (Pandey, Batlevi et al. 2007). HDAC6 mutations have been identified in AD patients (Cook, Gendron et al. 2012) and its overexpression has been associated with tumour formation and metastasis (Sakamoto, Aldana-Masangkay 2011). Although the precise mechanism by which HDAC6 links the two systems is still elusive, it has been suggested that it may link polyubiquitinated proteins with dynein motors on the cytoskeleton, facilitating their retrograde transport to the pericentriolar regions for autophagic clearance (Kawaguchi, Kovacs et al. 2003).

Based on this discussion the following model can be proposed for the physiological and pathological breakdown of α-synuclein:

- Normal α-synuclein exists in different forms and compartments that are degraded by more than one mechanism including endosomal, proteasomal and autophagic, pathways.
- Early Disease Stage: Primary dysfunction of protein degradation mechanisms due to ageing, environmental and genetic factors cause an excess level of α-synuclein and modified/aggregated α-Synuclein species. These aggregates/modified species may be cleared by selective (e.g. ubiquitin-dependent) or bulk autophagy involving specific adaptors at the cross-talk between UPS and autophagy.
- In the late stages, the imbalance occurs again since α-synuclein aggregated and pathologic species inhibit membrane fusion events or pathologically overactivate the autophagic pathways, leading to a failure of the degradation

pathways and further accumulation of the pathogenic protein. This in turn contributes to neuronal dysfunction and degeneration.

- This accumulation of α-synuclein aggregates could stimulate secretion of α-synuclein promoting seeding or templating that could contribute to the spread of pathology to adjacent cells.

Therapeutic strategies for neurodegenerative diseases could involve inhibition of translation of potentially toxic proteins or increased clearance. This is because there is a dynamic equilibrium between the normal protein conformation/localization and the aggregated/mislocalized form (Kim, Nollen et al. 2002, Caughey, Lansbury Jr. 2003, Haass, Selkoe 2007). Therefore, if the levels of production of the normal protein are decreased or the levels of breakdown of both the normal and aggregated protein are increased, the balance will be shifted to the left, thus more protein will be in its normal form. One could then hypothesize that possible therapeutic strategies could involve decreased α-synuclein synthesis by RNA interference (RNAi) strategies or gene therapy. Although these methods could prove very useful in gain-of-function mutations, they could raise safety issues regarding method of delivery and off-target effects (Rubinsztein 2006).

An alternative approach would be to increase protein breakdown. In order to increase the breakdown rates of α-synuclein, the basal proteasomal or lysosomal fluxes would have to be increased. This would be very problematic in the case of proteasomal breakdown enhancement. This is because the proteasomal system is responsible for the breakdown of short-lived cytosolic proteins and altering their breakdown could cause a myriad of problems in the cell (Rubinsztein 2006). The only way one could take advantage of the proteasomal breakdown system without

altering global protein breakdown would be to treat the cells with chaperones or chemical stabilizers to unfold and de-aggregate abnormal aggregates of α-synuclein and they would then be broken down by the efficient proteasomal system physiologically (Rubinsztein 2006). This theory has some initial experimental evidence (Sánchez, Mahlke et al. 2003, Davies, Sarkar et al. 2006). The other major target is to increase macroautophagy, a strategy with less toxic effects as autophagy degrades long-lived proteins. This can be done either by blocking the mTOR pathway with agents like rapamycin, or via an mTOR-independent manner, for example by increasing intracellular inositol levels or by administering trehalose (Rubinsztein 2006, Sarkar, Floto et al. 2005). Drugs used in bipolar disorder such as valproate, lithium and carbamazepine increase the intracellular inositol levels and induce autophagy (Sarkar, Floto et al. 2005). In addition, it has been shown that trehalose, a non-mammalian disaccharide is capable of inducing autophagy and clear α-synuclein as well as huntingtin (Sarkar, Davies et al. 2007). In addition, inhibition of the mTOR pathway with rapamycin, which induces autophagy, clears huntingtin (Ravikumar, Vacher et al. 2004), poly-glutamine and wild-type or mutant tau aggregates (Berger, Ravikumar et al. 2006). Enhancement of autophagy could also confer resistance to oxidative stress (Kiffin, Bandyopadhyay et al. 2006). However, as autophagy serves diverse functions, the ideal targeted therapy should involve the activation of a highly selective mechanism of α-synuclein degradation.

Ubiquitination of α-Synuclein

Ubiquitin immunoreactivity is a robust neuropathological hallmark of LB (Kuzuhara, Mori et al. 1988, Lowe, Blanchard et al. 1988) and a fraction of α-

synuclein in LB is ubiquitinated (Tofaris et al. 2003), suggesting that enzymes that regulate ubiquitin conjugation may contribute to the cell autonomous response to α-synuclein accumulation.

Since α-synuclein is ubiquitinated in Lewy bodies in Parkinson's disease, identifying enzymes that enhance or reduce the ubiquitination of α-synuclein, could potentially offer a highly selective way of tagging α-synuclein for degradation. According to one study, a fraction of α-synuclein is mono-ubiquitinated by the E3 ligase SIAH (seven in absentia homologues). In this system, α-synuclein monoubiquitination by SIAH promoted α-synuclein aggregation and inclusion formation (Engelender 2008). Previous work from our lab showed that the E3 ubiquitin ligase Nedd4 ubiquitinates α-synuclein almost exclusively by conjugating K63 ubiquitin chains promoting its degradation by the lysosome (Tofaris et al. 2011). Importantly, Nedd4 overexpression is protective against α-synuclein toxicity in yeast, *Drosophila*, rodent and iPSC models (Tardiff et al. 2013, Davies et al. 2014). The ubiquitination of α-synuclein by Nedd4 was replicated by other groups (Sugeno, Hasegawa et al. 2014, Wijayanti, Watanabe et al. 2015). However, pharmacologically it is more difficult to activate an E3 ubiquitin ligase than to inhibit a deubiquitinating enzyme, which performs the opposite function. Thus, it would be very important to identify deubiquitinating enzymes relevant to the pathology of α-synuclein aggregation in Parkinson's disease that could be inhibited pharmacologically.

1.4. Study of ubiquitination properties of α-synuclein in Lewy Bodies

An important unanswered question is the biochemical nature of ubiquitin chains on α-synuclein in Lewy bodies and whether they are equally detected in cortical and nigral neurons. To this end, previous work performed in our lab (by Dr Johannes Lang) assessed the regional differences in the pattern and composition of LB ubiquitination using immunohistochemistry in twenty cases of almost pure α-synuclein pathology (Alexopoulou et al. 2016). This study showed a significantly higher percentage of ubiquitinated α-synuclein aggregates in cortical areas (cingulate and entorhinal cortex) than in the SN **(Figure 1.6.).** Linkage-specific ubiquitin immunostaining showed that inclusions were immunoreactive with anti-K63 antibodies and the results with this antibody correlated with pan-ubiquitin staining, suggesting that differential expression or activation of proteins that regulate the trafficking or conjugation of K63-linked chains may be account for this pattern **(Figure 1.6.)**.

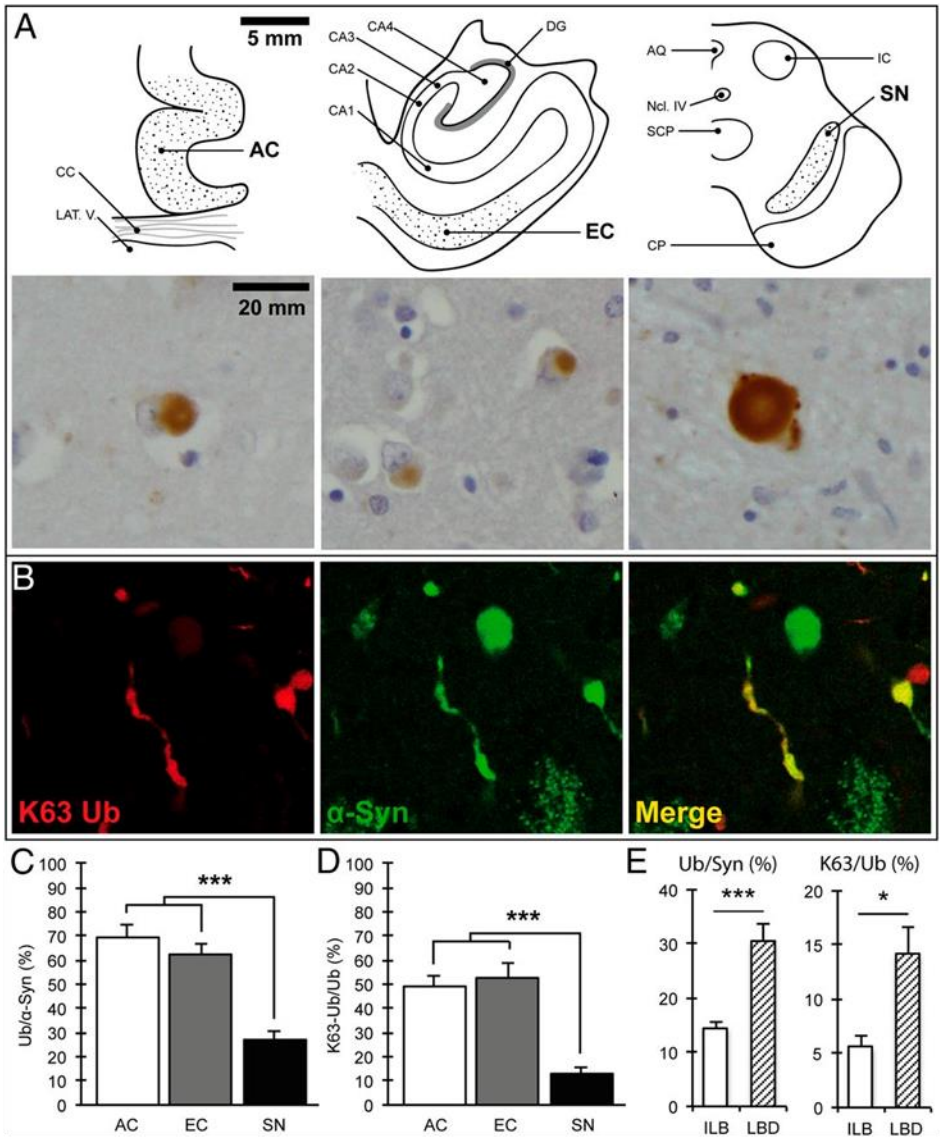


Fig. 1.6. K63-linked ubiquitin conjugates are detected in α-synuclein–positive inclusions and are reduced in the substantia nigra (Alexopoulou et al. 2016)**. (A)** Schematic view of the studied brain regions and corresponding light microscopy images showing K63-linked ubiquitinated inclusions. [Scale bars, 5 mm (schemes) and 20 mm (images).] **(B)** Confocal immunofluorescence showing colocalization of K63-linked ubiquitin chains and α-synuclein in Lewy bodies and Lewy neurites in nigral neurons. **(C)** Quantification of ubiquitin-positive inclusions as a percentage of α-synuclein–positive inclusions (Ub/α-Syn) in serial sections of the AC, EC, and SN; ***P < 0.0001, n = 14. **(D)** Quantification of K63-linked ubiquitinated inclusions as a percentage of ubiquitin–positive inclusions (K63-Ub/Ub) in serial sections of the AC, EC, and SN; ***P < 0.0001, n = 14. **(E)** The percentage of ubiquitinated inclusions in nigral neurons was low irrespective of the stage of

disease and lower in incidental Lewy body compared with Lewy body disease cases. Ub/Syn, ***$P < 0.0001$, $n = 14$; K63/Syn, *$P < 0.05$, $n = 14$. AQ, central aqueduct; CA1–4, cornu ammonis 1–4; CC, corpus callosum; CP, corticopontine and pyramidal tracts; DG, dentate gyrus; IC, inferior colliculus; LAT.V., lateral ventricle; Ncl IV, trochlear nucleus; SCP, superior cerebellar peduncle. Error bars correspond to standard error of the mean.

A targeted screen for potential interactors in K63-linked ubiquitin pathways performed in the laboratory revealed up-regulation and pathological localization of the deubiquitinase Usp8, which exhibited a striking inverse correlation with the extent of ubiquitin immunoreactivity in disease brains regions (**Figure 1.7.**). These data indicated that ubiquitination in Lewy bodies is comprised primarily of K63-linked conjugates and is regionally distinct. Importantly, they strongly implicated Usp8 in the pathogenesis of sporadic PD and K63-linked poly-ubiquitin signaling in LB biogenesis.

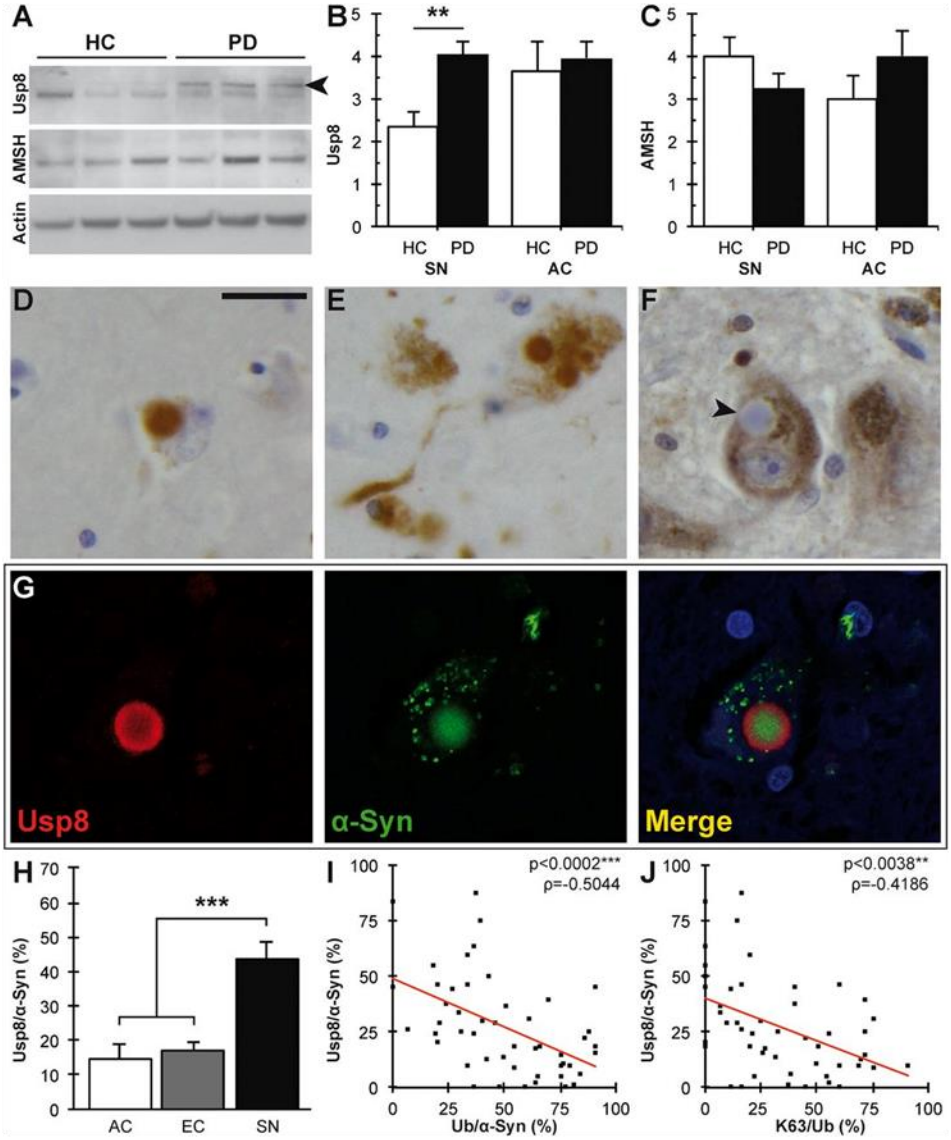


Fig. 1.7. Usp8 expression and localization inversely correlate with ubiquitinated inclusions in α-synucleinopathies (Alexopoulou et al. 2016)**. (A)** Representative immunoblot showing increased levels of Usp8 but not AMSH relative to the actin loading control in the SN from patients with LB disease; a specific band is indicated by an arrowhead. **(B)** Quantification of Usp8 protein level in the human brain showed a significant increase in the SN but not AC of patients with Lewy body disease compared with controls HC, healthy control (***P* = 0.0028, *n* = 8). Quantification of AMSH protein levels did not show a significant difference **(C).** Usp8-positive LBs and Lewy neurites in the AC **(D)** and SN **(E).** (Scale bar, 20 μm.) **(F)** No AMSH staining was seen in nigral LBs, as indicated by the arrowhead. **(G)** Double immunofluorescence and confocal imaging confirmed the colocalization of Usp8 (red) and α-synuclein (green) in nigral LBs. DAPI indicates

nuclear staining in blue. **(H)** Quantification of Usp8-positive as a percentage of α-synuclein–positive inclusions (Usp8/α-Syn) in serial sections showed a significant increase in the SN compared with cortical areas (AC, EC) (***P = 0.0001, n = 14). **(I)** Negative correlation between Usp8-positive and ubiquitin-positive inclusions in the SN (***P = 0.0002, ρ = −0.5044). **(J)** Negative correlation of Usp8-positive and K63-linked ubiquitinated inclusions shown as the ratio K63/Ub (**P = 0.0038, ρ = −0.4186). Error bars correspond to standard error of the mean.

1.5. Deubiquitinases and their relevance in Parkinson's disease.

There are five categories of DUBs, four of which correspond to cysteine proteases: (i) ubiquitin-specific proteases (USPs), (ii) ubiquitin C-terminal hydrolases (UCHs), (iii) ovarian tumour proteases (OTU) and (iv) Machado-Josephin domain proteases (MJDs)) and (v) metalloproteases (Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) (JAMM) domain proteases) (Clague et al. 2013). Cysteine proteases share a catalytic triad of cysteine, histidine and asparagine or aspartic acid (Storer, Menard 1994). Different DUBs have different selectivity for ubiquitin chains, although most USPs have low preference amongst ubiquitin chains. In particular, JAMMs, such as AMSH, show preference for deconjugating K63 chains (McCullough, Clague et al. 2004, McCullough, Row et al. 2006). In addition Ataxin 3, which belongs to the Josephine family deubiquitinates both K48 and K63 chains, but shows a preference for branched K63 chains (Winborn, Travis et al. 2008). Usp8, is a USP with slight preference for K63 chains, followed by K48 and K33 when assessed *in vitro* (Ritorto, Ewan et al. 2014). To achieve a highly fine-tuned selective deubiquitination of proteins in proteostasis and other cellular functions, DUBs are subjected to regulation. Some require multi-protein complex assembly, others allosteric activation, phosphorylation (like in Usp8), ubiquitination (e.g. Ataxin 3),

or substrate capture by other protein complexes in order to enhance their activity (Clague et al. 2013). Such an example is the endosomal interaction of Usp8 and AMSH with the ESCRT complexes (McCullough, Row et al. 2006, Row, Liu et al. 2007).

Many methods have been used for the identification of Ubiquitin-substrate and ubiquitin-ubiquitin interactions and linkage specificity, most of which have some limitations that have to be taken into account. Some of these methods include K0 (mutated ubiquitin containing no lysines), tagged concentrated ubiquitin binding domain constructs (e.g. TUBEs) which isolate poly-ubiquitin species and monoclonal antibodies recognizing diglycine (gly-gly) bonds on a lysine side chain with an isopeptide bond, followed by tandem mass spectrometry (Kessler 2013). More recently, chemoproteomics have been exploited in order to identify E3 ubiquitin ligase- and DUB –substrate interactions, linkage specificity and selectivity of small molecule DUB inhibitors. Direct co-immunoprecipitation of DUBs is more difficult as the affinity of DUB-substrate is not substantial and unless overexpressed, the substrate may not be co-immunoprecipitated. Other methods include probes with fluorescent species added to the N or C terminus. In 2009 Sowa et al developed a computational proteomic approach, using a new software platform termed CompPASS, by which they defined the DUB interaction landscape (Sowa, Bennett et al. 2009). With this method, they identified that various DUBs are involved in very diverse cellular functions, such as DNA replication and damage repair, transcription, translation, mitosis and apoptosis, as well as development, signal transduction, vesicle transport, protein folding and proteolysis.

DUBs have been implicated in many disease processes. For example, Usp8 mutations have been identified in certain cancers and Cushing's disease and AMSH

is downregulated in gastric cancers (Clague et al. 2013). It is no surprise that some DUBs have been associated with neurodegeneration since a major feature of such diseases is protein breakdown defects and aggregation, and this is no exception. Examples for other DUBs associated with neurodegenerative diseases include Ataxin 3, which causes Machado Joseph ataxia (SCA3) (Warrick, Morabito et al. 2005, Todi, Paulson 2011).

Below, I outline several DUBs that have been implicated with the pathophysiology of Parkinson's disease and their effects.

- *UCHL1*

  UCHL1 makes up 1-2% of the proteins expressed in the brain (Doran, Jackson et al. 1983, Jackson, Thompson 1981, Wilkinson, Deshpande et al. 1992). It interacts with and stabilizes mono-ubiquitin moieties (Osaka, Wang et al. 2003) and has also been shown to function at the synapse (Todi, Paulson 2011). The deactivating mutation UCHL I93M increases the risk for PD (Leroy, Boyer et al. 1998) but this requires replication in other families. However, it has been shown that the phenotype in mouse models results from gain of function of the mutant protein rather than the loss of function of the DUB (Leroy, Boyer et al. 1998, Setsuie, Wang et al. 2007). In addition, the S18Y SNP in its sequence confers protection against PD (Maraganore, Lesnick et al. 2004) but UCHL1 was not identified in genome-wide studies. Furthermore, proteomic studies in post-mortem PD brains showed that UCHL1 is a major target of oxidative damage (Clague et al. 2013, Choi et al. 2004). UCHL1 has also been shown to deubiquitinate α-synuclein (Liu, Meray et al. 2009) and it is also involved in neurodegeneration more widely. For example, soluble UCHL1 levels have been found to be reduced in post-mortem Alzheimer's disease brains (Setsuie, Wang

et al. 2007) and in Alzheimer's mouse models (Gong, Cao et al. 2006). The decrease in its levels is probably a result of its sequestration in neurofibrillary tangles (Setsuie, Wang et al. 2007). The overexpression of active enzyme (and thus the restoration of its function) but not catalytically dead rescues the cognitive defects of such mice (Gong, Cao et al. 2006). On the contrary, its aberrant expression has been associated with cancer in the pancreas, liver and colon (Hibi, Westra et al. 1999, Tezel, Hibi et al. 2000, Yamazaki, Hibi et al. 2002).

- *Usp9x*

  Usp9x has been associated with neurodegeneration. In MPTP-treated mice there was altered expression of Usp9x (Zhang, Zhou et al. 2010). Usp9X has been detected in Lewy-bodies but this was not quantified and its cytosolic levels were decreased in the SN of post-mortem PD brains. The same group also showed that Usp9X deubiquitinates α-synuclein *in vivo* and thus enhances its recycling (Rott, Szargel et al. 2011). Interestingly, a transcriptomic study of PD brains identified the closely-linked Usp9Y to be differentially expressed in PD brains (Riley, Gardai et al. 2014).

- *Usp30*

  Usp30 is located in the outer mitochondrial membrane. Its downregulation results in elongated interconnected mitochondria (Nakamura, Hirose 2008). Bingol et al in 2014 showed that Usp30 has an antagonistic effect in PINK1- and Parkin-induced mitophagy. More specifically, Usp30 removes ubiquitin chains on protein-substrates on mitochondria that were conjugated by Parkin. As expected, its knockdown rescues the mitophagy defects-even in Parkin or PINK1 deficient flies- and also rescues the dopaminergic neurons and prevents

the subsequent motor defects from paraquat-induced toxicity in the *Drosophila* (Bingol et al. 2014).

- *Usp24 and Usp40*

  Gene-linkage analysis suggested that variants in Usp24 and Usp40 increase the risk for developing late-onset Parkinson's disease (Li, Schrodi et al. 2006). More research is needed to identify the functional significance of this association, especially since Usp24 failed to replicate in genome-wide association studies (Do, Tung et al. 2011).

Collectively, this evidence suggests that DUBs are involved in pathways relevant to neurodegeneration and thus are possible targets for treatment. What makes them even more attractive targets is the cell-type and substrate specificity they possess.

Below, I will outline the function of several DUBs that I will use as controls in my experiments in **Chapters 3-5** for reference.

- *Usp14*

  Usp14 is associated with the 19S proteasome (Lee, Lee et al. 2010) and thus recycles ubiquitin moieties associated with proteasomal degradation of substrates. If Usp14 is mutated it causes ataxia in mice (Wilson, Bhattacharyya et al. 2002, Anderson, Crimmins et al. 2005) by reducing the ubiquitin recycling at the synaptic proteasomes. Usp14 function has also been linked to the neuromuscular junction (NMJ) development (Wilson, Bhattacharyya et al. 2002) and in central synaptic function by affecting the turnover and distribution of synaptic receptors (Lappe-Siefke, Loebrich et al. 2009). Usp14 inhibition enhances proteasomal degradation of ubiquitinated proteins through its active catalytic site. This was also the case for neurodegeneration, where Usp14

inhibition enhances the proteasomal clearance of tau and Ataxin3 which are both implicated in neurodegeneration (Lee, Lee et al. 2010). While proteasomal function is increased in malignancy, it is decreased in neurodegeneration. Thus, fine-tuning it pharmacologically with correct timing and precision, could keep a fine therapeutic balance for both diseases (Jara, Frank et al. 2013).

- *Usp7*

  USP7 is the closest DUB to Usp8. It is also known as HAUSP (Herpesvirus-associated ubiquitin-specific protease) because it was originally found to be associated with proteins of the Herpes Simplex Virus and later with Epstein-Barr virus (Holowaty, Frappier 2004). Like most USPs, it shows low ubiquitin linkage specificity, but has slightly higher activity against K63 and K11 chains (Ritorto, Ewan et al. 2014). Usp7 can stabilize and increase the function of p53 with anti-proliferative effects. It does this by antagonizing Mdm2, an E3 ubiquitin ligase which ubiquitinates and promotes the breakdown of p53 (Li, Chen et al. 2002). Thus, in malignancies Usp7 may have a tumour suppressor function by stabilizing p53. p53 is involved in the cell cycle regulation and prevents mutations in the genome. On another note, Usp7 interacts with Ataxin1 which causes SCA1 (Hong, Kim et al. 2002).

- *Usp47*

  Usp47 is not amongst the well-studied DUBs. Some evidence has shown that Usp47 inhibition decreases cell survival and increases the cytotoxic effects of anticancer drugs (Peschiaroli, Skaar et al. 2010). One of the reasons for the above observations could be the role of Usp47 in regulating DNA repair and maintaining genome integrity (Parsons, Dianova et al. 2011). Another effect of

Usp47 is to control axonal growth in development by rescuing katanin-p60 from degradation promoted by CHIP (Yang, Oh et al. 2013).

- *USP1*

  Usp1 has multiple functions but the best characterized is its regulatory role in the cellular response to DNA damage. It deubiquitinates a protein in the Fanconi anaemia DNA repair pathway (Nijman, Huang et al. 2005, Kim, Parmar et al. 2009). In addition, it could also be involved in regulation of cellular differentiation (Williams, Maecker et al. 2011). Its deregulation can result in cancer and its inhibition reverts cisplatin resistance to non-small cell lung cancer (Chen, Dexheimer et al. 2011, García-Santisteban, Peters et al. 2013).

- *AMSH*

  AMSH is also known as STAMBP (STAM binding protein) due to its association with STAM in the ESCRT pathway. As mentioned earlier, it is one of the 2 endosomally-associated DUBs- the other being Usp8 (McCullough, Clague et al. 2004) and is involved in the protein trafficking through the ESCRT machinery via its association with STAM (Agromayor, Martin-Serrano 2006). It is also involved in cytokine-mediated signaling for cell cycle progression and if mutated it causes microcephaly (McDonell, Mirzaa et al. 2013).

- *JOSD2*

  The function of this cytosolic DUB remains elusive. It is expressed in many tissues apart from the brain, including the skin, liver, heart, ovary and testis, lymphoid organs and the haematopoietic system. What has been identified is that it shows more preference for cleaving K-63 poly-ubiquitin chains than K-48 chains. (Seki, Gong et al. 2013).

1.7. Aims

Earlier work in the lab showed that Usp8 is upregulated in the substantia nigra of Parkinson's disease post-mortem brains and that its levels inversely correlated with the extent of ubiquitination in Lewy bodies and specifically with K63 chains. These observations firmly establish Usp8 as a relevant deubiquitinase in Lewy body disease and raise interesting mechanistic questions with regard to its role in the pathogenesis of the disease. Thus, my DPhil focuses on examining the effect of Usp8 on α-synuclein *in vitro* and *in vivo*. The overall objective of my thesis is to investigate the relevance of Usp8 in α-synuclein turnover and toxicity.

The specific **aims** are outlined below:

1. Does knockdown of Usp8 in the *Drosophila melanogaster* eye modify α-synuclein protein levels and toxicity?
2. Is the effect of Usp8 on α-synuclein toxicity specific when compared to other deubiquitinases or other toxic proteins (e.g. pathogenic expansion of Ataxin 3 or Huntingtin)?
3. Does knockdown of Usp8 in the dopaminergic neurons of the fly affect α-synuclein and toxicity using locomotion, cell counts and survival as readouts?
4. Is there a direct functional interaction between Usp8 and α-synuclein in mammalian cells?
5. Does overexpression or knockdown of Usp8 in mammalian cells have any effect on the ubiquitination, abundance and/or rate of degradation of α-synuclein?
6. Does Usp8 knockdown affect cellular stress or protein degradation more broadly?
7. Are Usp8 inhibitors effective regulators of α-synuclein levels?

2. Materials and Methods

2.1. *Drosophila* techniques

2.1.1. *Drosophila* genetics

The *UAS*-α-*synuclein* wild-type (8146) and A53T mutant (8148), *UAS-Usp8 RNAi* (38982), *UAS- Usp7 RNAi* (34708), *UAS-Usp14 RNAi* (53262), *UAS-Usp47 RNAi* (44645), and *UAS-Ataxin 3* (8150) and *UAS-HTT* (33808) with expanded polyQ were obtained from the Bloomington *Drosophila* Stock Center. The *UAS-JosD2 RNAi* (108379), and *UAS-AMSH RNAi* (108622) were obtained from the Vienna *Drosophila* Resource Center (VDRC). The drivers GMR Gal4 construct 12 and 15 were kindly provided by I. Davis lab (University of Oxford, Oxford, UK), the ddc Gal4 by A. Lin and G. Miesenbock (University of Oxford, Oxford, UK), the GMR Gal4 Construct 3 by G. Tofaris (University of Oxford, Oxford, UK), the *UAS-α-synuclein 120* by M. Feany (Harvard Medical School) (Periquet et al. 2007) and the MS-1096 Gal4, elav Gal4 and TH Gal4 by T. Fulga (University of Oxford, Oxford, UK). In addition, the UAS-GFP construct was provided by the T. Fulga lab ((University of Oxford, Oxford, UK).

For most experiments, the UAS GAL4 expression system was used to overexpress transgenes either in dopaminergic neurons at 25 °C using the *ddc*-GAL4 driver or specifically in the eye using the *GMR*-GAL4 (construct Davis 15) at 29 °C.

The following genotypes were used: (*i*) +/+; *ddc*-GAL4/+, (*ii*) +/+; *ddc*-GAL4/*UAS-A53T* α-*synuclein*, (*iii*) *UAS-Usp8 RNAi*/+; *ddc*-GAL4/+, (*iv*) *UAS-Usp8 RNAi*/+; *ddc*-GAL4/*UAS-A53T* α-*synuclein*, (*v*) *GMR*-GAL4/+, (*vi*) *GMR*-GAL4/+; *UAS*-α-*synuclein (WT or A53T or 120)*/+, (*vii*) +/+; *UAS-A53T or WT or 120* α-*synuclein*/+, (*viii*) *GMR*-GAL4/*UAS-Usp8 RNAi*; +/+, (*ix*) *UAS-Usp8 RNAi*/*GMR*-GAL4; *UAS*-α-*synuclein (WT or A53T or 120)*/+, (*x*) *MS1096-*

GAL4/Y; +/+; *UAS-Usp8 RNAi 39022/+*; (*xi*) *MS1096*-GAL4/Y, (*xii*) *MS1096*-GAL4/Y; *UAS-Usp8 RNAi 38982/+*; +/+, (*xiii*) *UAS-Usp47 RNAi/UAS-A53T α-synuclein,* (*xiv*) *UAS-Ataxin 3*/*GMR*-GAL4; *UAS-Usp8 RNAi*/+, (*xv*) *GMR*-GAL4/*UAS-Ataxin 3*; +/+, (*xvi*) *UAS-AMSH RNAi*; *ddc*GAL4/+, (*xvii*) *UAS-JOSD2 RNAi*;*ddc*Gal4/+, (*xviii*) *GMR*-GAL4/*UAS-AMSH RNAi*; +/+, (*xix*) *GMR*-GAL4/*UAS-AMSH RNAi*; *UAS-A53T α-synuclein*/+, (*xx*) *UAS-JOSD2 RNAi*/+; *UAS-A53T α-synuclein*/*ddc*GAL4, (*xxi*) *UAS-AMSH RNAi*/+; *UAS-A53T α-synuclein*/*ddc*GAL4, (*xxii*) *GMR*-GAL4/+; *UAS-Usp14 RNAi*/+, (*xxiii*) *GMR*-GAL4/+; *UAS-Usp47 RNAi*/+, (*xxiv*) *GMR*-GAL4/+; *UAS-Usp14 RNAi*/*UAS-A53T α-synuclein,* (*xxv*) *GMR*-GAL4/+; *UAS-Usp47 RNAi/UAS-A53T α-synuclein, (xxvi) +/+; ddc*GAL4*/UAS-Usp7 RNAi, (xxvii) +/+; ddc*GAL4*/UAS-Usp14 RNAi, (xxviii) GMR*-GAL4/+ ; *UAS-Usp7 RNAi/+, (xxix) GMR*-GAL4/+ ; *UAS-Usp7 RNAi/UAS-A53T α-synuclein, (xxx) GMR*-GAL4/*UAS-JosD2 RNAi; +/+, (xxxi) GMR*-GAL4/*UAS-JosD2 RNAi; UAS-A53T α-synuclein*/+, *(xxxii) GMR*-GAL4/+; *UAS-HTT RNAi/+, (xxxiii) GMR*-GAL4/+; *UAS-HTT RNAi/UAS-A53T α-synuclein, (xxxiv) UAS-Usp8 RNAi/+; TH-GAL4/+, (xxxv) elav-GAL4/y; UAS-Usp8 RNAi/+; +/+, (xxxvi) UAS-AMSH RNAi/+; UAS-A53T α-synuclein, (xxxvii) UAS-JosD2 RNAi/+; UAS-A53T α-synuclein, (xxxviii) +/+; UAS-Usp8 RNAi/UAS-A53T α-synuclein, (xxxviv) +/+; UAS-Usp7 RNAi/UAS-A53T α-synuclein, (xxxv) +/+; UAS-Usp14 RNAi/UAS-A53T α-synuclein.*

The experiments were carried out with the experimenter blinded to the sample genotypes throughout the analysis.

2.1.2. Preparation of *Drosophila* food containing compounds

Food was prepared separately for the purpose of testing the effect of compounds when feeding flies. Standard food was purchased from the Department of

Biochemistry, University of Oxford, which was prepared according to their standard recipe. It was then heated in a microwave oven until melted. Compounds were diluted in the food in the following concentrations: 0.1μM, 0.33μM, 1, 3.33μM, 10μM and put in vials. Food was left to dry for 24 hours before vials were used for experiments.

2.1.3 Feeding flies with compounds

Virgin females and males were put in vials containing the diluted compounds as denoted above. In order to increase protein expression, flies needed to be nurtured at 29ºC. Therefore, flies were crossed at 25ºC and 24 hours after crossing, adult flies were discarded and vials were transferred to 29ºC. Eye images were taken 24 hours post-eclosion using light microscopy. Female flies only were used for microscopy.

2.1.4. Quantification of Drosophila eye roughness

Quantification of the roughness seen in different flies could have been carried out using a special software (e.g. Flynotyper http://flynotyper.sourceforge.net/) to objectively determine which flies had rougher eyes. Such methods quantify the degree of distortion of ommatidia and eye bristles. However, the change in roughness was dramatic enough in the case of Usp8 knockdown (rescue of the eye phenotype) that there was no reason to use additional software for validation.

2.1.5 Dissection of adult fly brains

Male fly brains were dissected at DAE (days after eclosion) 25. They were dipped in ethanol for 10 seconds, washed in PBS and transferred to ice-cold Schneider's medium for dissection. An anterior dissection approach was adopted, pinning the flies with two minutiens, one in the thorax and one in the abdomen. The provoskis was removed and the retina peeled off taking care not to remove the optic lobes. The remaining cuticle and then the trachea were carefully removed. Once the brains

were isolated and intact, they were transferred to 4% paraformaldehyde (PFA) for 20 minutes. They were then washed in PBS containing 0.05% Triton X-100 (PBS-T), blocked in goat serum for 1 h, and then incubated for 48 h at 4 °C with monoclonal mouse anti-TH (1:500) followed by PBS-T washes and Alexa 488-coupled goat anti-mouse IgG (1:1,000) for a further 48 h at 4 °C. Stained brains were mounted in ProLong Gold antifade mountant. Z stacks of the brain were obtained (explained below).

2.2. Molecular Biology techniques

2.2.1. qPCR quantification

Five fly heads from each line were homogenized for 30 seconds using a tissue homogenizer. RNA was extracted (Qiagen; RNeasy Mini Kit) and reverse-transcribed (Qiagen; QuantiTect Reverse Transcription Kit) according to the manufacturer's instructions. Real-time qPCR was performed using Roche LightCycler 480 II and LightCycler 480 SYBR Green I Master. The primers used were 5′-GGCCAAGGAGGGAGTTGTGGC-3′ and 5′-TGCTGTCACACCCGTCA

CCA-3′ for α-synuclein and 5′-TGCTAAGCTGTCGCACAAATGGC-3′ and 5′-CGATCCGTAACCGATGTTGGGC-3′ for rp49, which was used for normalization.

2.2.2. Plasmids and Cell transfection of plasmid DNA

Dr Rebecca Perrett designed and produced the plasmids and I used them for cell transfection. Usp8 cDNA was amplified from FLAG-HA-Usp8 in pDEST_Tet_ON_CMV_N_FLAG_HA_PGK_puro (Addgene; plasmid 22608) using forward (FLAG in italics) 5′-CGATCAGCTAGC*CACCATGGACTACAAGG*

*ATGACGATGACAAG*ATGCCTGCTGTGGCTTCAGTTCCTAAAGAACTCTAC CTCAGTTCTTC-3′ and reverse 5′-GTCTCTGGATCCTTATGTGGCTACATCAG TTACTCGTGGTC-3′ primers. The Usp8 MIT deletion mutant was amplified from FLAG-HA-Usp8 in pDEST_Tet_ON_CMV_N_FLAG_HA_PGK_puro using forward 5′-CGATCAGCTAGCTCTGCCACCATGGACTACTATGAAGAAGCT GAAGTCCGGAAAAAACTT-3′ and the reverse primer listed above. The products were digested with BamHI and NheI (underlined) and subcloned into the corresponding digests of pIRES2-EGFP (Clontech). The Cys786-to-Ala mutation was introduced using the QuikChange site-directed mutagenesis protocol (Stratagene). HA-ubiquitin, HA-K63–only lysine ubiquitin, and HA-K48–only lysine ubiquitin were purchased from Addgene (plasmids 17608, 17606, and 17605, respectively). Human α-synuclein in pIRES2-EGFP was a kind gift of M. G. Spillantini, University of Cambridge, Cambridge, UK.

Cells were transfected with plasmid DNA using Lipofectamine 2000 and Optimem Reduced Serum Medium (ThermoFischer Scientific) at 60–80% confluency for 6 hours. Thirty-six hours after transfection, cells were rinsed with PBS and scraped into ice-cold lysis buffer (see below).

2.2.3. Design, Construction and Transduction of Usp8 or Scrambled shRNA Lentiviral Vectors.

Dr Rebecca Perrett designed and constructed the shRNA lentiviral vectors and I tested them for Usp8 knockdown and I used them in the experiments.

The rat Usp8 sequence (accession no. NM_001106502.1) was used to generate the Usp8 shRNA (5′-CCGCTCGAGAAAAAAGCTGAGATCTCAAGGCTTTCTTGA

CAGGAAGAGAAAGCCTTGAGATCTCAGCCAAAACAAGGCTTTTCTCCAA GG-3′), which was predicted to knock down the human protein as well as a scrambled shRNA (5′-CCGCTCGAGAAAAAAGGCACATTAGGAACCATACA

TTGACAGGAAGATGTATGGTTCCTAATGTGCCCAAAACAAGGCTTTTCT CCAAGG-3′). A short hairpin expression cassette comprised a sense and an antisense (antisense specific to the target mRNA) strand spaced by a loop sequence, RNA polymerase III transcription termination signal, and XhoI restriction site. A sequence coding for the mouse U6 promoter was inserted upstream of the antisense strand with a SpeI site. A PCR-based method was used to generate double-stranded shRNA expression cassettes using the pSilencer 1.0-U6 expression vector (Life Technologies) as a transcription template and reverse primer encoding the shRNA oligonucleotides. These were subcloned into the lentiviral backbone pRRLsincppt.U6.CMV.EGFP.wpre predigested with SpeI and XhoI, generating the pRRLsincppt.U6.rUsp8shRNA.CMV.EGFP.wpre or pRRLsincppt.U6.scrambled

shRNA.CMV.EGFP.wpre construct. The constructs were tested for efficiency in human cell lines and found to be effective in reducing the content of Usp8.

