ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

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''ROLE OF THE S18Y POLYMORPHISM OF UCH-L1 PROTEIN ON NEUROPROTECTION''

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ΚΑΜΑΡΑΤΟΥ ΜΑΡΤΙΝΑ

Επιβλέποντες καθηγητές: Δ. Καραγογέως, Α. Γραβάνης

Επιστημονικός Υπεύθυνος: Λ. Στεφανής

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Τριμελής επιτροπή:

Λ. Στεφανής

Α. Γραβάνης

Δ. Καραγωγέως

Τόπος διεξαγωγής ερευνητικής εργασίας:

Τομέας βασικών νευροεπιστημών, Εργαστήριο Νευροεκφυλιστικών Νόσων, Ίδρυμα Ιατροβιολογικών Ερευνών Ακαδημίας Αθηνών

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ΠΡΟΛΟΓΟΣ

Η παρούσα διπλωματική εργασία εκπονήθηκε στο εργαστήριο Νευροεκφυλιστικών Νόσων, στον τομέα Βασικών Νευροεπιστημών του Ιδρύματος Ιατροβιολογικών Ερευνών της Ακαδημίας Αθηνών. Η εργασία πραγματοποιήθηκε υπό την καθοδήγηση και επίβλεψη του Δρ. Λ. Στεφανή, στα πλαίσια του Μεταπτυχιακού Προγράμματος Ειδίκευσης '' Μοριακή και Κυτταρική Αιτιολογία, Διαγνωστική και Θεραπευτική των Νοσημάτων του Ανθρώπου''. Θα ήθελα να ευχαριστήσω τα μέλη της τριμελούς επιτροπής, τον καθηγητή Α. Γραβάνη, την καθηγήτρια Δ. Καραγογέως και τον καθηγητή Λ. Στεφανή. Ιδιαίτερα θα ήθελα να ευχαριστήσω τον υπεύθυνο καθηγητή Λ. Στεφανή για την ευκαιρία που μου έδωσε να πραγματοποιήσω την παρούσα διπλωματική εργασία στο εργαστήριο του και την υποστήριξη του καθ' όλη τη διάρκειά της. Οφείλω να ευχαριστήσω θερμά την ερευνήτρια Μ. Ξυλούρη για την πολύτιμη καθοδήγηση και την συμπαράσταση της και την ουσιαστική συμβολή της στην ολοκλήρωση της εργασίας αυτής. Τέλος, θα ήθελα να ευχαριστήσω όλα τα μέλη του εργαστηρίου για την βοήθεια και την υποστήριξη τους.

CONTENTS

ΠΕΡΙΛΗΨΗ

Η νόσος του Parkinson είναι μια χρόνια νευροεκφυλιστική νόσος και η δεύτερη πιο συχνή νευρολογική διαταραχή μετά τη νόσο Alzheimer. Η ασθένεια αυτή χαρακτηρίζεται από κινητικά και μη κινητικά συμπτώματα. Τα κύρια κινητικά συμπτώματα είναι ο τρόμος, η βραδυκινησία, η έλλειψη εκούσιας κίνησης και η μυϊκή δυσκαμψία. Η παθολογία της νόσου χαρακτηρίζεται από τη σταδιακή απώλεια των ντοπαμινεργικών νευρώνων στη συμπαγή μοίρα της μέλαινας ουσίας του εγκεφάλου και την παρουσία κυτταροπλασματικών εγκλείστων στους εναπομείναντες νευρώνες, τα οποία περιέχουν συσσωματώματα πρωτεϊνών και ονομάζονται σωμάτια Lewy Bodies (LBs). Την πλειονότητα των περιπτώσεων με τη νόσο Parkinson αποτελούν οι σποραδικές περιπτώσεις, ενώ μόνο το 5% είναι οικογενείς. Στην περίπτωση των σποραδικών περιπτώσεων εμπλέκονται τόσο περιβαλλοντικοί όσο και γενετικοί παράγοντες προδιάθεσης. Έχει βρεθεί ότι στην παθοφυσιολογία της νόσου εμπλέκονται διάφορες παθολογικές καταστάσεις, όπως η βλάβη των μιτοχονδρίων, η δυσλειτουργία του συστήματος αποικοδόμησης πρωτεϊνών, το οξειδωτικό στρες και η φλεγμονή.

Όσον αφορά τις οικογενείς περιπτώσεις, αρκετά γονίδια έχουν σχετιστεί με τη νόσο και ακολουθούν αυτοσωμικό επικρατή ή υπολειπόμενο τύπο κληρονομικότητας. Ο γενετικός τόπος PARK-5 που κωδικοποιεί για την πρωτεΐνη UCH-L1 είναι ένα από τα γονίδια επικρατούς κληρονομικότητας. Η πρωτεΐνη UCH-L1 βρίσκεται σε αφθονία στον εγκέφαλο όπου εντοπίζεται σε προ- και μετα-συναπτικές περιοχές. Ανήκει στην οικογένεια υδρολασών καρβοξυτελικού άκρου ουμπικουιτίνης και αποτελεί το μόνο μέλος της οικογένειας αυτής με ικανότητα διμερισμού. Η πρωτεΐνη UCH-L1 έχει *in vitro* ενεργότητα υδρολάσης του καρβοξυτελικού άκρου της ουμπικουιτίνης και ενεργότητα λιγάσης της ουμπικουιτίνης μετά από διμερισμό. Ακόμη, έχει βρεθεί πως συνδέεται με τη μονομερή ουμπικουιτίνη, αποτρέποντας την αποικοδόμησή της και σταθεροποιώντας τα επίπεδα της στα κύτταρα. Παρ' όλη την αφθονία της πρωτεΐνης στα νευρικά κύτταρα, ο ρόλος της *in vivo* παραμένει αδιευκρίνιστος. Η γενετική συσχέτιση της πρωτεΐνης UCH-L1 με τη νόσο Parkinson έγινε για πρώτη φορά το 1999, με την ανακάλυψη της μεταλλαγής I93M UCH-L1 σε μια γερμανική οικογένεια στην οποία η νόσος του Parkinson εμφάνιζε αυτοσωμικό επικρατή τύπο κληρονομικότητας. Το 1999 ανακαλύφθηκε ο πολυμορφισμός S18Y της πρωτεΐνης UCH-L1, ο οποίος προτάθηκε πως ασκεί μια προστατευτική