For knockdown experiments using lentiviruses expressing shRNA, SH-SY5Y cells were transduced for 24 h using conditions that achieved transduction in 90% of cells and at least 80% knockdown. Cells were lysed 7 d after transduction.

2.3. Cell Culture

2.3.1. Culture of SH-SY5Y and HEK293T cell lines

HEK-293T cells endogenously expressing α-synuclein were maintained at 37 °C and 5% CO_2 in DMEM containing 10% (vol/vol) FCS (Sigma) and 1% (vol/vol) penicillin/streptomycin/amphotericin B (Life Technologies). SH-SY5Y cells were maintained at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle's Medium/Nutrient

Mixture F-12 Ham (Sigma) containing 10% (vol/vol) FCS (Sigma) and 1% (vol/vol) penicillin/streptomycin/amphotericin B (Life Technologies).

Cells were grown to 60–80% confluency and transfected with plasmid DNA using Lipofectamine 2000. Thirty-six hours after transfection, cells were rinsed with PBS and scraped into ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 1 mM *N*-ethylmaleimide, complete protease inhibitor mixture; all from Sigma). Lysates were rotated for 20 min at 4 °C and then centrifuged at 4 °C at 13,000 × *g* for 5 min. For knockdown experiments using lentiviruses expressing shRNA, SH-SY5Y cells were transduced for 24 h and lysed 7 d after transduction.

2.3.2. Culture of primary rat cortical neurons

Primary rat neurons were dissected and cultured according to an adapted protocol derived from Fath et al 2009 and Beaudoin et al 2012 (Fath, Ke et al. 2009, Beaudoin III, Lee et al. 2012). More specifically, embryonic rat cortical neurons were dissected at day E19 and placed at ice-cold HBSS. The meninges were removed and brain was cut in small pieces. It was incubated at 37˚C in HBSS and trypsin for 20 mins. 50µL of 100mg/mL DNase I was added and trypsin was removed. Brain tissue was triturated well with decreasing size of pipettes in MEM medium. The medium was discarded following a 5-minute centrifugation at 1100rpm and the tissue was re-triturated in fresh MEM medium (Invitrogen) containing 10% (vol/vol) FCS (Sigma) and 1% (vol/vol) penicillin/streptomycin/amphotericin B (Life Technologies). Cells were counted and plated accordingly in plates. 96-well plates were plated at 4×10^4 cells/well and 6-well plates at 8×10^5 cells/well. The medium was changed to Neurobasal medium (NB) (Invitrogen) containing 2% (vol/vol) B27 (50X) supplement (Invitrogen),

0.3% L-glutamine 200mM (Invitrogen) and 1% (vol/vol) penicillin/streptomycin/amphotericin B (Life Technologies) after 3 hours in MEM medium that allowed for the cells to seed in the PLL-coated plates. Maintenance of neurons was achieved by changing half the medium every 3 days with fresh NB (Invitrogen) containing 2% (vol/vol) B27 (50X) supplement (Invitrogen), 0.3% L-glutamine 200mM (Invitrogen) and 1% (vol/vol) penicillin/streptomycin/amphotericin B (Life Technologies). At DIV (day *in vitro*) 3, during the first medium change, the mitotic inhibitor AraC was added at 4μM to stop glial growth.

2.3.3. Measurement of cell viability

2.3.3.1. MTS Viability Assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

The MTS assay from Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay was used. 20μL of the MTS solution were added in each well of the 96-well cell culture plate and the plate was incubated at 37°C in a humidified, 5% CO2 atmosphere for 2 hours for the SH-S5Y5 cells and 4 hours for the primary rat cortical neurons. Absorbance was recorded at 490nm on a plate reader.

2.3.3.2. LDH (Lactate Dehydrogenase) Assay

The Pierce LDH Cytotoxicity Assay Kit from ThermoFischer Scientific was used to measure cytotoxicity according to protocol. Briefly, lysis buffer (SDS) is added to the maximum LDH activity control wells (maximum death) and the plate is incubated at 37˚C for 35 minutes. 50μL from each well is transferred to an empty 96-well plate (containing the LDH in the medium). 50 μL of reaction mixture is transferred to each well and the plate is incubated at room temperature for 30 minutes protected from light. 50μL of Stop solution is added in each well and

absorbance is read at 490nm and 680 (background absorbance). Background toxicity is subtracted and % cytotoxicity is calculated by the following formula:

$$\%\ Cytotoxicity = \frac{Compound\ treated\ LDH\ activity - Spontaneous\ LDH\ activity}{Maximum\ LDH\ activity - Spontaneous\ LDH\ activity} \times 100$$

2.3.4. Assays of cell stress

Assays of cell stress were performed and cell survival was measured with the MTS assay and cell death with the LDH assay (see above). Below, the production of various forms of cellular stress is explained.

2.3.4.1. Proteotoxic stress

Heat shock causes protein misfolding and upregulation of the expression of Heat Shock Proteins as an attempt of the cells to recover from this form of proteotoxic stress. For this assay, cells were incubated for 30 mins either at 37˚C or 45 ˚C. Heat-stressed cells were allowed to recover for 15 minutes at 37˚C before viability was measured.

Proteotoxic stress is also caused with prolonged inhibition of the proteasome due to accumulation of misfolded or unwanted proteins. To this end lactacystin was added to the culture medium for 12 hours (with DMSO as control). Lactacystin is an irreversible inhibitor of the catalytic 20S core of the proteasome. It covalently modifies the amino-terminal threonine of the proteasomal subunit X/MB1 altering the proteasomal protease activity (Fenteany, Standaert et al. 1995). I tested the following doses: 0.5, 1 and 2μM.

2.3.4.2. Oxidative stress

Oxidative stress was produced in three different ways, by adding to the culture medium dopamine, hydrogen peroxide or arsenite. Each agent was dissolved in the culture medium for 24 hours.

Dopamine causes toxicity by producing toxic metabolites such as dopamine quinones and DopaL. For dopamine toxicity, the cells were treated with 25, 50, 100, 200 and 400μM of dopamine.

Hydrogen peroxide is a strong oxidizing agent and causes permanent cell growth arrest and cell necrosis. The doses I tested were 25, 50, 100, 200 and 400μM of hydrogen peroxide.

Sodium arsenite generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) which among others cause DNA damage and alter gene expression. Arsenite was added to the cells at the following doses 2, 5, 10, 20 and 30μM.

2.3.5. Treatment of cells with compounds

For the purpose of assessing the effect of Usp8 inhibitors and control compounds on SH-SY5Y cells and neurons, different concentrations were added to the medium. For SH-SY5Y cells, compounds were left in the medium for 48 hours, when the medium was changed to new including the same dose of freshly made compounds. This was left for another 24 hours prior to lysis. For neurons, the compounds were left for 3 days without change in the medium. For toxicity studies the compounds were diluted in warm medium at the following concentrations using serial dilutions: 0.05μM, 0.1μM, 0.33μM, 1, 3.33μM, 10μM.

2.4. Biochemical techniques

2.4.1. Immunoblotting

2.4.1.1. Western Blotting

Cells were homogenized in ice-cold lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM sodium chloride, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, 1 mM *N*-

ethylmaleimide; all from Sigma) and centrifuged for 5 min at 13,000 × *g* at 4 °C and either fractionated (see below) or directly immunoblotted. The protein content of the lysates/resulting supernatants from fractionation was measured using the BCA assay (Thermo Fisher Scientific). Equal amounts of protein were mixed with NuPAGE Tris-Acetate SDS Running Buffer (Life Technologies) containing NuPAGE Sample Reducing Agent (Life Technologies) and heated at 95 °C for 10 min before being separated by 4–12% SDS/PAGE (NuPAGE Novex Bis-Tris Gels; Life Technologies) and electrotransferred onto a nitrocellulose membrane (Amersham Protran 0.45; GE Healthcare) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol]. After blocking with 4% nonfat powdered milk in PBS containing 0.1% Tween 20 (Sigma) for 1 h, primary antibodies were incubated with the membrane overnight at 4 °C and binding was visualized after washing with peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies (1:10,000; GE Healthcare) and enhanced chemiluminescence (GE Healthcare). Relative quantitation of the protein of interest to the loading control was measured using ImageJ (NIH).

2.4.1.2. ELISA for detection of α-synuclein levels

Two versions of ELISA were used to detect extracellular α-synuclein levels.

The first one is the Human α-synuclein ELISA kit from Invitrogen. This is a solid-phase sandwich ELISA. Briefly, 50µL were added from standards, diluted samples or controls. 50µL of the detector antibody was added in each well except chromogen blank and incubated for 3 hours at room temperature. The plate was washed four times. 100µL of anti-rabbit IgG HRP were added in all wells (except chromogen blank) and the plate was incubated at room temperature for 30 mins. The plate was washed 4 times. 100µL of chromogen were added in all wells and the

plate was incubated at room temperature in the dark for 30 mins. 100µL of stop solution were added in all wells and the absorbance was read at 450nm immediately. A 4-parameter standard curve was constructed from the absorbance reading from the standards and values were interpolated using Graphpad Prism.

2.4.1.3. ELISA for detection of Dopamine levels

The LDN Dopamine ELISA kit was used as per protocol. Briefly, on the extraction plate 10µL of standards, controls or samples were added and topped up to 100µL with double distilled water. 25µL of TE buffer was added in all wells. The plate was shaken at 600rpm at room temperature for 60 mins. It was blotted dry on an absorbent paper. The plate was washed twice (1mL of wash buffer was added and shaken for 5 mins and then blot dry). 150µL of acetylation buffer and then 25µL was added into all wells and the plate was shaken for 20 mins. It was blot dry and washed twice (as above). 100µL of hydrochloric acid was added in all wells and the plate was shaken for 10 mins. 90µL were transferred to the respective wells of the microtitre plate and 25µL of Enzyme solution was added in all wells. The plate was incubated at 37˚C for 2 hours. 100µL were transferred to microtitre strips and 50µL of dopamine antiserum were added. The plate was incubated at 4˚C for 18 hours. The supernatant was discarded and the plate was washed and blotted dry four times (as above). 100µL of enzyme conjugate was added and the plate was shaken for 30 mins at room temperature. The plate was washed 4 times and 100µL of substrate was added. The plate was covered in aluminum foil and shaken for 30 mins at room temperature. 100µL of stop solution were added in all wells. Absorbance was read at 450 nm and the background values at 630nm.The calibration standard curve from which the concentrations in the samples were read was obtained by plotting the absorbance readings measured for the standards (linear, y axis) against the

corresponding standard concentrations (logarithmic, x-axis) with r^2= 99.3. The use of a non-linear regression for curve fitting (spline) was used.

2.4.2. Tissue and cell fractionation

All steps were performed at 4 °C. Tissue lysates or five male adult heads from each *Drosophila* genotype were homogenized in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, complete protease inhibitor mixture, 1 mM *N*-ethylmaleimide). Fly head lysates were directly fractionated, whereas SH-SY5Y cell lysates were further homogenized. Further homogenization involved syringing with a 25-gauge needle 10 times on ice followed by a brief vortexing, and the process was repeated three times with 2-min intervals on ice. In both cases, crude lysates were centrifuged at 4,000 × *g* for 5 min. Supernatants were then centrifuged at 100,000 × *g* for 1 h. The resulting supernatant was designated the cytosolic fraction, and the pellet was resuspended in lysis buffer containing detergents (150 mM NaCl, 10 mM Tris, pH 7.4, complete protease inhibitor mixture, 1 mM PMSF, 1 mM *N*-ethylmaleimide, 1% Nonidet P-40, 0.1% SDS) and centrifuged at 100,000 × *g* for 30 min. The supernatant was designated the membranous fraction and the remaining pellet was discarded.

2.4.3. Immunoprecipitation

Cell-lysate supernatants were incubated overnight at 4 °C with anti-HA or anti-FLAG antibodies (Sigma) or 2F12 anti–α-synuclein antibodies. Equilibrated protein G Sepharose beads (P3926; Sigma) were then added for a further 2 h at 4 °C. The beads were washed three times with Tris-buffered saline containing 0.1% Triton X-100, resuspended in NuPAGE Tris-acetate SDS running buffer (Life Technologies) containing NuPAGE Sample Reducing Agent (Life Technologies), and heated at 95 °C for 10 min before loading onto the gel.

2.4.4. Protein degradation assay

Protein degradation was assayed by immunoblotting after treatment with 20 μg/mL cycloheximide for 7 h before lysis. No obvious cell death or stress was detected under these conditions.

2.5. Microscopy

2.5.1. Immunofluorescence

Confocal Imaging of Fly Brains: Fly brains were dissected at 25 d post-eclosion in ice-cold Schneider's fly medium and fixed in 4% paraformaldehyde (PFA). They were washed in PBS containing 0.05% Triton X-100 (PBS-T), blocked in goat serum for 1 h, and then incubated for 48 h at 4 °C with monoclonal mouse anti-TH (1:500) followed by PBS-T washes and Alexa 488-coupled goat anti-mouse IgG (1:1,000) for a further 48 h at 4 °C. Stained brains were mounted in ProLong Gold antifade mountant. Z stacks of the brain were obtained on a Zeiss LSM 780 confocal microscope using a 25× objective (1.4× digital zoom) at 1-μm steps and Z-projected. Dopaminergic neuronal clusters were identified and counted.

2.5.2. Electron microscopy

Age-matched male flies were fixed in 70% ethanol. The flies were then dehydrated in 100% ethanol, dried, and mounted on SEM stubs. The samples were then sputter-coated with gold and imaged using a JEOL JSM-6390 scanning electron microscope at the Dunn School of Pathology. Eyes were examined for abnormal bristle orientation, ommatidial fusion or pitting, and disorganization of the ommatidial array.

2.6. Behavioural Assays

2.6.1. Climbing Assays

Locomotor function was assayed using a startle-induced negative geotaxis assay. Ten male flies were placed in each vial and five vials were used per line in each experiment (total 50 flies per line). Each vial was tapped 10 times and the percentage of flies above 6 cm was recorded after 4 s.

2.7. Statistical Analysis

The statistical analysis was performed using Prism (GraphPad). All data were examined for distribution, and statistical tests were chosen accordingly. For normally distributed data, a *t* test or a one-way ANOVA was used. For nonparametric data, the Wilcoxon–Mann–Whitney and Kruskal–Wallis rank-sum tests were performed to assess differences in mRNA or protein levels and for climbing assays. The null hypothesis was rejected at a significance level of $P = 0.05$. Exponential data were log-converted if reasonable before statistical calculation was performed.

3. Genetic manipulation of Usp8 *in vivo* in *Drosophila melanogaster*

3.1. Introduction

3.1.1. Drosophila genetics

Drosophila melanogaster is an excellent model organism that has proven very useful in identifying basic biological processes and the genetic basis of diseases. It is a simple organism, having only 4 chromosomes and allows for the study of molecular interactions *in vivo* between different genes/proteins. Throughout the years, it has proven an essential tool to geneticists not only studying neurodegenerative diseases, but many other rare and common diseases.

In order to study genetic interactions, several crosses are performed to obtain the desired fly genotype. In this, several markers and balancers are used, such as TM3 (shortened bristles (stubble) on the back of the fly), CyO (curly wings), if (altered eye structure) and TM6 (more bristles near the eye and tubby body). Male flies show no recombination, whereas scientists have developed balancer chromosomes to control recombination in female flies[1]. Therefore, the progeny either gets the balancer (as an entire chromosome) or the homologue. The balancers and markers can signify the presence or absence of a specific gene in the progeny. Therefore, by crossing male with female flies of specific genotypes with carefully designed crosses, the progeny can express or have silenced a number of selected genes. This becomes particularly useful when producing double mutants, where one can study the interaction between two genes, for example silencing of Usp8 and expression of α-synuclein.

Some of the advantages of utilizing Drosophila for genetic studies include its short life cycle, easy handling, the large number of individuals generated at a relatively

[1] http://www.cureffi.org/2014/10/27/genetics-16/

low cost and the evolutionary conservation of important genes (Bier 2005). About 75% of human genes have a *Drosophila* homologue/orthologue (Reiter, Potocki et al. 2001). Importantly, the protein degradation pathways such as the ESCRT-mediated endosomal (Leung, Dacks et al. 2008), autophagic and proteasomal systems, which are pertinent to my research questions are highly conserved.

An essential tool in fly genetics is the UAS-Gal4 system **(Fig 3.1)**. This is a binary expression system discovered in 1993 by Andrea Brand. Gal4 is a yeast transcription activator protein and UAS is an upstream activation enhancer to which Gal4 binds to activate transcription. Thousands of Gal4 lines exist, which express Gal 4 in a tissue specific manner. When a fly with a tissue-specific Gal4 is crossed to another with a transgenic construct linked to UAS, the progeny will express the transgene in a tissue specific manner, enabling targeted gene expression or ablation in cells of interest (Dietzl, Chen et al. 2007). Gal4 lines have been produced as either enhancer-traps with random integration of Gal4 followed by validation of the expression of a UAS gene, or with Enhancer-Fusion, where Gal4 is fused with a characterized tissue-specific promoter (Dietzl, Chen et al. 2007).

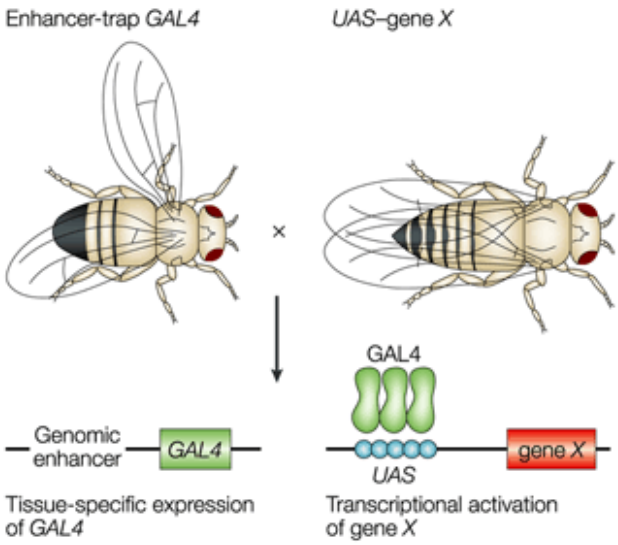


Fig 3.1 The UAS-Gal4 system in *Drosophila melanogaster***.** When Gal4 flies are crossed with UAS-gene X flies, the progeny have both genes: UAS-gene X and Gal4. Gal4 is then expressed only in the tissue of interest and binds to UAS which is ubiquitously expressed. Therefore, UAS is activated and gene X is expressed only in the tissue of interest. Figure adapted from St. Johnston et al (St Johnston 2002).

3.1.2. *Drosophila melanogaster* models of Parkinson's disease

Drosophila melanogaster has been extensively used to model various diseases and pathogenic processes. Notable examples of neurological conditions studied include the spinocerebellar ataxias, Huntington's disease, Alzheimer's and Parkinson's diseases, Fragile X syndrome and Spinal muscular atrophy. Other well-studied conditions include cardiac (e.g. congenital heart diseases and venous malformations), cancer (e.g. melanoma, B-cell leukaemia) and dysmorphologies (e.g. synpolydactyly) (Bier 2005).

An important example of the value of *Drosophila* genetic models in Parkinson's disease includes the delineation of the Pink1/Parkin pathway. It is well documented that Pink1 acts upstream of Parkin, which it recruits to enable mitophagy upon defective mitochondrial structure/function/stress. Parkin knockdown flies

demonstrate mitochondrial defects (swollen mitochondria with broken cristae) (Greene, Whitworth et al. 2003, Pesah, Pham et al. 2004), muscle, and dopaminergic neuron degeneration (Whitworth, Theodore et al. 2005). The fact that the PINK1 phenotypes are due to loss of function is because knockdown of endogenous PINK1 and expression of a PD-related mutant form of PINK1 have similar phenotypes (Yang, Gehrke et al. 2006, Yun, Cao et al. 2008). Rigorous genetic studies in *Drosophila* have established that the relation between PINK1 and Parkin is linear and that PINK1 acts upstream of Parkin to maintain mitochondrial integrity and function. Parkin overexpression rescues the PINK1 mutant phenotypes whereas PINK1 overexpression does not rescue the Parkin mutant ones (Clark et al. 2006, Park et al. 2006, Yang et al. 2006). Either PINK1 or Parkin *Drosophila* and mice knockouts have no milder phenotype than a combined mutant (Clark et al. 2006, Park et al. 2006, Yang et al. 2006, Kitada et al. 2009). They were also shown to have a physical interaction (Kim, Park et al. 2008, Xiong, Wang et al. 2009, Sha, Chin et al. 2009). In addition, both mutations in humans produce clinically undistinguishable syndromes. In addition, more lines of evidence show that these findings in *Drosophila* can be translated to humans. Cells from humans with PINK1 or parkin have defective mitochondria and oxidative phosphorylation (Gegg, Cooper et al. 2009, Schmidt, Linnartz et al. 2011, Greene, Whitworth et al. 2003, Gautier, Kitada et al. 2008). This is also recapitulated in neurons derived from iPC cells from the same patients (Seibler, Graziotto et al. 2011).

Drosophila does not have the α-synuclein gene, so transgenic expression is required. The orthologue of Usp8 in *Drosophila* is UBPY (here referred to as Usp8) with very similar functions. Scientists have taken advantage of *Drosophila's* genetics in order to model molecular interactions of α-synuclein toxicity both in

phenotypic and behavioural assays. A very good example of this is the rough eye *Drosophila* phenotype, initially described by Mel Feany at Harvard. Transgenic expression of α-synuclein in the fly eye caused retinal degeneration with vacuolization and architectural distortion (Feany, Bender 2000). The fly eye consists of 760 unit eyes, the ommatidia, which contain photoreceptors and bristles (Mishra, Knust 2013). This rough eye phenotype is easily assayed and can be utilized to identify second site modifiers, that is, proteins functioning in the same pathway of α-synuclein that ameliorate or worsen its toxicity phenotype. Since the development of the rough eye phenotype, many modifiers have been identified, implicating or verifying the interaction of gene families with α-synuclein (Bier 2005).

Apart from a molecular interaction between α-synuclein and potential modifiers, the *Drosophila* model recapitulates several cardinal features of Parkinson's disease: when synuclein was expressed under the control of elav-Gal4 (pan-neuronal driver) in flies, it led to the development of cytoplasmic punctuate inclusions and thread-like neurite inclusions, mimicking the Lewy bodies observed in the human brain of PD patients. The inclusions have a core and radiating filaments into a halo and appear after day 30 (Feany, Bender 2000). Moreover, when α-synuclein is expressed in the dopaminergic neurons (both α-synuclein WT and A53T mutation) it causes dopaminergic cell degeneration with ageing specifically in the dorsomedial (PPM1) cluster of *Drosophila* dopaminergic neurons (Feany, Bender 2000). This is one of several clusters of dopaminergic neurons that control locomotion, the others implicated in olfaction, learning and memory. The locomotive defect in the synuclein flies is best documented as a loss of climbing abilities in a behavioural negative geostatic response. This deterioration in locomotion is age-dependent

(Feany, Bender 2000, Kong et al. 2015). Thus, expression of α-synuclein in the fly dopaminergic neurons causes α-synuclein aggregation, neuronal loss and adult-onset locomotor defects (Feany, Bender 2000).

Apart from genetics, *Drosophila* has been utilized to investigate environmental effects on Parkinson's disease and drug screens. Such examples are rotenone causing loss of dopaminergic neurons and defects in locomotion (Coulom, Birman 2004) and MPTP and paraquat causing selective dopaminergic loss (Przedborski, Jackson-Lewis et al. 2000, Thiruchelvam, Brockel et al. 2000). Furthermore, drug screens of therapeutic agents with potential application to human disease have been performed. Rapamycin, histone deacetylase inhibitor SAHA and Stress upregulator geldanamycin have all been tested in flies in the context of Parkinson's disease (Bilen, Bonini 2005).

Following the models above, I developed single and double transgenic lines including the UAS-α-synuclein, the eye specific (GMR-Gal4) or dopaminergic specific (ddc-Gal4) drivers and UAS-Ups8 RNAi as a second site modifier to assess the effect of Usp8 knockdown in α-synuclein toxicity *in vivo* and their appropriate controls.

The primary objectives of this chapter were to assess:

1. the interaction of α-synuclein and knockdown of Usp8 using the *Drosophila melanogaster* rough eye phenotype
2. whether the knockdown of other DUBs produce similar effects
3. whether Usp8 knockdown affects the toxicity from unrelated proteins (e.g. pathogenic expansion of Ataxin 3 and Huntingtin)
4. whether the protein levels of α-synuclein are modified

5. whether Usp8 knockdown alters the locomotive defects caused by the expression of α-synuclein in the dopaminergic neurons
6. whether Usp8 knockdown affects the dopaminergic neurons that are decreased with the expression of α-synuclein

3.2. Knockdown of Usp8 in the *Drosophila* eye

The first aim was to assess the effectiveness of knockdown of the UAS-Usp8 RNAi construct. It is well-documented in the literature that knockdown of Usp8 in the *Drosophila* wing causes a specific wing defect (Mukai et al. 2010). This is because Usp8 regulates Wg/Wnt and Hedgehog signaling. Firstly, the development of sensory bristles on the wing depends on Wg/Wnt signaling. Mukai et al showed that gain/loss of function of Usp8 up/downregulates canonical Wg/Wnt signaling, thus Usp8 is required for the sensory bristle formation in the fly wing (Mukai et al. 2010). Secondly, the fly wing has margin and longitudinal veins (L2-L5). The space between L3 and L4 is dictated by Hedgehog signaling and the space between L2 and L3 and between L4 and L5 depends on BMP (Bone morphogenic protein) signaling (Blair 2007). Therefore, any interference with these pathways has an effect on the wing shape. Xia et al (Xia, Jia et al. 2012) have showed that Usp8 knockdown increases Smoothened ubiquitination and inhibits Hedgehog-induced smoothened accumulation which in turns leads to decreased Hedgehog activity. Therefore, by knocking down Usp8 specifically in the fly wing, we expect to see a deformed wing (Mukai, YamamotoHino et al. 2010).

I knocked down Usp8 (BL38982) in the fly wing using the MS1096-GAL4 driver and observed the deformed phenotype as expected (**Fig 3.2**).

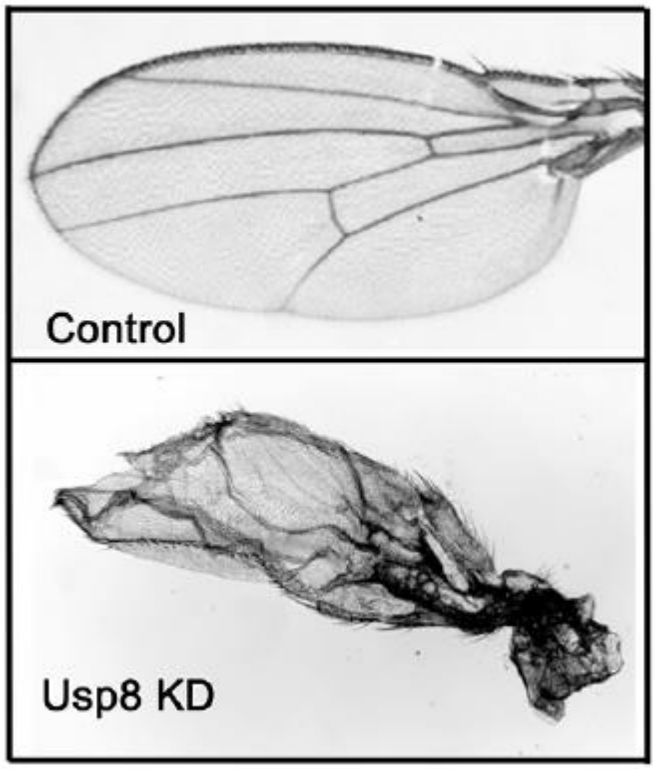


Figure 3.2: The functional efficiency of Usp8 knockdown was confirmed by detecting the previously documented wing defect when Usp8 RNAi was expressed under the *MS1096*-GAL4 driver. n=14

3.3. Modification of α-Synuclein-induced eye toxicity

3.3.1. Identification of optimal experimental conditions

My first aim was to identify the optimal experimental conditions for the expression of α-synuclein in the *Drosophila* eye as a readout of toxicity. For all experiments, male flies were used, unless mentioned otherwise.

Temperature selection: It is well-known that when flies develop at higher temperatures, the gene expression is enhanced. I therefore investigated whether higher expression of the transgene could cause a more tractable eye-toxicity phenotype. I therefore let the larvae develop at 29˚C which compared to flies at 25 ºC had a more prominent rough eye phenotype (see below). An important element is

that when adult flies are placed at 29˚C they may become sterile and not reproduce. To avoid this possibility, adult flies were crossed at 25˚C and the next day adults were removed from the vial/bottle, which was transferred to the 29˚C incubator to allow larvae to develop at this higher temperature.

Driver selection: A very important factor is the selection of the appropriate driver of Gal4 in the tissue of interest. This is because various drivers have different expression spatiotemporally and different efficiencies. I therefore tested the expression of three different variants of GMR-Gal4 (eye drivers) using the UAS-α-synuclein A53T construct according to the cross below:

$$\frac{GMR\ Gal4}{GMR\ Gal4};\frac{+}{+}\ \times\ \frac{+}{+};\frac{\alpha\ synuclein\ A53T}{\alpha\ synuclein\ A53T}\ \rightarrow\ \frac{GMR\ Gal4}{+};\frac{a\ synucleinA53T}{+}$$

Driver **A**: Kindly obtained from Dr. Gero Miesenbock

Drivers **B** (Davis 15) and **C** (Davis 12) were kindly provided by the Ilan Davis lab (Oxford).

Drivers A and C did not express adequately α-synuclein in order to cause a rough eye phenotype. On the other hand, driver B caused a rough eye phenotype (**Fig. 3.3.**)

Therefore, I performed all subsequent experiments using driver B (Davis 15).

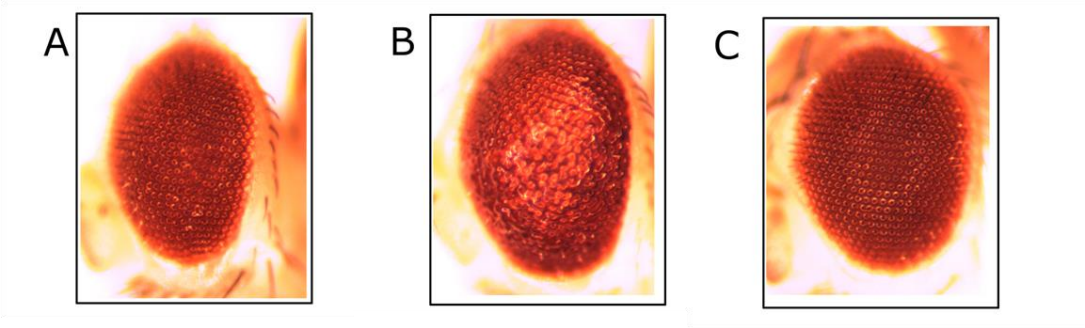


Figure 3.3 Drivers **A** and **C** did not cause high enough expression of α-synuclein in the fly eye and did not produce a rough eye phenotype. On the other hand, driver **B** (Davis 15) causes the documented rough eye phenotype. n=20, magnification: 6.3x

α-synuclein construct selection: Different constructs/mutants of α-synuclein were tested to identify which ones have a rough eye phenotype. I tested three constructs of α-synuclein: α-synuclein wild-type (WT), α-synuclein A53T and α-synuclein 120. α-syn A53T is a human mutation causing an alanine to threonine substitution at position 53, and is more pathogenic than the WT protein in a variety of assays (Stefanis, Larsen et al. 2001, Lu, Sun et al. 2015, Huang, Chegini et al. 2012). α-syn 120 is a truncated form of α-synuclein at the carboxy-terminus with 120 amino acids. This fragment has been isolated from Lewy bodies (Tofaris et al., JBC 2003) and considered a pathological form that *in vitro* (Crowther, Jakes et al. 1998, Serpell, Berriman et al. 2000, Murray, Giasson et al. 2003) and in mouse and fly models has been associated with increased aggregation propensity (Serpell et al. 2000, Periquet et al. 2007, Tofaris et al. 2006, Fares et al. 2016). In addition, it lacks the degradation signal (amino acids 120-133) that promotes the clearance of α-synuclein by Nedd-4 dependent ubiquitination (Tofaris et al. 2011).

The genotypes used for these experiments were:

$$\frac{GMR\ Gal4}{+}\ ;\ \frac{\alpha\ synuclein\ WT}{+}$$

$$\frac{GMR\ Gal4}{+}\ ;\ \frac{\alpha\ synuclein\ A53T}{+}$$

$$\frac{GMR\ Gal4}{+}\ ;\ \frac{\alpha\ synuclein\ 120}{+}$$

As observed, all 3 constructs produce a rough eye phenotype, so all 3 were used for further experiments **(Fig. 3.4).** Importantly, both α-synuclein A53T and 120 mutants showed slightly more roughness than the WT construct, which is consistent with the human pathology.

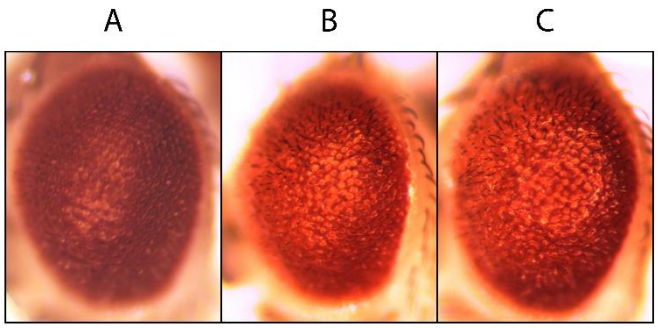


Figure 3.4 The rough eye phenotype with different α-synuclein constructs using the GMR Gal4 driver. **A:** α-synuclein WT, **B:** α-synuclein A53T, **C:** α-synuclein 120 mutant. n= 20-30, magnification: 6.3x

Temporal selection: Another important consideration is the effect of time on the roughness of the eyes. To assess this effect, I studied three timepoints and imaged the eyes of different lines at day 1, day 10 and day 20 post-eclosion. There is no

apparent worsening of the rough eye phenotypes of α-synuclein WT and A53T between the 3 timepoints (**Fig. 3.5**).

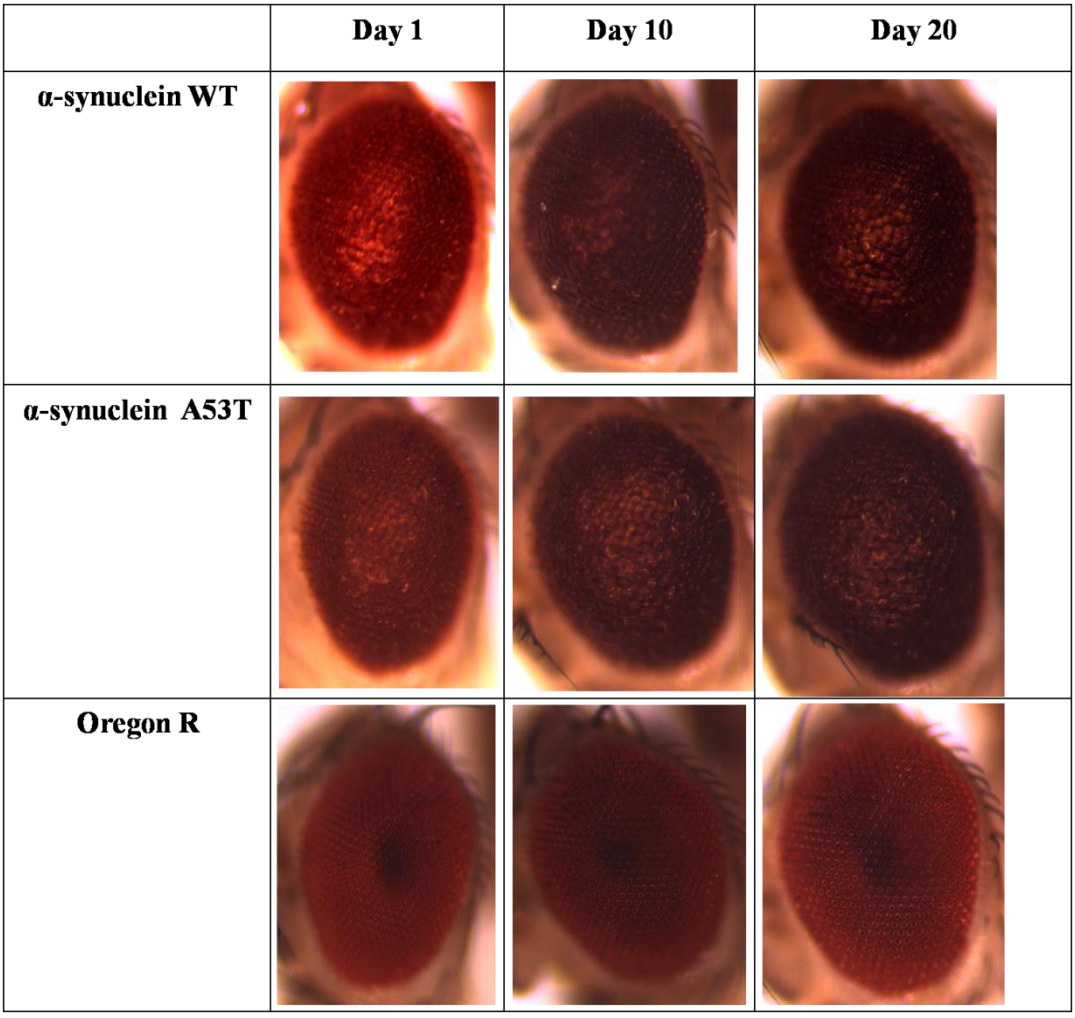


Fig 3.5: α-synuclein WT and A53T were imaged at days 1, 10 and 20 post-eclosion. Oregon R was included as a control wildtype fly for reference. There is no apparent worsening of the rough eye phenotypes of α-synuclein WT and A53T between the 3 timepoints. n=9, magnification: 6.3x

It is important to note that I also checked the double transgenics for roughness $\left(\frac{\text{GMR Gal4}}{\text{UAS Usp8 RNAi)}} ; \frac{\text{UAS α synuclein}}{+}\right)$ at days 1, 10 and 20 and saw no

difference; therefore, I will only include eye photos from day 1 in the rest of my thesis.

Other important controls: Other essential controls include the following (some are included in section 3.3.3.-e.g. other DUBs):

- UAS-Usp8 RNAi was expressed in the eye in the absence of α-synuclein expression to test whether Usp8 knockdown *per se* could cause a rough eye phenotype. For this I used the following genotype $\frac{GMR\ Gal4}{UAS\ Usp8\ RNAi}; \frac{+}{+}$.

  The expression of the UAS-Usp8 RNAi construct alone does not cause a rough eye phenotype, so it can be used for further experiments **(Fig. 3.6).**

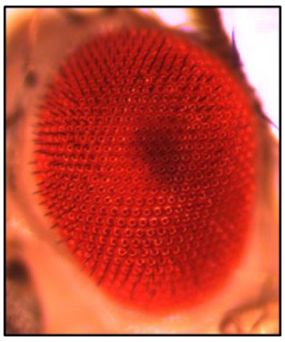


Fig 3.6: The expression of the UAS-Usp8 RNAi construct alone does not cause a rough eye phenotype. n=15, magnification: 6.3x

- GMR Gal4 was crossed with a WT fly to get progeny with one copy of the GMR Gal4 construct to ensure no rough eye phenotype is caused by the expression of the driver alone. The $\frac{GMR\ Gal4}{GMR\ Gal4}; \frac{+}{+}$ fly is stronger expresser than the $\frac{GMR\ Gal4}{+}; \frac{+}{+}$ fly which carries only one copy of the gene. One copy of the GMR Gal4 is going to be used in the experiments to follow, thus it is important to have $\frac{GMR\ Gal4}{+}; \frac{+}{+}$ as a control. The expression of the UAS-GMR

Gal4 construct alone does not cause a rough eye phenotype, so it can be used for further experiments **(Fig. 3.7).**

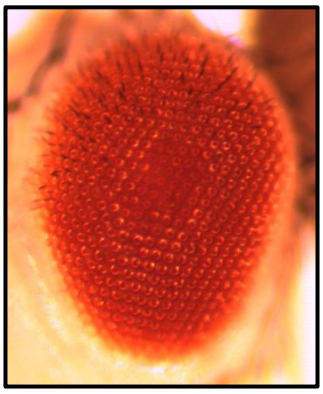


Fig 3.7: The expression of the UAS-GMR Gal4 construct alone does not cause a rough eye phenotype. n=11, magnification: 6.3x

3.3.2. Effect of eye-specific Usp8 knockdown against α-synuclein toxicity

Having established the optimal conditions for my experiments, I then first tested the effect of Usp8 knockdown on the α-synuclein-induced rough eye phenotype. To this end, I performed light microscopy (as shown above) and Scanning Electron Microscopy (SEM) with the help of Dr Myriam Elschami in our laboratory to assess the morphology at higher resolution.