δράση έναντι της εμφάνισης της νόσου Parkinson σε διάφορους πληθυσμούς και ιδιαίτερα στις σποραδικές περιπτώσεις. Από τότε μέχρι σήμερα έχουν πραγματοποιηθεί αρκετές μελέτες και γονιδιακές αναλύσεις ευρείας κλίμακας, κάποιες εκ των οποίων δεν υποστηρίζουν τη γενετική συσχέτιση του πολυμορφισμού S18Y με τη νόσο του Parkinson. Πρόσφατες μελέτες έδειξαν πως η πρωτεΐνη S18Y UCH-L1 προστατεύει έναντι του H_2O_2 και της τοξίνης MPTP η οποία χρησιμοποιείται ευρέως ως μοντέλο τοξικού παρκινσονισμού. Παρ' όλα αυτά, ο ακριβής μηχανισμός της αντιοξειδωτικής αυτής δράσης παραμένει σε μεγάλο βαθμό αδιευκρίνιστος.

Ο στόχος της παρούσας εργασίας ήταν η διευκρίνιση του μηχανισμού της νευροπροστατευτικής δράσης του S18Y πολυμορφισμού της UCH-L1 πρωτεΐνης σε νευρικά κύτταρα. Χρησιμοποιώντας μια μεταλλαγμένη μορφή της S18Y UCH-L1 πρωτεΐνης (μεταλλαγή στην αλληλουχία φαρνεσυλίωσης), που έχει προταθεί πως συνδέεται σε μικρότερο βαθμό στις μεμβράνες, βρέθηκε πως η παρεμπόδιση της μετα-μεταφραστικής αυτής τροποποίησης δεν επηρέασε την πρόσδεση του S18Y πολυμορφισμού στις μεμβράνες, ούτε την προστατευτική του δράση έναντι στην νευροτοξικότητα επαγόμενη από MPP^+. Ακόμη, η υπερεκφρασμένη πρωτεΐνη S18Y UCH-L1 ανιχνεύθηκε σε παρόμοια επίπεδα στις υποκυττάριες περιοχές (κυτταρόπλασμα, πυρήνες, μιτοχόνδρια, ενδοσώματα) συγκριτικά με την υπερεκφρασμένη WT UCH-L1, υπό φυσιολογικές συνθήκες. Προκειμένου να διερευνήσουμε εάν ο πολυμορφισμός S18Y προστατεύει επιλεκτικά έναντι οξειδωτικών παραγόντων ή εάν χαρακτηρίζεται από μια πιο ευρεία προστατευτική δράση, βρήκαμε πως δεν έδρασε προστατευτικά ενάντια σε νευροτοξικότητα επαγόμενη από γλουταμινικό οξύ ή από υπερέκφραση της αγρίου τύπου α-συνουκλεΐνης. Τα ευρήματα αυτά προτείνουν μια επιλεκτική δράση του συγκεκριμένου πολυμορφισμού, εστιασμένη σε ερεθίσματα που επάγουν οξειδωτικό στρες. Καθώς ο πολυμορφισμός S18Y ανιχνεύθηκε στα μιτοχόνδρια και στα ενδοσώματα, οργανίδια τα οποία συμμετέχουν στη ρύθμιση της οξειδοαναγωγικής ομοιόστασης, είναι πιθανόν η προστατευτική του δράση να επιτελείται μετά από μεταφορά του στα οργανίδια αυτά υπό συνθήκες οξειδωτικού στρες. Το οξειδωτικό στρες αποτελεί παράγοντα που εμπλέκεται στην παθοφυσιολογία του Parkinson και άλλων νευροεκφυλιστικών διαταραχών. Επομένως, περαιτέρω διερεύνηση του μηχανισμού μέσω του οποίου ο πολυμορφισμός S18Y UCH-L1 ασκεί τη νευροπροστατευτική του δράση θα συμβάλλει στην ανάπτυξη νέων θεραπευτικών προσεγγίσεων για την αντιμετώπιση της νόσου του Parkinson και άλλων νευροεκφυλιστικών ασθενειών.

SUMMARY

Parkinson's disease (PD) is a chronic neurodegenerative disease and the second most common neurological disorder after Alzheimer disease. It is characterized by motor and non motor symptoms. The main motor symptoms include tremor at rest, slowness or absence of voluntary movement, rigidity (increased resistance to passive movement of patient's limbs) and freezing. The neuropathological hallmarks of PD are the progressive loss of the dopaminergic neurons in the substantia nigra pars compacta and the presence of intraneuronal proteinacious deposits within the surviving neurons, termed Lewy Bodies. The majority of PD cases (95%) are sporadic, whereas only 5% of cases are familiar. As far as sporadic PD is concerned, evidence implicates mitochondrial damage, dysfunction of protein degradation systems, oxidative stress and inflammation in disease development.

For the familiar forms of PD, several dominant and recessive inheritance genes have been linked to the disease. PARK-5 locus is one of the PD-linked genes with an autosomal dominant manner of inheritance and codes for the protein UCH-L1. UCH-L1 belongs to the Ubiquitin C- terminal Hydrolase family and is the only member of this family reported to dimerize. It is one of the most abundant proteins in the brain and is localized in the pre- and postsynaptic regions. It is also normally expressed in testes and ovaries. Experimental evidence suggests that UCH-L1 protein possesses hydrolase and ligase activity *in vitro*. In has been also reported to bind to mono-ubiquitin *in vitro,* thus inhibiting its degradation and stabilizing its intracellular levels. Although UCH-L1 is abundant in neuronal cells, the physiological role of this protein *in vivo* remains elusive. The genetic linkage of UCH-L1 protein to PD was reported in 1998, when the I93M mutation in the UCH-L1 gene was found in a German family with autosomal dominant PD. The S18Y UCH-L1 polymorphism was discovered in 1999 and reported to be protective against sporadic forms of PD, a finding that has been questioned by many studies, while others suggest an age-dependent protective effect. Even though the S18Y UCH-L1 polymorphism was recently found to exert a neuroprotective role against well-established oxidants, such as MPP+ and H_2O_2, the precise mechanism via which this is accomplished, remains elusive.