In order to achieve simultaneous expression of α-synuclein and knockdown of Usp8 in the *Drosophila* eye I performed multiple crosses to get the following final genotypes:

$$\frac{GMR\ Gal4}{UAS\ Usp8\ RNAi}\ ;\ \frac{UAS\ \alpha\ synuclein\ WT}{+}\ \text{vs}\ \frac{GMR\ Gal4}{+}\ ;\ \frac{\alpha\ synuclein\ WT}{+}$$

$$\frac{GMR\ Gal4}{UAS\ Usp8\ RNAi}\ ;\ \frac{UAS\ \alpha\ synuclein\ A53T}{+}\ \text{vs}\ \frac{GMR\ Gal4}{+}\ ;\ \frac{\alpha\ synuclein\ A53T}{+}$$

$$\frac{GMR\ Gal4}{UAS\ Usp8\ RNAi}\ ;\ \frac{UAS\ \alpha\ synuclein\ 120}{+}\ \text{vs}\ \frac{GMR\ Gal4}{+}\ ;\ \frac{\alpha\ synuclein\ 120}{+}$$

Concomitant expression of α-synuclein with Usp8 knockdown rescued the rough eye phenotype caused by the expression in the *Drosophila* eye of α-synuclein WT and A53T (**Fig. 3.8 and 3.9**) and α-synuclein 120 (**Fig 3.10**).

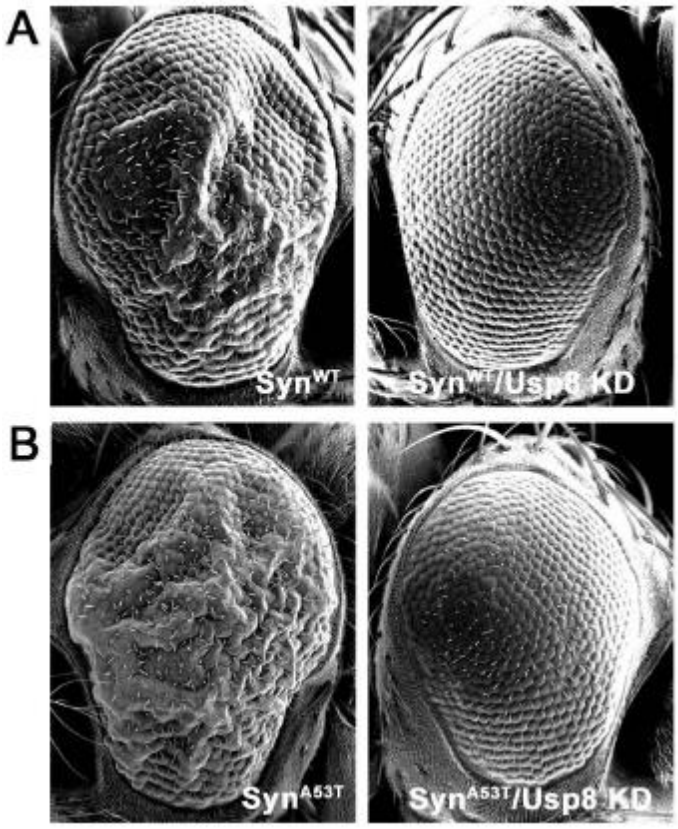


Fig 3.8: Knockdown of endogenous Usp8 in *Drosophila* protects against α-synuclein–induced toxicity. Overexpression of α-synuclein caused a rough eye phenotype, which was more severe in flies expressing A53T mutant α-synuclein, as detected by SEM. This phenotype was rescued in double-transgenic flies with eye-specific Usp8 knockdown and either wild-type **(A)** or A53T mutant α-synuclein **(B).** n=4-5 flies

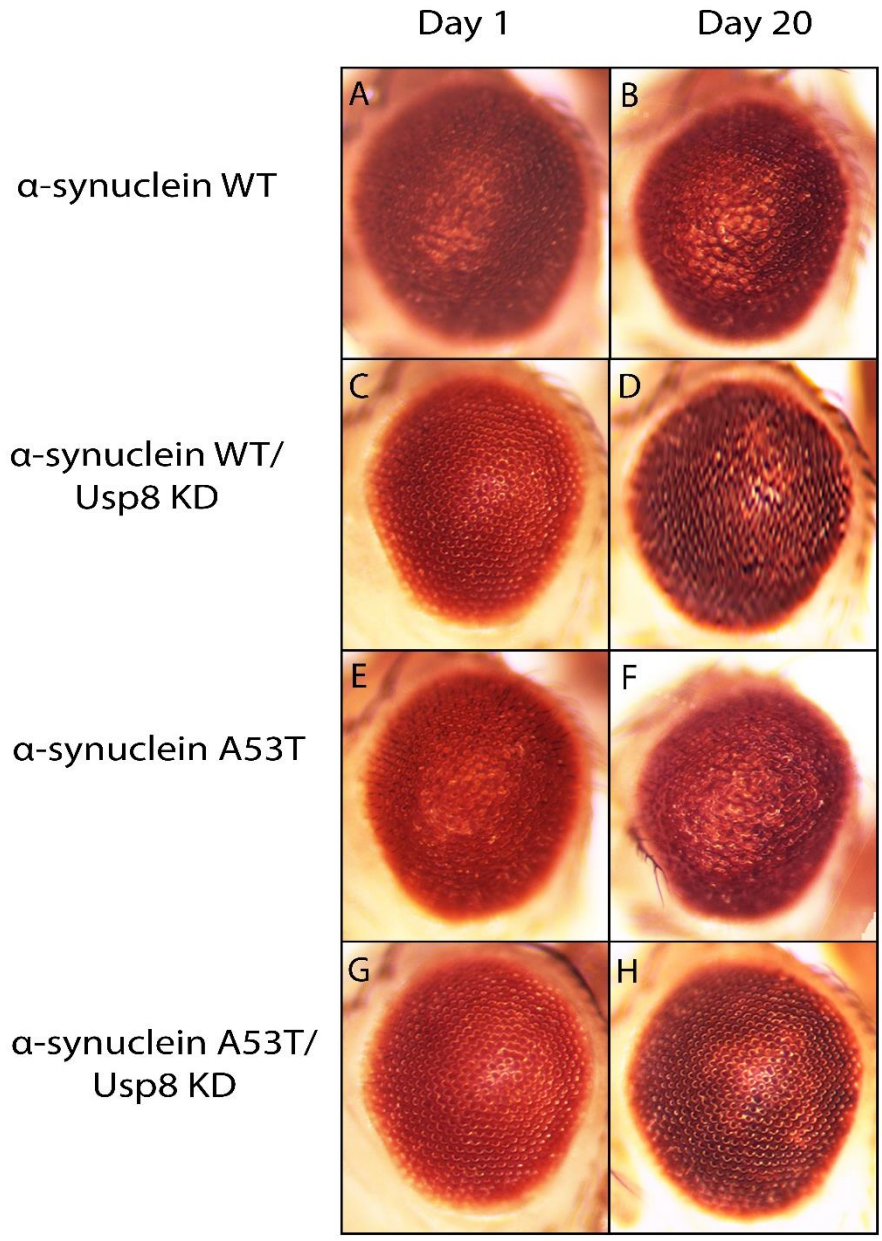


Fig 3.9: Light microscopy images of single and double transgenic flies with α-synuclein WT and A53T at days 1 and 20 post-eclosion. As observed, the concomitant knockdown of Usp8 rescues the rough eye phenotype caused by α-synuclein toxicity recapitulating the results observed by SEM above. n=13, magnification: 6.3x

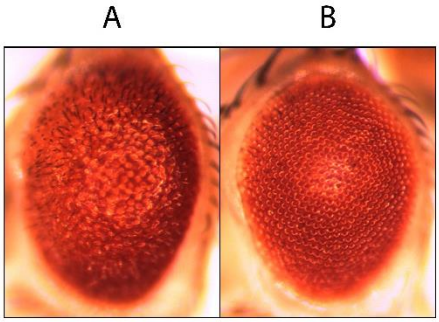


Fig 3.10: Expression of α-synuclein 120 causes a rough eye phenotype **(A)** which is rescued by concomitant Usp8 knockdown **(B)**. n=14, magnification: 6.3x

Collectively, my data from the experiments using three different lines of synuclein demonstrate that Usp8 knockdown is protective against various forms of α-synuclein toxicity.

I next examined whether the aforementioned rescue of the rough-eye phenotype was due to a dilutional effect of the GMR Gal 4 driver. This is sometimes possible when two UAS constructs are driven by the same driver, causing decreased expression and thus an apparent rescue of the phenotype due to the lower expression of α-synuclein. To exclude this possibility, I used qPCR to measure the level of expression (RNA levels) of α-synuclein WT and A53T (**Fig 3.11**). In both cases the mRNA levels were not decreased, which indicates that the absence of α-synuclein toxicity is not due to lack of expression of α-synuclein, but rather due to the Usp8 knockdown (second transgene). Also, using the same driver GMR Gal4, I co-expressed α-synuclein together with the unrelated construct GFP and showed that the rough eye phenotype was still present (**Fig. 3.12**).

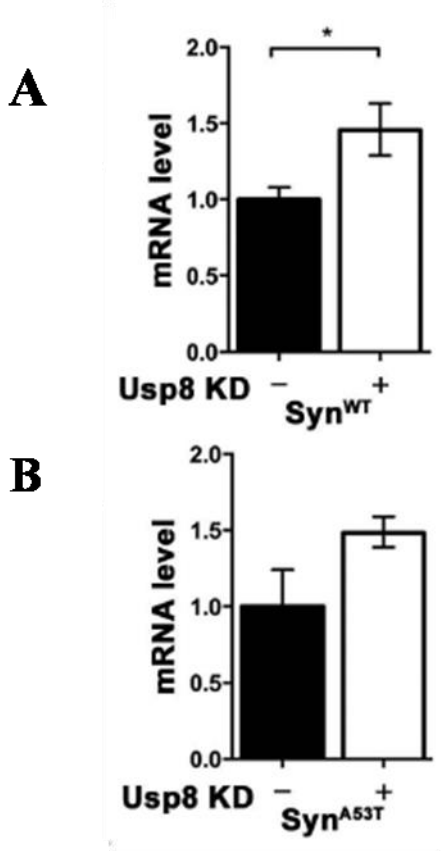


Fig 3.11: Quantitative PCR did not show a reduction in α-synuclein mRNA levels between single- and double-transgenic lines of α-synuclein WT **(A)** or α-synuclein A53T **(B)** to explain the rescue of the rough eye phenotype. **P* < 0.05

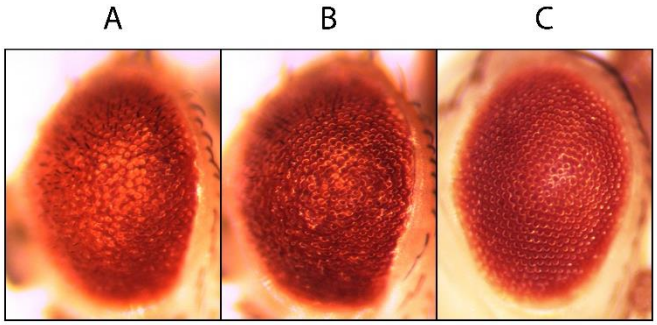


Fig 3.12: The rough eye phenotype caused by α-synuclein toxicity **(A)** is not ameliorated by the addition of a non-relevant UAS construct, GFP **(B),** therefore there is no dilutional effect of the GMR Gal4 driver. **(C)** shows the rescue by the knockdown of Usp8 for reference. n=7 magnification: 6.3x

3.3.3. Assessment of other deubiquitinases

To assess the specificity of Usp8 as a critical deubiquitinase in the α-synuclein-induced eye toxicity phenotype, I then assessed the effect of other deubiquitinases. Ideally, one would perform an unbiased screen of all deubiquitinating enzymes using Mass Spectrometry (e.g. MS/MS) to identify which DUBs are active against recombinant α-synuclein (currently under investigation in the laboratory) and measure the abundance of all DUBs in post-mortem brains of Parkinson's patients when compared with normal controls. However, these experiments were beyond the scope of my thesis. Therefore, I used as controls additional DUBs, which belong to different categories and have fly orthologues. These DUBs are Usp7, Usp14, Usp47, AMSH and JosD2. The reasons I selected these DUBs for a targeted screen are explained in the introduction. None of the DUBs knockdowns tested rescued the rough eye phenotype caused by α-synuclein (**Fig. 3.13**).

I crossed the flies in single and double transgenic lines to get the following genotypes:

$$\frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ Usp7\ RNAi}{+}\ \text{and}\ \frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ Usp7\ RNAi}{\alpha\ synuclein\ A53T}$$

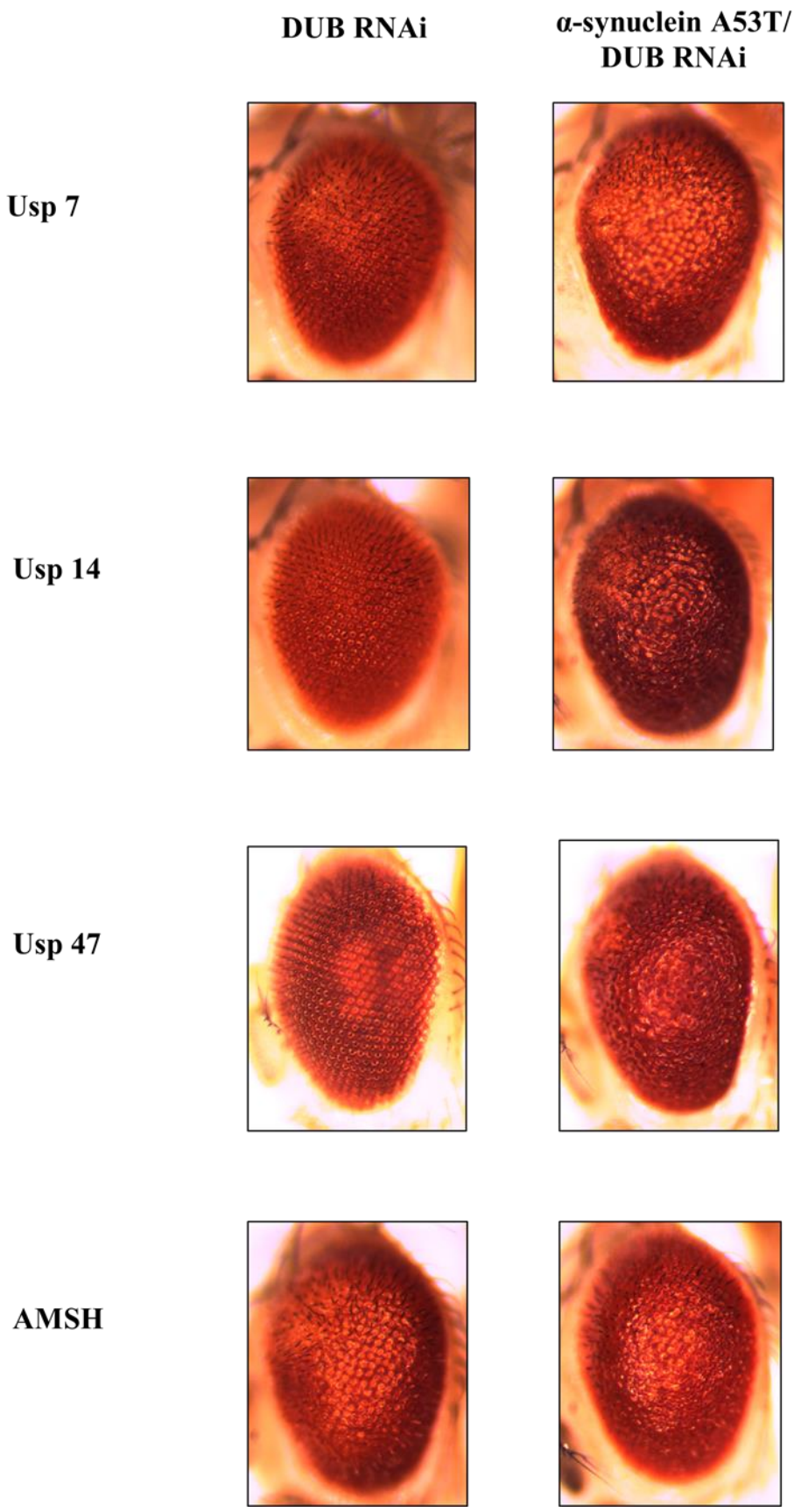
$$\frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ Usp14\ RNAi}{+}\ \text{and}\ \frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ Usp14\ RNAi}{\alpha\ synuclein\ A53T}$$

$$\frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ Usp47\ RNAi}{+}\ \text{and}\ \frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ Usp47\ RNAi}{\alpha\ synuclein\ A53T}$$

$\frac{GMR\ Gal4}{UAS\ AMSH\ RNAi}\ ;\ \frac{+}{+}$ and $\frac{GMR\ Gal4}{UAS\ AMSH\ RNAi}\ ;\ \frac{\alpha\ synuclein\ A53T}{+}$

$\frac{GMR\ Gal4}{UAS\ JosD2\ RNAi}\ ;\ \frac{+}{+}$ and $\frac{GMR\ Gal4}{UAS\ JosD2\ RNAi}\ ;\ \frac{\alpha\ synuclein\ A53T}{+}$

The AMSH single transgenic ($\frac{GMR\ Gal4}{UAS\ AMSH\ RNAi}\ ;\ \frac{+}{+}$) had a mild rough eye phenotype by itself in the absence of α-synuclein, thus the effect of AMSH knockdown cannot be assessed in modifying α-synuclein toxicity.


DUB RNAi
α-synuclein A53T/
DUB RNAi
Usp 7
Usp 14
Usp 47
AMSH

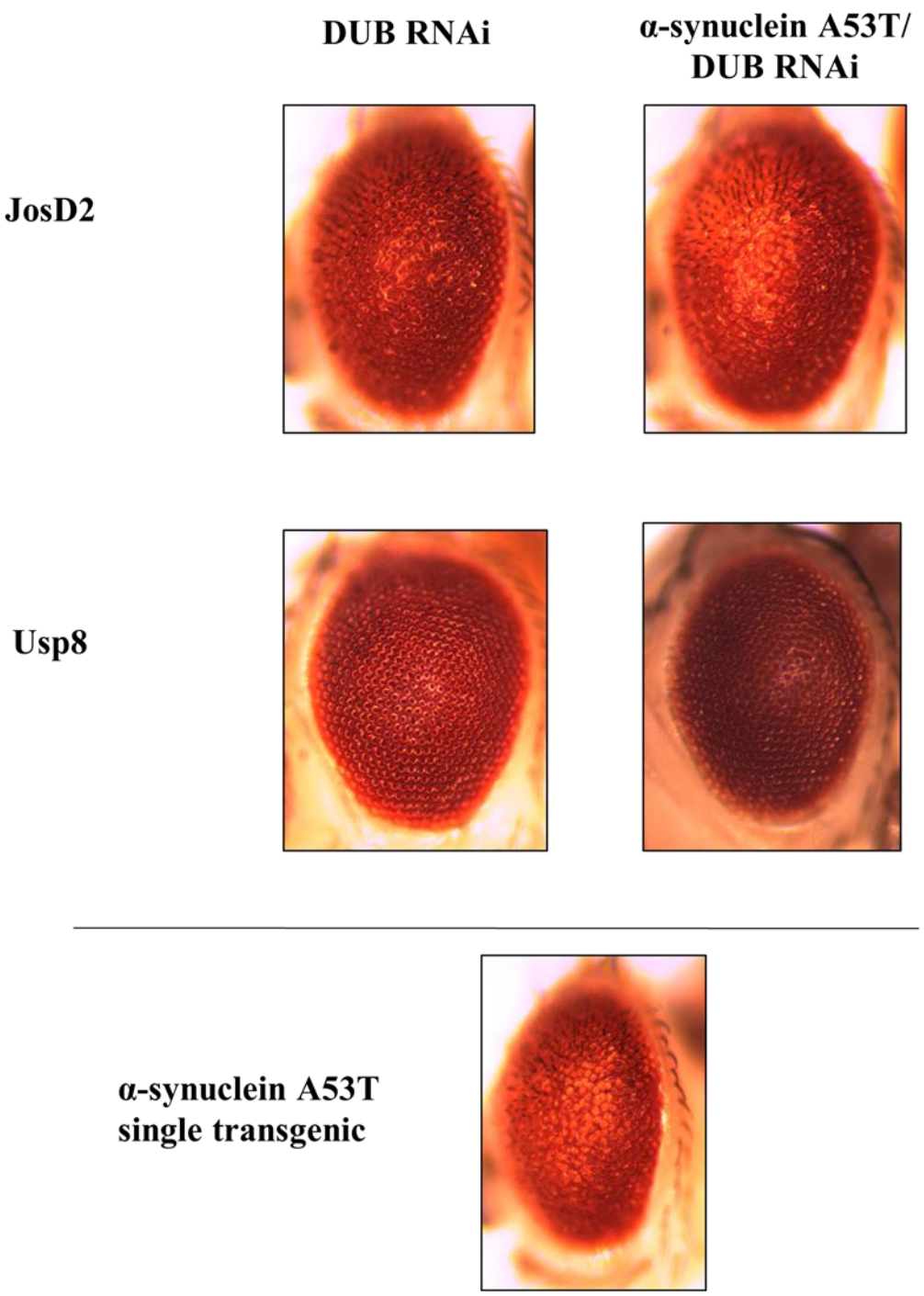


Fig 3.13a: Usp8 but not the other DUBs tested rescue the rough eye phenotype caused by α-synuclein A53T toxicity. Here, Usp7, Usp14, Usp47, AMSH and JosD2 were tested. α-synuclein A53T single transgenic is included for reference. n=7-12, magnification: 6.3x

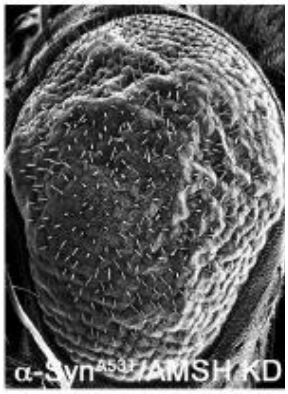


Fig 3.13b: SEM of $\frac{GMR\ Gal4}{UAS\ AMSH\ RNAi}$; $\frac{UAS\ \alpha\ synuclein\ A53T}{+}$ showing no rescue of the phenotype similarly to the light microscopy image above. Image included for light microscopy/SEM images comparison. n=4 flies

3.3.4. Investigation of the specificity of the phenotype

An important next step was to investigate whether the effect of Usp8 knockdown is specific for α-synuclein or also mitigates the toxicity of other toxic proteins implicated in neurodegeneration. For this purpose, I tested the pathogenic expansion of Ataxin3 and Huntingtin, which cause Spinocerebellar ataxias (SCAs)/Machado-Joseph disease (MJD) and Huntington's disease respectively. The selected constructs contain an expanded polyglutamine (polyQ) repeat sequence, which when expressed in the *Drosophila* eye is known to cause a rough eye phenotype (Jackson et al. , Warrick et al. 2005, Bonini 1999). More specifically, pathogenic Ataxin 3 expansion causes late onset progressive degeneration, forming abnormal nuclear inclusions (abnormal protein aggregates), whereas the control Ataxin 3 is cytoplasmic (Bonini 1999, Warrick, Paulson et al.). In addition, the HTT *Drosophila* model recapitulates characteristics of the disease in humans, such as neuronal degeneration, which together with the age of onset and severity correlate

with the polyglutamine repeat length. Moreover, the nuclear localization of HTT precedes neuronal degeneration (Jackson, Salecker et al.). The construct of Ataxin3 has 78 expansions and of human Huntingtin (HTT) has 128; both were expressed under UAS control.

The construct of pathogenic UAS-Ataxin 3 expansion is on the 2nd chromosome. However, both the GMR Gal4 and UAS-Usp8 RNAi (BL38982) constructs are on the 2nd chromosome too. Thus, it is impossible to recombine 3 alleles on 1 chromosome. Therefore, I needed to use a different construct of either GMR Gal4 or UAS-Usp8 RNAi for it to be on the 3rd chromosome. I chose to keep the GMR Gal4 on the 2nd chromosome as I know it is a strong driver and decided to test another UAS-Usp8 RNAi (BL39022) which is on the 3rd chromosome. However, as mentioned above I first need to ensure adequate Usp8 knockdown again testing for the characteristic wing phenotype, which was obtained with the BL39022 UAS-Usp8 RNAi **(Fig.3.14).** I used this Usp8 RNAi construct only for this section of experiments with Ataxin 3 and HTT.

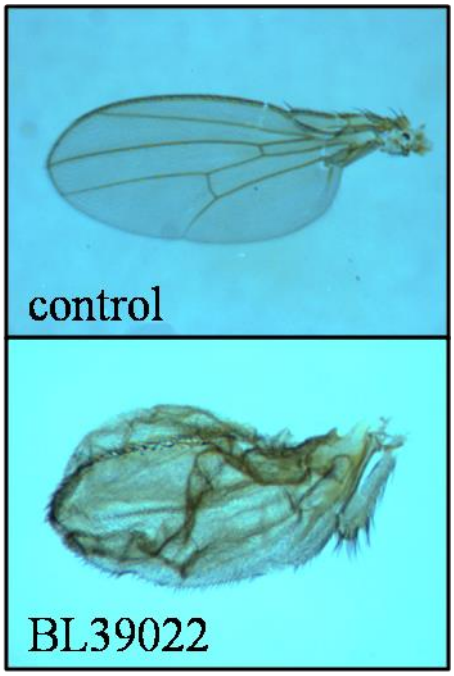


Figure 3.14: The functional efficiency of BL39022 UAS-Usp8 RNAi construct was confirmed by detecting the previously documented wing defect when the RNAi was expressed under the *MS1096*-GAL4 driver. n=18

I crossed the flies with the above constructs to obtain the following genotypes:

$$\frac{UAS\ Ataxin\ 3}{GMR\ Gal4}\ ;\ \frac{+}{+}\ \text{vs}\ \frac{UAS\ Ataxin\ 3}{GMR\ Gal4}\ ;\ \frac{UAS\ Usp8\ RNAi}{+}$$

$$\frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ HTT}{UAS\ Usp8\ RNAi}\ \text{vs}\ \frac{GMR\ Gal4}{+}\ ;\ \frac{UAS\ HTT}{UAS\ Usp8\ RNAi}$$

Usp8 knockdown did not rescue the toxicity caused by the pathogenic poly-Q expansion of Ataxin 3 (**Fig 3.15**) and Huntingtin (**Fig. 3.16**).

Fig 3.15: Usp8 knockdown did not rescue the toxicity caused by the pathogenic poly-Q expansion of Ataxin 3. Image is in grayscale as the colour image was too bright. n=8, magnification: 6.3x

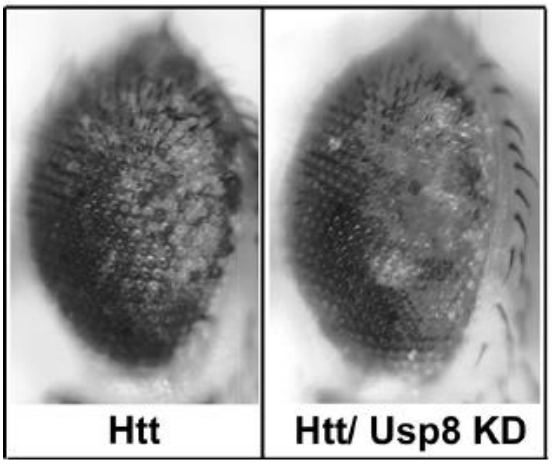


Fig 3.16: Usp8 knockdown does not rescue the toxicity caused by the pathogenic poly-Q expansion of Huntingtin. Image is in grayscale as the colour image was too dark. n=12, magnification: 6.3x

Therefore, the beneficial effects of Usp8 knockdown could be specific for rescuing α-synuclein toxicity. More pathogenic phenotypes should be screened in the future to assess Usp8 KD effects, but this is beyond the scope of my thesis.

3.3.5. Quantitation of α-synuclein protein levels in single and double transgenic flies

Since Usp8 is a deubiquitinase, one possible mechanism by which it mitigates α-synuclein toxicity is by regulating α-synuclein levels. To assess this possibility, I performed serial fractionation of fly head lysates and measured the intensity of the monomeric α-synuclein band as a ratio to the actin loading control in the cytosolic and pelleted fractions of single- and double-transgenic flies as measure of α-synuclein abundance. I found that in Usp8 depleted tissues, both wild-type and A53T α-synuclein protein levels were reduced **(Fig 3.17)**.

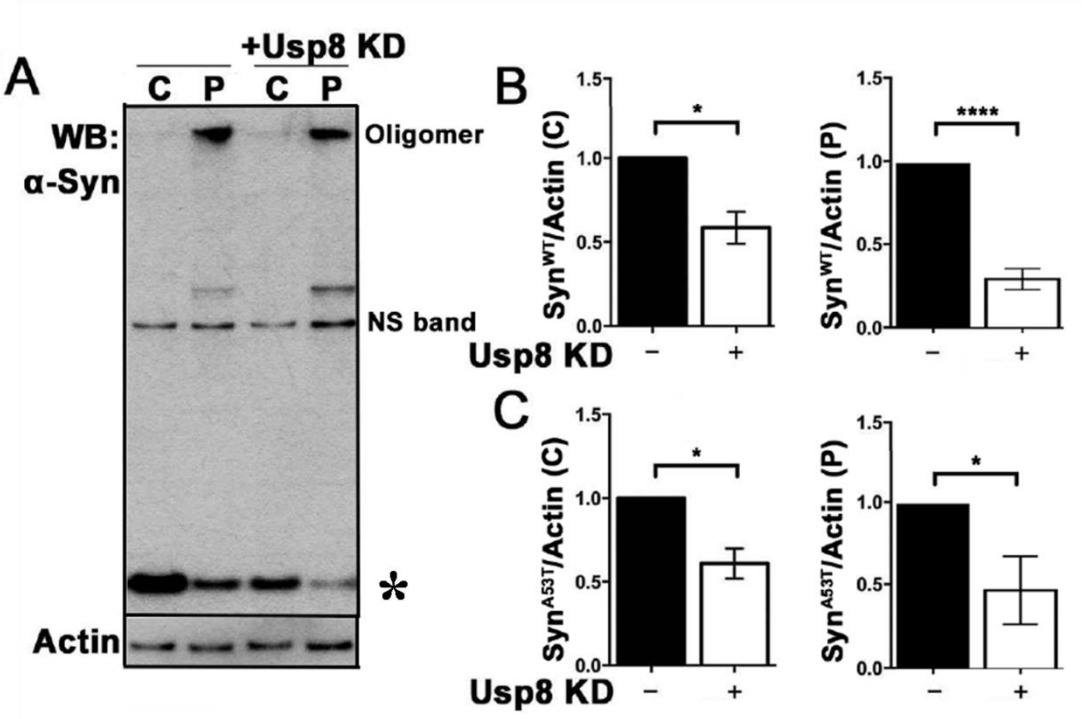


Fig 3.17: (A) Representative immunoblot of fractionated head lysate (C, cytosol; P, pellet) from A53T mutant α-synuclein flies and flies expressing A53T mutant α-synuclein with Usp8 knockdown (+Usp8 KD). Quantitative band densitometry showed that, relative to the actin loading control, protein levels of either wild-type **(B)** or A53T **(C)** mutant monomeric α-synuclein (indicated by an asterisk) were significantly reduced in flies coexpressing Usp8 RNAi (n = 4 biological replicates)

in the absence of a reduction in mRNA levels, as in **Fig 3.16**. *$P < 0.05$; ****$P < 0.0001$. Antibodies used: α-synuclein: C20, Actin: Sigma AldrichA1978

Interestingly, oligomers of α-synuclein were also visualized as shown at the top of the gel, and were also reduced in double compared to single α-synuclein transgenics. Given that the mRNA level of α-synuclein was unchanged (see earlier **section 3.3.2**), these data suggest that Usp8 knockdown rescues the α-synuclein toxicity by lowering its protein levels.

3.4. Knockdown of Usp8 and other deubiquitinases in the *Drosophila* dopaminergic neurons

3.4.1. Identification of optimal experimental conditions

My studies using the rough eye phenotype as a measure of α-synuclein toxicity strongly suggest that Usp8 is a modifier of α-synuclein pathobiology. In order to assess the relevance of α-synuclein-Usp8 interaction in a cell-type and readout that is a more representative model for Parkinson's disease, I then expressed α-synuclein in the *Drosophila* dopaminergic neurons. In order to do this, I first had to identify the most suitable driver. The options included the use a pan-neuronal driver (elav Gal4), a Tyrosine Hydroxylase driver (TH Gal4) or a Dopamine Decarboxylase driver (ddc Gal4). The ddc Gal4 driver in *Drosophila* drives expression in dopaminergic and serotonergic neurons since it is involved in the last step of both dopamine and serotonin synthesis in *Drosophila* **(Fig 3.18)** (Beall, Hirsh 1987, Monastirioti 1999). On the other hand, TH is more specific for dopaminergic neurons (Friggi-Grelin, Coulom et al. 2003). When I knocked down Usp8 in *Drosophila* with the elav Gal4 driver it was lethal since no flies eclosed. Thus, for further experiments I used the ddc Gal4 driver, kindly provided by the Miesenbock lab.

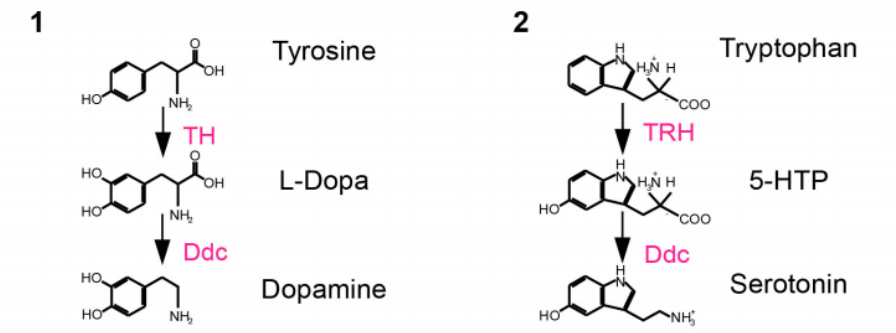


Fig 3.18. Dopamine **(1)** and Serotonin **(2)** biosynthesis pathways in *Drosophila*. ddc is involved in both pathways at the last enzymatic conversion. Figure adapted from Riemensperger et al (Riemensperger, Issa et al. 2013).

To investigate locomotion in *Drosophila* I used a behavioural assay, the climbing assay, assessing their negative geotactic response following startle. When a vial of flies is tapped down, their normal response is to quickly climb back up the vial walls to the top. This normal response is decreased with ageing (Gargano, Martin et al. 2005, Arking, Wells 1990).

For this assay, the flies were maintained at 25˚C. For each trial, I transferred ten male flies in an empty vial without food. Each vial was tapped 10 times and the percentage of flies above 6cm was recorded after 4 seconds, denoted as the Climbing Index. This was repeated with five vials per line in each experiment (total 50 flies per line).

An example of the climbing assay is shown in **Figure 3.19.**

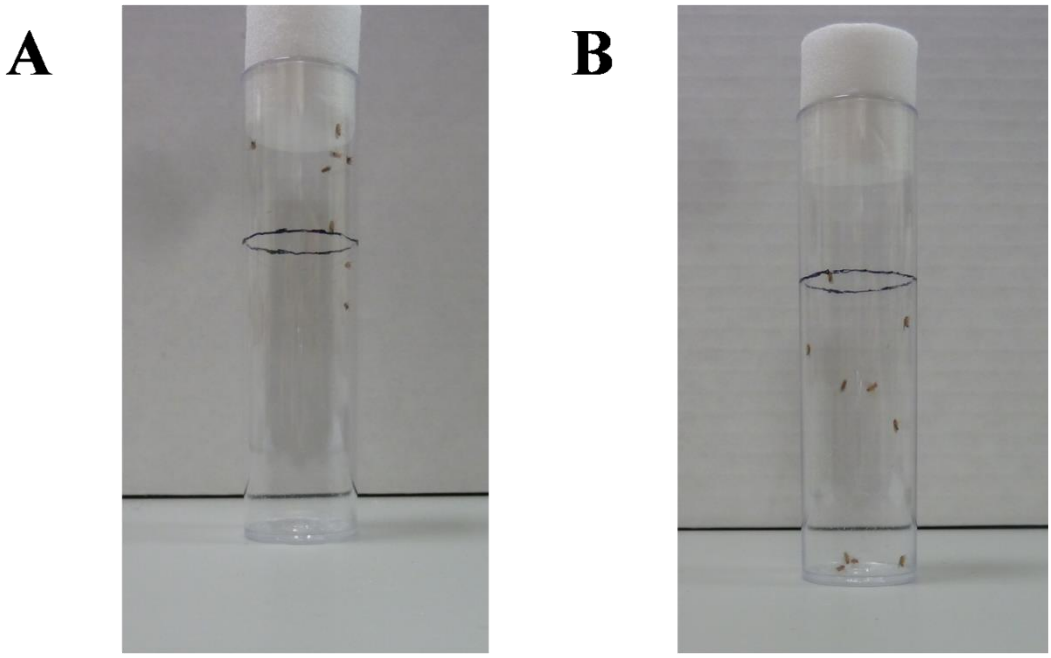


Fig 3.19: Climbing assay showing Wild Type **(A)** young flies –day 1 post eclosion- and **(B)** older flies –day 45.

An important control experiment here is to test whether the expression of UAS Usp8 RNAi construct causes any defects in locomotion in itself, before its effect can be combined with that of α-synuclein co-expression. The expression of UAS Usp8 construct in the dopaminergic and serotonergic neurons of the *Drosophila* with the ddc Gal4 driver does not impair locomotion as compared to WT flies (**Fig. 3.20).**

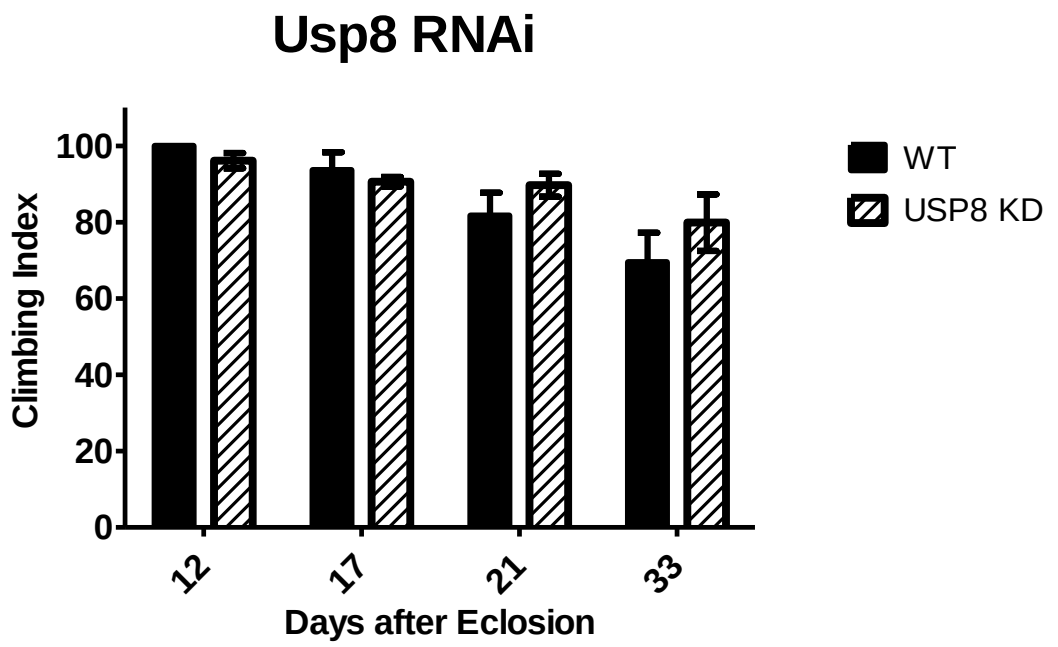


Fig 3.20 The expression of UAS Usp8 construct in the dopaminergic and serotonergic neurons of the *Drosophila* with the ddc Gal4 driver does not impair locomotion as compared to WT flies. n=50

3.4.2. Usp8 prevents the age-dependent α-synuclein-induced climbing defect

It is well-documented in the literature that when α-synuclein is expressed in the dopaminergic neurons of *Drosophila*, it causes a decrease in locomotive activity. In addition, this is age-dependent, suggesting a degenerative mechanism that more closely mimics Parkinson's disease (Feany, Bender 2000). Thus, the next step in my experimental strategy was to recapitulate this readout and ask whether Usp8 knockdown mitigates this locomotor defect.

For this purpose, I crossed the flies in single and double transgenics to get the following genotypes:

$\frac{+}{+}$; $\frac{UAS\ \alpha\ synuclein\ A53T}{ddc\ Gal4}$ (denoted A53T)

$\frac{UAS\ Usp8\ RNAi}{+}$; $\frac{UAS\ \alpha\ synuclein\ A53T}{ddc\ Gal4}$ (denoted A53T/Usp8 KD)

I also included the driver ddc Gal4 as the genetic background (control). In order to have 1 copy of the ddc Gal4 construct, I crossed the homozygous ddc Gal4 with a control fly w^{1118} with a resulting genotype $\frac{ddc\ Gal4}{+}$ (denoted ddc).