The aim of the present study was to examine the mechanism of the potential neuroprotective effects of the S18Y UCH-L1 protein in neuronal cells. Using the double farnesylation mutant S18Y

C220S UCH-L1, which is reported to bind to a lower extent to membranes, we found that inhibition of farnesylation did not alter the membrane binding or the protective effect of the S18Y polymorphism against MPP^+-induced toxicity. In addition, overexpressed S18Y UCH-L1 protein localized at similar levels in cytosol, nuclei, mitochondria and endosomes, compared to overexpressed WT UCH-L1, under normal conditions. In an attempt to investigate whether the protective properties of S18Y polymorphism are selective against oxidative stress-evoked insults or might be observed, against other neurotoxic stimuli, we found that S18Y UCH-L1 was not protective against excitotoxic cell death induced by L-Glutamate, or neurotoxicity mediated by α-synuclein overexpression. These results suggest that the S18Y UCH-L1 polymorphism exerts a specific anti-oxidant neuroprotective role. Since S18Y UCH-L1 localized both in lysosomes and mitochondria, organelles known to contribute to the regulation of redox homeostasis, this protein may exert a protective effect following its translocation to one of these compartments, upon oxidative stress conditions. As oxidative stress is implicated in pathophysiology of PD and other neurodegenerative conditions, the elucidation of the mechanism(s) mediating the observed anti-oxidant effect of the S18Y UCH-L1 protein, may pave the way for the development of new therapeutic strategies for the treatment of PD and related disorders.

INTRODUCTION

Clinical characteristics of Parkinson's disease

Parkinson's disease (PD), originally described by James Parkinson in 1817, is the second most common neurodegenerative disease after Alzheimer Disease (AD). It is a movement disorder that affects 1–2% of the population over age 60 (Lang & Lozano 1998a; Lang & Lozano 1998b). Majority of PD is sporadic accounting to 95% of the cases, while in the remaining cases the disease is inherited. The typical clinical characteristics of PD include tremor at rest, slowness or absence of voluntary movement, rigidity and freezing (the inability to begin a voluntary movement such as walking) (Parkinson et al. 1817). Paucity of normal facial expression, drooling (failure to swallow without thinking about it), decreased size and speed of handwriting represent a variety of motor symptoms observed in PD. PD patients also present non-motor symptoms such as depression, dementia (especially in older patients), autonomic dysfunction, sleep difficulties and pain (Don, C.N., 1996; Kumar et al., 2002; Calne et al., 1992).

Pathology of Parkinson's disease

The neuropathological hallmarks of PD are the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of intraneuronal proteinacious deposits termed Lewy Bodies (LBs) (Figure 2). Substantia nigra pars compacta is part of the basal ganglia, a brain region responsible for voluntary and involuntary movement. Brains of PD patients are characterized by loss of dopaminergic neurons, accompanied by a dramatic decrease of dopamine levels in striatum, due to the nigrostriatal pathway degeneration (**figure A** depicts the normal nigrostriatal pathway) (Trétiakoff et al. 1919). Production of neuromelanin in the dopaminergic neurons results in the normal pigmentation of the SNpc. Depigmentation of SNpc is a result of the marked loss of dopaminergic neurons and subsequently of the dark-brown pigment neuromelanin. Degeneration of the nigrostriatal pathway due to dopamine loss leads to failure of normal movement and development of clinical motor symptoms of PD patients.

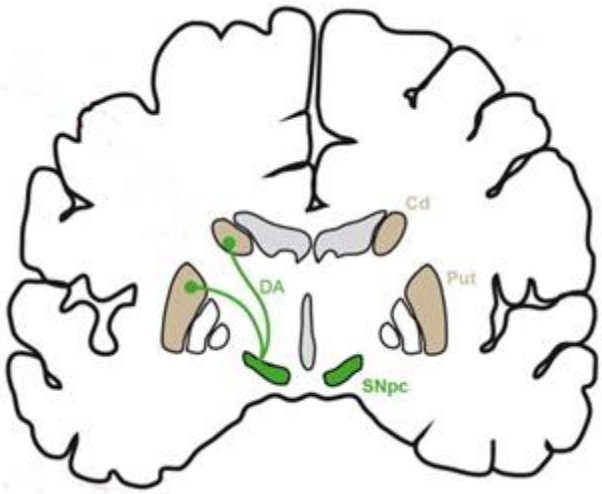

Figure 1: Schematic representation of the normal nigrostriatal pathway (in green). The nigrostriatal pathway is composed of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc;in green). These neurons project (green lines) in the striatum [i.e., putamen (Put) and caudate nucleus (Cd)] (D' Anglemont de Tassigny et al., 2015).

The second characteristic of PD is the presence of LBs in the surviving neurons of SNpc (Lewy et al., 1912). LBs are spherical eosinophilic cytoplasmic protein inclusions, resulting from the aggregation of misfolded proteins, such as the neuronal protein α-synuclein. α-synuclein is a core pathological feature of PD due to its presence in LBs and its genetic linkage to the disease. LBs are also characterized by the presence of ubiquitin, neurofilament and phosphorylated, oxidized and ubiquitinated proteins (Spillantini et al., 1997, 1998). LBs or other abnormal deposits of α-synuclein are also present in other neurodegenerative diseases, such as Multiple System Atrophy (MSA), Dementia with LBs (DLB) and Alzheimer's disease (AD). Although definite diagnosis of PD requires the identification of both LBs and nigral dopaminergic neuron loss, LB presence is not always associated to the disease (Takahashi et al., 1994; Mori et al., 1998; Wszolek et al., 2004). Notably, several studies suggest a protective role for LBs in that they accumulate and isolate misfolded, potentially toxic proteins that have not been successfully degraded by the proteolytic degradation systems (Wakabayashi et al., 1999; Van Duinen et al., 1999).

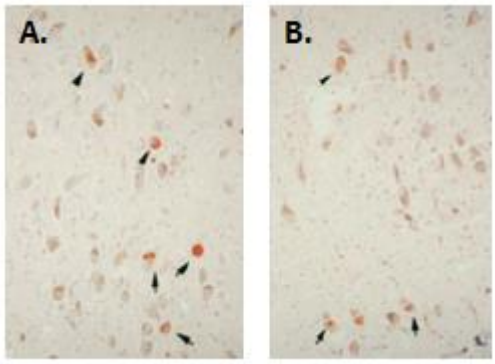

Figure 2: Immunohistochemical labeling of α-synuclein and ubiquitin reveals the presence of LBs in nigral dopaminergic neurons in the brains of PD patients (Tofaris et al., 2003).

Neurodegeneration and LB formation are also found in noradrenergic (locus coeruleus), serotonergic (raphe nucleus) and cholinergic (nucleus basalis of Meynert, dorsal motor nucleus of vagus) systems and in the cerebral cortex, the olfactory bulb and the autonomic nervous system (reviewed by Hornykiewicz and Kish, 1987). Moreover, Braak et al. in 2003 suggested a staging procedure for the development of PD; the pathogenesis firstly appears in the dorsal motor nucleus of the vagus and the anterior olfactory nucleus. Neurodegeneration spreads gradually to less vulnerable brain regions until it affects the SNpc at later stages of the disease (Braak et al., 2003).

Therapeutic strategies

Currently, the therapeutic approach used for PD is based on the alleviation of the motor symptoms of the disease and includes L-DOPA (Levodopa, a precursor molecule of dopamine), dopamine receptor agonists and monoamine oxidase inhibitors. However, the effectiveness of these therapeutic approaches is limited in time with a consequent loss of the effect. In addition, the patients with advanced disease may manifest unsuccessful response to the treatment and side effects, such as neuropsychiatric manifestations and dyskinesias (Jancovic, J., 2005). The existence of separate initial pathways that lead to disease manifestation highlights the need for specific targeting of the affected pathway and the stratification of patients in order to select the appropriate treatment. In this way, the initial causes that lead to neuronal dysfunction and

death in PD will be targeted efficiently.

Etiology of Parkinson's disease

The majority of PD cases (95%) are sporadic, of unknown etiology, with implications for a role of environmental factors. Although only 5% of cases are familiar, they manifest very similar clinical symptoms, thus serving as a useful tool for the elucidation of the pathophysiology of sporadic PD, which is the most common form of the disease. Notably, investigation of the genetic factors causative to PD propose common molecular pathways through which familiar and sporadic forms develop. Subsequently, genetic factors may act synergistically with sporadic, resulting in differential propensity to PD manifestation.

Familiar forms of Parkinson's disease

A. Autosomal dominant inheritance genes

α-SYNUCLEIN (PARK-1; PARK4)

Although the exact function of α-synuclein is yet to be clarified, the main function of the protein is suggested to be the regulation of synaptic activity (Chandra et al, 2005) and the control of neurotransmitter release (Lundvig et al., 2005). Expression of α-synuclein is induced during neuronal development, following establishment of synaptic connections and induction of proteins involved in synaptic structure (Murphy et al. 2000). Six point mutations in the SNCA gene encoding for α-synuclein, A53T, A30P, E46K, H50Q, G51D, and A53E, cause autosomal dominant PD (Polymeropoulos et al. 1997; Kruger et al. 1998; Zarranz et al. 2004; Kiely et al., 2013; Fujioka et al., 2014; Pasanen et al., 2014). Moreover, α-synuclein multiplication cases have also been reported, suggesting that overexpression of the wild-type form of α-synuclein may be causative to PD (Singleton et al. 2005; Farrer et al. 2004;Ibanez et al. 2004; Nishioka et al. 2006; Fuchs et al. 2007). The most prevalent suggestion for the pathogenicity of the above mutations and multiplication cases is that they are more prone to aggregation, an event that may lead to

neurodegeneration and cell death. In PD patients deposits of α-synuclein have been identified in pathological aggregates in LBs, in particularly in the halo of the inclusions (Spillantini et al. 1997, 1998) and in LNs. Amongst the proposed pathological mechanisms via which α-synuclein exerts its neurotoxic function, are inhibition of protein degradation pathways, mitochondrial fragmentation, impairment of synaptic function, and alteration of calcium homeostasis (reviewed in Vekrellis et al, 2011)

LRRK2 (PARK-8)

LRRK2 (leucine-rich repeat kinase 2) is a large protein coded by the genetic locus PARK8. Mutations in LRRK2 protein are the most common cause for autosomal dominant PD. LRRK2 has functional GTPase and kinase activities flanked by multiple protein interaction domains and has been reported to regulate lysosomal positioning and autophagy, synaptic vesicle endocytosis, synaptogenesis, cytoskeleton and neurite outgrowth, protein synthesis, Golgi sorting, and retromer function (reviewed by Dawson T.M., et al., 2014; Rideout and Stefanis, 2014). Amongst the identified LRRK2 mutations, the G2019S mutation, which causes increased LRRK2 kinase activity, is probably the single most common mutation linked to the disease.

UCH-L1 (PARK-5)

Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), also known as PGP9.5, is a protein of 223 amino acids (Wilkinson et al. 1989) (molecular mass 24.8 kDa). Ubiquitin C-terminal hydrolases (UCHs) belong to the family of DUBs together with Ubiquitin-specific proteases (UBPs). Both classes are cysteine proteases that hydrolyze the isopeptide bond (either α- or ε- linked) between the substrate and the C-terminal Gly76 of ubiquitin. To date four isoforms (L1-L4) of UCH proteins have been identified in mammals (Mayer, Wilkinson 1989; Wilkinson 1995;Osawa et al., 2001). Among them, UCH-L1 is neuron specific (Wilkinson et al., 1992; Wilkinson et al., 1989).