Accelerated loss of climbing ability was seen in transgenic flies expressing human A53T mutant α-synuclein in dopaminergic neurons (*ddc*-GAL4 driver) with increasing age. The climbing ability of double-transgenic lines expressing A53T α-synuclein with Usp8 knockdown in dopaminergic cells was significantly improved (shown with asterisks) compared with A53T α-synuclein–expressing flies and was similar to the control genotype, *ddc*-GAL4/+ **(Fig.3.21).**

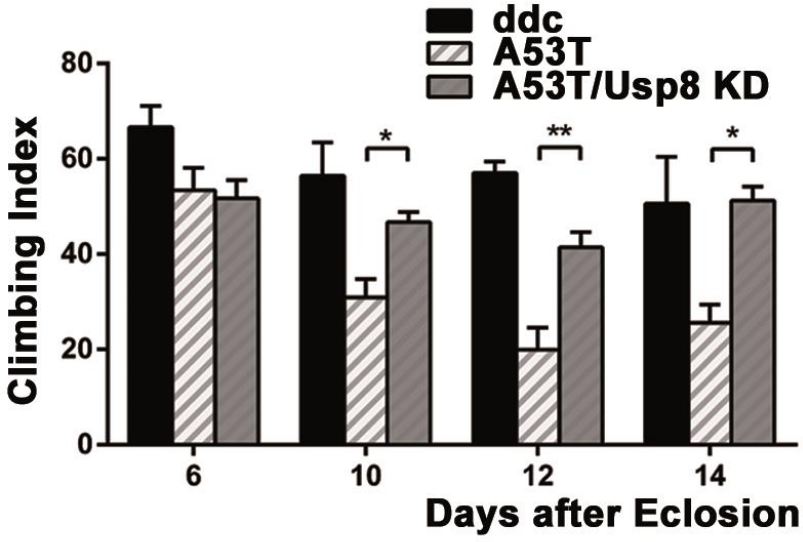


Fig 3.21. The accelerated loss of climbing ability of α-synuclein flies was rescued by concomitant Usp8 knock down (shown with asterisks). **P* = 0.0381 for day 10; ***P* = 0.01 for day 12; **P* = 0.0159 for day 14; *n* = 50 flies per group.

3.4.3. Unlike Usp8, other deubiquitinases do not prevent the α-synuclein-induced climbing defect

To assess whether the locomotor defect is modified by other DUBs and further confirm the specificity of Usp8/synuclein interaction as was shown using the rough eye phenotype, I knocked down in the dopaminergic neurons expressing α-synuclein the other DUBs. The choice of DUBs used is discussed in the **Introduction.** Initially, I tested the effect of expression of the DUB RNAi alone, without the expression of α-synuclein. Expression with ddc Gal4 of the Usp7 RNAi and Usp14 RNAi constructs but not of AMSH RNAi, JosD2 RNAi and Usp8 RNAi reduces the locomotive activity of the flies **(Fig. 3.22 and 3.23).**

I crossed the flies to get the following genotypes:

$\frac{+}{+}; \frac{UAS\ Usp7\ RNAi}{ddc\ Gal4}$ (denoted Usp7 KD)

$\frac{+}{+}; \frac{UAS\ Usp14\ RNAi}{ddc\ Gal4}$ (denoted Usp14 KD)

$\frac{UAS\ AMSH\ RNAi}{+}; \frac{ddc\ Gal4}{+}$ (denoted AMSH KD)

$\frac{UAS\ JosD2\ RNAi}{+}; \frac{ddc\ Gal4}{+}$ (denoted JosD2 KD)

$\frac{UAS\ Usp8\ RNAi}{+}; \frac{ddc\ Gal4}{+}$ (denoted Usp8 KD)

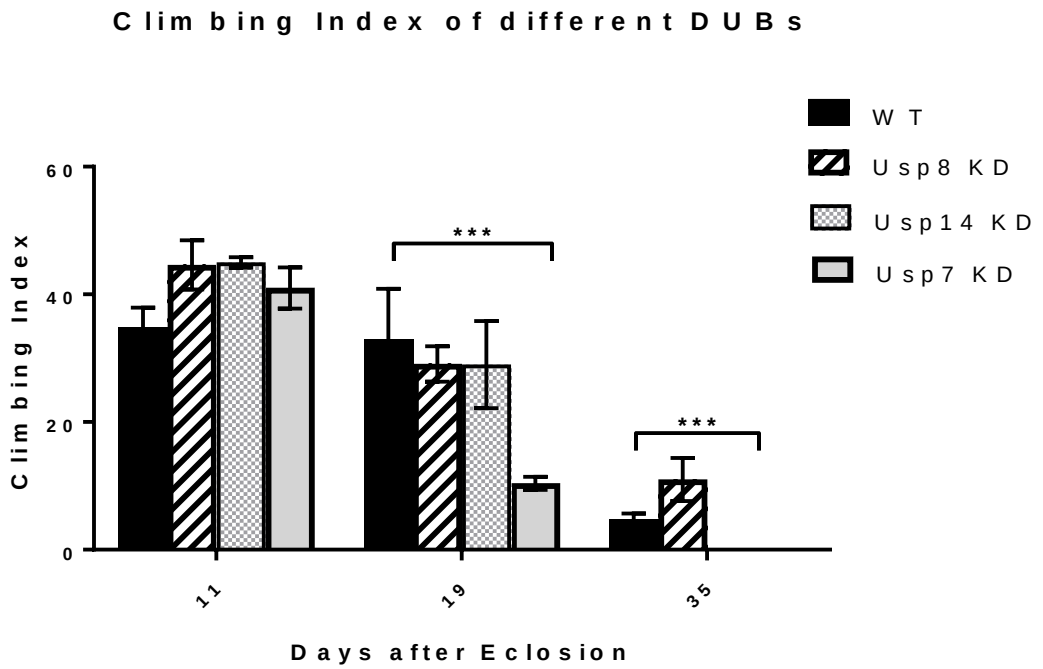


Figure 3.22: Expression with ddc Gal4 of the Usp7 RNAi (Usp7 KD) and Usp14 RNAi (Usp14 KD) constructs but not of Usp8 RNAi (Usp8 KD) reduces the locomotive activity of the flies. This effect is age-dependent. The climbing index for Usp7 and Ups14 is zero at day 35. n=50, *** $p<0.001$ between WT and Usp7 and Usp14 KD, 1-way ANOVA

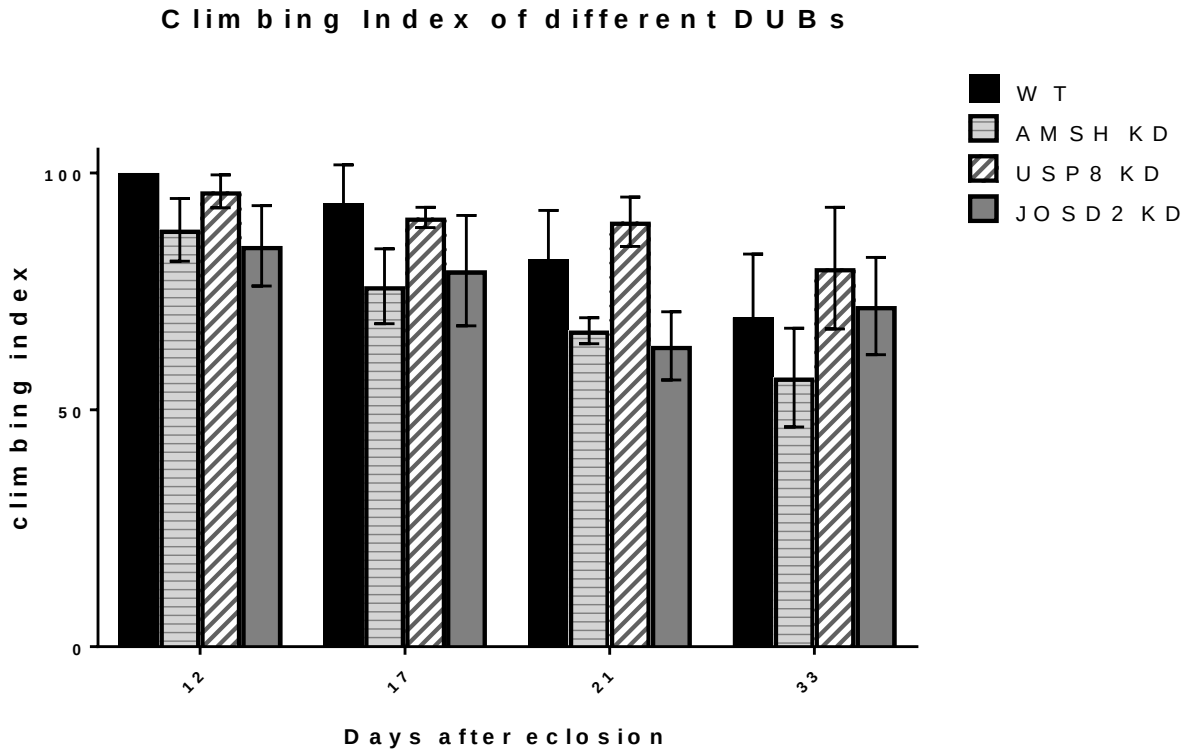


Figure 3.23 Expression with ddc Gal4 of the AMSH RNAi (AMSH KD) and JosD2 RNAi (JosD2 KD) constructs similarly to Usp8 RNAi (Usp8 KD) did not reduce the locomotive activity of the flies. n=50, no significant difference with 1-way ANOVA

As demonstrated in **Figure 3.22**, the expression of the constructs of Usp7 RNAi and Usp14 RNAi themselves cause a decrease in locomotion, therefore they could not be used further to assess any combinatorial effect with α-synuclein. The decrease of locomotion with the Usp7 and Usp14 knockdown might be because these two DUBs are essential, in that Usp7 stabilizes p53 and Usp14 is required for the deubiquitination of substrates for proteasomal breakdown. Thus, this locomotive defect could reflect these essential functions. In **Figure 3.23** it is evident that the expression of both AMSH and JosD2 do not affect locomotion until day 33,

therefore they can be further tested as double transgenics with α-synuclein in comparison to Usp8.

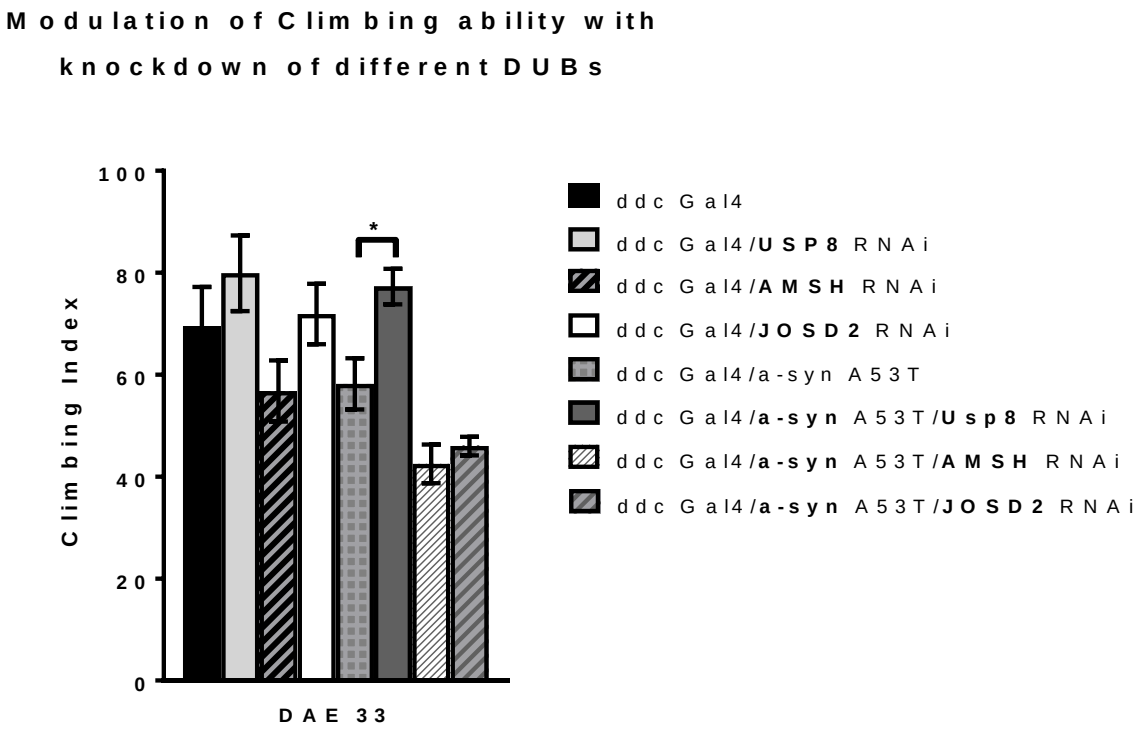


Fig 3.24. Knockdown of AMSH or JosD2 in dopaminergic neurons slightly worsened the A53T mutant α-synuclein phenotype, instead of rescuing it like Usp8 KD did **(A)**. $p<0.05$, n=50

These data show that knockdown of AMSH, the second endosomal DUB or JosD2 which is unrelated to the pathway of interest do not rescue the α-synuclein phenotype.

3.4.4. Quantitation of TH-positive dopaminergic neurons

α-Synuclein expression in the fly brain was shown in several studies to reduce the number of dopaminergic neurons (Davies et al. 2014, Feany, Bender 2000, Auluck et al. 2002). I therefore asked whether the rescue of the locomotor defect by Usp8

KD was also associated with protection against the loss of dopaminergic neurons. To this end, I dissected adult male fly brains at 25 days post-eclosion using the protocol described in Wu and Luo 2006 (Wu, Luo 2006). Dopaminergic neuronal clusters were identified and counted. I found that Usp8 knockdown in dopaminergic cells reduced the loss of the TH-immunoreactive PPM1/2 cluster of dopaminergic neurons **(Fig. 3.25)** that is most vulnerable in the *Drosophila* α-synuclein model and typically correlates with the locomotor deficit (Chen, Periquet et al. 2009).

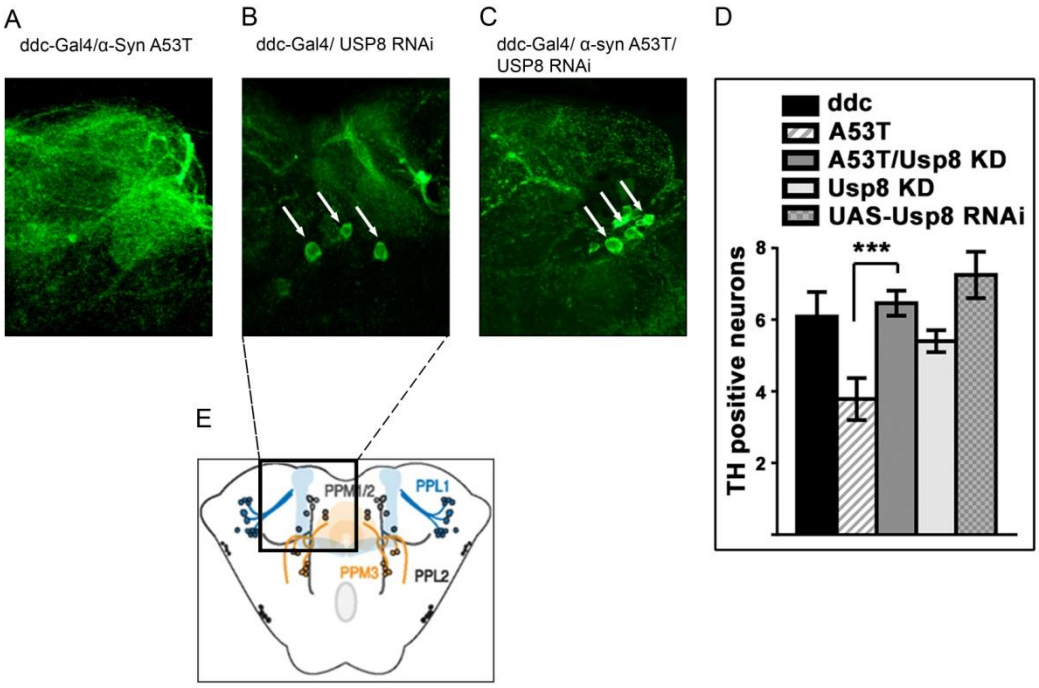


Fig 3.25. Expression of A53T mutant α-synuclein **(A)** but not control constructs (Usp8 KD in itself) **(B)** in dopaminergic neurons (*ddc*-GAL4 driver) led to loss of TH-immunoreactive neurons in the PPM1/2 cluster, which was prevented by concomitant Usp8 knockdown **(C).** PPM1/2 neurons quantified in **(D). (E)** schematic showing the clusters of dopaminergic neurons in the *Drosophila* adapted from White et al. (White, Humphrey et al. 2010) (***$P <$ 0.001), n=50, magnification: 25× with 1.4× digital zoom. TH antibody: Merck Millipore MAB318 Clone LNC1

3.4.5. Survival of single and double transgenic flies

I also asked whether the survival of the flies is affected by α-synuclein expression as previously reported (Haywood, Staveley 2004, Büttner, Habernig et al. 2013). In this assay 10 male flies were kept at 25˚C in each vial, with 3 vials per line. Alive flies were counted every 2 days and changed to a new vial. The experiment was performed twice. The expression of α-synuclein with the ddc Gal4 driver was associated with lower life expectancy in *Drosophila* **(Fig 3.26, Fig 3.27, Fig 3.28, Fig 3.29),** which was rescued by Usp8 KD, AMSH KD and JosD2 KD **(Fig 3.28, Fig 3.29)**. However, since the knockdown of JosD2 and ASMH failed to rescue the toxicity from α-synuclein, it is unlikely that the rescue of the reduced survival comes from rescue of the pathology. Thus, it is likely that this effect is due to genetic background changes.

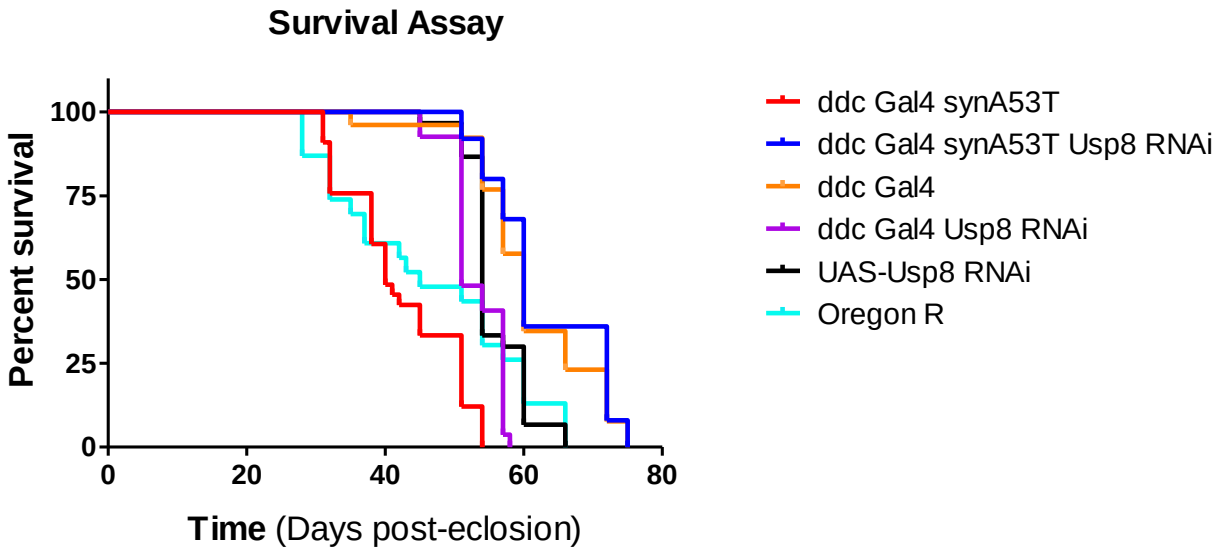


Fig 3.26 Kaplan-Meier survival curve in *Drosophila.* The survival assay showed a decreased lifespan of α-synuclein flies, which was rescued with concomitant knockdown of Usp8. Oregon R is a WT fly. n=70

	Oregon R	ddc Gal4	ddc Gal4/ α-syn A53T	UAS Usp8 RNAi	ddc Gal4/ Usp8 RNAi	ddc Gal4/ α-syn A53T/ Usp8 RNAi
Median Survival (Days)	45	60	40	54	51	60

Fig 3.27. Median survival. The expression of α-synuclein reduces the *Drosophila* lifespan to 40 days, which is increased to 60 days by the concomitant knockdown of Usp8. Calculated from the Kaplan Meier curve using Prism (GraphPad). n=70

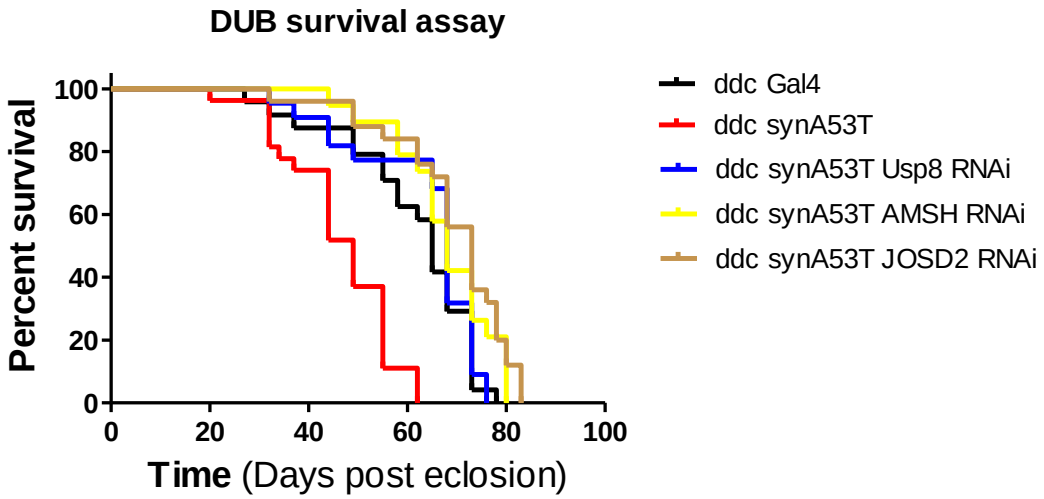


Fig 3.28 Kaplan-Meier survival curve. AMSH and JosD2 knockdown also rescued the locomotive defect caused by α-synuclein expression. n=70

	ddc Gal4	Ddc Gal4/ α-syn A53T	Ddc Gal4/ α-syn A53T/ Usp8 RNAi	Ddc Gal4/ α-syn A53T/ AMSH RNAi	Ddc Gal4/ α-syn A53T/ JosD2 RNAi
Median Survival (Days)	65	49	68	68	73

Fig 3.29. Median survival. The expression of α-synuclein reduces the *Drosophila* lifespan to 49 days, which is increased to 68 days by the concomitant knockdown of either Usp8 or AMSH or JosD2. Calculated from the Kaplan Meier curve using Prism (GraphPad). n=70

3.4.6. Measurement of Dopamine levels

In Parkinson's disease, there is a decrease in dopamine levels in the striatum (Dauer, Przedborski 2003). In order to assess if this is also the case in the flies, I tested the dopamine levels in *Drosophila* heads in different lines.

To this end, I used the LDN Dopamine ELISA kit as per protocol. My analysis suggests that in the *Drosophila* there is no decrease of dopamine levels **(Fig 3.31)** upon expression of α-synuclein. This may be related to the sensitivity of the assay that may not detect changes from loss of a small number of neurons. Interestingly, there was a dramatic increase in dopamine levels in head lysates where Usp8 was knocked down in the dopaminergic and serotonergic neurons **(Fig 3.30).** This 3-fold increase in dopamine levels disappears with concomitant expression of α-synuclein. As investigated above, this increase in dopamine levels is not associated with an enhanced locomotion of the specific line (not enhanced when compared to control

flies). This increase in dopamine levels is going to be further discussed in the discussion.

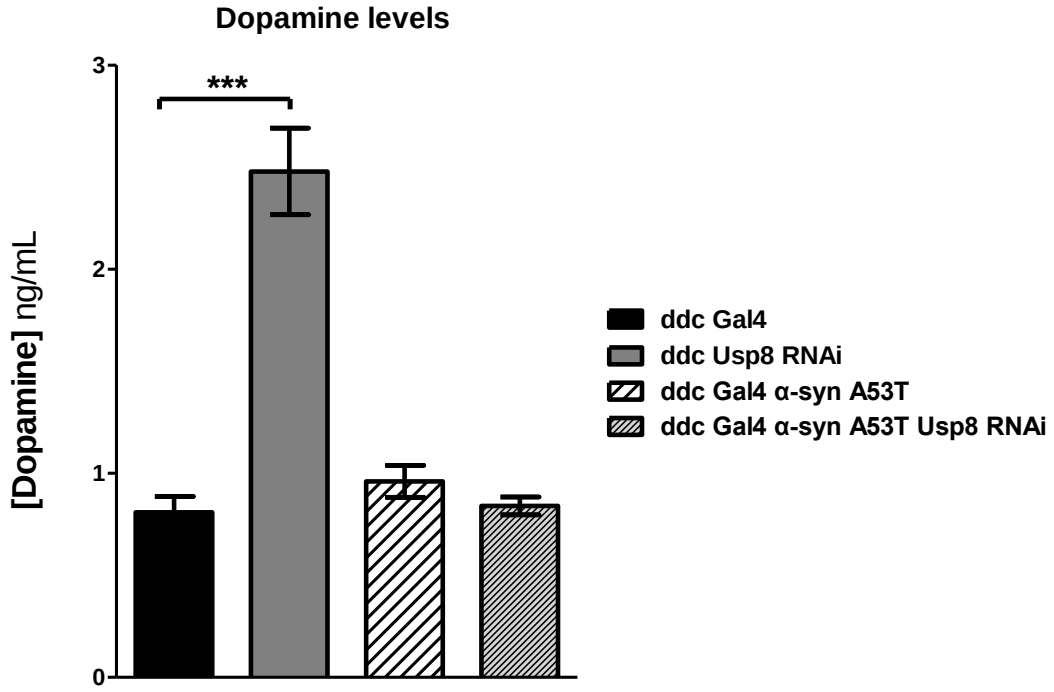


Fig 3.30 Dopamine levels as assessed by ELISA. The expression of α-synuclein in the dopaminergic neurons does not cause a decrease in dopamine levels. There is a great increase in dopamine levels in knockdown of Usp8. P<0.001 2-tailed unpaired t-test, n=3 biological replicates

3.5. Discussion

The *Drosophila* model of Parkinson's disease has been extensively used to study α-synuclein pathobiology. It has proven very useful in investigating second site modifiers such as Parkin and Pink1. Taking advantage of this model, I investigated *in vivo* the effect of Usp8 knockdown on α-synuclein. Usp8 knockdown rescued the rough eye phenotype, the locomotor defect and the loss of dopaminergic neurons caused by α-synuclein toxicity. The fact that Usp8 knockdown also rescues the

roughness caused by α-synuclein 120 means that Usp8 doesn't act via the C-terminus of α-synuclein, like Nedd4 does.

Importantly, the effects of Usp8 were specific for α-synuclein, not affecting the toxicity of other toxic proteins such as expanded Ataxin 3 or Huntingtin. This might suggest that there is a degradation pathway involving α-synuclein and Usp8 which is different from poly-glutamine expansion diseases. Light is shed from the fact that K-63 association with Huntingtin increases its aggregation and decreases its degradation. In contrast, association of HTT with K-48 ubiquitin chains increase its proteasomal degradation (Bhat, Yan et al. 2014). In our case, Usp8 knockdown could increase the K-63 ubiquitination of HTT and thus increase its aggregation, decreasing its breakdown. In addition, knockdown of Usp7, Usp14, Usp47 and JosD2 did not rescue the rough eye phenotype. When expressed in the dopaminergic neurons of the fly, RNAi against Usp7 and Usp14 *per se* reduced the climbing ability of WT flies, whereas knockdown of AMSH and JosD2 did not cause any locomotive defect. I therefore generated double transgenic flies with synuclein and either AMSH or JosD2 knockdown and found that unlike the knockdown of Usp8, they do not rescue the locomotive defect. This makes the interaction between Usp8 and α-synuclein stand out from other DUBs tested. Importantly, AMSH is the second endosomal DUB. The lack of effect from knocking down this specific enzyme could signify that the effect from Usp8 knockdown is not exclusively mediated through its endosomal association.

My findings provide corroborative evidence that Usp8 knockdown is beneficial in this model organism of synuclein toxicity and thus potentially in PD pathomechanisms. This benefit may be due to direct interaction between Usp8 and α-synuclein and/or an indirect effect. In **Chapter 4** I discuss evidence of a direct

interaction between the two proteins; therefore, the discussion here will not be focused on their relationship. Apart from a direct relationship, the data suggests a relation between Usp8 KD and dopamine levels; thus, this will be discussed in more detail here.

An unexpected finding in my studies was that Usp8 knockdown *per se* increased dopamine levels, though this increase was abolished with concomitant expression of α-synuclein. However, the latter needs further investigation as it is of furthermost importance. It may be that in addition to protection against α-synuclein toxicity, which is independent of the cell-type, this finding raises the exciting possibility that Usp8 may also be involved in dopamine homeostasis. This is important because (a) the major clinical features of PD are due to dopamine deficiency and (b) current dopamine replacement therapies are limited by side-effects. In this context, modulation of dopamine levels by Usp8 could be useful for symptomatic as well as neuroprotective therapies. An important consideration at this stage is whether this phenotype is solely due to the effect of dopamine in neural networks involved in locomotion and relevant to the mammalian nervous system.

One theory states that in PD, defective dopamine biogenesis produces O_2^- -a superoxide anion-, dopamine auto-oxidation produces dopamine-quinones and dopamine breakdown produces toxic metabolites such as DOPAL, which are all toxic for the cells (Goldstein, Sullivan et al. 2013). The dopamine metabolic pathway involves dopamine production from L-Tyrosine, packing it into vesicles by VMAT-2, release in the synaptic cleft and the activation of its receptors. It is then either taken up by the high affinity DAT or the low affinity PMAT transporters (and repackaged in vesicles by VMAT-2) or broken down by enzymes such as COMT, MAO and ALDH**.** (**Fig 3.31**)

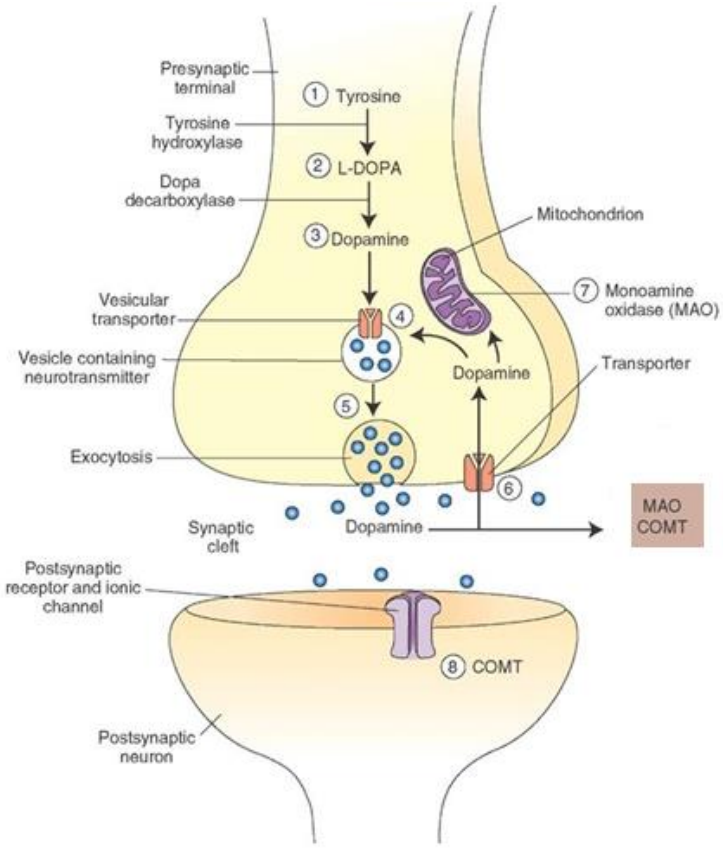


Fig. 3.31 Dopamine metabolic pathway[2]

Reduced $VMAT_2$ activity would shift dopamine metabolism towards breakdown instead of storage and thus would lead to the production of toxic metabolites. In fact, in the putamen of PD brains elevated DOPAL reflects an 89% decreased vesicular uptake of cytosolic dopamine and a 70% decreased detoxification by ALDH (Goldstein, Sullivan et al. 2013). Furthermore, it is known that dopamine-associated α-synuclein is more toxic and more resistive to breakdown, which could also contribute to PD pathology (Martinez-Vicente, Talloczy et al. 2008). My findings could potentially be linked with these hypotheses. For example, it could be that Usp8 increases the production of dopamine (e.g. through upregulating

2 Adapted from: http://what-when-how.com/wp-content/uploads/2012/04/tmp1472_thumb1.jpg

Catecholamine Up (Chaudhuri, Bowling et al. 2007, Wang, Ferdousy et al. 2011)) and shift its levels from cytosolic to vesicle-bound forms (via increasing $VMAT_2$). However, this is purely a hypothesis that would need investigation.

The breakdown of dopamine generates toxic products of metabolism, thus causing cellular toxicity. Some studies have shown that treatment with L-Dopa is toxic to dopaminergic neurons following the activation of oxidative toxic pathways by dopamine (Stokes, Hastings et al. 1999, Pardo, Mena et al. 1995, Walkinshaw, Waters 1995). This is very interesting as L-dopa is the most commonly used compound offered to Parkinson's disease patients for symptomatic treatment. However, dopamine elevation *per se* is actually beneficial for neuron survival. It has been documented that flies with enhanced dopamine production are protected against paraquat exposure instead of being at higher risk from dopamine oxidative stress (Chaudhuri, Bowling et al. 2007). In addition, flies with compromised dopamine synthesis are more susceptible to paraquat stress (Chaudhuri, Bowling et al. 2007). Furthermore, overexpression of the *Drosophila* orthologue of VMAT2 rescues the degeneration caused by mutant Parkin overexpression and its knockdown exacerbates this degeneration (Sang, Chang et al. 2007). This indicates that free cytoplasmic dopamine worsens the phenotypes of neurodegeneration in Parkin mutant flies. It has also been shown that reduced vesicular storage/reuptake of dopamine by knocking down VMAT-2 causes progressive nigrostriatal neurodegeneration (Caudle, Richardson et al. 2007), suggesting that promoting the uptake of dopamine in vesicles after internalization reduces its cytosolic exposure to oxidants and thus preventing neurodegeneration.

In addition to effects on neural degeneration, dopamine also has effects on locomotion. Previous studies have shown that in *Drosophila*, increased dopamine

levels cause increased locomotor activity (Yellman, Tao et al. 1997). Accordingly, pharmacological or genetic manipulation of the dopaminergic system causes hyperactivity if dopamine levels are increased or hypoactivity if they are decreased (Draper, Kurshan et al. 2007, Ueno, Masuda et al. 2012, Pendleton, Rasheed et al. 2002). For example, overexpression of DVMAT-A (*Drosophila* orthologue of VMAT2) increases locomotion that can be reversed by reserpine (blocking DVMAT activity) and haloperidol (DA receptor antagonist) (Chang, Grygoruk et al. 2006).

Dopamine levels are essential for locomotion and signaling, but to avoid toxicity, dopamine should be kept intracellularly in its storage vesicles. This is why when VMAT2 is overexpressed, it reduces dopamine toxicity and enhances locomotion. Therefore, by increasing simultaneously the dopamine levels and VMAT expression neurotoxicity and locomotive defects could be prevented. However, this needs further investigation. It will be interesting in the future to examine biochemically the localization of dopamine in order to assess what percentage is cytoplasmic (not in vesicles) and what percentage is chemically dopamine quinones (toxic species).

One theory that warrants further investigation could support that Usp8 knockdown increases total dopamine levels but might also increase VMAT-2 activity (e.g. by enhancing its transcription or increase its activity allosterically) which reduces dopamine breakdown thus decreasing cytosolic toxic metabolites such as superoxide anions, dopamine quinones and DOPAL therefore reducing oxidative stress and cellular toxicity. By reducing cytosolic dopamine, it could also prevent the association of toxic dopamine metabolites (quinones) to α-synuclein and thus facilitate its breakdown, especially under the pathological condition of PD. This

action of Usp8 would be in addition to a direct effect of Usp8 on α-synuclein, as will be investigated in chapter 4.

The ddc Gal4/α-synuclein A53T/UAS-Usp8 RNAi flies have comparable lifespan to ddc Gal4, to ddcGal4/UAS-Usp8 RNAi and to wild type flies, but the ddc Gal4/α-synuclein A53T flies have significantly lower lifespan. It is well documented that *Drosophila* models of Parkinson's disease exhibit reduced lifespan. Interestingly, this is the case both in familial PD mutations (e.g. GBA mutations (Davis, Trinh et al. 2016)) and in sporadic models (α-synuclein phosphorylation (Zhang, Xie et al. 2015)). Therefore, my data is consistent with the literature findings. Importantly, this reduction in lifespan is rescued by Usp8 knockdown. **Figure 3.16** showed that concomitant Usp8 knockdown lowers the levels of α-synuclein, therefore this is consistent with also rescuing the shortening lifespan.

Furthermore, Usp8 knockdown appeared to be more specific in rescuing the α-synuclein toxicity than the knockdown of the other 5 DUBs tested. It is possible that various DUBs will be involved in the pathogenesis of PD, each to different extent creating a biochemical signature. It will be interesting to identify them and create compounds targeting different DUBs to different extent. It is known that DUB inhibitors are not very specific in distinguishing between individual DUBs (Ritorto, Ewan et al. 2014). In this way, one compound will be able to inhibit our desired cocktail of DUBs to different extent and be effective. This is the ultimate aim following this study. However, it is beyond the scope of my thesis to explore further the relative effect of all DUBs on α-synuclein toxicity.

One limitation of using *Drosophila* as a model for PD is that *Drosophila* does not have endogenous α-synuclein and therefore the mechanism by which it handles this

protein may significantly differ from mammalian biology. In addition, *Drosophila* being a simpler organism than humans, it has less genes. About 75% of human genes have a *Drosophila* homologue/orthologue (Reiter, Potocki et al. 2001). Therefore, one specific DUB that has a single function in humans, it might have multiple functions in *Drosophila* to compensate for the absence of another DUB that is not expressed in *Drosophila*. Thus, it might be that in *Drosophila,* Usp8 has multiple functions that are absent in mammals. Therefore, my results must be recapitulated in mammalian cells to verify our hypothesis. However, not only have I used the *Drosophila* as a model of molecular interactions in α-synuclein toxicity but this model also recapitulates many characteristics of the human pathology. Important examples are the age-dependent phenotype of neuronal loss and locomotor defects, the accumulation of α-synuclein aggregates and the predilection of neurotoxicity for dopaminergic neurons. In addition, I have driven α-synuclein expression in 2 different cell types in order to overcome any issues with results being cell-type-specific.

On the other hand, Drosophila as well as other simple model organisms offer a tractable model system to investigate evolutionarily conserved pathways in PD pathomechanisms that are ideally complemented by experiments in mammalian cells. For example, yeast is very good in studying cell autonomous processes and molecular interactions, having a very high replication time. Yeast has been used to elucidate interactions between proteins and second site modifiers in neurodegeneration and more specifically in Parkinson's disease (Miller-Fleming, Giorgini et al. 2008, Outeiro, Lindquist 2003). For example, Suzan Lindquist using yeast identified druggable interactors of the E3 ligase Nedd4, which ameliorates α-synuclein toxicity and specifically the compound NAB (Tardiff, Jui et al. 2013).

The *Drosophila* or *Caenorhabditis elegans* can be used to study cell/tissue interactions and then mammals such as mice or rats can be utilized to come closer to human physiology. Therefore, a multi-organism approach in PD research would be ideal, screening or investigating second site modifiers in yeast, investigating and validating them in *Drosophila* and further validating them in mice/rats (Bier 2005).

3.6. Conclusions

In summary, in this chapter I investigated the *in vivo* functional interaction between α-synuclein expression and Usp8 knockdown. For this purpose, I used the well-documented *Drosophila melanogaster* model of Parkinson's disease.

I have optimized the previously documented readout of α-synuclein toxicity when ectopically expressed in the *Drosophila* eye and the dopaminergic neurons. I also demonstrated that expression in dopaminergic neurons causes an age-dependent locomotive defect. Concomitant knockdown of Usp8, but not of other DUBs tested, rescued these phenotypes. Importantly, the effect was specific for α-synuclein as opposed to poly-glutamine repeat diseases. In addition, I found that Usp8 knockdown increased dopamine levels which could have implications for Usp8 in dopamine metabolism.

These data show for the first time that reducing the levels of a deubiquitinase which is a druggable target, can be beneficial in an *in vivo* model of α-synuclein toxicity.

4. Knockdown and overexpression of Usp8 in human cell lines by genetic manipulation

4.1. Introduction

Usp8 biology

Usp8 is expressed in many tissues, including the brain. Its expression is especially prominent in the substantia nigra, hippocampus, septum, preoptic nucleus, ventral tegmental area, cochlear nucleus, cerebellar granular cells and the periventricular nucleus of the hypothalamus (Bruzzone, Vallarino et al. 2008). This differential expression might represent a specialized function of Usp8 in different cellular processes. Various research groups have employed *in vitro* and *in vivo* techniques to elucidate the function of Usp8, which is going to be discussed in this introduction.

Usp8 has recently been implicated in neurodegenerative diseases, such as Parkinson's disease, Amyotrophic lateral sclerosis (ALS) and Alzheimer's disease. Usp8 was found to be involved in Parkin-dependent mitophagy. Parkin auto-ubiquitinates itself in many lysine positions, having an auto-inhibitory effect. Usp8 de-ubiquitinates Parkin at K6 and removes this auto-inhibition, allowing it to access PINK1 and initiate mitophagy of defective mitochondria (Durcan, Tang et al. 2014). Usp8 knockdown delays the recruitment of Parkin to the damaged mitochondria via its deubiquitinating role and not due to ESCRT destabilization or any other effects of Usp8 on mitochondria. This is because delayed Parkin recruitment was not observed when STAM was knocked down and the structure, dynamic and depolarization of mitochondria were not affected in Usp8 knockdown (Durcan, Tang et al. 2014). The above findings might suggest that Usp8 knockdown dysregulates mitophagy, however there is another theory that could apply. Usp8

knockdown would increase Parkin auto-ubiquitination and thus rescue it from proteasomal or lysosomal degradation, enhancing its bioavailability (Shang, Deng et al. 2005, McLelland, Soubannier et al. 2014). Thus, the exact mechanism of the effect of Usp8 in mitophagy via Parkin recruitment would need to be further investigated. It is important to mention that these findings have only been tested in cell culture, did not have a sustained effect with time and have not been validated *in vivo*. Instead, other DUBs have been shown to be involved in mitophagy *in vivo* (e.g. Usp30 (Bingol et al. 2014) and Usp15 (Cornelissen, Haddad et al. 2014)). In addition, Usp8 knockdown has been shown to be protective *in vivo* in *Drosophila.* Parkin normally ubiquitinates mitofusin, enhancing the mitochondrial interaction with the ER and contributes to physiological mitophagy. In Parkin deficient cells, the interaction between the ER and mitochondria is compromised. Usp8 knockdown in *Drosophila* increased the levels of mitofusin enhancing mitochondrial fission. This effect was even able to rescue the locomotive defects caused by PINK1 mutations in *Drosophila*[3].