UCH-L1 is one of the most abundant proteins in the brain (1–2% of the total soluble protein) and is localized both in the pre and postsynaptic regions (Liu et al. 2002) (Cartier et al. 2009). It is

also normally expressed in testes and ovaries, but not in the other tissues due to silencing by methylation. It is abnormally expressed in lung cancer lines and colorectal cancer (Yamazaki et al. 2002) (Sasaki et al. 2001) (Hibi et al. 1998).

Role on I93M mutation in Parkinson's disease pathology

The genetic link of UCH-L1 protein with PD was first described in 1998 when the I93M mutation in the UCH-L1 gene was identified in a German family with autosomal dominant PD (the mutant displayed ~ 80% penetrance) (Leroy et al., 1998). In this family, four of seven family members were affected with PD, although, except for the two siblings, the family members were not genotyped. Since then, a number of studies examined the role of the I93M mutation in the development of PD. The I93M mutant form of the UCH-L1 protein displays significant decreased hydrolase activity *in vitro* (~55% of the WT type protein), implying that loss of UCH-L1 function may contribute to a decrease in the availability of free ubiquitin, and an impaired clearance of proteins by the UPS. Transgenic mice that overexpress the I93M UCH-L1 mutation show an age-dependent loss of nigral dopaminergic neurons and a significant reduction in the dopamine content in the striatum (Setsuie et al. 2007).

Although the I93M UCH-L1 mutation has been associated with PD, several pieces of evidence suggest that simple loss of hydrolytic function of 193M does not render it causative to the disease. Firstly, the mutation in the family case was not 100% penetrant. In addition, as mentioned above, gracile axonal dystrophy (gad) mice, which lack the UCH-L1 protein, do not manifest a PD phenotype, although they do develop neurological abnormalities (Setsuie et al. 2007). Importantly, in contrast to WT UCH-L1-Tg mice, viral-mediated overexpression of α-synuclein enhanced the loss of nigral dopaminergic neurons in I93M UCH-L1-Tg mice, suggesting that 193M UCH-L1 augmented the toxic effects of α-synuclein (Yasuda,et al, 2009).

The effects of I93M mutation are not associated with the enzymatic activities of the protein, but are suggested to be caused by changes in its structure, although minor in scale. More specifically, UCH-L1 protein is a major target of oxidative damage in PD as it undergoes reversible oxidative modifications at methionine residues (Choi et al., 2004). The differential

structural conformation of I93M UCH-L1 may render it more prone to such modifications, thus explaining its contribution to the disease.

Structure of UCH-L1

The UCH-L1 monomer is composed of two lobes, one consisting of five α-helices (α1, α3, α4, α5, and α6), and the other consisting of two helices (α2 and α7) and six β-strands (β1–β6). These structures together form a helix-β-helix sandwich fold **(Figure 5)**. The active site cleft is composed of three secondary structure elements: a helix (α3), a strand (β3), and a loop (L9) on which the members of the putative catalytic triad Cys90, His161, and Asp176 reside, respectively (Das et al., 2006).

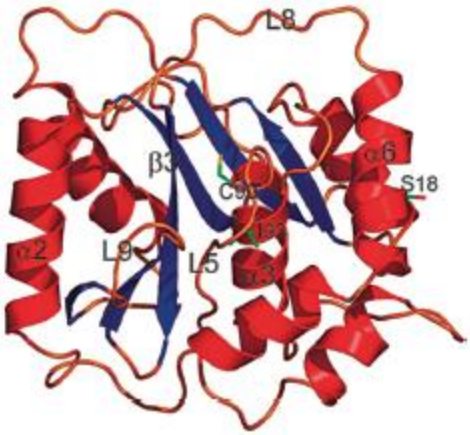

Figure 3: Stereo-projection of a ribbon representation of UCH-L1 monomer. Helices are colored red, sheets are blue, and the rest are orange. Side-chain atoms of residues C90, I93, and S18 are shown as sticks (Das et al., 2006).

In the crystal structure, the side chains of these residues are not close enough for catalytic activity, suggesting that in the absence of substrate, UCH-L1 is in an inactive form. In the presence of substrate, the secondary structures in the active site can move to form a productive triad; helix α6 can unravel to allow loop L8 to move away from the active-site cleft, thereby exposing the active site to its substrates.

Molecular functions of the UCH-L1 protein

1. Hydrolase activity *in vitro*

UCH-L1 catalyzes hydrolysis of C-terminal ubiquityl esters and amides *in vitro* and the latter are the preferred substrates (Larsen et al. 1998). More specifically, UCH-L1 can hydrolyze bonds between ubiquitin and small adducts or unfolded polypeptides. It can also cleave ubiquitin gene products *in vitro*, either tandemly-conjugated ubiquitin monomers (UbB, UbC), or ubiquitin fused to small ribosomal protein (S27a), very slowly, to yield free ubiquitin (Larsen et al. 1998). Hydrolase activity of UCH-L1 is presumed to be critical for cytoplasmic protein degradation, recycling free ubiquitin by cleaving ubiquitinated peptides that are the products of the proteasomal degradation of polyubiquitinated proteins (Larsen et al. 1996) (Larsen et al. 1998).

2. Ubiquitin ligase activity *in vitro*

In 2002, another *in vitro* enzymatic activity of UCH-L1, the ubiquitin ligase activity, was identified (Liu et al. 2002). UCH-L1 was shown to exhibit hydrolase activity in monomeric form and a dimerization-dependent ubiquitin ligase activity. UCH-L1 is the only member of the UCH family that has been reported to dimerize. More specifically, this study demonstrated that the UCH-L1 dimer exhibits an ATP-independent ubiquitin ligase activity that inhibits ubiquitinated (at Lys 63) α-synuclein degradation (Liu et al, 2002). This ligase activity is thought to be at least partly pathogenic because Lys-63-linked polyubiquitination may inhibit Lys-48 ubiquitination-mediated α-synuclein degradation, leading to accumulation and aggregation of α-synuclein (Hofmann and Pickart, 2001). Addition of ubiquitin at Lys48 destines ubiquitinated proteins for proteasomal degradation, while addition at Lys63 stabilizes them. In contrast to the ubiquitination pathway (using E1, E2 and E3 ligases), which requires ATP to activate free ubiquitin in order to conjugate ubiquitin to the substrate, UCH-L1 does not require ATP, a notable characteristic of this ligase. Importantly, both activities detected *in vitro* are significantly lower than those of any other known ubiquitin hydrolase or ligase (Liu 2002).