In certain types of motor neuron degeneration, oligodendroglia are heavily immunostained with Usp8, which might suggest a role of Usp8 in neuron-glia relationship in neurodegeneration (Paiardi, Pasini et al. 2014). In addition, the same authors also identified partial colocalization of Usp8 with EEA-1 (early endosomal marker) and with Vps54 (part of the retrograde recycling mechanism), thus suggesting a role for Usp8 in early endosomal trafficking and retrograde transport (Paiardi, Pasini et al. 2014).

Importantly, it was recently shown that USP8 knockdown increases BACE1 ubiquitination (Yeates, Tesco 2016). BACE1 is the enzyme involved in the rate-

[3] https://www.michaeljfox.org/foundation/grant-detail.php?grant_id=1445

limiting step in the production of Amyloid-β (Aβ) in Alzheimer's disease (Hussain et al. 1999, Sinha et al. 1999, Vassar et al. 1999, Yan et al. 1999, Lin et al. 2000). Thus, Usp8 seems to be involved critical pathways that are linked to pathology in diverse neurodegenerative diseases.

Using the CompPASS platform as explained above, Sowa et al identified that Usp8 is involved in translation, development and ubiquitin processing and that it is primarily located in the cytoskeleton (Sowa, Bennett et al. 2009). Usp8 has also been localized around microtubules in neuronal axons and is especially expressed pre-synaptically. Despite these diverse associations, the principal function of Usp8 is in the endosomal pathway. Usp8 contains an MIT domain for endosomal association and interacts with components of the ESCRT-0 (STAM) and ESCRT-III (CHMP) complexes stabilizing HRS and STAM (McCullough, Row et al. 2006, Agromayor, Martin-Serrano 2006, Tanaka, Kaneko et al. 1999, Kato, Miyazawa et al. 2000, Tsang, Connell et al. 2006, Ross, Lindsay et al. 2007, Solomons, Sabin et al. 2011). In flies Usp8 knockdown has been shown to result in enlarged endosomes (Zhang, Du et al. 2014). However, in a study by Mukai et al in 2010, the authors suggest that the enlarged endosomes that they themselves also found represent accelerated membrane trafficking from early to late endosomes rather than an endosomal defect (Mukai, Yamamoto-Hino et al. 2010, Seto, Bellen 2006). In our hands, using HEK293T cells (Alexopoulou et al. 2016) and iPSC-derived neurons (unpublished lab observations) Usp8 knockdown did not lead to enlarged endosomes. Deubiquitination by Usp8 in the endosomal sorting has been a matter of debate as it was suggested to either stabilize cargo such as EGF receptor or promote its trafficking to lysosomes: For example, deubiquitination by Usp8 stabilizes certain receptors (e.g. Frizzled, Smoothened, EGFR and MET) and promotes the

degradation of others (e.g. $ErbB_3$). In the case of Frizzled, the receptor for the WNK pathway, removal of ubiquitin by Usp8 recycles Frizzled to the plasma membrane, enhancing the cellular response to WNK signaling. It also enhances hedgehog (Hh) signaling by halting Smoothened ubiquitination and preventing its degradation (Xia, Jia et al. 2012). Furthermore, the endolysosomal breakdown of tyrosine kinase receptors is an essential part for the termination of their signaling when activated. In the case of EGFR (Epidermal Growth Factor Receptor), Usp8 has been shown to deubiquitinate it on endosomes and thus recycle it back to the plasma membrane instead of breaking it down (Mizuno, Iura et al. 2005). A similar effect is observed with the E3 Ligase Nrdp1 which ubiquitinates and promotes the breakdown of ErbB3 receptors. Usp8 mediates Nrdp1 stabilization, thus indirectly contributing to the breakdown of ErbB3 receptors (Wu, Yen et al. 2004).

The clinical significance of inhibition of Usp8 is evident both with EGF and MET receptors. One common treatment strategy in non-small cell lung cancer is EGFR inhibition, e.g. with gefitinib. However, eventually resistance occurs in the majority of patients. Byun et al used genetic and pharmacological Usp8 inhibition showing that lower Usp8 levels increased the sensitivity to gefitinib and killed the cancerous cells, but had very little toxicity to normal cells (Byun, Lee et al. 2013). Furthermore, a portion of corticotroph adenomas that cause Cushing's disease were found to have an activating Usp8 mutation which promoted the stability of EGF-R and thus EGF signaling causing pro-opiomelanocortin promoter activation and Cushing's disease (Reincke 2015). These somatic mutations were in the 14-3-3 protein-binding motif of Usp8 and shown to activate the enzyme by (i) preventing 14-3-3 binding-induced inhibition and/or (ii) promoting cleavage by a yet unknown protease. (Reincke 2015). The fact that cleavage of Usp8 enhances its activity is

very important, since so far studies haven't focused on different Usp8 species and their specific effects on the protein/pathway studied. Thus, this is an important consideration for future studies.

In addition, MET is clinically significant since it promotes tumour invasion. A MET antibody, SAIT301, promotes LRIG1 ubiquitination which in turn degrades MET and itself, through the endolysosomal route following an interaction with Hrs. Usp8 was found to have an antagonistic action to SAIT301 by deubiquitinating LRIG1. Thus, a possible therapeutic strategy for certain cancer invasions would be SAIT301 combined with Usp8 inhibitors (Oh, Lee et al. 2014).

Usp8 has also been shown to deubiquitinate proteins unrelated to the endocytic pathway, such as TDP-43 (Hans, Fiesel et al. 2014) and CLOCK proteins (Hans, Fiesel et al. 2014, Luo, Li et al. 2012). In addition, Usp8 has anti-inflammatory effects. Luteolin inhibits microglial neuroinflammation by increasing the production of Usp8. Usp8 was found to decrease the levels of LPS (lipopolysaccharide), COX (cyclooxygenase) and prostaglandins (Zhu, Bi et al. 2015).

Usp8 is an essential gene as evidenced from early studies in Usp8 knockout mice. Such animals had defective embryogenesis and embryonic lethality (Niendorf, Oksche et al. 2007). This suggests that Usp8 has a very important function in development of certain tissues. Conditional knockouts in adult mice resulted in liver failure (Niendorf, Oksche et al. 2007). However, these models involved complete gene deletion in development or even during adult life. As in other examples, (e.g. proteasome inhibitors in the treatment of myeloma), it is possible that partial Usp8 inhibition within the appropriate therapeutic window may lead to desirable

beneficial effects in certain disease states, whilst maintaining its core essential functions intact.

Considering the neuropathological data from the preliminary studies, it will be interesting to assess the effect of Usp8 on α-synuclein pathology. If Usp8 proves to be a valid target for addressing α-synuclein pathology, its inhibition could prove therapeutic in Parkinson's disease.

Aims of this chapter:

One of the main aims of my thesis is to investigate whether Usp8 has a direct effect on α-synuclein levels. In this chapter I have attempted to address the following specific questions:

1. Is there a direct interaction between Usp8 and α-synuclein?

2. Does Usp8 deubiquitinate α-synuclein?

3. Does Usp8 regulate the degradation of α-synuclein in human cells by the proteasome or the lysosome?

4.2. Overexpression of Usp8 in cell culture

4.2.1. Colocalization of Usp8 with α-synuclein

To investigate a possible direct interaction between Usp8 and α-synuclein several methods could be adopted. Our lab utilized 3 of them, 2 were performed by 2 postdocs and I performed the third one. Firstly, Usp8 and α-synuclein colocalized by triple labeling in human iPS-derived dopaminergic neurons with TH (dopaminergic neurons), Usp8 and α-synuclein, as shown in **Fig. 4.1.**

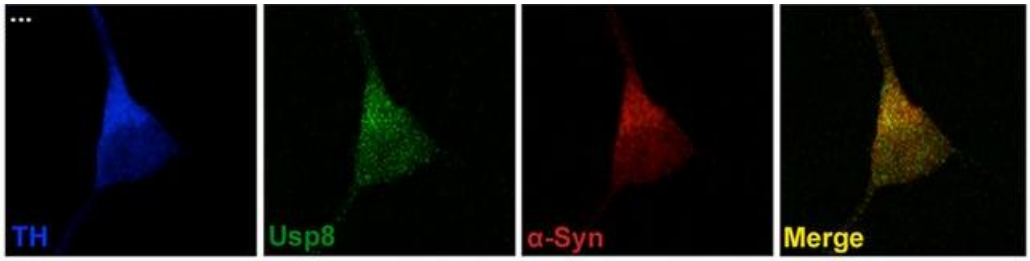


Fig. 4.1. Localization of Usp8 in relation to α-synuclein in human iPSc-derived dopaminergic neurons: triple labeling of neurons with TH (for detection of dopaminergic neurons; first column, blue), Usp8 (second column, green), and α-synuclein (third column, red). *n* = 50 neurons per condition. Images obtained by Dr Myriam Elschami. Antibodies used: TH: PA1-4679, Usp8: HPA004869, α-synuclein: Syn1

In addition, bimolecular fluorescence complementation assay (BiFC) was used to investigate the spatial relation between Usp8 and α-synuclein. BiFC is one method to determine protein interaction, where N- and C-terminal ends of fluorescent proteins are conjugated to each of the two proteins of interest. If the reporter protein fluoresces, it signifies that it has regained its 3D structure, thus the two proteins of interest must be in close proximity to allow for the two fragments to interact. This

experiment performed in the lab by Dr Rebecca Perrett, produced spatial evidence of Usp8–α-synuclein interaction **(Fig. 4.2).**

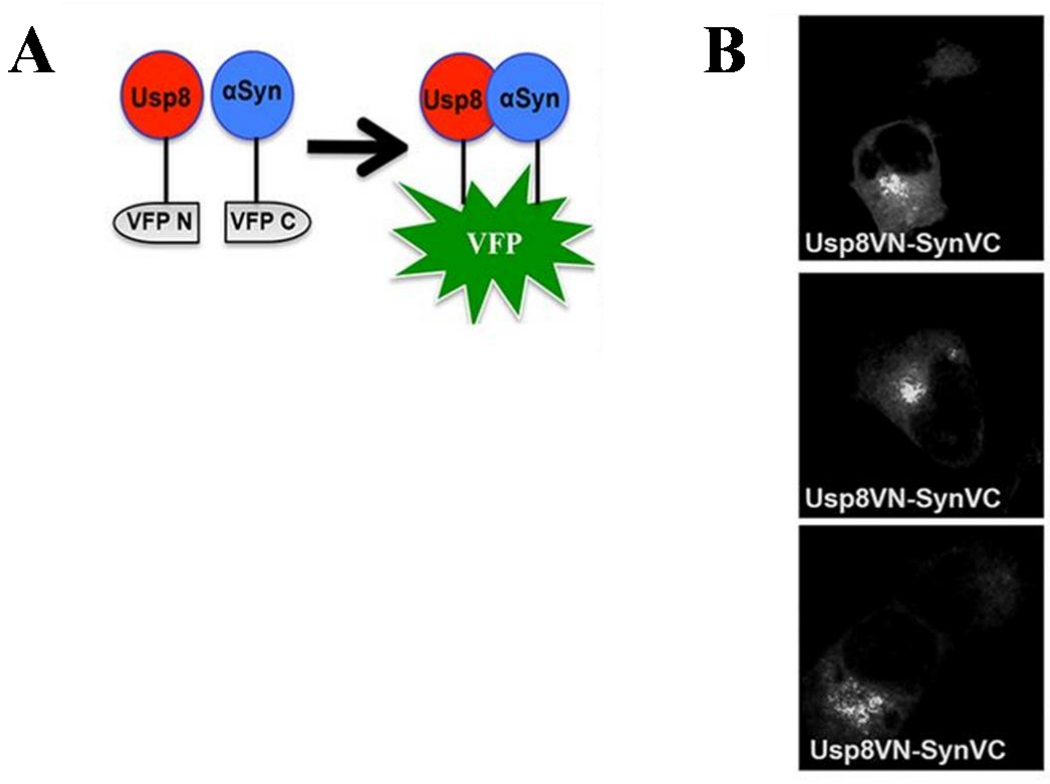


Fig. 4.2. Bimolecular fluorescence complementation assay. Usp8 was tagged to the N-terminus of Venus fluorescent protein and α-synuclein to its C-terminus **(A).** Colocalization between α-synuclein and Usp8 as documented from resulting fluorescence of different cells **(B).** Experiment performed by Dr Rebecca Perrett.

Apart from immunofluorescence, a third way to investigate a potential interaction between Usp8 and α-synuclein is with immunoprecipitation. Therefore, I first co-transfected FLAG-tagged wild-type Usp8 ($Usp8^{WT}$) or catalytically inactive (Cys786 to Ala) Usp8 ($Usp8^{CA}$) or an empty vector and untagged human α-synuclein in HEK-293T cells. When overexpressed, α-synuclein was immunoprecipitated from lysates of cells expressing wild-type or catalytically inactive Usp8 with anti-FLAG–tagged antibodies but not from lysates of a control

expressing empty vector **(Fig. 4.3).** Overexpression was required because of the low levels of α-synuclein that HEK-293T cells express endogenously. Of note, the amount of α-synuclein that co-immunoprecipitated with Usp8 was relatively low because such interactions are transient.

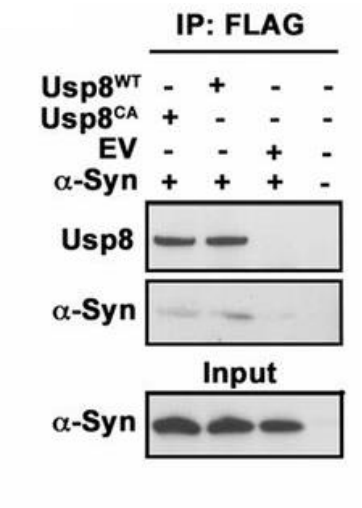


Fig. 4.3. Representative immunoblot showing co-immunoprecipitation of transiently expressed α-synuclein with FLAG-tagged $Usp8^{WT}$ or $Usp8^{CA}$ but not EV in HEK-293T cells. IP, Immunoprecipitation, Antibodies used: α-synuclein: C20, Usp8: HPA004869

4.2.2. Effects on α-synuclein ubiquitination

The experiments above suggest that the two proteins interact but do not prove any direct functional significance. Therefore, I next investigated the effects of Usp8 overexpression on α-synuclein ubiquitination.

I co-transfected FLAG-tagged wild-type Usp8 or catalytically inactive Usp8 or an empty vector and HA-tagged ubiquitin in HEK-293T cells endogenously expressing α-synuclein. I immunoprecipitated with an anti-HA antibody, pulling down all the ubiquitinated proteins and immunoblotted for α-synuclein. These experiments

showed that overexpressed Usp8^{WT} deubiquitinated α-synuclein whereas expression of the catalytically inactive form where the catalytic site cysteine was mutated to alanine, termed Usp8^{CA}, or empty vector did not affect the ubiquitination of α-synuclein. The smears indicate different lengths of polyubiquitinated α-synuclein. The input shows effective overexpression of the Usp8 constructs **(Fig. 4.4).**

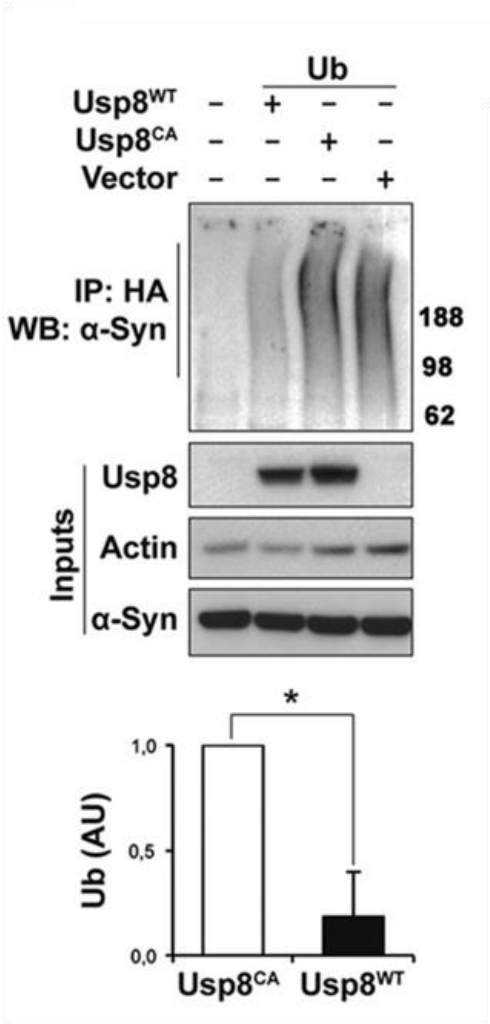


Fig. 4.4. Expression of Usp8^{WT} caused robust deubiquitination of endogenous α-synuclein compared with expression of Usp8^{CA} or empty vector. Quantification of ubiquitinated α-synuclein in Usp8^{WT}- relative to Usp8^{CA}-expressing cells shown as arbitrary units (AU; $*P = 0.0410$, $n = 3$ biological replicates). IP, Immunoprecipitation. WB, Western blot. Antibodies used: α-synuclein: C20, Usp8: HPA004869, Actin: A1978, HA: H3663

To further investigate this interaction and understand the role of Usp8 in α-synuclein biology, I expressed mutant ubiquitin constructs which express only one lysine residue. I used K48 and K63 single lysine ubiquitin constructs (Addgene) which when linked to form chains dictate degradation through the proteasome or the lysosome respectively. Together with the mutated ubiquitin, I co-transfected HEK-293T cells with Usp8 wild type, catalytically inactive (CA) or an empty vector control. All ubiquitinated proteins were pulled down with anti-HA antibody and ubiquitinated α-synuclein was visualized with an anti-α-synuclein antibody (C20). This experiment showed that $Usp8^{WT}$ preferentially deubiquitinated K63 over K48 ubiquitin chains **(Fig. 4.5)**. The smear in the immunoprecipitation panel represents the ubiquitinated α-synuclein. Importantly, anti-ubiquitin staining showed equal ubiquitination of total proteins, suggesting that Usp8 activity against K63 chains on α-synuclein is specific.

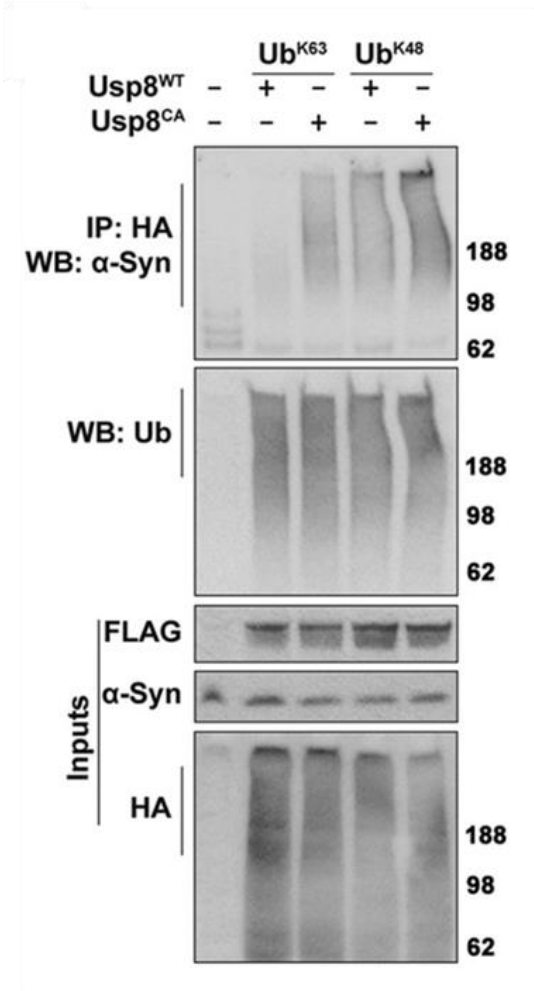


Fig. 4.5. Coexpression of Usp8 with either K63 or K48 single-lysine ubiquitin showed that it deconjugated preferentially K63-linked chains on α-synuclein without any obvious difference in the total amount of ubiquitinated proteins between the immunoprecipitated samples (representative of $n = 3$ biological replicates). Antibodies used: α-synuclein: C20, HA: H3663, FLAG: F725, Poly-ubiquitin: DAKO Z0458

Bimolecular fluorescence studies from our lab showed that at least partly Usp8 and α-synuclein colocalize with Rab proteins which means that at least partly they interact in the endosomal membranes (data not shown). To further investigate whether Usp8 deubiquitination of α-synuclein involves endosomal localization I used a mutant Usp8 construct which lacks the MIT domain ($Usp8^{\Delta MIT}$, generated in the laboratory by Anna Stachowicz). The MIT domain is required for endosomal

localization of Usp8 (Row, Liu et al. 2007). Usp8 $^{\Delta MIT}$ or Usp8^{WT} were overexpressed in HEK-293T cells. The Usp8 $^{\Delta MIT}$ had reduced activity against K63-specific α-synuclein deubiquitination **(Fig. 4.6)** suggesting that endosomal association of Usp8 enhances the deubiquitination of α-synuclein. In addition, expression of Usp7 (kindly provided by Prof Benedikt Kessler), did not deubiquitinate α-synuclein in cells.

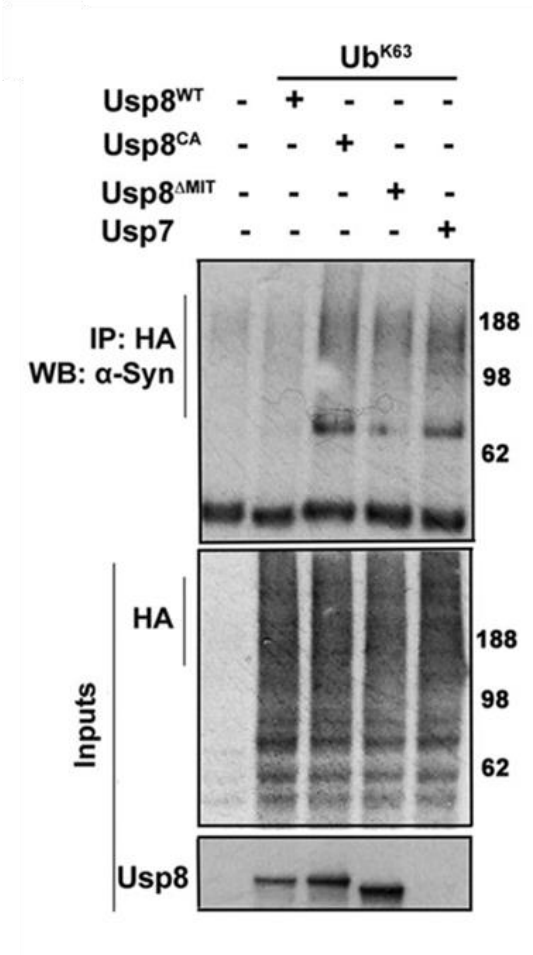


Fig. 4.6. Expression of a deletion mutant of Usp8 lacking the MIT domain required for endosomal localization (Usp8$^{\Delta MIT}$) reduced its activity against K63-linked ubiquitin chains on α-synuclein compared with Usp8^{WT}. Usp7 had reduced activity against α-synuclein compared with Usp8 in cells (n = 5 biological replicates). Antibodies used: α-synuclein: C20, HA: H3663, Usp8: HPA004869

4.2.3. Measurements of α-synuclein rate of degradation

To further investigate whether the deubiquitination of α-synuclein is linked to degradation I measured the effect of Usp8 on endogenous α-synuclein half-life. To this end, I performed a cycloheximide chase experiment of α-synuclein. Cycloheximide blocks mRNA translation on ribosomes and thus inhibits protein synthesis which enables the assessment of the rate of clearance of proteins. I first transfected FLAG-tagged Usp8^{WT} and empty vector (EV) in HEK-293T cells and added the cycloheximide to stop protein synthesis, 24h after transfection. Cells were lysed at times 0 and 7 hours post-cycloheximide addition and immunoblotted for α-synuclein. This assay showed that Usp8^{WT} significantly prolonged the half-life of α-synuclein when compared to empty vector **(Fig. 4.7).**

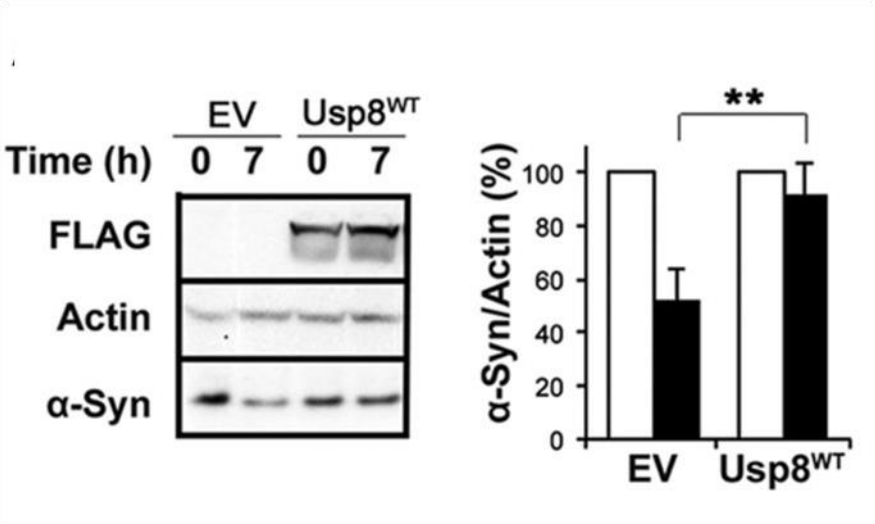


Fig. 4.7. Cycloheximide chase of endogenous α-synuclein showed that its rate of clearance was reduced at 7 h when wild-type Usp8 was expressed in HEK-293T cells (**P = 0.0091, n = 3 biological replicates). Antibodies used: α-synuclein: C20, FLAG: F725, Actin: A1978

In order to gain more information about the mode of interaction between Usp8 and α-synuclein, I transiently expressed in HEK 293T cells α-synuclein fragments

together with Usp8^{WT} or EV. More specifically, I expressed full length (FL), and the carboxyl-terminal truncated forms α-synuclein 1-130 amino acids (130) and α-synuclein 1-120 amino acids (120). Previous work in the lab had shown that the C-terminal region (approximately amino acids 120-133) is required for ubiquitination by the E3 ligase Nedd4. The constructs were generated in the laboratory by Anna Stachowicz. In these experiments, the half-life of overexpressed α-synuclein was much prolonged compared to the endogenous protein as expected from previous studies and was not degraded significantly even at 14h post cycloheximide treatment. However, it is noteworthy that under these conditions, compared to empty vector, Usp8^{WT} expression increased the baseline levels of full-length α-synuclein but not the c-terminal fragment, especially α-synuclein 1-120 **(Fig 4.8.)**.

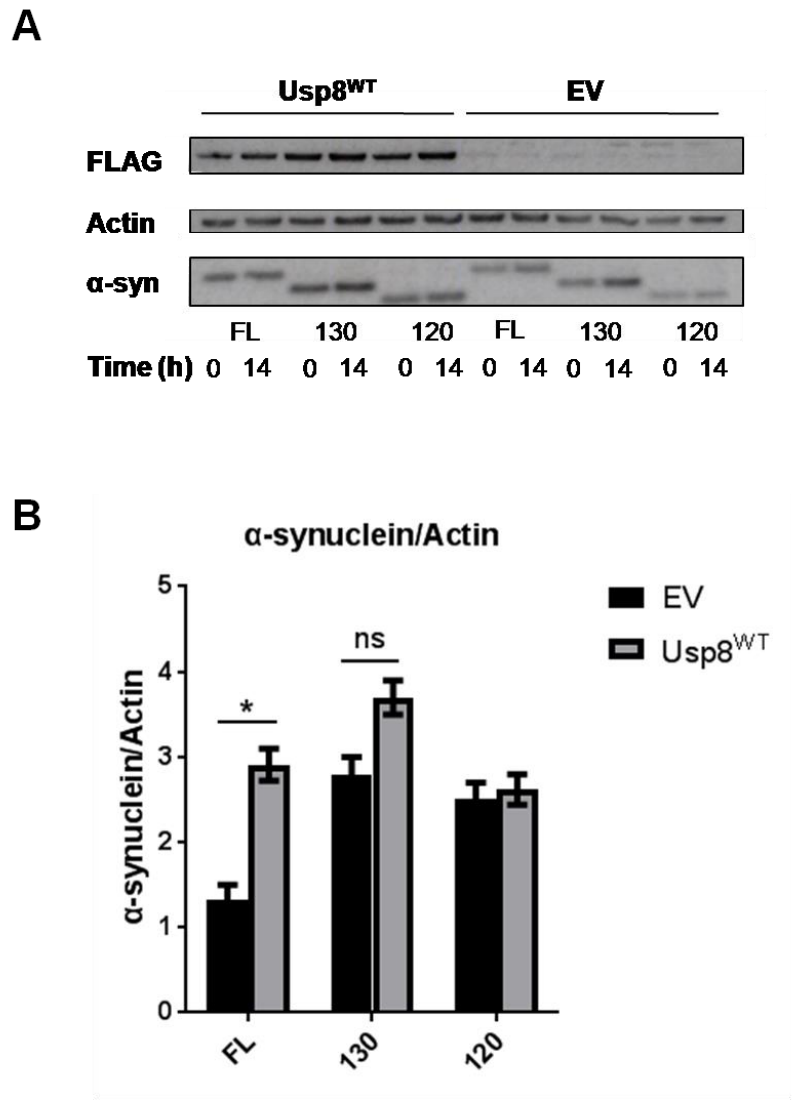


Fig. 4.8. HEK-293T cells were co-transfected with either Usp8^{WT} or Empty vector (EV) and either α-synuclein Full length (FL), synuclein 1-130 amino acids (130) or α-synuclein 1-120 amino acids (120). **(A)** Overexpression of Usp8^{WT} increased the levels of α-synuclein FL. p=0.0258. **(B)** Representative blot including baseline (Time 0) and 14 hours post cycloheximide (Time 14). n=3 biological replicates. Antibodies used: α-synuclein: C20, FLAG: F725, Actin: A1978

Collectively, the experiments so far indicate that Usp8 colocalizes with α-synuclein and its overexpression deubiquitinates and stabilizes α-synuclein by reducing its rate of clearance. However, these experiments are based on Usp8 overexpression

studies. To further evaluate endogenous interactions, Usp8 knockdown experiments were performed as detailed below.

4.3. Lentiviral-mediated shRNA knockdown of Usp8 in SH-SY5Y cells

4.3.1. Effective knock down of Usp8 by lentiviral vectors

As mentioned above, a knockdown of Usp8 would be much more informative and physiologically relevant than its overexpression. For this purpose, I used lentiviral shRNA (constructs generated by Rebecca Perrett in the laboratory) to transduce SH-SY5Y cells, a neuroblastoma cell line with dopaminergic characteristics. The use of lentiviral construct enabled effective Usp8 knockdown, which persisted at 8 days post-transduction (**Fig 4.9**), which is important as endogenous α-synuclein in these SH-SY5Y cells and most neurons is a long-lived protein.

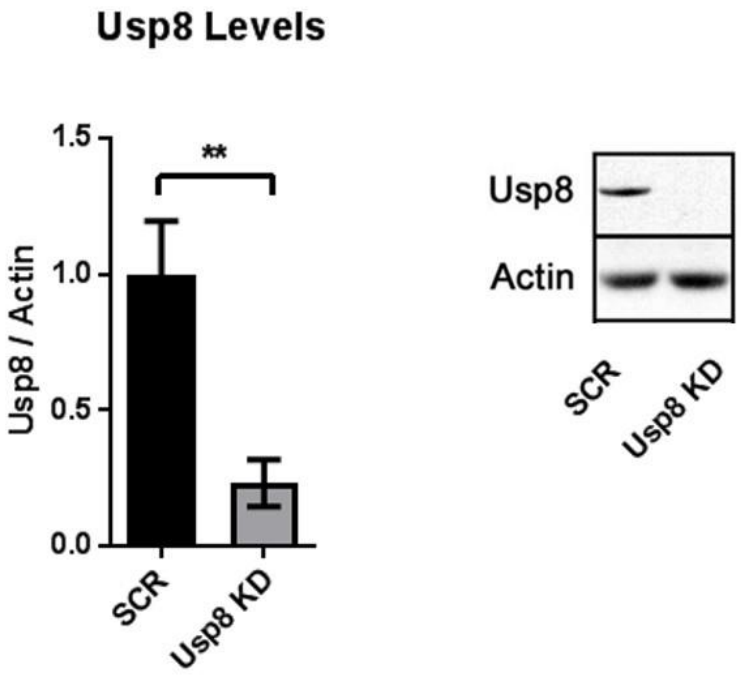


Fig. 4.9. Effective knockdown of Usp8 levels 8 days post-transduction of SH-SY5Y cells with lentiviral Usp8 shRNA. Cytosolic fraction shown after fractionation. n=3

biological replicates, p=0.0226 using t-test. Antibodies used: Usp8: HPA004869, Actin: A1978

Another (indirect) way to show that Usp8 levels are decreased in the Usp8 knockdown is by measuring a canonical substrate of Usp8, STAM. It is known that Usp8 knockdown reduced but did not deplete STAM levels (Niendorf, Oksche et al. 2007, Berlin, Higginbotham et al. 2010, De Ceuninck, Wauman et al. 2013), which was detected in my experiments **(Fig 4.10).**

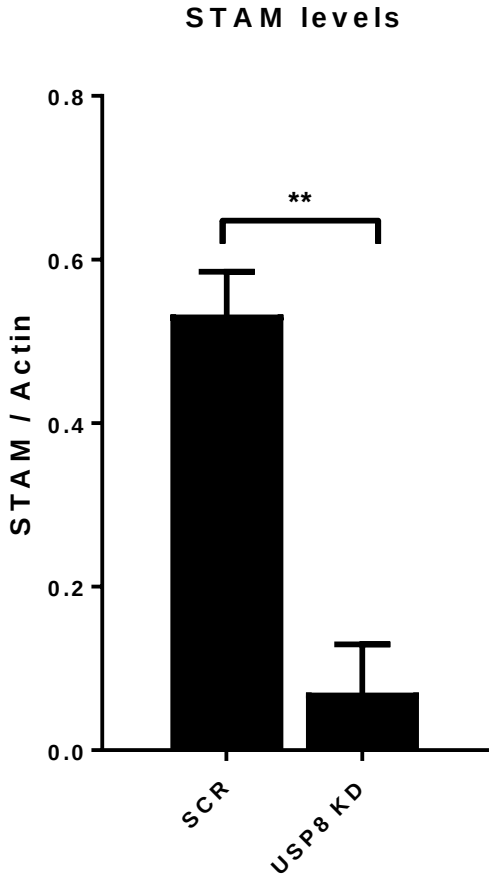


Fig. 4.10. Usp8 shRNA decreases STAM levels. Statistical significance was reached with t-test. n=3 biological replicates, p= 0.0042

4.3.2. Effect of Usp8 knockdown on cell viability

In order to assess whether Usp8 knockdown at the level achieved here is harmful in SH-S5Y5 cells, I performed an MTS assay 8 days post transduction, which showed that reduction of Usp8 levels did not affect viability but rather suggested an increase when compared to Scr shRNA-treated cells **(Fig. 4.11)** as assessed by the MTS assay**.** The MTS assay uses a colorimetric method to measure mitochondrial metabolism, which is directly proportional to cell viability. This kit uses a novel tetrazolium compound and an electron coupling reagent. The tetrazolium compound is bioreduced by cells into a soluble coloured formazan product. This reaction produces NADPH or NADH by dehydrogenase enzymes. The resulting formazan is directly proportional to the number of viable cells. Here, it should be noted that acute addition of any compound, including the MTS solution itself, temporarily increases the metabolism of the cell, therefore produces values above 100% which may seem impossible, but in fact they signify increased mitochondrial metabolism rather than viability above 100%. However, this effect quickly abolishes. The background was subtracted from all raw measurements. If a compound/other treatment was to be added, the values of no treatment/no compounds would be set to 100% viability. However, here we have 2 genetic modifications and no other treatment. Therefore, one way of calculating the amount of cells is to correct the MTS absorbance values to the fluorescence obtained by DAPI staining of the wells. I then set the SCR shRNA as 1 and I compared the viability of Usp8 shRNA with this.

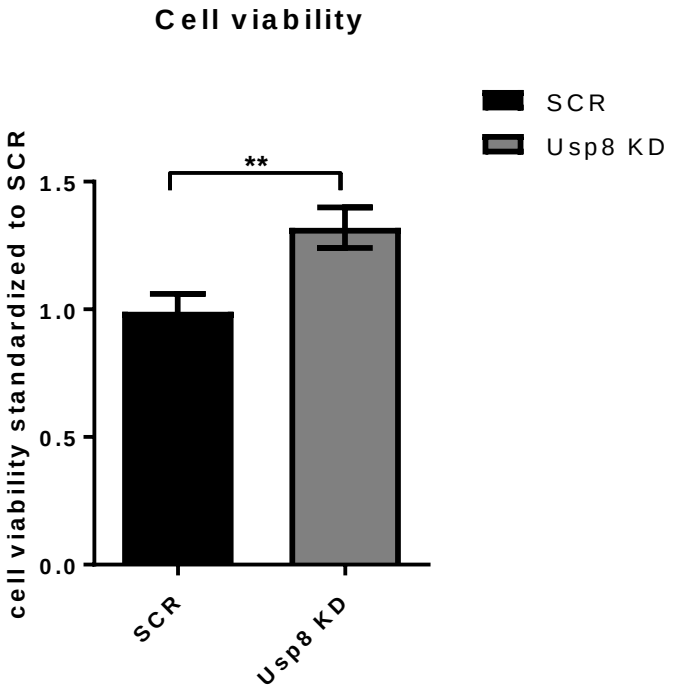


Fig. 4.11. Viability of SH-SY5Y cells following treatment with Usp8 shRNA or Scr shRNA. Values are standardized to SCR=1. Usp8 shRNA shows increased viability when compared to Scr shRNA. n=3 biological replicates, p=0.0078 with t-test.

4.3.3. Measurement of intracellular and secreted α-synuclein levels

Since I showed that the Usp8 shRNA was effective in reducing Usp8 levels in SH-SY5Y cells and that the cells are viable, the next step is to assess its effect on α-synuclein levels. For this purpose, I transduced SH-SY5Y cells with Usp8 or Scr shRNAs and I lysed them at day 8. This is because endogenous α–synuclein has a very long half-life in neurons and neuronal cell lines. I immunoblotted total lysates with C20 anti-α-synuclein antibody, anti-actin antibody as a loading control and anti-Usp8 to ensure effective knockdown. My results showed that 70% knockdown of Usp8 in cells causes a 35% decrease in intracellular α-synuclein levels **(Fig. 4.12).**

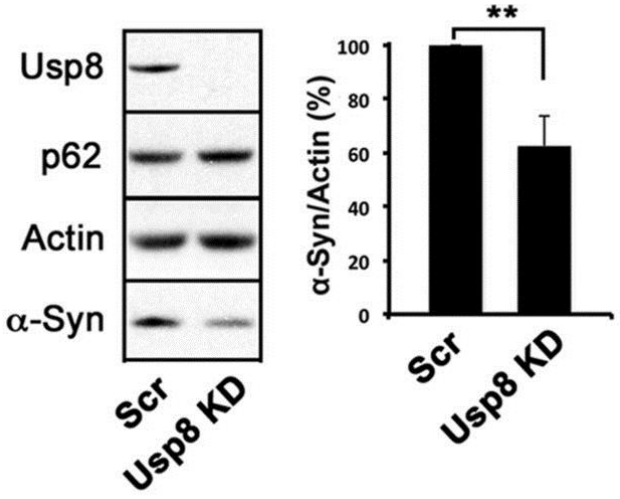


Fig. 4.12. Lentiviral-mediated shRNA knockdown of Usp8 in SH-SY5Y cells reduced endogenous α-synuclein levels by 35% when compared to Scr shRNA controls (**P = 0.0031, n = 5 biological replicates). The immunoblot shows the levels of various proteins at low exposure, which explains the lack of a Usp8 band. Antibodies used: α-synuclein: C20, Actin: A1978, Usp8: HPA004869, p62: Abcam ab56416

In order to ensure that this effect was not due to increased secretion of α-synuclein, I measured the extracellular levels of α-synuclein. For this purpose, and because the concentration of extracellular α-synuclein is usually very low, I performed an ELISA, with the ThermoFischer Scientific human α-synuclein ELISA kit. However, as shown in **Fig. 4.13.**, the sensitivity of this ELISA kit was 0.23ng/mL of α-synuclein minimum detectable dose, and most of my samples produced absorbance readings below the lowest concentration of the standard solutions, from which the standard curve was calculated. Therefore, this experiment was inconclusive. For this reason, Miss Dimitra Mazaraki, performed an ELISA for human α-synuclein of the conditioned media I had collected, in the lab of Professor Vekrellis. This ELISA was developed in-house and has much higher sensitivity, especially for low

concentrations of α-synuclein. For this experiment cells were treated with lentiviral Usp8 or Scr shRNA for 7 days with either chloroquine or lactacystin added for 4 hours prior to cell lysis in addition to baseline. Although further confirmation may be warranted as this experiment was based on three technical replicates, extracellular α-synuclein was not increased when Usp8 was knocked down **(Fig. 4.14)**.