Mono-Ubiquitin stabilizer *in vivo*

UCH-L1 also manifests a mono-ubiquitin stabilizing effect *in vivo,* independent of its enzymatic activity (Osaka et al. 2003). A large amount of mono-ubiquitin was shown to be tightly associated with UCH-L1 in lysosomes, inhibiting the degradation of mono-ubiquitin in the brain. Overexpression of UCH-L1 protein extended the half-life and the levels of mono-ubiquitin *in vitro* and *in vivo* (Osaka et al., 2003). Moreover, gad mice showed a decreased level, and UCH-L1-WT transgenic mice showed an increased level of mono-ubiquitin comparing to wild-type mice. Thus, UCH-L1 is suggested to function as an ubiquitin stabilizing factor, regulating the pool size of mono-ubiquitin *in vivo*. Notably, this mono-ubiquitin stabilizing effect of UCH-L1 was independent of its enzymatic activity, as the C90S mutant that lacks enzymatic activity but retains its ubiquitin-interacting ability, still showed a mono-ubiquitin stabilizing effect in cells (Osaka et al., 2003). A recent study confirmed that UCH-L1 protein plays a key role in the control of ubiquitin homeostasis, which is important for normal synaptic activity. More specifically, pharmacological inhibition of UCH-L1 activity led to an impairment in synaptic transmission and maintenance of long-term potentiation (LTP) (Gong et al., 2006), a form of synaptic plasticity that is involved in learning and memory in the hippocampus. Many targets of the Ubiquitin Proteasome System (UPS) exist at synapses (Patrick, 2006; Yi and Ehlers, 2007) and the stability of synaptic proteins was shown to change depending on UCH-L1 activity and/or levels of monomeric ubiquitin (Gong et al., 2006).

Other *in vivo* activities of UCH-L1

As a member of the DUB family, UCH-L1 plays important roles in many cellular processes, including proteasomal and lysosomal protein degradation, DNA damage response, DNA repair, endoplasmic-reticulum-associated protein degradation, cell cycle regulation, kinase modification, apoptosis, learning and memory (Osaka 2003; Harada 2004; Kwon 2004; Komander 2009). Nevertheless, the precise role of UCH-L1 in these processes has not been elucidated yet.

UCH-L1 protein and its relation to Parkinson's disease

As mentioned above, UCH-L1 is almost exclusively expressed in neurons (Wilkinson et al. 1989) and is one of the most abundant proteins in the brain. More specifically, its expression is high in SNpc (Solano et al., 1990; Xilouri et al., 2012) and the I93M mutation causes autosomal dominant PD (Leroy et al, 1998). The significance of the UCH-L1 protein in the maintenance of the central nervous system has been highlighted in a recent study, where an early-onset progressive neurodegeneration was reported in two siblings of a five-member family (Bilguvar K., et al., 2012). Following exome sequencing, a homozygous missense mutation was identified, affecting a glutamic acid residue within the ubiquitin binding domain of the UCHL1 gene (UCH-$L1^{GLU7ALA}$). This mutation co-segregated with the phenotype and was absent in the other unaffected members of the family (Bilguvar K., et al., 2012). It completely abolished UCH-L1 hydrolase activity and reduced the ubiquitin binding affinity of the enzyme, thus causing a potentially dysfunctional ubiquitin pathway and subsequently inadequate protein degradation, both of which are associated with the pathogenic mechanisms of PD. This degenerative syndrome affects multiple pathways in the nervous system including the optic system, cerebral cortex, cerebellum, and spinal cord, proposing a fundamental role for UCH-L1 protein in the integrity of the nervous system. It should be noted however that the phenotype in the human cases reported above did not include extrapyramidal features.

a) UCH-L1 & Oxidative Stress

As discussed above, neurons are highly susceptible to lipid peroxidation. Thus, the phospholipid metabolism in neurons should be highly active in order for the integrity of the plasma membrane to be maintained. Abnormal metabolism of phospholipids has been reported in neurodegenerative diseases. In brains of AD patients the composition of phospholipids and the activities of phospholipid-metabolizing enzymes are altered (Ross et al., 1998; Wells et al., 1995) and the activity of phospholipid synthesizing enzymes is elevated in SNpc of PD patients (Ross et al., 2001). In a recent study, UCH-L1 protected neurons from lipid peroxidation (Wada K. et al., 2010). The precise mechanism of this role of UCH-L1 is still unclear, but the subcellular localization of UCH-L1 to the inner side of the plasma membrane and its interaction with

phosphatidic acid may associate with this physiological function of UCH-L1. In several neurodegenerative diseases, including AD and PD, extensive oxidative modification and down-regulation of UCH-L1 have been observed, further supporting the reciprocal relationship between UCH-L1 protein and oxidative stress (Castegna et al., 2002; Choi et al., 2004; Butterfield et al., 2006). Indeed, the activity of the UCH-L1 protein was studied in response to oxygen-glucose deprivation (OGD) and re-oxygenation *in vitro*. OGD/re-oxygenation causes energy withdrawal and the subsequent generation of ROS that activates two pathways depending on the severity of the insult; either a stress-adaptation survival response or a cell death pathway (Liu Q.Y. et al., 2002). In this study, mild stress upregulated expression of UCH-L1, whereas severe stress caused apoptosis and down-regulation of the protein. On the other hand, down regulation of endogenous UCHL1 increased cell sensitivity to the OGD/re-oxygenation stress, confirming the interrelationship between oxidative stress and UCH-L1 protein (Liu Q.Y. et al., 2002).

b) UCH-L1 & Protein Degradation Systems

As far as the UCH-L1 protein is concerned, when proteasomal or autophagic pathways are disrupted, levels of this protein rise and the pool of damaged UCH-L1 also increases. As a result protein aggregates start to form leading to further proteasome and autophagy inhibition and increased levels of oxidative stress.

Ubiquitin Proteasome System (UPS)

Apart from its participation in the protein degradation through the UPS, UCH-L1 is itself a UPS substrate and malfunction of this pathway may result in accumulation of the protein. Under normal conditions, the PD-linked I93M form of UCH-L1 was shown to have an inherent ability to form inclusions, in contrast to WT and S18Y UCH-L1 (Ardley H.C et al., 2004). Aberrant UPS system led to aggregation of WT and I93M UCH-L1, a finding similar to those observed with Parkin and α-synuclein (Stefanis et al. 2001; Junn et al. 2002; Lee et al. 2002; Ardley et al. 2003; Cookson et al. 2003; Muqit et al. 2004). Importantly, inclusion formation was reduced by

overexpression of the S18Y polymorphism of UCH-L1 protein. These findings are in agreement with the potential protective effect of S18Y polymorphism against PD.

Chaperone mediated autophagy (CMA)

In an effort to identify a potential relationship between the UCH-L1 protein and the CMA machinery, Kabuta et al., discovered a physical interaction between WT UCH-L1 and LAMP-2A receptor, CMA's rate-limiting step (Kabuta et al., 2008). UCH-L1 was also shown to interact with the CMA chaperones Hsc70 and Hsp90, suggesting further a potential role for this enzyme in CMA. This characteristic was irrespective of the hydrolase and ligase activities of the enzyme. The familial PD associated protein I93M UCH-L1 abnormally interacted with LAMP-2A, Hsc70, and Hsp90 and caused aggregation of α-synuclein. These findings suggest that an increase in the amount of α-synuclein protein resulting from CMA inhibition via an aberrant interaction between the I93M UCH-L1 and the CMA machinery underlies one of the causes of familial PD associated with mutant UCH-L1 protein.

Macroautophagy

Although WT UCH-L1 physically interacts with LAMP- 2A, it is not a substrate for CMA because it does not contain a KFERQ-like motif, which is required for substrate proteins to be degraded by CMA (Dice 1990). Instead, WT UCH-L1 is mainly turned over by macroautophagy (Kabuta et al., 2008). Indeed, in a recent study, Chin et al., discovered that UCH-L1 is K63-polyubiquitinated and subsequently degraded by the autophagy-lysosome pathway, an event mediated by Parkin (Chin et al., 2013). More specifically, elevated levels of UCH-L1 protein were reported in Parkin knock-out mouse, proposing an increase in UCH-L1 levels in PD patients with Parkin mutations.

Study of loss of UCH-L1 protein in gad mice

The gracile axonal dystrophy (gad) mice have a naturally occurring, spontaneous mutation in the *UCH-L1* gene that causes loss of detectable UCH-L1 expression (Saigoh et al., 1999). Gad mice exhibit severe sensory ataxia at early stages of pathogenesis caused by axonal degeneration in

the gracile tract, followed by motor paresis at later stages. Gad mice do not develop a Parkinsonian disorder as there is no degeneration in SNpc, nor they are more susceptible to parkinsonian toxins such as the MPTP toxin (Xilouri et al, 2012). Thus, a loss or decrease in the levels of UCH-L1 does not appear to be associated with PD. However, ubiquitin decrease and the consequent inadequate ubiquitination of proteins may trigger increased levels of proteins that should undergo ubiquitin-dependent degradation, resulting in the accumulation of such proteins within spheroids observed in gad mice. For example in gad mice there is aggregation of the amyloid APP and β-amyloid, proteins that characterize the inclusions observed in brains of AD patients (Ichihara et al. 1995). UPS dysfunction is one of the factors that may contribute to the neurodegeneration in PD and other diseases, rendering gad mice a useful model to study UPS-related neurodegeneration.

In an effort to interpret the above controversial findings regarding the function of UCH-L1, Cartier et al. recently showed that this protein exerts differential effects in the context of normal and pathologic conditions (Cartier et al., 2012). Under normal conditions, inhibition of UCH-L1 activity reduced distribution and protein levels of presynaptic α-synuclein, which in turn could have detrimental effects on normal neuronal function. However, loss of UCH-L1 function was shown to be beneficial against α-synuclein mediated disruption of the autophagy pathway (Cartier et al., 2012). Thus, the role of UCH-L1 protein in normal and pathologic conditions needs further examination. Several mutations and polymorphisms of the PARK5 gene coding for UCH-L1 protein have been linked to PD, further confirming the significant role of this protein in the disease, and will be discussed in the next chapter.

The S18Y polymorphism

In the course of a search for the role of UCH-L1 and its genetic linkage to PD, a previously unrecognized polymorphism in the UCH-L1 gene, the S18Y polymorphism, was discovered and found to be linked to decreased susceptibility to sporadic forms of PD (Maraganore et al., 1999; Wintermeyer et al., 2000; Satoh et al. 2001a; Momose et al., 2002). The association of this polymorphism with PD is suggested to be dependent on the S18Y allele dosage; homozygotes are at significantly lower risk (relative risk of 0.31) than are heterozygotes (relative risk between

0.55 and 0.81) (Maraganore et al., 2004). This polymorphism is relatively rare in the European population but common in the Japanese and Chinese populations (Liu et al., 2002). On the other hand, decreased susceptibility to PD has been questioned by many studies (Mellick et al. 2000; Levecque et al. 2001; Healy et al., 2006; Zhang et al., 2008; Wang et al 2011), while others suggest an age dependent protective effect (Elbaz et al. 2003; Xiromerisiou et al., 2011). The discrepancies in the literature concerning the role of S18Y polymorphism in PD development, suggest that further studies are required.