	α-synuclein concentration (ng/mL) (Interpolated values)	**Raw optical density**
Scr_1	0.224	0.186
Scr_2	NK	0.163
Scr_3	NK	0.170
Usp8 KD_1	NK	0.153
Usp8 KD_2	NK	0.158
Usp8 KD_3	NK	0.166
Scr Cycloheximide	1.015	0.227
Usp8 KD Cycloheximide	NK	0.148

Fig. 4.13. Extracellular α-synuclein ELISA performed with the ThermoFischer Scientific kit, yielding mostly undetectable levels. α-synuclein concentrations were interpolated from the standard curve constructed from standards of known concentrations, plotted in GraphPad Prism. NK=Not Known=Undetectable

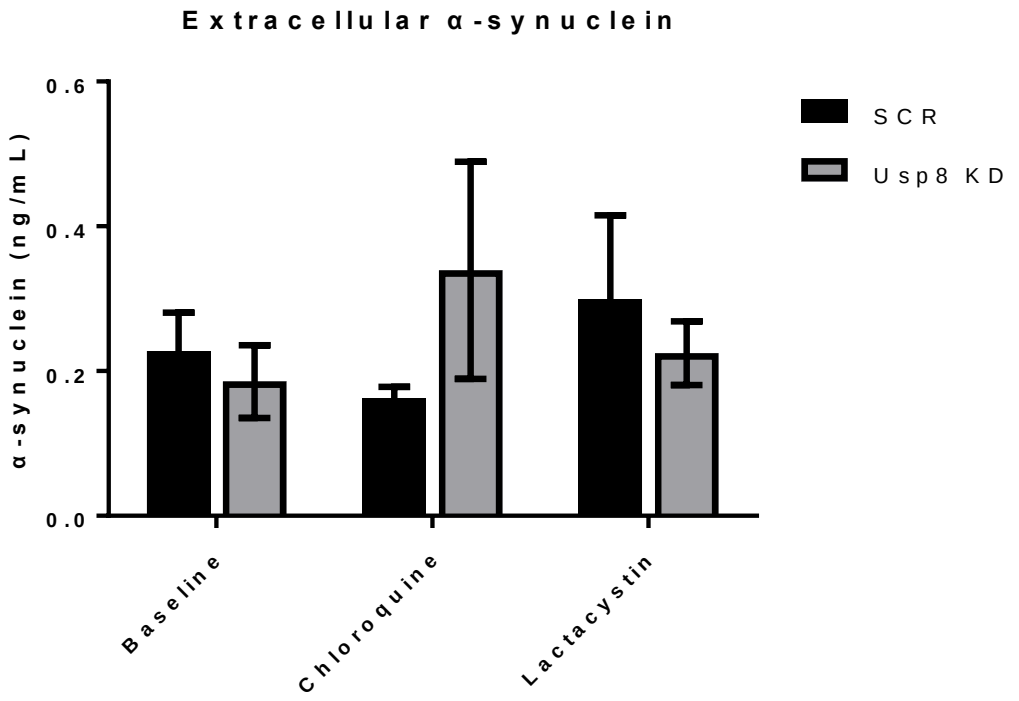


Fig. 4.14. Extracellular α-synuclein ELISA performed with the ELISA kit from Prof. Vekrellis. Usp8 knockdown does not cause an increase in extracellular α-synuclein levels, indicating no increase in its secretion. n=3 technical triplicates

Another way to have performed this experiment if the high-sensitivity ELISA of Prof. Vekrellis' was not available would have been to incubate the cells in culture in less volume than the 2mL of medium used: eg in 1.5mL of medium. However, this would have introduced other variables in this experiment, as the cells could have been stressed having less medium and nutrients, which by itself could change the normal physiology of the cells and thus possibly alter the α-synuclein metabolism and its possible excretion. Thus, the experiment with the high-sensitivity ELISA is a much better option.

4.4.4. Investigation of α-synuclein ubiquitination upon acute Usp8 depletion

To assess the effect of endogenous Usp8 knockdown on ubiquitination of endogenous α-synuclein, I immunoprecipitated α-synuclein with anti-α-synuclein antibody (2F12) and immunoblotted with pan-Ubiquitin antibody and with anti-K63-specific antibody. These experiments showed that when Usp8 was knocked down, endogenously ubiquitinated α-synuclein could be detected in cells (**Fig. 4.4, Fig. 4.5.**), providing further validation of my earlier findings using HA-tagged ubiquitin (**Fig. 4.18**).

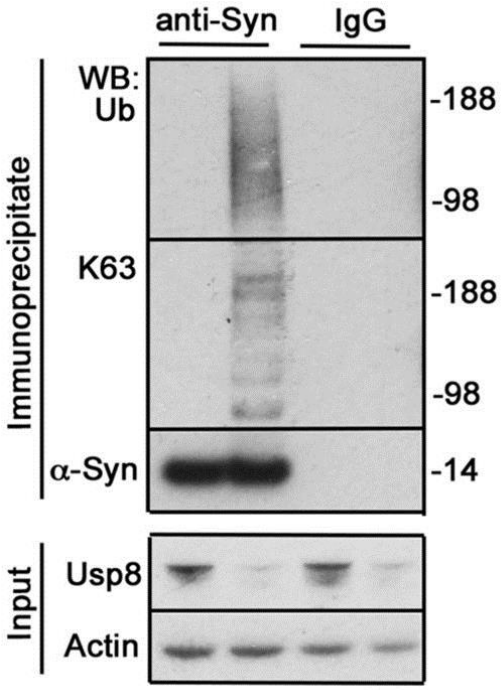


Fig. 4.18. Usp8 knockdown increased the amount of ubiquitinated α-synuclein as evidenced by immunoblotting of immunoprecipitated α-synuclein with anti-ubiquitin and anti-K63 antibodies. No smear was seen when anti-HA was used as an IgG control. Antibodies used: α-synuclein: 2F12, Actin: A1978, Usp8: HPA004869, Poly-ubiquitin: Merck Millipore MAB1510, K-63 ubiquitin: BMLPW0600-0100

4.4.5 Effect on total protein ubiquitination

To investigate the effect of Usp8 more globally on ubiquitination and protein degradation pathways, I first assessed total ubiquitination after Usp8 knockdown for 8 days compared to Scr shRNA **(Fig 4.19.).** This showed that overall the poly-ubiquitin smear which is indicative of poly-ubiquitinated substrates or free chains was not significantly different between the two conditions. In addition, treatment of cells with lactacystin increased poly-ubiquitin in both conditions similarly suggesting that proteasomal degradation is intact in these cells.

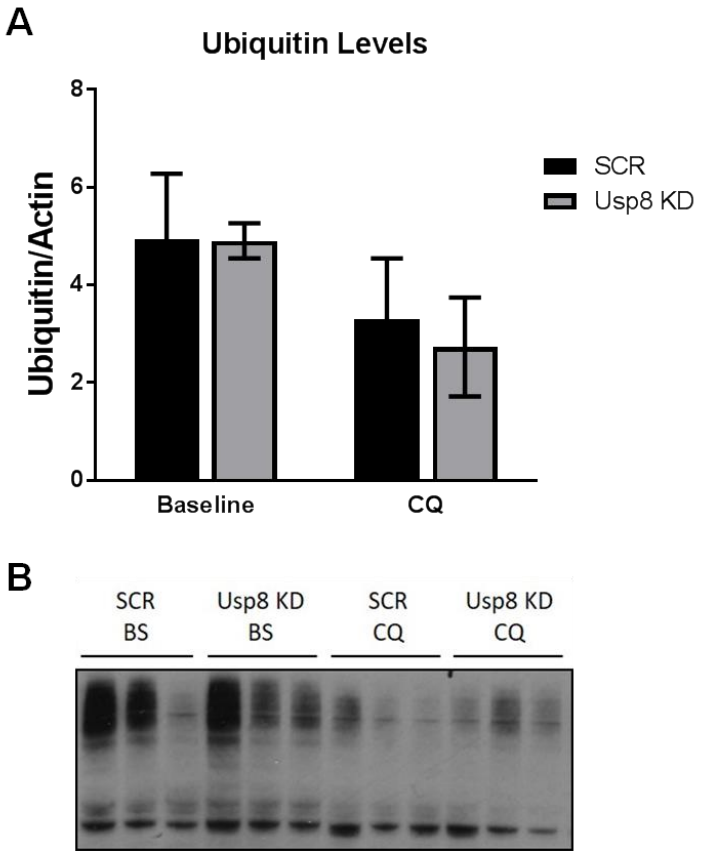


Fig. 4.19. (A) Total ubiquitination levels are unchanged in Usp8 knockdown when compared to Scr shRNA. **(B)** Representative blot. n=5 biological triplicates. Antibodies used: Actin: A1978, Poly-ubiquitin: Merck Millipore MAB1510

4.4.6. Assessment of autophagic and proteasomal flux and pathways related to Parkinson's Disease

A static assessment of the proteasomal or lysosomal states is not enough to assess the function of Usp8 on proteasomal or lysosomal breakdown levels. It is essential to assess the proteasomal and lysosomal fluxes. To this end, one should block the proteasome and the lysosome. In my studies, I used lactacystin to block the proteasome and chloroquine to inhibit lysosomal function. Lactacystin inhibits the three peptidase activities of the proteasome (mainly chymotrypsin-like but also trypsin-like and peptidylglutamyl-peptide hydrolyzing activities) to different extents, the first two irreversibly (Fenteany, Standaert et al. 1995). It also inhibits peptide hydrolysis by the 26S complex and inhibits ubiquitin/proteasome-mediated degradation of proteins (Craiu, Gaczynska et al. 1997). Chloroquine raises the lysosomal pH causing inhibition of fusion of autophagosomes and lysosomes and thus inhibits autophagy (Ohkuma, Poole 1978, Gonzalez-Noriega, Grubb et al. 1980).

To identify the degradative pathway involved in Usp8-mediated α-synuclein degradation, I assessed the relative abundance of α-synuclein monomeric band at baseline and following 8h inhibition of the proteasome (5μM lactacystin) or lysosome (50μM chloroquine) in SH-SY5Y cells 8 days after transduction with lentiviral Usp8 shRNA or Scr control. For these experiments, cell lysates were fractionated into cytosolic and membranous fractions to study the corresponding pools of α-synuclein separately and increase the sensitivity of this assay. The membranous fraction contains the endolysosomal/autophagic compartments. In this fraction, immunoblotting with α-synuclein revealed an accumulation of the α-

synuclein band in Usp8 knockdown cells treated with chloroquine **(Fig. 4.20.).** This suggests that there was accelerated lysosomal degradation of α-synuclein in these cells. Decreased levels of α-synuclein were seen in both fractions as detected earlier in total lysates (**Fig. 4.12**).

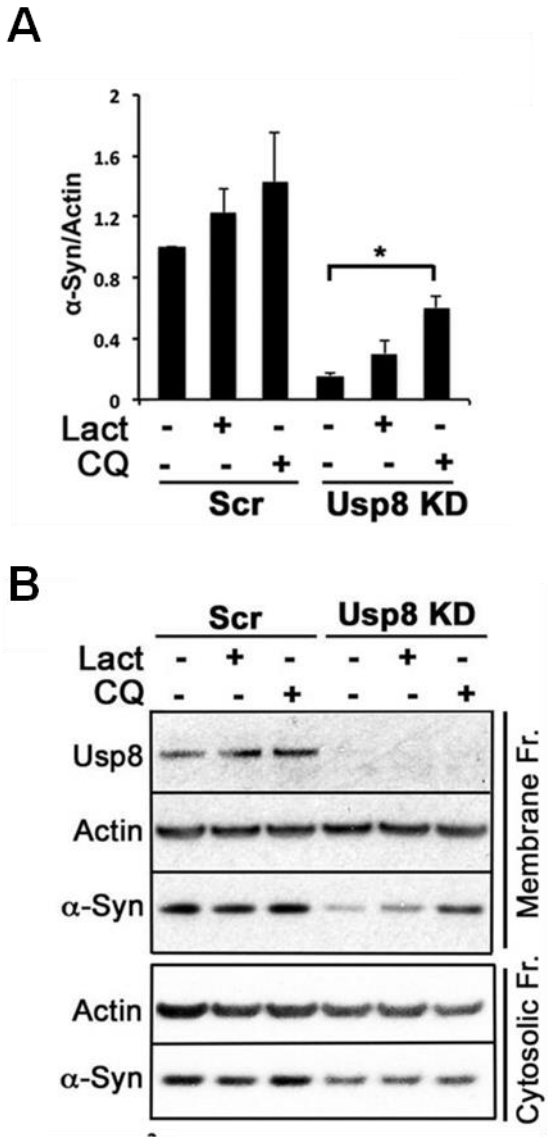


Fig. 4.20. Lysates isolated from Usp8 knockdown and Scr shRNA-treated SH-SY5Y cells were fractionated into cytosolic and membrane fractions and tested for α-synuclein levels at baseline and following 8 h of treatment with either 50 μM chloroquine (CQ) or 5 μM in (Lact). **(A)** Accumulation of α-synuclein was observed in the membrane fraction in Usp8 knockdown cells treated with chloroquine, suggesting that there is accelerated lysosomal degradation of α-synuclein in these

cells (*P = 0.019, n = 3). Error bars correspond to standard error of the mean. **(B)** representative blot. Antibodies used: α-synuclein: C20, Actin: A1978, Usp8: HPA004869

In order to further investigate more broadly lysosomal function under conditions of Usp8 knockdown, I also assessed markers of macroautophagy such as p62 and $LC3_{II}$, endosomal/lysosomal markers such as LAMP2 and GBA and the pS6/S6 ratio, which is a marker of mTOR activity.

These experiments were performed in SH-SY5Y cells 8 days post-transduction with lentiviruses expressing Usp8 shRNA or Scr shRNA and total lysates were immunoblotted. The exception was for $LC3_{II}$ which was assessed in the membrane component of fractionated samples in order to increase sensitivity of detection. At baseline, there was a small increase in $LC3_{II}$ in Usp8 KD versus the control which could be either due to inhibition of autophagosome fusion or increased autophagosome formation. To dissect this out I measured autophagic flux after addition of 50μM choroloquine for 4h and calculated by dividing the $LC3_{II}$/actin levels in Usp8 KD with those of Scr for normalization across different experiments. This revealed a 2.87 fold increase in autophagic flux in Usp8 KD cells **(Fig. 4.21 B.**). To further investigate this result I measured p62 levels. Despite increased $LC3_{II}$ levels in Usp8 knockdown cells, there was no increase in p62 levels in total lysates **(Fig. 4.22.)**, suggesting that there is an increase in autophagy rather than a defect in autophagic pathways. Importantly, p62 which is degraded by both autophagic and lysosomal pathways was increased in Usp8 KD cells after addition of chloroquine. GBA and LAMP2 levels seemed to be overall unaffected by the knockdown of Usp8 across various treatments **(Fig. 4.23., Fig.4.24).** However, there was a big variation across the replicates, thus creating big error bars. GBA and LAMP2 are

markers of lysosomes. Usp8 knockdown did not increase pS6/S6 levels at baseline as compared to Scr shRNA (**Fig. 4.25**).

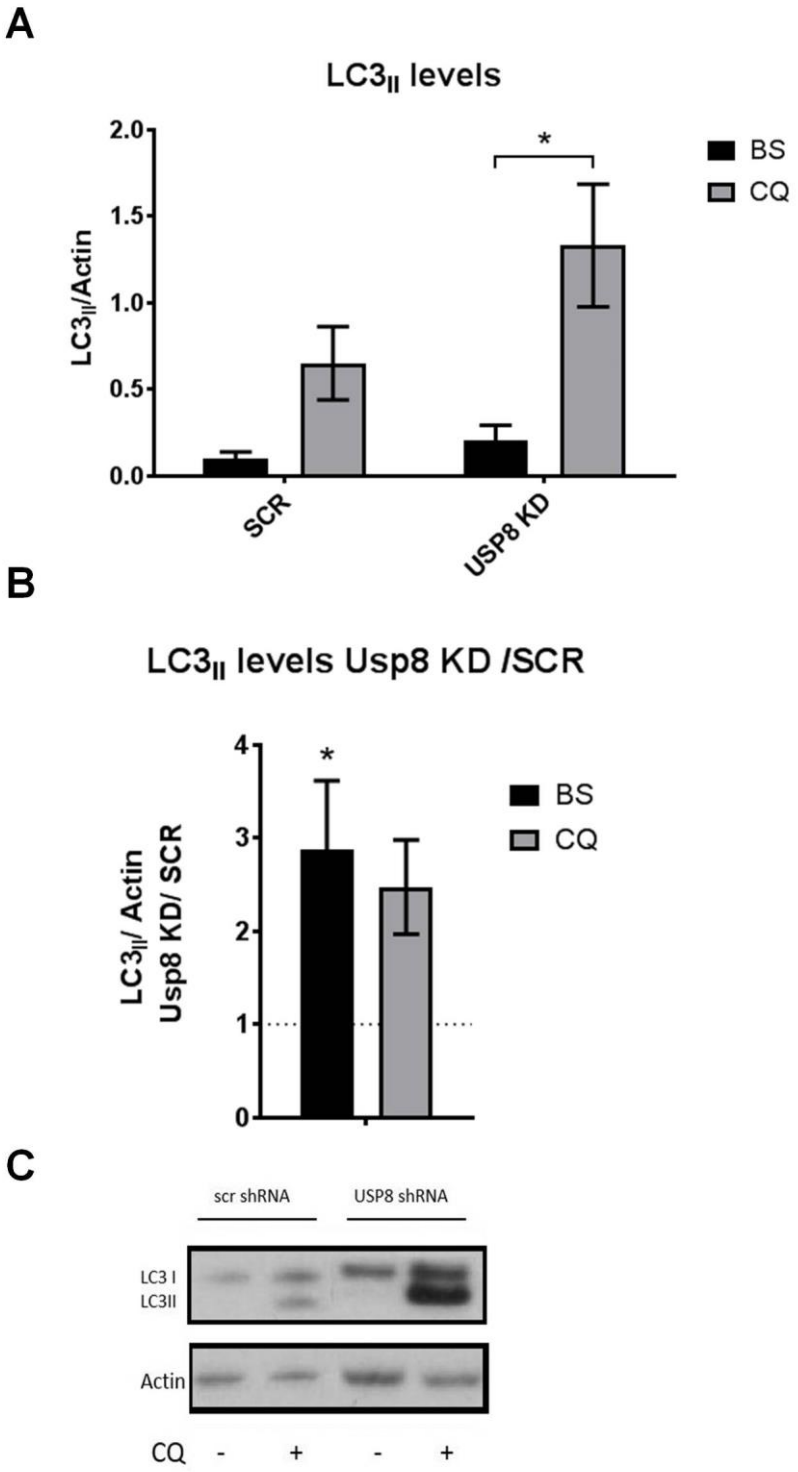


Fig. 4.21. LC3II levels are increased with the addition of chloroquine (inhibition of the lysosome) both in SCR and Usp8 KD conditions **(A)**. Usp8 Knockdown causes a 2.87 fold increase in the levels of $LC3_{II}$ at baseline and 2.47 with chloroquine **(B)**.

The ratios were calculated by dividing the actin corrected $LC3_{II}$ levels in Usp8 KD with those of Scr for normalization. **C:** representative blot. * denotes statistical significance as follows: in panel **A** (P=0.0131) significant difference in LC3II levels in Usp8 knockdown between baseline and chloroquine addition. In panel **B**: (p=0.0262) significant difference between Usp8 from SCR (located at y=1). n=7 biological replicates. Antibodies used: Actin: A1978, LC3 NB100-2220

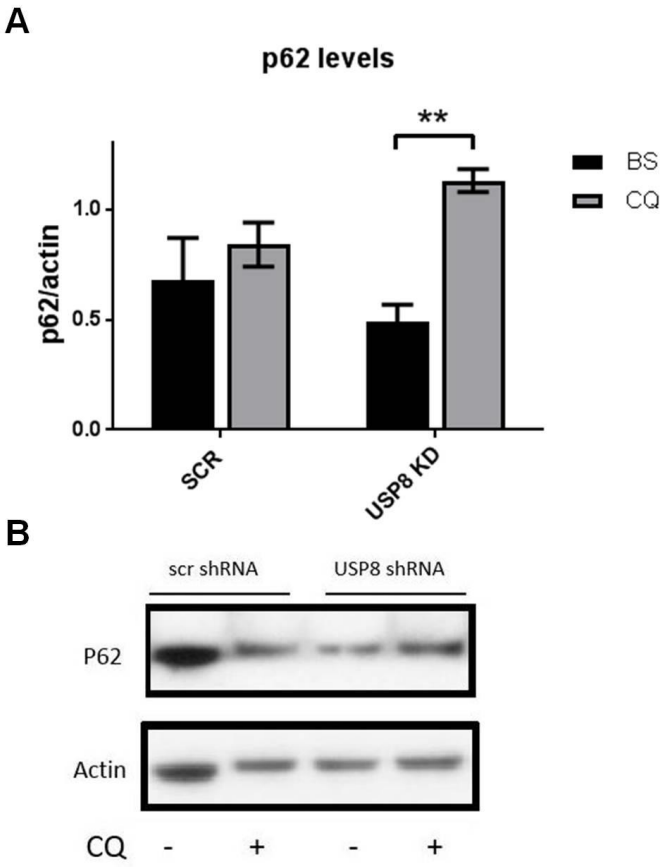


Fig. 4.22. A: Usp8 knockdown increased significantly the levels of p62 during chloroquine treatment. P62 levels were unchanged from SCR to Usp8 KD at baseline. **B:** Representative blot. n=3 biological replicates. Antibodies used: Actin: A1978, p62: Abcam ab56416

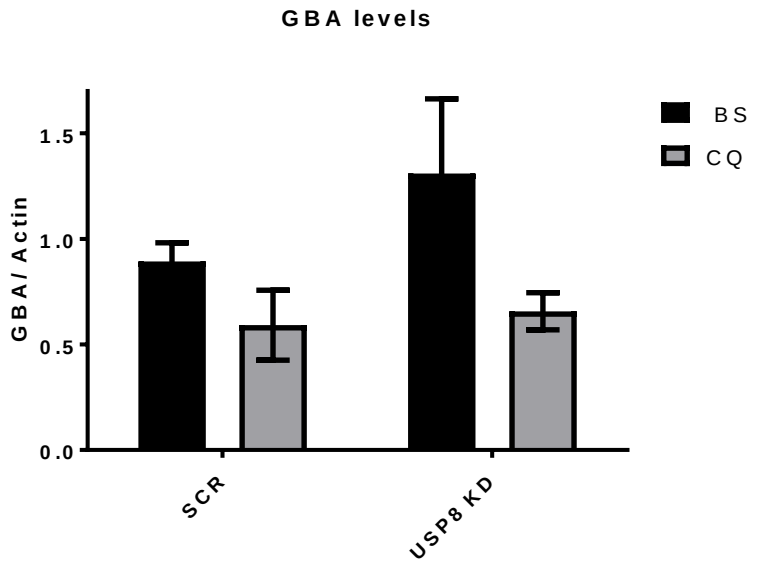


Fig. 4.23. Usp8 knockdown did not affect the levels of GBA across different treatments. n=8 biological replicates. Antibodies used: Actin: A1978, GBA: Sigma Aldrich G4171

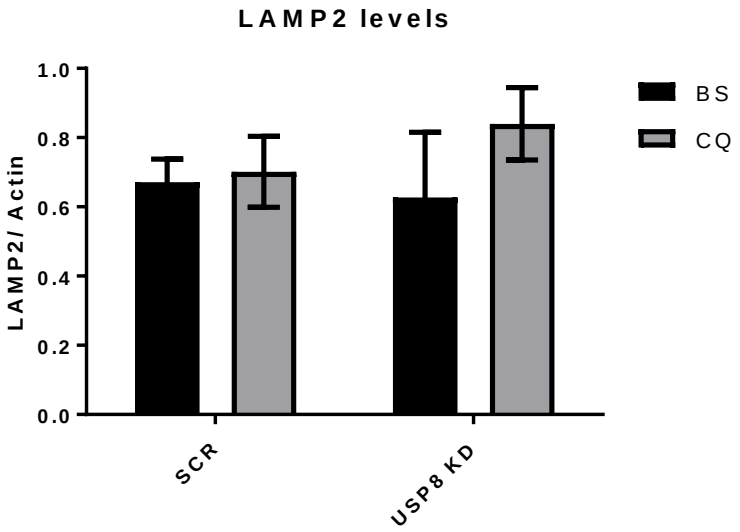


Fig. 4.24. Usp8 knockdown did not affect the LAMP2 levels, n=8 biological replicates. Antibodies used: Actin: A1978, LAMP2: DSHB H4B4-S

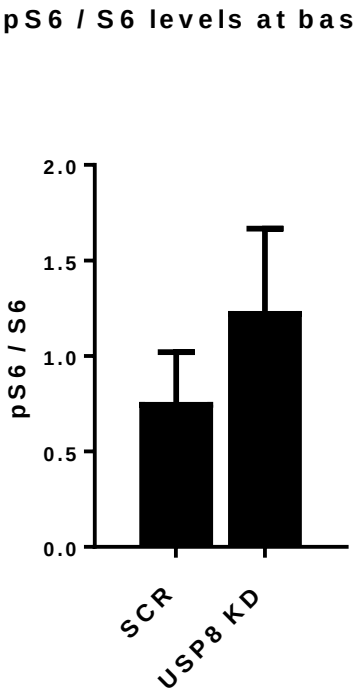


Fig. 4.25. Usp8 knockdown did not increase pS6/S6 levels at baseline as compared to Scr shRNA, n=3 technical replicates. Antibodies used: Actin: A1978, pS6: Cell Signaling 2211S, S6: Santa Cruz C-8 SC74459

4.4.7 Effect of Usp8 knockdown under conditions of cellular stress

To further assess whether Usp8 KD has any protective or harmful effects under different conditions, I performed viability and cell death assays following oxidative or proteotoxic stress. To this end, I knocked down Usp8 in SH-SY5Y cells and 8-days post-transduction I treated the cells with various toxins and assayed them using the MTS and LDH assays. Typically, MTS assay monitors the percentage of viable cells, whereas LDH release is used as a measure of the percentage of dead cells.

For assessing cell death (cytotoxicity), I used a colorimetric LDH assay. Lactate dehydrogenase (LDH) is a cytosolic enzyme. When cells die, the plasma membrane is destroyed/perforated and LDH is released in the culture medium. The kit contains

lactate which is transformed to pyruvate by LDH with concomitant reduction of NAD^+ to NADH. The kit also contains a tetrazolium salt and an enzyme, diaphorase which in the presence of NADH transforms to formazan, which is measured at 490nm absorbance. The formazan is directly proportional to the LDH released, therefore measuring directly cell death.

There are various ways to stress cells. Five different forms of cell stress were used to assess whether Usp8 knockdown is protective across a wide range of conditions. More specifically, I tested three forms of oxidative stress (dopamine, sodium peroxide and arsenite toxicity) and two forms of proteotoxic stress (heat shock and proteasomal inhibition).

1) Firstly, I stressed the cells by adding in the medium increasing doses of dopamine for 24 hours. It is known that excessive dopamine is toxic by producing toxic dopamine quinones (Goldstein, Sullivan et al. 2013) as further explained in **Chapter 3**. I treated the cells with 25, 50, 100, 200 and 400μM of dopamine.
2) In addition, I induced oxidative stress by adding hydrogen peroxide (H_2O_2) for 24 hours. At very low doses (3-15μM) hydrogen peroxide was reported to cause growth stimulation in C6 glioma cells, at higher doses (120-150μM) temporary growth arrest, with increasing doses (250-400μM) causing a permanent growth arrest. At high doses (≥ 1 mM) it kills the cells via necrotic cell death (Gülden, Jess et al. 2010). I treated the cells with 25, 50, 100, 200 and 400μM of hydrogen peroxide.
3) I induced oxidative stress by adding arsenite. Arsenite generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) which among other effects,

also cause DNA damage and alter gene expression (Shi, Shi et al. 2004). Arsenite was added to the cells at 2, 5, 10, 20 and 30μM for 24 hours.

4) To investigate proteotoxic stress, I used two different conditions that are known to induce accumulation of misfolded proteins: prolonged proteasomal inhibition and heat shock. For the former, cells were treated for 12 hours with lactacystin, which inhibits irreversibly the 20S proteasome and therefore inhibits protein proteasomal degradation (Myung, Kim et al. 2001). The doses I tested are 0.5, 1 and 2μM of lactacystin or DMSO.
5) To induce heat stress, I incubated the cells for 30 mins at 45 ˚C. The cells recovered for 15 minutes at 37˚C before the MTS or LDH solutions were added (to avoid quicker metabolism of MTS and a quicker reaction in the heat shock samples due to increased temperature). The results were compared to cells that were always kept at 37˚C. Following heat shock and protein misfolding, the cell changes its gene expression and metabolism in order to switch to survival mode and be able to handle misfolded proteins. Heat shock proteins (HSPs) are upregulated and act as chaperones, refolding damaged proteins, and assist in the ubiquitination of other proteins to destine them for breakdown (Vabulas, Raychaudhuri et al. 2010, Haslbeck, Franzmann et al. 2005).

Usp8 knockdown was neither harmful nor protective against toxicity from dopamine, hydrogen peroxide, arsenite or lactacystin **(Fig. 4.15. Fig. 4.17).** However, there was a clear protective effect of Usp8 knockdown on cellular stress resulting from heat shock **(Fig. 4.16.).**

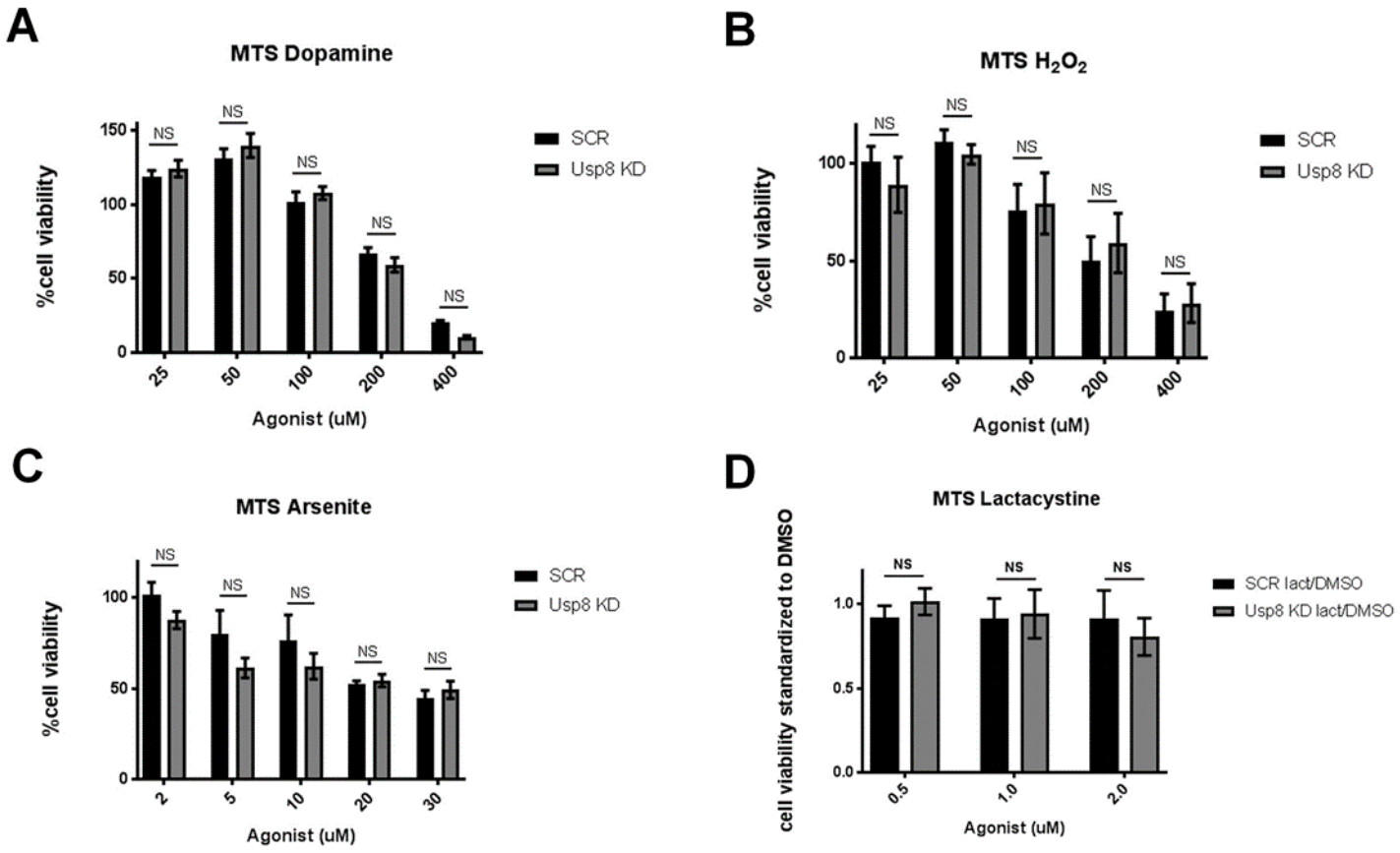


Fig. 4.15. Viability of SH-SY5Y cells treated with lentiviral Usp8 shRNA or Scr shRNA for 8 days following different toxic treatments. There was no difference in cell survival between Usp8 KD and Scr shRNA following treatments with toxic doses of dopamine, Hydrogen Peroxide, Arsenite and Lactacystin. Error bars represent standard error of mean, n=4-6 biological replicates

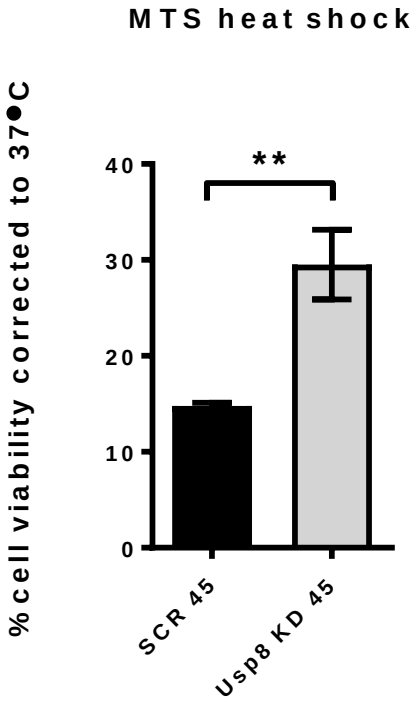


Fig. 4.16. Usp8 knockdown was protective against cellular stress caused by heat shock. Cells were incubated either at 45˚C or at 37˚C for 30 mins. Both Usp8 KD and Scr shRNA as shown as percentage change when standardized to their corresponding viability at 37˚C. p= 0.0012, with t-test, n=4 biological replicates,

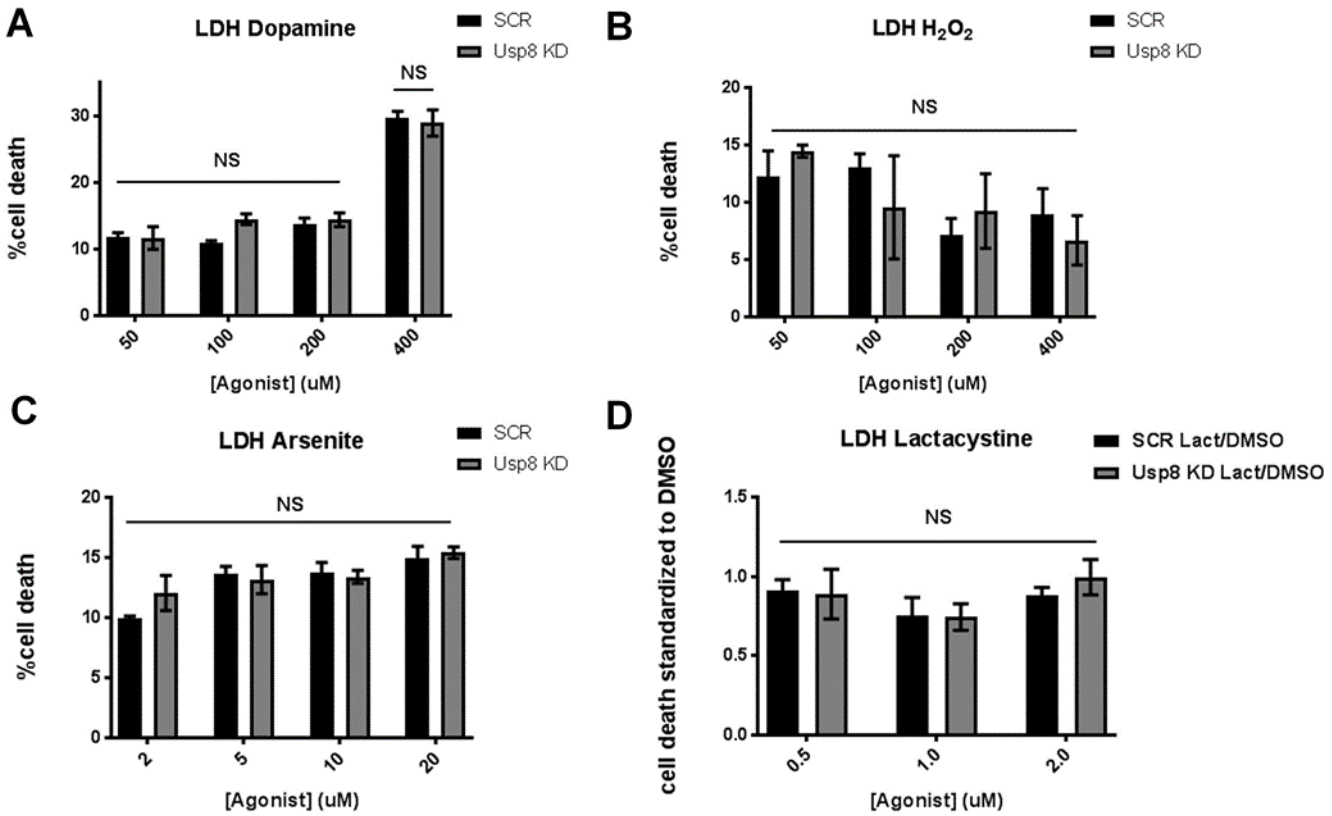


Fig. 4.17. Death of SH-SY5Y cells treated with lentiviral Usp8 shRNA or Scr shRNA for 8 days following different toxic treatments. There was no difference in cell death between Usp8 KD and Scr shRNA following treatments with toxic doses of dopamine, Hydrogen Peroxide, Arsenite and Lactacystin (proteasomal blockade). Error bars represent standard error of mean, n=4-6 biological replicates

4.4 Discussion

Our *in vitro* studies have shown that α-synuclein and Usp8 co-immunoprecipitate when overexpressed in HEK-293T cells and co-localize in human iPS-derived dopaminergic neurons. The bimolecular fluorescence complementation assay produced fluorescence implying co-localization in SH-SY5Y cells. This shows a direct interaction between the two proteins.

The next step was to assess a possible effect of Usp8 on ubiquitination of α-synuclein. The experiments above show that overexpressed Usp8 robustly de-ubiquitinates α-synuclein. In addition, Usp8 (although a DUB with some preference for K63 ubiquitin chains in recombinant assays), has a preference for de-ubiquitinating K63 linked chains from α-synuclein in cells, without changing the total levels of protein ubiquitination. This functional interaction was seen with endogenous proteins as ubiquitinated α-synuclein could be immunoprecipitated in total lysates only when Usp8 was knocked down. The next aim would be to assess the effect of Usp8 on the levels of α-synuclein. We showed that Usp8 overexpression decreases the rate of clearance of α-synuclein in a protein synthesis inhibition assay (cycloheximide chase). In addition, lentivirally-mediated shRNA of Usp8 decreased the levels of α-synuclein by 35% which is not a negligible reduction for the purposes of preventing the development of symptoms of Parkinson's disease (see below). When investigating the way that Usp8 knockdown enhances the breakdown of α-synuclein, we identified that it acts through the lysosome. Lysosomal blockade increased the levels of α-synuclein in cells with Usp8 knockdown. Therefore, Usp8 KD increases the lysosomal flux. This was also demonstrated by the fact that Usp8 knockdown increases the levels of $LC3_{II}$ with Usp8 knockdown but with no simultaneous increase in p62 levels, which signifies

an increased flux rather than a blockade of autophagy. This evidence will thoroughly be discussed below.

For the purpose of investigating the interaction of Usp8 with α-synuclein *in vitro*, a variety of cellular and molecular biology approaches were used. We first used overexpression assays. To this end, $Usp8^{WT}$, $Usp8^{CA}$ (an inactive Usp8 analogue), $Usp8^{MIT}$ (a Usp8 analogue with the endosomal localization domain MIT missing) or EV (Empty vector) were overexpressed together with or without α-synuclein WT, 130 truncation or 120 truncation. $Usp8^{WT}$ in contrast to the inactive $Usp8^{CA}$ deubiquitinated α-synuclein and showed preference for K63 ubiquitin chains. This is important, as the absence of a deubiquitinating activity of $Usp8^{CA}$ shows that $Usp8^{WT}$ acts via its catalytic domain (the cysteine protease). In addition, when $Usp8^{MIT}$ was overexpressed, part of the deubiquitinating ability of Usp8 was lost, but not completely abolished. This would signify that although part of its activity happens through its association with the endosomal compartments, it also acts independently in de-ubiquitinating α-synuclein. Interestingly, full-length but not truncated forms of α-synuclein were increased when co-expressed with $Usp8^{WT}$, compared to empty vector control. This might suggest that α-synuclein 1-120 is no longer responsive to Usp8-mediated regulation. However, this comes in contrast to the finding in flies that Usp8 knockdown rescued the toxicity produced by α-synuclein 1-120. Further investigation would be needed to assess whether in mammalian cells knockdown of Usp8 lowers the levels of co-transfected α-synuclein 120.

For the purpose of the knockdown experiments, lentiviral shRNA knockdown was used with Scr shRNA as a control. To begin with, an effective knockdown was proven by western blot as well as by a reduction of Usp8's canonical substrate

STAM (Niendorf, Oksche et al. 2007, Berlin, Higginbotham et al. 2010, De Ceuninck, Wauman et al. 2013). It was also important that cells treated with both viruses had a good and comparable viability, therefore the knockdown of Usp8 was not deleterious to cells. Transduction was performed 7-8 days prior to lysis. Although this may allow for some compensatory mechanisms to be activated, it more closely resembles our ultimate aim in the context of targeted therapies and takes into consideration the long half-life of α-synuclein in these cells. Moreover, the fact that Usp8 knockdown does not increase the global levels of ubiquitinated proteins signifies that it does not perturb ubiquitin homeostasis more broadly.

The fact that the levels of α-synuclein are reduced by 35% with the Usp8 shRNA is not unexpected, as α-synuclein is degraded via multiple pathways. Therefore, enhancing one pathway would only reduce the levels by a small fraction. However, this is very promising as reducing α-synuclein levels may be beneficial to halt the progression of Parkinson's disease (Tofaris 2012). This is because increasing α-synuclein levels by only 1.4 fold due to gene multiplications is enough to trigger Parkinson's disease, the severity of which is proportional to the initial level of expression (Ross, Braithwaite et al. 2008, Ikeuchi, Kakita et al. 2008). Transgenic mice that were immunized against α-synuclein produced high affinity antibodies, showed less accumulation of aggregated α-synuclein and less neurodegeneration (Masliah, Rockenstein et al. 2005) and this reduced synuclein expression was shown to be protective against toxins such as prolonged MPTP exposures (Dauer, Kholodilov et al. 2002, Drolet, Behrouz et al. 2004). On the other hand, it would not be desirable to deplete α-synuclein as its absence in neurons may be harmful. For example, α-synuclein knockout mice showed reduced learning abilities, lacked good working and spatial memory (Kokhan, Afanasyeva et al. 2012). In addition, they

exhibited neurotransmitter release deficits including reduced striatal dopamine levels and altered dopamine release (Abeliovich, Schmitz et al. 2000).

Our data indicate that knockdown of Usp8 decreases the levels of α-synuclein by enhancing its lysosomal degradation. This is evident both by the fact that Usp8 overexpression preferentially deubiquitinates K63 over K48 ubiquitin chains but also because Usp8 knockdown causes increased α-synuclein levels upon lysosomal inhibition (with chloroquine). Although Usp8 functions in the endosomal processing of ubiquitinated substrates, our results suggest that it may also be implicated in autophagic degradation as evidenced by increased autophagic flux in cells where Usp8 levels were reduced. This effect may have relevance to other conditions where activation of autophagy may be beneficial but requires further investigation of the relevant mechanism. My initial analysis, suggests that this effect of Usp8 knockdown on autophagy is not under mTOR regulation as it was detected without any change in phosphorylation of S6, which is a substrate of mTOR kinase activity. It is also worth noting that in a study by Jacomin et al, bafilomycin treatment (causing lysosomal blockade) in HeLa cells increased the $LC3_{II}$ levels without an increase in p62 levels in Usp8 knockdown cells. They too concluded that autophagy was increased in Usp8 knockdown cells (Jacomin, Bescond et al. 2015). However, this was not the case in the *Drosophila melanogaster* fat body, where they found that Usp8 inactivation blocked the autophagy flux and caused lysosomal defects (e.g. reducing their size). The authors conclude that in *Drosophila*, Usp8 knockdown could both activate autophagy and also cause degradation defects and we are only able to see the degradation defects by the accumulation of the autophagolysosomes and we cannot see the increased autophagy in the first place. In mammalian HeLa cells, there is no defect in degradation, thus we can see the

increase in autophagy. Another plausible explanation they offer is that DUBs in mammals have different actions than DUBs in insects. This is because humans have around 100 DUBs in their genome (Hutchins, Liu et al. 2013), whereas flies have only 41 (Nijman, Luna-Vargas et al. 2005). Thus, another human DUB other than Usp8 could have an effect on lysosomal biogenesis, replacing this function of Usp8 in the flies (Jacomin, Bescond et al. 2015). However, my data in flies suggests otherwise, so this could also be a tissue-specific phenomenon.

Another interesting controversy regarding differences of activators/depressors of autophagy in the fat body of the *Drosophila* comes from Blommart and collegues and Scott and collegues. This relates to conflicting evidence as to whether p70S6K is inhibitory for or activates autophagy. Evidence by Blomaart et al suggests that phosphorylation of S6 inhibits autophagy and that the degree of S6 phosphorylation regulates the autophagic vs protein synthesis flux (Blommaart, Luiken et al. 1995). In contrast, Scott et al showed that in the *Drosophila melanogaster* fat body, mTOR inhibits autophagy, whereas p70S6K is required for the induction of autophagy (Scott, Schuldiner et al. 2004). These two lines of evidence may not be contradicting. Because the activity of p70S6K depends on mTOR, p70S6K could be the limiting step for autophagy upon mTOR inhibition (Scott, Schuldiner et al. 2004). In addition, p70S6K was found to be necessary but not sufficient to induce autophagy, as shown by the failure of activated p70S6K to induce autophagy in wild-type animals (Scott, Schuldiner et al. 2004). Thus, it is possible that the fat body of *Drosophila* is not an appropriate model to study mammalian autophagy, thus these findings could not be translated to humans.

The precise mechanism of Usp8 effect on the lysosomal degradation of α-synuclein requires further clarification. Although some of our data indicate a role of

endosomal association (e.g. MIT domain and colocalization with Rabs), the fact that degradation occurs despite reduced STAM levels (ESCRT-0) indicates that additional adaptors may be involved in this process. In this respect our finding that Usp8 knockdown increases autophagic flux may be relevant.

Alternatively, chaperone-mediated or endosomal-microautophagy (e-MI) may be involved. At this stage, these pathways are understood to function via heat shock chaperones at the lysosome or endosomes respectively without prior ubiquitination (Cuervo 2010, Sahu, Kaushik et al. 2011). In this respect Usp8 knockdown was protective against heat shock toxicity, suggesting that Usp8 may antagonize the function of heat shock proteins (HSPs) and thus its knockdown may relieve the HSP inhibition improving the cellular response to heat stress. These mechanisms may be relevant to Parkinson's disease, where upregulation of HSPs, especially HSP70, have been shown to be protective in animal models (McCormack, Mak et al. 2010). An alternative explanation could be that Usp8 knockdown contributes to the degradation of misfolded proteins in an HSP-independent fashion thus preventing their accumulation and proteotoxicity.

Thus, in order to assess CMA one would have to look at the levels of LAMP2A or knockdown LAMP2A, whereas to assess e-MI function, one should knockdown ESCRT components. It is unlikely that Usp8 knockdown increases α-synuclein breakdown via stimulating e-MI because Usp8 knockdown decreases the levels of STAM which is a component of ESCRT, thus it can't enhance ESCRT-dependent breakdown of proteins. Because blocking the lysosome with inhibitors would inhibit both forms of autophagy one could not differentiate between the 3 pathways (Klionsky, Abdalla et al. 2012). Thus, in the future it will be interesting to

investigate how Usp8 affects lysosomal LAMP2A levels, to assess for a possible increase in CMA function on top of the increase in macroautophagy.

However, if Usp8 knockdown was acting purely via increasing chaperone-mediated autophagy, it would have improved cellular survival in the oxidative stress assays (hydrogen peroxide, arsenite and dopamine) because CMA enhances survival in oxidative stress situations. Thus, there is a possibility that Usp8 knockdown could also act via enhancing CMA, but this is not the sole method of action as it does not completely explain the findings.

Finally, an interesting observation is that Usp8 appears to function in a pathway that opposes the ubiquitin ligase Nedd4. Nedd4 overexpression was shown firstly by Dr Tofaris and validated by others to increase the ubiquitination and clearance of α-synuclein and also be protective against α-synuclein toxicity *in vitro* and *in vivo* models of Parkinson's disease (Tofaris et al. 2011, Tardiff et al. 2013, Sugeno et al. 2014, Wijayanti et al. 2015). It primarily functions by increasing α-synuclein degradation by the lysosome and it preferentially ubiquitinates α-synuclein with K63-linked ubiquitin chains which are removed by Usp8 (Tofaris et al. 2011, Alexopoulou et al. 2016). There has been a study that identified that Usp8 acts antagonistically to Nedd4 in the recycling of AMPA receptors and thus determining synaptic strength (Scudder, Goo et al. 2014). It is therefore interesting to further investigate the relationship between Nedd4 and Usp8 and their downstream adaptors as well as their therapeutic potential.

4.5. Conclusions

1. α-Synuclein and Usp8 co-immunoprecipitate when overexpressed in mammalian cells and co-localize in mammalian and human iPS-derived dopaminergic neurons.
2. Usp8 overexpression, deubiquitinated α-synuclein and showed preference for K63 over K48 ubiquitin chains.
3. Usp8 knockdown reduced α-synuclein levels by 35% and increased the amount of ubiquitinated (and K-63 ubiquitinated) α-synuclein when immunoprecipitated.
4. Usp8 knockdown accelerated the clearance of α-synuclein via the lysosome and increased autophagic flux as well as resistance to heat-stress.

5. Pharmacological inhibition of Usp8 in human cell lines, rat primary cortical neurons and *Drosophila melanogaster*

5.1. Introduction

The previous chapters assessed the effects of Usp8 on α-synuclein and on cellular toxicity in *in vitro* and *in vivo* models. These results suggested that overexpressed Usp8 deubiquitinates α-synuclein and decreases its rate of clearance. In contrast, genetic knockdown of Usp8 enhances the ubiquitination of α-synuclein and increases its breakdown via the lysosome. It would now be interesting to assess the effect of pharmacological inhibition of Usp8 on α-synuclein levels and on general cellular toxicity. This question would be of particular importance; as such an inhibition, may have therapeutic value in Parkinson's disease. Of course, such an endeavour would first need further investigation for target validation in more complex systems, lead identification and optimization and assessment of toxicity from potential off target effects. Pharmacological targeting of ubiquitination holds promise for such therapies as evidenced by a major chemical-genetic screen in yeast and iPSC cells which identified the neuroprotective compound NAB2 as an activator of the E3 ligase Nedd4 (Tardiff, Jui et al. 2013, Chung, Khurana et al. 2013). In this regard, deubiquitinating enzymes are more druggable compared to E3s for reasons including having a catalytic domain and accessible pocket and the fact that inhibiting a protein (eg DUB) is more achievable than activating another (eg. E3 ligase) (Lopez-Castejon, Edelmann 2016). Therefore, if Usp8 and Nedd4 proved to have an antagonistic effect in Parkinson's disease, targeting α-synuclein it would be advantageous.

Aims of this chapter

1. Assess the toxicity of Usp8 inhibitors and controls in SH-SY5Y cells and primary rat neurons
2. Identify the TD50 values of the compounds for the aforementioned cell types
3. Assess the effect of non-toxic doses of DUB inhibitors on α-synuclein levels
4. Assess the effect of non-toxic doses of DUB inhibitors against oxidative stress
5. Assess the effect of non-toxic doses of DUB inhibitors on total protein ubiquitination
6. Assess the effect of non-toxic doses of DUB inhibitors on autophagic markers
7. Assess the effect of DUB inhibitors *in vivo* in *Drosophila melanogaster* investigating the eye phenotype and α-synuclein levels *in vivo.*

Results

I tested whether Usp8 inhibitors have an effect on the cellular levels of α-synuclein or exert a protective effect against cellular stress *in vitro*. Usp8 inhibitors and control compounds were provided by MRC-Technology. The Usp8-specific inhibitors were identified by Hybrigenics by high-throughput screening of 65092 compounds. Compounds with a halogen atom on the phenyl ring were found to be potent Usp8 and Usp7 inhibitors (IC_{50} in the sub-micromolar range). The addition of O-alkyloxime moieties at position 9 of the tricyclic scaffold added specificity for Usp8 over Usp7 and other DUBs tested (e.g. compounds 91 and 92- HBX90397 and HBX90659) (Colombo et al. 2010, Guédat, Colland 2007). Similarly, HBX41108 (compound 50) was found to be an uncompetitive reversible inhibitor of Usp7 and consequently also reduce the Usp7-mediated p53 ubiquitination in vitro and in vivo, thus increasing p53 levels (Colland et al. 2009). Another group was screening commercial libraries using a Ub-PLA2 (Ub-CHOP) reporter assay trying to identify Usp7 and Usp47 inhibitors as potential anti-cancer therapies. They identified compound D38813 (Compound 49) that exhibited joint Usp7 and Usp47 activity (Weinstock et al. 2012). Furthermore, Dexheimer et al discovered and optimized compound ML323 (Compound 51), which reversibly inhibits Usp1/UAF1 with high selectivity in the nanomolar ranges (Liang et al. 2014). For my research I used the Usp8 specific compounds from Hybrigenics, the Usp8/7 inhibitor and the Usp7/47 and Usp1 inhibitors as controls, as shown in **Table 5.1**. It should be noted that compound 50 has been found in the literature to also inhibit Usp1, Usp2, Usp16, Usp27x, Usp28, Usp36, Usp20, Usp25, OTUB1, A20 and vOTU at 1μM and nearly all Usps at 10μM (Ritorto, Ewan et al. 2014).

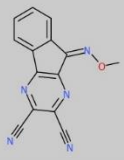
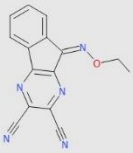
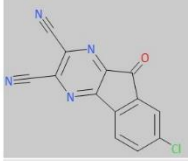
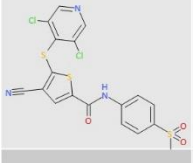
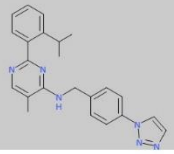
CHEMISTRY	Name	Molecular Weight	Supplier ID	Reference	Pharmacological IC 50 for Usp8 (μM)	Pharmacological IC 50 for OTHER DUBs (μM)
	**92**	261	MRT00251292	Colombo 2010 PMID:20186914	**1**	USP7 >100
	**91**	275	MRT00251291	Colombo 2010 PMID:20186914	**0.28**	USP7 >100
	**50**	266	HBX41108	Colland 2009 PMID:19671755	**0.096 OR 0.21**	USP7 0.42OR 6
	**49**	484	D38813	Weinstock 2012 PMID:24900381	**>30**	USP7: 0.43 USP47: 1 >30 against USP2/5/21/28
	**51**	384	ML323	Dexheimer 2014 PMID:25229643	**No inhibition**	USP1 0.076 No inhibition of USP2/5/7/45

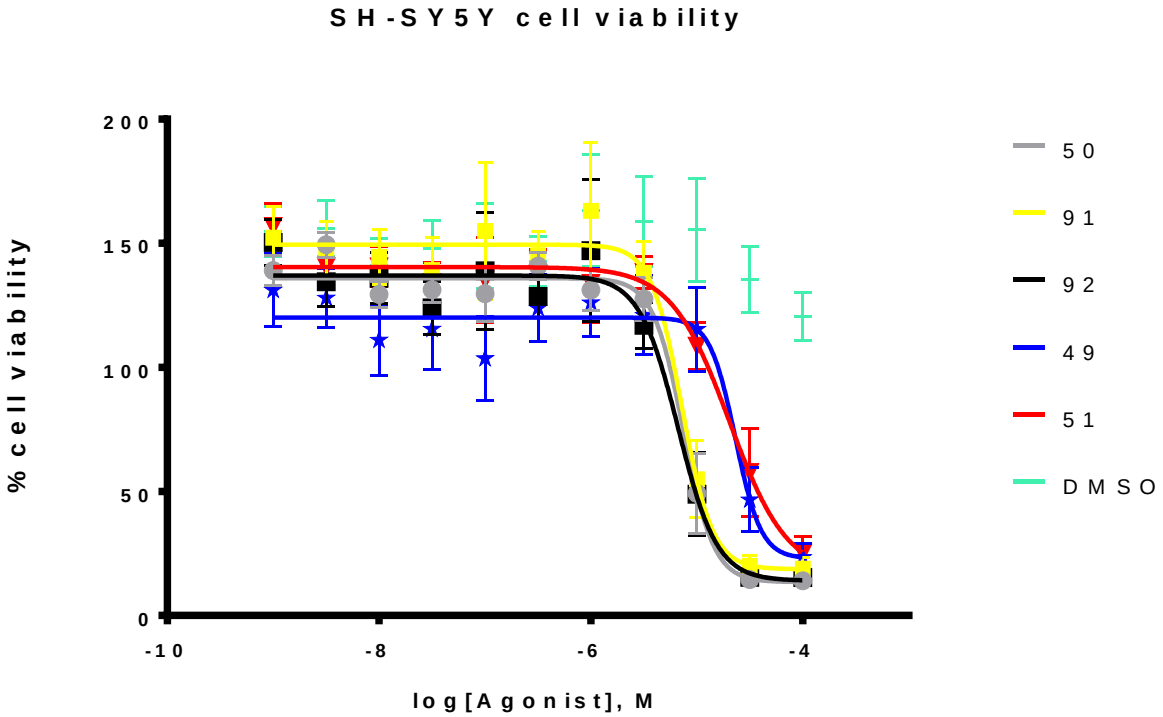
Table 5.1.: Usp8 inhibitors and control compounds: chemistry and characteristics

It should be noted that inhibitors of deubiquitinating enzymes are not entirely selective in their action and although they have improved specificity, depending on the concentration used they eventually inhibit the activity of other DUBs to some extent (Ritorto, Ewan et al. 2014, Daviet, Colland 2008). This is expected, as about 70 DUBs are cysteine specific proteases with a high degree of homology in their catalytic core. Allosteric USP inhibitors are more likely to be specific because of higher conformational specificity and not dependence just on the catalytic site which is core in most USPs and relying only on some additional conformational specificity around the pocket (Daviet, Colland 2008).

5.2. Identification of TD50 for five inhibitors of deubiquitination

5.2.1. Studies in SH-SY5Y cells

In order to identify non-toxic doses for my studies in SH-SY5Y cells I performed cell viability assays. For this purpose, cells were treated with compounds for 3 days with a change of medium and addition of new compounds once at 48 hours post-initial treatment. Cell viability was assayed with the MTS assay as explained in materials and methods. The MTS compound (Aqueous One solution) was incubated with the cells for 2 hours prior to measurement of absorbance at 490nm. DMSO was not toxic to the cells at the doses tested **(Fig 5.1.).** As expected, the compounds exhibited a dose-dependent toxicity and the calculated TD_{50} doses for different compounds are displayed in **Figure 5.1.**



Compound number	Viability TD50 (µM)
92	6.67
91	7.23
50	7.45
49	23.08
51	20.26

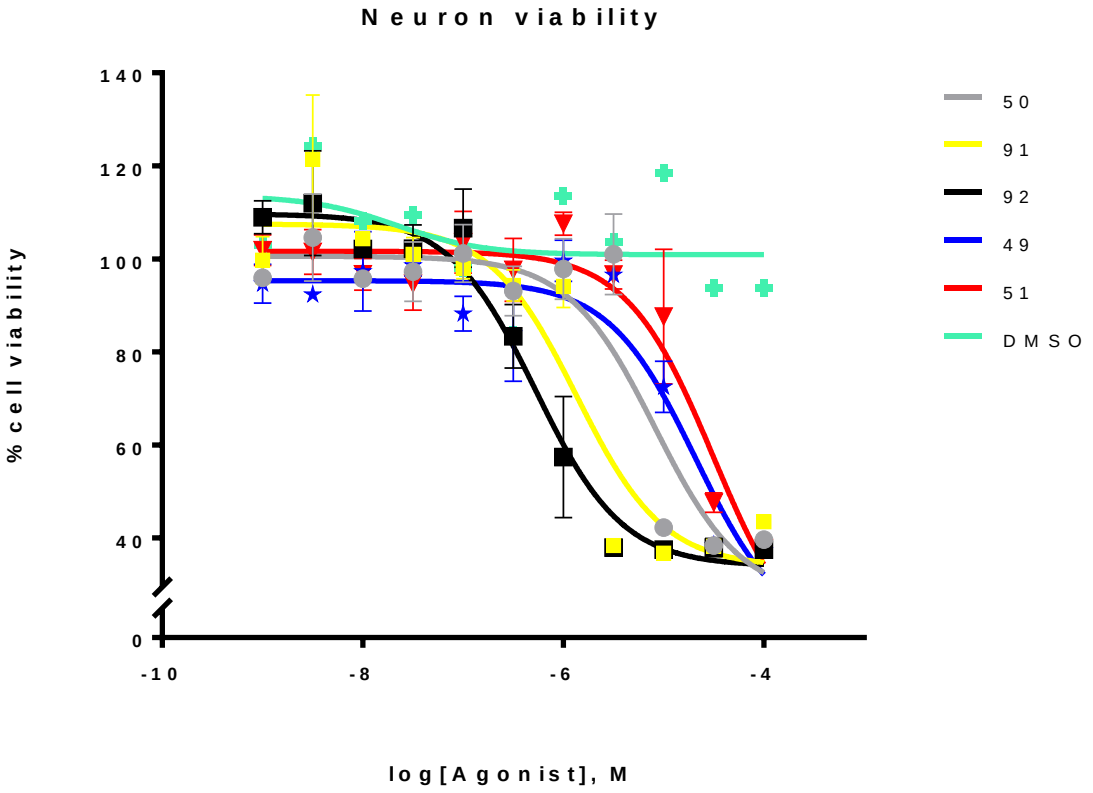
Fig. 5.1. Cell viability of SH-SY5Y cells treated with different doses of the compounds and Viability TD50. The compounds followed a dose-toxicity curve as expected. Compounds 49 and 51 showed less toxicity even at higher doses as compared to the rest of the compounds tested. DMSO did not show toxicity as assayed by cell viability.

For the remaining experiments described here the conditions were used as in this initial study, i.e. two days of initial compound treatment, medium change with fresh

compound addition for further 24 hours followed by lysis. Given the doses required for pharmacological inhibition (IC50) and the doses causing toxicity to the cells (TD50), the following doses were tested in the remainder of experiments: 0.1μM, 0.3μM, 1μM, 3μM, 6μM.

5.2.2. Studies in primary rat cortical neurons

To prepare future experiments in primary neurons, I also assessed the effect of the compounds on the viability of primary rat cortical neurons. Primary rat cortical neurons were obtained from embryonic day 18 rat embryos (E18) and cultured according to the protocol explained in **section 2.3.2.** Neurons were cultured to day 11 to ensure that they expressed adequate α-synuclein (Emmanouilidou, Melachroinou et al. 2010, Clough, Dermentzaki et al. 2011, Xilouri, Vogiatzi et al. 2009) when the compounds were added. Because dividing cells have a higher metabolic rate than primary cells (Warburg 1956), the compounds were added to neurons for 3 days without change in media and the MTS compound (Aqueous Solution One) was incubated with the cells for 3 hours 15 mins prior to measurement of absorbance.The MTS assay showed that compound 92 was more toxic than the others in neurons, with a lower TD_{50} value at 0.529. DMSO was not toxic at the tested doses, as expected **(Fig. 5.2).** The calculated TD50 doses for different compounds are displayed in **Figure 5.2.**



Compound number	Viability TD 50 (µM)
92	0.529
91	1.354
50	8.198
49	20.9
51	31.5

Fig. 5.2. Cell viability of rat primary cortical neurons treated with different doses of the compounds and viability TD50 values. The compounds followed a dose-toxicity curve as expected. Compound 92 was the most toxic amongst the compounds tested. DMSO did not show toxicity as assayed by cell viability.

These experiments showed that in primary neurons compound 92 is toxic at doses that are below the therapeutic doses (the TD50 is less than the IC50), thus limiting its further use for therapeutic purposes. However, for completeness of my thesis I have included it in further testing in SH-SY5Y cells at lower doses.

5.3. Effect of non-toxic doses of DUB inhibitors on α-synuclein levels

Initial experiments were focused on SH-SY5Y cells as an initial screening tool for assessment of Usp8 inhibitors with the aim to further validate potentially effective compounds in primary rat cortical neurons. In order to assess α-synuclein levels in SH-SY5Y cells, cells were treated with compounds at doses 1μM, 3μM and 6μM. The exception was compound 92, where lower doses were tested due to its higher toxicity to cells; therefore the doses used were adjusted to 0.5μM, 1μM and 3μM. The lysates were fractionated in cytosolic and membranous compartments according to the protocol described in **section 2.4.1.1**. There was a reduction in both cytosolic and membranous fractions of α-synuclein with compounds 91, 49 and 51 (**Fig 5.3 and 5.4**).

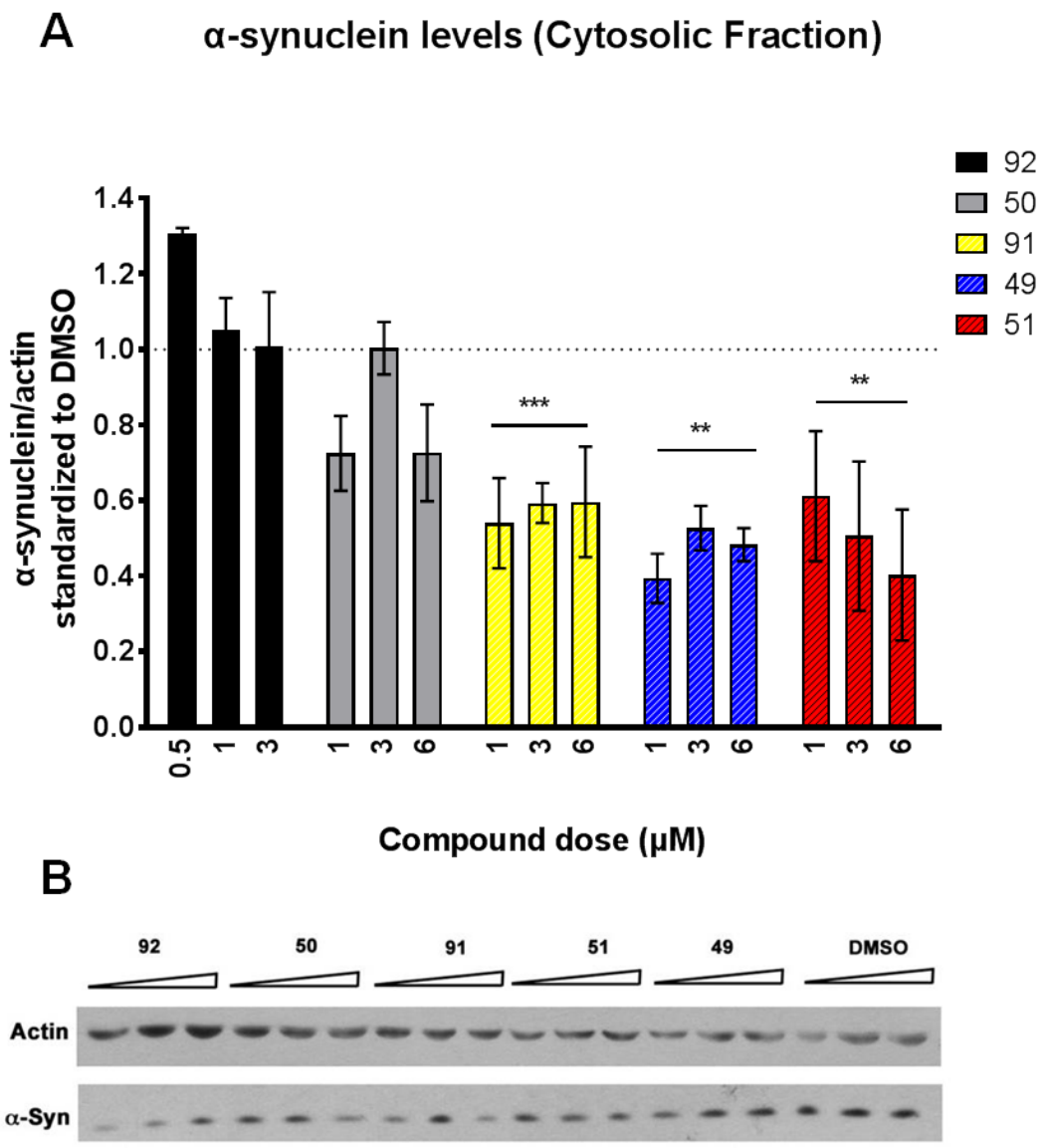


Fig. 5.3. α-synuclein levels in the cytosolic fraction of cells treated with compounds. **(A)** There is a decrease in α-synuclein levels with compounds 91, 49 and 51 as compared to control. **(B)** Blot. n=5-9 biological replicates. P values calculated with 1-way ANOVA when compared to control (DMSO): p = 0.0003 for compound 91, p = 0.0014 for compound 49, p = 0.0074 for compound 51. Antibodies used: Actin: A1978, α-synuclein: C20

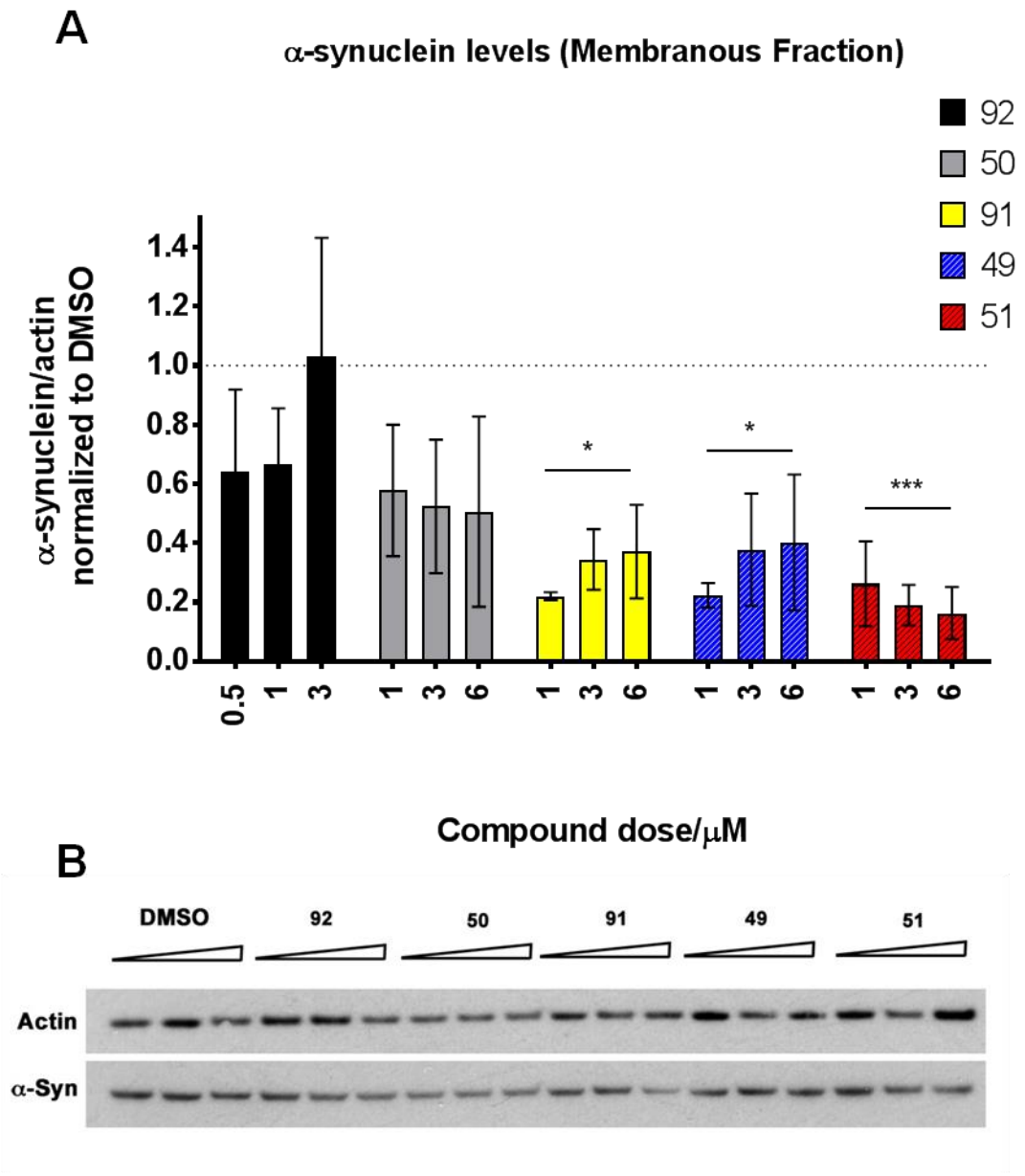


Fig. 5.4. α-synuclein levels in the membranous fraction of cells treated with compounds. **(A)** There is a decrease in the levels of α-synuclein in the membranous fraction of cells treated with compounds 91, 49 and 51 as compared to DMSO-treated cells. **(B)** Blot n=4 biological replicates. P values calculated with 1-way ANOVA when compared to control (DMSO): p = 0.0153 for compound 91, p = 0.0409 for compound 49, p = 0.0006 for compound 51. Antibodies used: Actin: A1978, α-synuclein: C20

Since in my thesis I am investigating the effect of Usp8 inhibition and not of other DUBs and for other reasons further discussed in **Section 5.8** (discussion), I will mostly further investigate the effects of Compound 91. To briefly explain, compound 49 increases the extracellular excretion of α-synuclein and compound 50 is very unstable being metabolized within minutes (data not shown, investigated by the DDI[4]), thus multiple lines of evidence suggest that neither is a good candidate for further investigation. This is the reason that I focused my investigations on compounds 91 (as a Ups8 inhibitor) and 51 as a control.

In order to examine if the effect of compound 91 is through Usp8 inhibition, we were provided with a catalytically-inactive compound structurally very similar to 91 but without effect on Usp8 (compound z7). This time, the lysates were immunoblotted without fractionation. It seems that the effect of 91 in lowering α-synuclein levels was abolished with compound z7, although the experiment was performed only twice, so statistical significance was not reached **(Fig. 5.5.).** Thus, this needs to be verified in the future.

[4] DDI= Alzheimer's UK Oxford Drug Discovery Institute, with which we collaborate

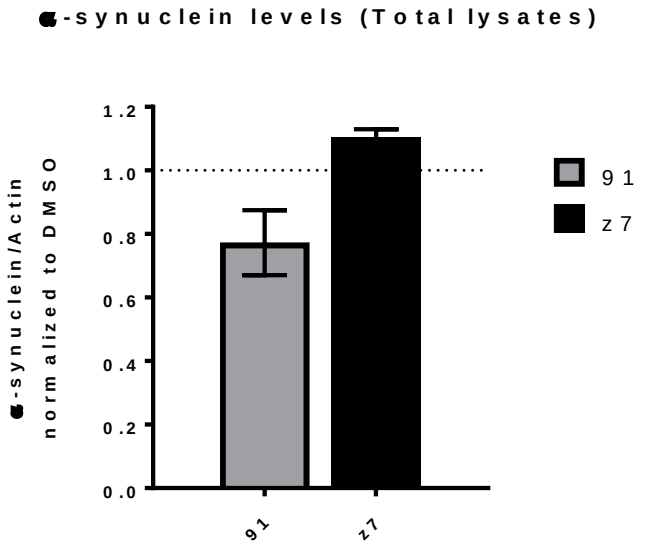


Fig. 5.5. The effect of compound 91 in lowering the levels of α-synuclein was abolished with compound z7 (when its effect on Usp8 was abolished). The results have been standardized to DMSO. n=2 biological replicates. Antibodies used: Actin: A1978, α-synuclein: C20

5.4. Assessment of non-toxic doses of DUB inhibitors against oxidative stress

The response to oxidative stress in cells treated with different compounds was assessed under conditions of oxidative stress induced by arsenite, dopamine and hydrogen peroxide as explained before. Cell viability was assayed using the MTS assay (**Fig. 5.6)** and cell death using the LDH assay (**Fig. 5.7**). There was no effect of the different compounds tested on cell viability across different toxic environments (**Fig. 5.6**), but Usp8 inhibition seemed to reduce death rates from toxicity caused by higher concentrations of dopamine, hydrogen peroxide and arsenite (**Fig 5.7**). For this part of experiment, only cells treated with compounds inhibiting Usp8 were assessed (specific and non-specific compounds) as well as DMSO-treated cells as control.

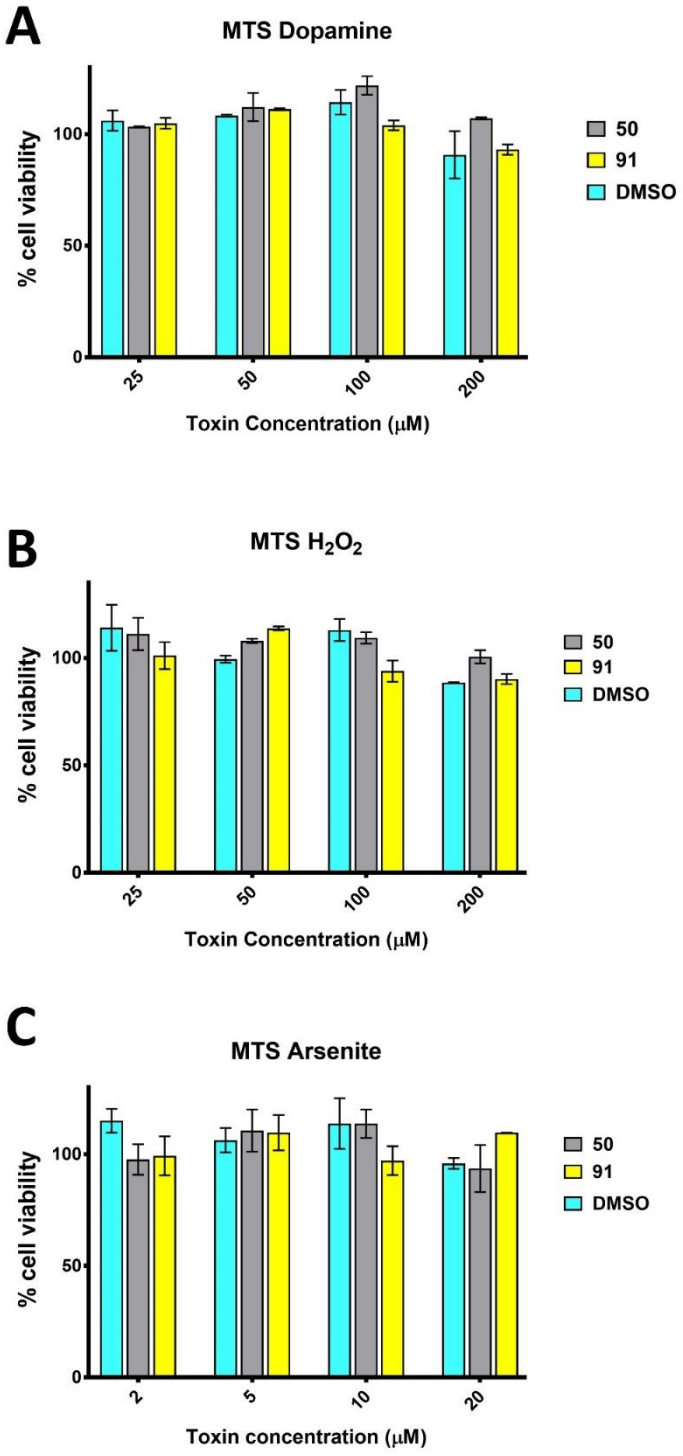


Fig 5.6. Cell viability of SH-SY5Y cells treated with compounds and subjected to different forms of oxidative stress as assayed by the MTS assay. Cell viability was similar across different compound treatments when cells were subjected to dopamine **(A)**, hydrogen peroxide **(B)** and arsenite **(C).** All compounds were used at 3μM, n= 2 technical replicates.

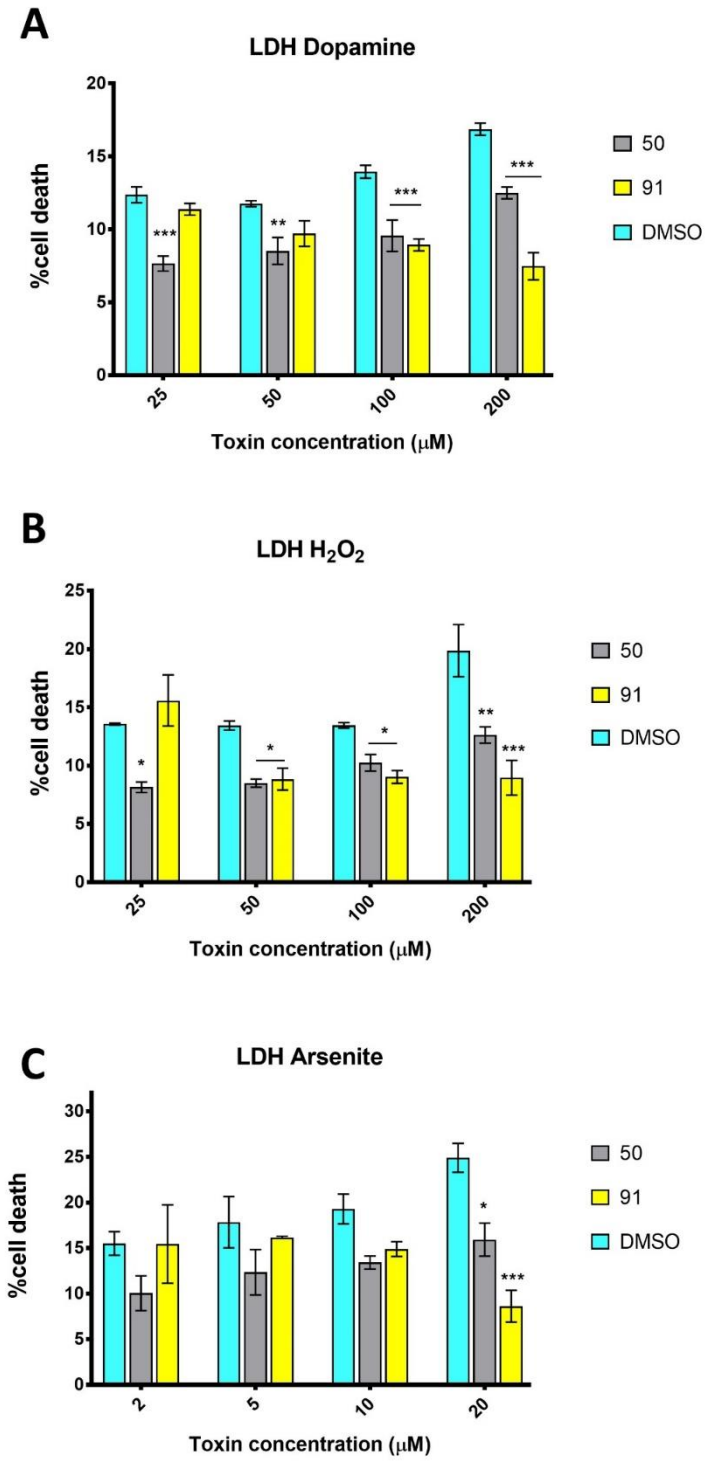


Fig. 5.7. Cell death of SH-SY5Y cells treated with compounds and subjected to different forms of oxidative stress as assayed by the LDH assay. **(A)** Cell death was reduced significantly by Usp8 inhibition across higher concentrations of dopamine treatment (100, 200μM). At lower doses of dopamine compound 50 was protective. **(B)** compounds 50 and 91 were protective against increasing doses of hydrogen peroxide. **(C)** Inhibition of Usp8 was protective against toxicity from 20μM

arsenite. n=2 technical replicates. All compounds were used at 3μM. Statistical significance calculated with 1-way ANOVA *denotes $0.1 \geq p > 0.01$, **denotes $0.01 \geq p > 0.001$, ***denotes $p < 0.001$.

5.5. Effect of non-toxic doses of DUB inhibitors on total protein ubiquitination

Given that these compounds inhibit multiple DUBs especially at higher concentrations, I assessed whether total ubiquitination is altered in cells. If the inhibitor is specific for only one or two DUBs, the prediction is that total ubiquitination would be unaffected. Alternatively, if there is inhibition of multiple DUBs or secondary cellular stress, a change in total ubiquitination would be detected. I found that increasing concentrations of compound 50 increased the levels of global ubiquitination. In contrast, with compound 91 the global ubiquitination levels were reduced overall in all doses tested (**Fig 5.8 and 5.9**).

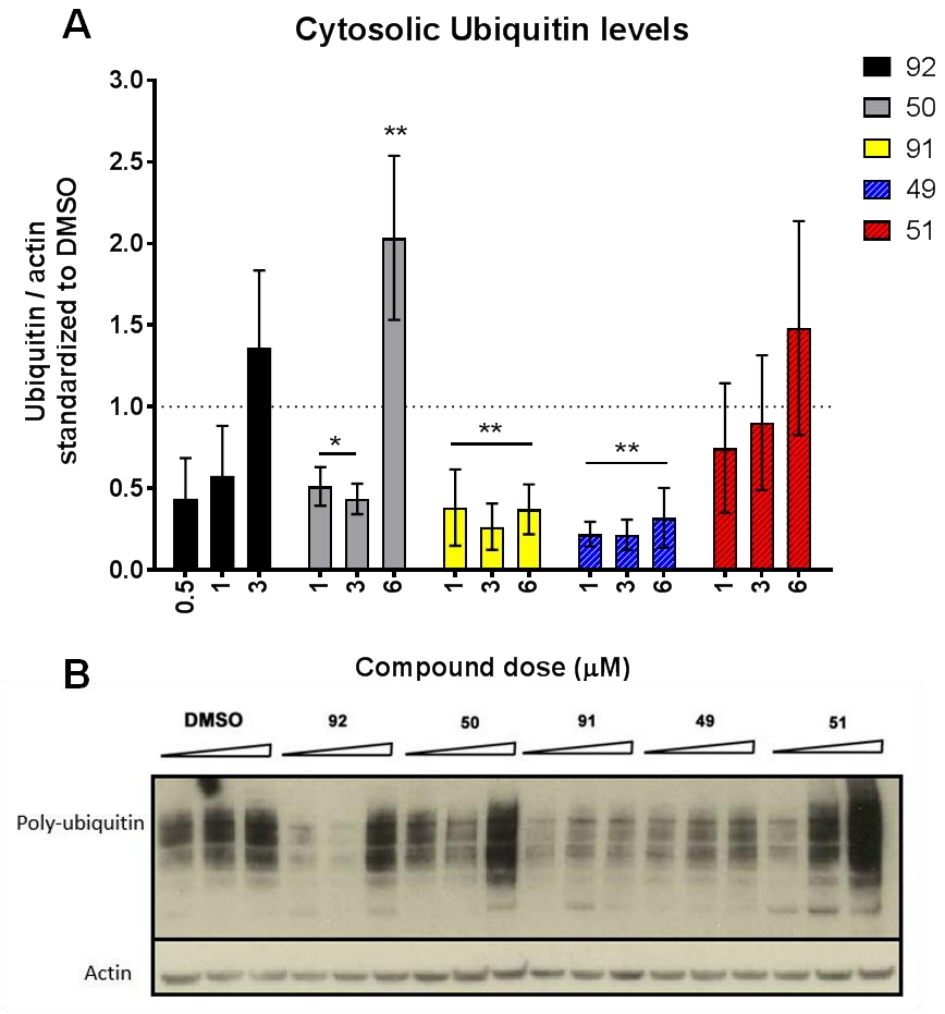


Fig. 5.8. Poly-Ubiquitin levels in the cytosolic fraction of cells treated with compounds. **(A)** There is a decrease in ubiquitination levels in the cytosolic fraction of cells treated with compounds 91 and 49 and at doses 1 and 3μM of compound 50 and an increase in ubiquitination levels in cells treated with compound 50 at 6μM as compared to DMSO-treated cells. **(B)** Representative blot. n=4 replicates. P values when compared to control (DMSO): p = 0.0032 for compound 91, p = 0.0021 for compound 49, p = 0.0468 for compound 50 at 1 and 3μM and p=0.092 for compound 50 at 6μM. Antibodies used: Actin: A1978, poly-ubiquitin: Merck Millipore MAB 1510

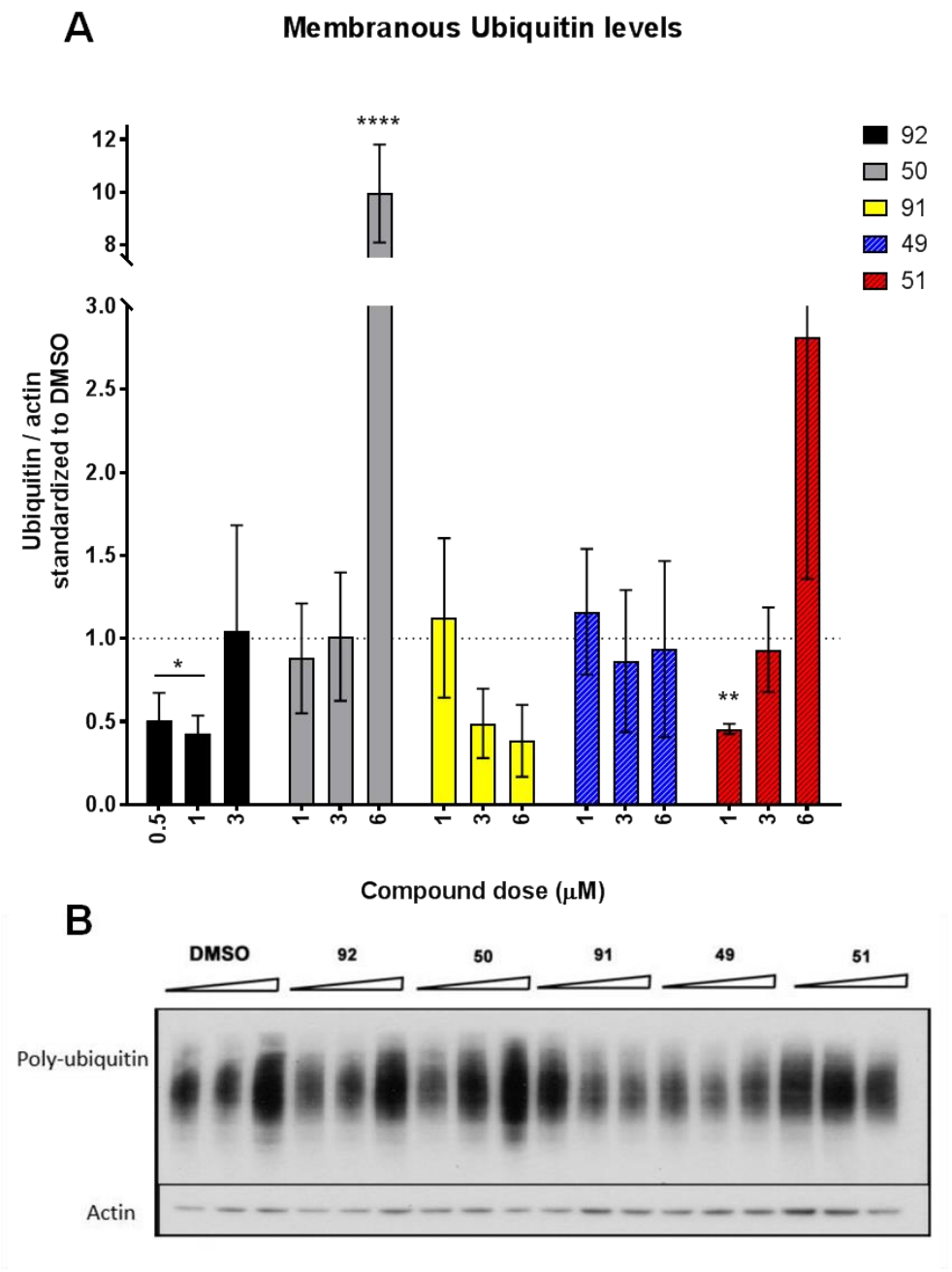


Fig. 5.9. Poly-Ubiquitin levels in the membranous fraction of cells treated with compounds. **(A)** There is a decrease in ubiquitination levels in the membranous fraction of cells treated with 1 and 3μM of compound 91 and 1μM of compound 51, a very significant increase in ubiquitination levels of cells treated with 6μM of compound 50 and a non-statistically significant increase with 6μM of compound 51 as compared to DMSO-treated cells. **(B)** Representative blot. n=3 replicates. p values when compared to control (DMSO): p = 0.0451 for compound 92, p = 0.0038 for compound 51 and p<0.0001 for compound 50 at 6Mm. Antibodies used: Actin: A1978, poly-ubiquitin: Merck Millipore MAB 1510

5.6. Effect of non-toxic doses of DUB inhibitors on autophagic markers

I also assessed the autophagy markers p62 and $LC3_{II}$ in cells treated with compounds to test whether they change in a similar fashion as seen with Usp8 knockdown. At the concentrations used (1, 3, and 6μM except for compound 92 where 0.5, 1 and 3μM were tested), $LC3_{II}$ levels did not appear to change significantly (**Fig 5.10**). Therefore, there was no overall change of $LC3_{II}$ with Usp8 inhibition. In addition, p62 levels were not changed with any of the compounds tested (**Fig 5.10**). This might suggest that autophagy was not significantly altered with the pharmacological inhibition of Usp8. However, we have not established the extent of Usp8 inhibition at 3 days post-addition of the compounds; therefore we may have had partial or short-lived inhibition of Usp8 with some of the compounds. Therefore, we cannot definitively establish whether some compounds increase autophagy or not, all we can conclude is that autophagy is not changed at 3 days post-addition of the compounds. Further research is needed to address this issue.

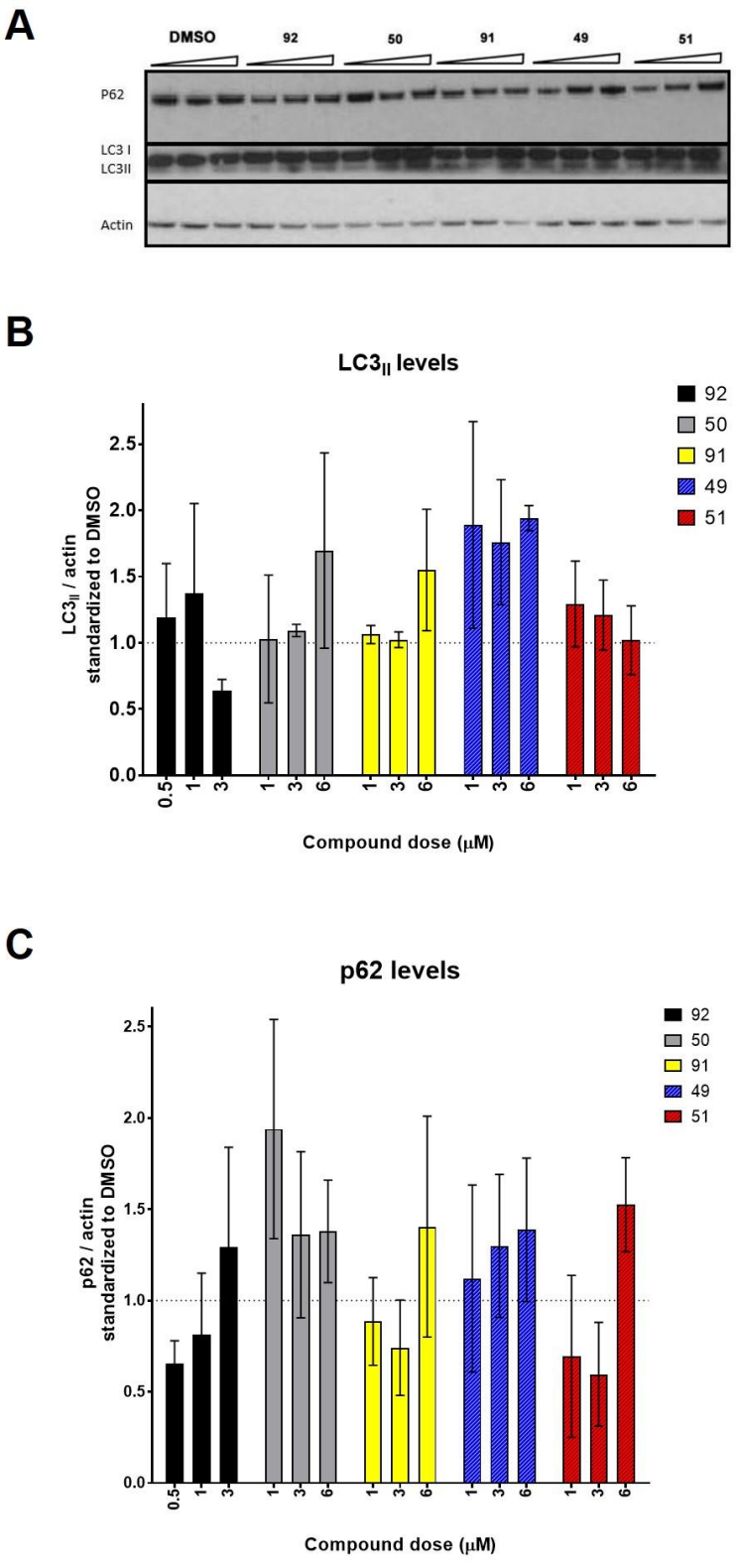


Fig 5.10. $LC3_{II}$ and p62 levels post addition of compounds. **(A)** Representative blots **(B)** $LC3_{II}$ levels: there is no significant change between control (DMSO) and all the other compounds tested at different doses. **(C)** p62 levels: there is no significant

change between control (DMSO) and all the other compounds tested at different doses. n=3 biological replicates, 1-way ANOVA performed for statistical analysis. Antibodies used: Actin: A1978, LC3: nb100-2220, p62: Abcam ab56416

5.7. Investigation of DUB inhibitors *in vivo* in *Drosophila melanogaster*

5.7.1. Effect on the eye phenotype

In order to assess the effect of different compounds *in vivo*, I used the *Drosophila melanogaster* eye model of Parkinson's disease, as explained in **Chapter 3**. My aim was to pharmacologically inhibit Usp8 and compare the rough eye phenotype of control-treated α-synuclein flies with that of different compounds at different doses. To this end, I prepared food for the flies containing different doses of all compounds and controls as explained in the materials and methods. The doses used represented an initial screen, as the doses for future experiments should be determined according to the toxicity after survival assays are to be performed and after carefully assessing the uptake of compound and bioavailability in *Drosophila*. Thus, briefly, I prepared food with 5 doses of each compound: 0.1μM, 0.33μM, 1 μM, 3.3 μM, 10 μM. All flies were either expressing α-synuclein or huntingtin (used as a control) in the eye with the GMR-Gal4 driver. Female flies were imaged at day 1 post-eclosion as it was determined in **Chapter 3** that the phenotype does not worsen further with increasing age. None of the lines tested showed an ameliorated rough eye phenotype at any of the doses tested (α-synuclein flies in **Fig. 5.11.** and Huntington flies in **Fig. 5.12).**

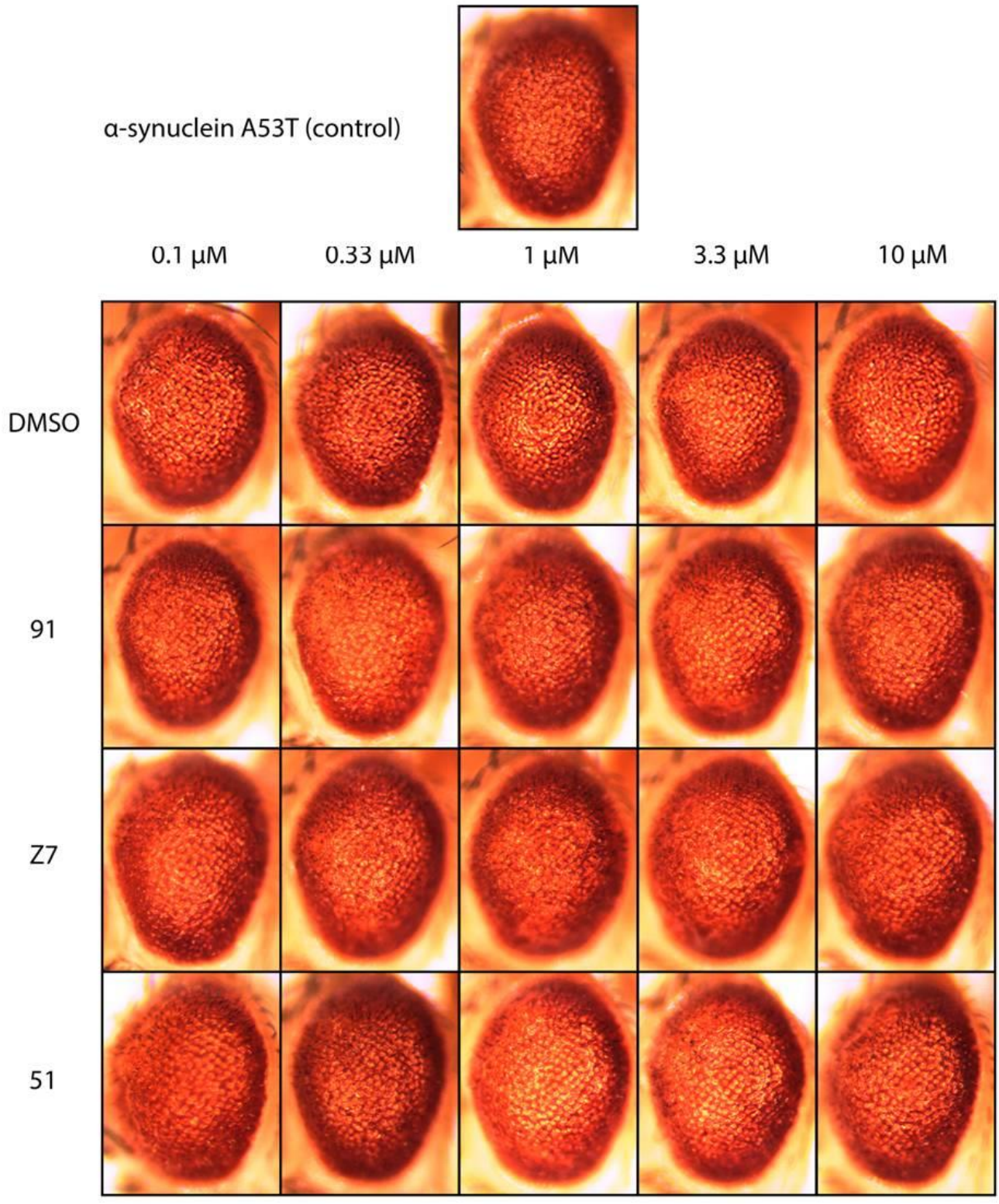


Fig. 5.11. Eyes of 1 day-post eclosion female flies expressing α-synuclein A53T fed with different doses of compounds or DMSO. n=8-30 flies, magnification 6.3x

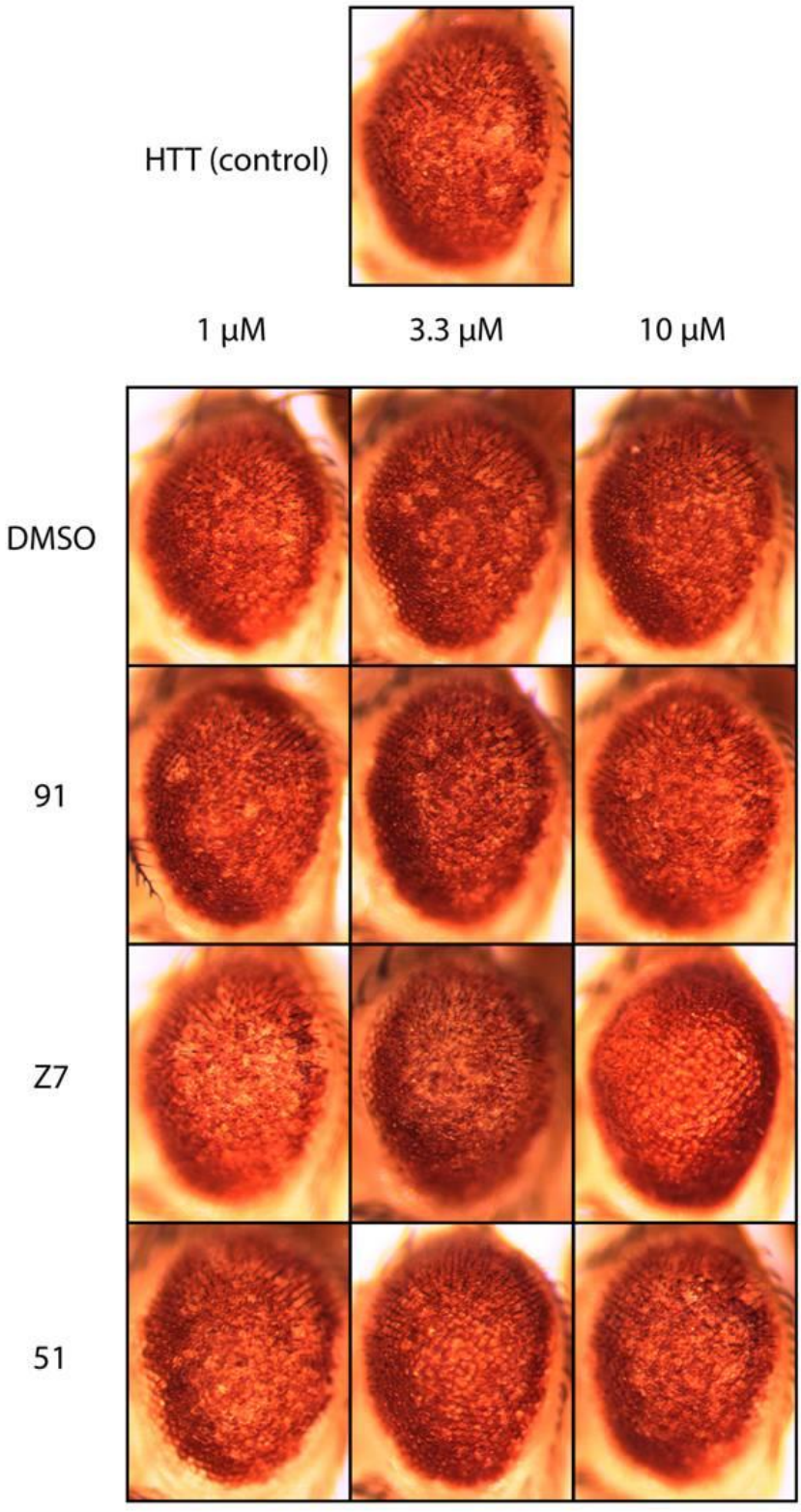


Fig. 5.12. Eyes of 1 day-post eclosion female flies expressing HTT in the eye fed with different doses of compounds or DMSO. n=8-30 flies, magnification 6.3x

5.8. Discussion

The cell viability and cell death assays (MTS and LDH) have to be interpreted together as they both represent two faces of the same coin. For this purpose, a compound that causes a drop in the viability of cells at a specific dose of toxin should increase proportionally the cell death in the LDH assay. However, this was not the case in the experiments here for all conditions. For example, judging from the MTS assays, compound 91 appears to be slightly more toxic at 200μM of hydrogen peroxide and 2.5μM of arsenite. But judging from the LDH assays, it appears to be protective against most doses of dopamine applied and against 20μM of arsenite. One of the reasons that a compound may appear to increase death in LDH assay but not decrease viability in the MTS assay is that the MTS assay represents cellular metabolism and thus, with the addition of any compound (toxic or not), cellular metabolism increases at the beginning to above 100%. Thus, a toxic compound, would abolish this increase and would show values just below 100% which might actually be more toxic than values at 110%. Therefore, these assays have to be interpreted in context. Overall, these studies suggest that Usp8 inhibition at the concentrations used does not compromise viability of cells under conditions of cellular stress. These findings mostly agree with studies in Usp8 knockdown cells as detailed in **Chapter 4.3.2** where I found that lack of Usp8 did not compromise the viability of SH-SY5Y cells under oxidative stress. It would be interesting to assess in the future the effect of pharmacological Usp8 inhibition on the heat shock response, where knockdown of Usp8 was protective.

These initial results suggest that compounds 91, 49 and 51 lower the cytosolic and membranous-bound levels of α-synuclein across all doses tested. Compound 50

lowered the membranous-bound α-synuclein only at higher doses, which were associated with increased global ubiquitination, and thus potentially an off target or toxic secondary effect. In order to conclude about the α-synuclein lowering properties of each compound, one needs to check the extracellular levels of α-synuclein in these experiments as well as the total levels using lower concentrations to establish whether there is a dose-response effect. To this end, Dimitra Mazaraki, an MSc student in the laboratory, has measured extracellular α-synuclein in media collected from my initial experiments. She found that compound 49 significantly raised the extracellular level of α-synuclein, suggesting that this compound may affect α-synuclein secretion rather than degradation. From my own data, I can conclude that compounds 91 and 51 lower the total amount of alpha synuclein. Compound 91 is a specific Usp8 inhibitor, but compound 51 is a specific Usp1 inhibitor. This could implicate both Usp8 and Usp1 inhibition in lowering the levels of α-synuclein. However, from additional experiments by Dimitra Mazaraki, using lower compound concentrations (ranging from 0.1μM to 1μM) and daily media changes to counteract the spontaneous conversion of these compounds to inactive intermediates that occurs within 24h (data obtained at the Oxford DDI), she found that compound 91 was the only compound that reproducibly reduced total intracellular α-synuclein. The fact that compound 92 (specific Usp8 inhibitor) and compound 50 (mixed Usp8 and Usp7 inhibition) don't lower the levels of α-synuclein could be due to a number of reasons. Firstly, compound 92 is more toxic for the cells, suggesting that this compound may have multiple off target effects. In addition, the medicinal chemists at the Oxford DDI who we collaborate with, established that compound 50 is very short acting within cells and metabolised within minutes. Thus, in retrospect, compound 50 would not have been active for

the duration of my initial incubation experiments. In contrast, compound 91 was found to be slowly metabolized, thus being stable for at least 24 hours. Overall, my initial experiments and subsequent work in the laboratory suggest that pharmacological inhibition of Usp8 with small molecules that share the specificity, stability and toxicity profile of compound 91 can lower the intracellular levels of α-synuclein.

In addition, when I tested the effect of Ups8 inhibition by feeding the flies with inhibitors, I did not observe any amelioration of the phenotype with any of the compounds or doses tested. This is most probably because the eye disc of the drosophila has already been formed before it starts eating. Thus, since it is a developmental phenotype we would not be able to alter it by intervening with their nutrition. A suggested next step would be to feed ddcGal4/UAS α-synuclein flies (expressing α-synuclein in the dopaminergic neurons) compounds and assess an age-related phenotype, for example the age-dependent decrease in locomotion caused by the α-synuclein toxicity, using the climbing assay. This would be a better experiment because this phenotype does not produce structural defects which are permanent, but it can assess (locomotive) function which could be altered with Usp8 inhibition in adulthood.

The next step would be to test compound 91 in rat cortical neurons or iPSC-derived dopaminergic neurons to assess its effect on α-synuclein levels in primary cells that are relevant to the disease pathogenesis. If Usp8 proves a valid target at this stage, the next step would be to proceed to lead optimization and assessment of pharmacokinetics and safety in animals and also include assessing global changes in protein expression to assess for differences and compensation (e.g. with Mass

Spectrometry). When this will have been assessed, experiments must be done with an equivalent dose *in vivo* in mammalian models of Parkinson's disease (e.g. rats or mice with increased α-synuclein levels) to assess if mammalian organisms have a similar reaction to pharmacological Usp8 (or Usp1) inhibition. As part of *in vivo* testing, the compound's pharmacokinetic and pharmacodynamic properties must be assessed to achieve the desired metabolism, distribution, plasma protein binding, solubility, permeability etc. Therefore, although my results are very early studies in such a pipeline, they could inform the development of therapeutic compounds in Parkinson's disease and other synucleinopathies. Importantly, a specific and effective Usp8 inhibitor could lead to better therapies in other conditions where Usp8 is relevant such as certain forms of drug-resistant cancers and Cushing's disease.

The advantage of targeting Usp8 in Parkinson's disease is that in principle, it is pharmacologically druggable more so than an E3 ubiquitin ligase (like Nedd4). Although efforts are being made to produce more specific small molecule inhibitors, it may be beneficial to target multiple DUBs which may be acting in disease-relevant pathways. Multiple DUB inhibitors are currently being investigated in preclinical studies.

DUBs are involved in many pathologies, ranging from neurodegeneration and cancer to viral infections. Thus, it is a very promising therapeutic area. New technologies (e.g. assessing DUB selectivity and specificity with MALDI-TOF Mass Spectrometry) and improving older techniques such as ubiquitin C-terminal 7amido-4-methylcoumarin (Ub-AMC) will allow rapid progress in the identification of specific and selective inhibitors.

5.9. Conclusion

My main conclusion based on my initial experiments and other work in the laboratory is that pharmacological Usp8 inhibition with a specific and potent compound has the potential to lower the levels of intracellular α-synuclein without causing toxicity. Thus, pharmacological Usp8 inhibition recapitulates the findings of genetic Usp8 knockdown. The viability and toxicity experiments performed show that partial Usp8 inhibition is viable for the cells. Although more specific compounds would be valuable, if a DUB signature is identified in synucleinopathies, a DUB inhibitor targeting several DUBs to different extent might also be a good strategy.

6. Discussion

Parkinson's disease is a multi-factorial disease that incorporates pathologies ranging from protein aggregation and dopamine dysregulation to mitochondrial dysfunction and oxidative stress. Integration of research in multiple systems would be needed to bring together the apparent distinct pathologies of Parkinson's disease and to find the links between sporadic and genetic Parkinson's disease. The different arms of protein homeostasis communication and integration will need to be studied first in order to get a wider picture of what the overall mechanism is in Parkinson's pathogenesis (Narayan, Ehsani et al. 2014). Furthermore, this integrative approach needs to be extended beyond the intracellular homeostasis, to interneuronal connections in the brain and beyond (eg enteric nervous system) and to neuronal-glial interactions in order to explain the disease progression. This is particularly important in order to elucidate the pathophysiology of the progression of PD from the site of initiation (e.g. enteric nervous system and olfactory bulb) and the possible prion-like mechanism of α-synuclein transmission.

Current licenced therapies in Parkinson's disease (eg levodopa) are symptomatic, targeting its motor symptoms. However, there is an increasing need for the development of disease-modifying therapies that will target the cause of the disease and prevent or delay cell death that primarily targets the nigrostriatal dopaminergic neurons in PD. In my thesis, I identified and assessed a potential target, which functions via regulating α-synuclein levels. When considering proteostasis and protein clearance mechanisms, there are various ways one could utilize to reduce the amount of protein aggregates. One could reduce protein production (e.g. with antisense oligonucleotides -ASOs), enhance protein breakdown or shift the

equilibrium from protein aggregates to monomers that could be removed easier than the aggregates. Usp8 knockdown and inhibition decreases the levels of α-synuclein by increasing its breakdown.

When considering ways to lower the levels of α-synuclein, targeting a DUB would be a valid approach, since DUBs are involved in many parts of protein breakdown and amongst the 100 DUBs in the human genome, there is scope for specificity and selectivity. DUBs are druggable targets and new methods and approaches are being undertaken currently to develop specific and potent DUB inhibitors. The E3 Ubiquitin ligase Nedd4 has been identified and validated to be a target against α-synuclein-induced toxicity by our laboratory and others in diverse systems including yeast, *Drosophila*, rodent and iPSC-derived neuronal models (Tofaris et al. 2011, Tardiff et al. 2013, Davies et al. 2014, Alexopoulou et al. 2016, Chung et al. 2013). The discovery of NAB2 as neuroprotective agent that activates Nedd4 lends support to the proposition that targeting ubiquitin signalling could be a useful therapeutic intervention in synucleinopathies ((Tardiff, Jui et al. 2013, Chung, Khurana et al. 2013). Identifying Usp8, a DUB which acts antagonistically to Nedd4 at least in some synapses (Scudder, Goo et al. 2014) would offer an alternative and more druggable target for such therapies. Our proposed model identifies Usp8 to have opposing functions to those of Nedd4 in α-synuclein degradation and toxicity (**Fig 6.1**).

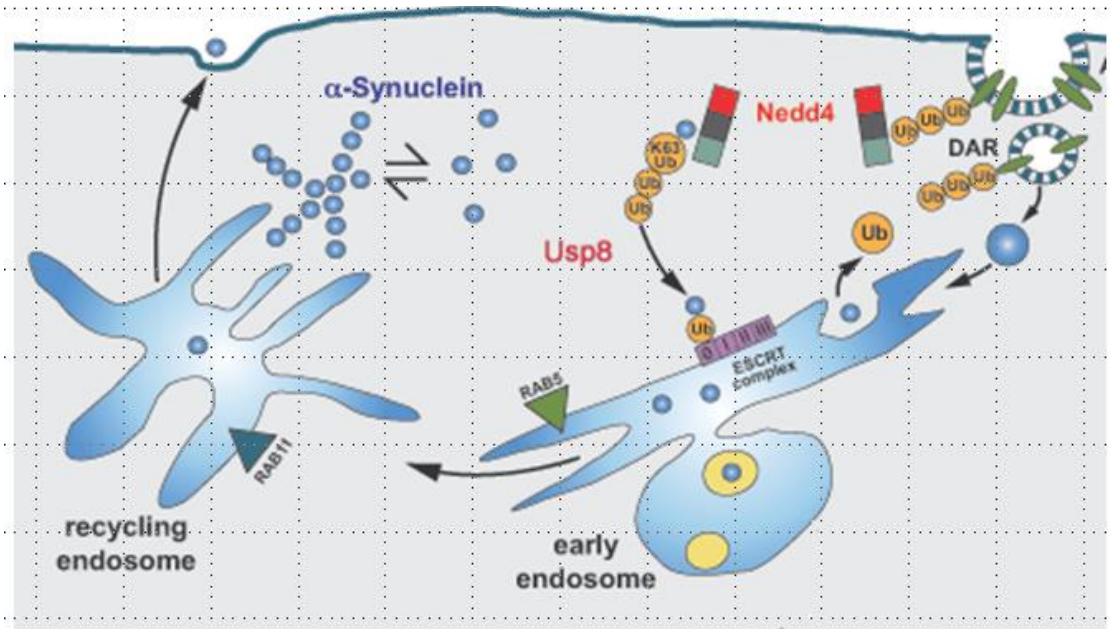


Fig 6.1 Usp8 acts antagonistically to Nedd4. It deubiquitinates α-synuclein and prevents its breakdown via the endosomal-autophagic-lysosomal route by recycling it back to the cytoplasm. On the other hand, Nedd4 ubiquitinates α-synuclein enhancing its breakdown. Figure adapted from (Perrett, Alexopoulou et al. 2015).

The first two result chapters of my thesis established Usp8 as a target for α-synuclein lowering strategy both *in vitro* and *in vivo* using genetic techniques (knockdown and overexpression) and the third chapter suggests that under optimal conditions, similar effects can be obtained with pharmacological Usp8 inhibition. Thus, the next steps could be target validation, lead optimization and further preclinical studies including pharmacokinetic and pharmacodynamic assessment before progressing to clinical trials.

Our starting screen in the substantia nigra of post-mortem Parkinson's disease brains was a targeted screen for possible interactors of K-63 chains, where Usp8 was identified. It is possible that additional DUBs may be involved in the pathogenesis of Parkinson's disease. Thus, the next step would be to perform an

unbiased screen for all DUBs in human pathological specimens and other Parkinson's disease-relevant cellular readouts. Of those the essential DUBs or DUBs that are involved in crucial processes such as tumour suppression stabilization or DNA damage repair would need to be excluded, but the rest could make up a "Parkinson's disease signature" that could be targeted pharmacologically if further validated. This would be certainly possible, since about 70 out of 95 DUBs share a similar catalytic domain (cysteine proteases) and current inhibitors were shown to target more than one DUB to a variable degree. Taking advantage of these properties of such therapeutics, we could target the "Parkinson's disease signature" of DUBs with a single or with two compounds.

A final issue that needs to be considered is that Usp8 is an essential DUB for development and when knocked out it is embryonic lethal. This would not be the case when it is partially inhibited pharmacologically in advanced age (such as in Parkinson's disease). Furthermore, an additional strategy that could be implemented is to target Usp8 inhibitors to dopaminergic neurons, e.g. by attaching it to an agonist to the DAT receptor, thus internalizing the compound. By this way any unwanted side-effects from targeting non-dopaminergic neurons could be avoided.

7. Conclusion

In my thesis, I explored a way to enhance the breakdown of α-synuclein. I started from evidence from post-mortem brains of Parkinson's disease that showed an elevated level of the DUB Usp8 in the substantia nigra over cortical areas which inversely correlated with total and K-63 levels of ubiquitination. I then evaluated the function of Usp8 in relation to α-synuclein. I found that Usp8 has a direct interaction with α-synuclein and it is able to deubiquitinate it and increase its half-life. Its knockdown increases α-synuclein ubiquitination and specifically K63-associated ubiquitination and decreases its levels. I also found that reducing Usp8 activates, autophagy which may be relevant to the observed effect on α-synuclein. In addition, Usp8 knockdown is protective against heat shock in cell cultures. For further validation, experiments were performed *in vivo* in *Drosophila melanogaster*. α-Synuclein expression in the fly eye caused a rough eye phenotype that is rescued by concomitant Usp8 knockdown. This effect is specific to α-synuclein and does not affect the toxicity of other unrelated proteins causing rough eye phenotypes, for example pathogenic ataxin3 expansion or huntingtin repeats. In addition, this effect on α-synuclein was specifically seen with Usp8 knockdown and not with the knockdown of other DUBs tested. Furthermore, expression of α-synuclein in the dopaminergic neurons of the fly causes an age-dependent locomotive defect, a loss of a specific cluster of dopaminergic neurons and decreased lifespan. These were all rescued with concomitant knockdown of Usp8 in the dopaminergic neurons. All the above suggest that knockdown of Usp8 is protective against α-synuclein toxicity *in vitro* and *in vivo* by lowering its levels. Initial experiments with compound inhibitors of Usp8 at non-toxic specific concentrations suggest that it is possible to decrease the levels of α-synuclein pharmacologically. Taken together, work

included in my thesis indicates that Usp8 may be a potential target for disease-modifying therapies in Parkinson's disease. However, further assessments are required in the areas of target validation, lead optimization, and preclinical studies including *in vivo* studies with pharmacokinetic and pharmacodynamic approaches before we can progress to clinical trials. This thesis could be the beginning of a long process of investigation of a promising disease-modifying target.

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