The S18Y polymorphism is a result of a cytosine (C) to adenine (A) substitution, converting the serine (S) at residue 18 to tryptophan (Y). Residue S18 is in helix α1 and is exposed to solvent. The primary effect of the S18Y mutation on UCH-L1 is to slightly reduce its stability without significantly affecting the structure. The mechanism by which the S18Y polymorphism of UCH-L1 may confer a decreased susceptibility to PD has not been yet clarified. Interestingly, S18Y UCH-L1 has decreased ligase activity and increased hydrolase activity comparing to WT UCH-L1 (Liu et al., 2002). However, the theory that S18Y is protective due to these differences in activities is inconsistent with the predicted location of residue 18 on the protein surface, distal from the active site and the binding site **(Figure 6)** (Johnston et al., 1997, 1999). Furthermore, the fact that position 18 is one of only a few residues that are not conserved between human and mammals (horse, mouse, and rat have Ala at position 18) suggests that residue 18 is not involved in the normal "biological" activity of UCH-L1 but confers a distinct UCH-L1 activity.

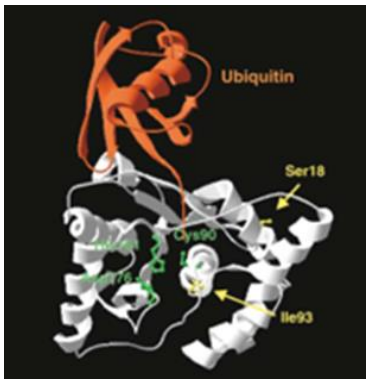

Figure 4: A structural model for the UCH-L1-Ubiquitin complex does not explain the protective effect of the S18Y polymorphism. Residue 93 is proximal to the active site nucleophile (C90), while S18 is distal from the active site and from the ubiquitin-binding site (Johnston et al., 1997, 1999).

In an attempt to discover the mechanisms by which the S18Y polymorphism confers a decreased susceptibility to PD pathogenesis, Kyratzi et al., proposed an anti-oxidant role for this polymorphism, *in vitro* (Kyratzi et al., 2008). In more detail, the S18Y polymorphism protected SH-SY5Y cells from cation 1-methyl-4- phenyl-pyridinium (MPP^+) induced toxicity, by reducing the levels of ROS production, an effect independent of ubiquitin-binding competency and hydrolase activity of the enzyme. Overexpression of WT UCH-L1 did not rescue the cells from oxidative damage and lack of mouse UCH-L1 did not render neurons more susceptible to oxidative stress, suggesting that the anti-oxidant effect of S18Y polymorphism is not due to an enhancement of a latent protective capacity of UCH-L1. Following evidence suggesting a facilitation of proteasomal function by the UCH-L1 enzyme (Hegde et al., 1997), this group examined for a possible effect on the UPS. UCH-L1 was not found to physically interact with the proteasome and neither WT nor S18Y UCH-L1 overexpression rescued the cells after proteasomal inhibition- or DNA damage- induced cell death. Lastly, the S18Y UCH-L1 polymorphism appeared to have less propensity to induce protein aggregation and cell death comparing to the WT UCH-L1. In addition, in order for the potential neuroprotective effects of the S18Y UCH-L1 polymorphism to be also studied *in vivo*, the same group recently examined this polymorphism in the dopaminergic system of gad mice (Xilouri et al., 2012). Nigral overexpression of S18Y but not of WT UCH-L1 led to significant protection against MPTP-induced toxicity. In humans and mice, MPTP produces a parkinsonian syndrome characterized by selective degeneration of the nigrostriatal pathway and it is thus a useful tool to study the nigrostriatal degeneration that occurs in PD (Dauer and Przedborski, 2003; Przedborski and Vila, 2003.

Regulation of UCH-L1: Post-translational modifications

1. Monoubiquitination

Changes in post-translational modifications of UCH-L1 may contribute to its role in neurodegenerative diseases. Meray and Lansbury discovered a monoubiquitination that negatively regulates UCH-L1 by preventing binding to ubiquitinated targets or free ubiquitin (Meray and Lansbury, 2007). Monoubiquitination was found to occur at lysines near the UCH-L1

active site and to be dependent on the enzyme hydrolytic activity. It was also shown that this modification is reversible as the lifetime of ubiquitin binding was regulated by a supposedly intramolecular auto-deubiquitination. In the same study, the I93M mutant but not the S18Y polymorphism, eliminated the monoubiquitination of UCH-L1. These findings indicate that a functional ubiquitin-binding site and catalytic activity are essential for the stability of monoubiquitination and for normal cellular levels of monoubiquitinated UCH-L1. A potential role for transient monoubiquitination of UCH-L1 is to facilitate yet unidentified protein-protein interactions, regulated by auto-deubiquitination of the enzyme. Alternatively, transient monoubiquitination may be related to the ubiquitin homeostatic role of UCH-L1. As it was mentioned earlier, UCH-L1 has a mono-ubiquitin effect *in vivo* (Osaka et al., 2003). This activity may provide a readily accessible pool of ubiquitin when cellular ubiquitin concentrations drop; transiently monoubiquitinated UCH-L1 may release free monomeric ubiquitin, at a rate dependent on its auto-deubiquitination reaction. As ubiquitin-dependent processes are sensitive to perturbations in free ubiquitin levels (Swaminathan et al., 1999; Burbea et al., 2002), a system of ubiquitin homeostasis may be particularly useful to neurons, in which the ubiquitin system is highly active.

2. Glycosylation

In 2001, Cole and Hart discovered a post-translational modification, cytosolic O-glycosylation (O-GlcNAc) on UCH-L1 protein (Cole and Hart, 2001). O-GlcNAc is a single N-acetylglucosamine attached to the hydroxyls of serines or threonines in numerous intracellular proteins. O-GlcNAc modification was shown to be abundant in the nerve terminals, suggesting a regulatory role for this modification in nerve terminal proteins, such as UCH-L1.

3. Oxidative modification

Choi et al. discovered that human brain UCH-L1 exists as three different isoforms: a full-length form (isoform #1) and two amino-terminally truncated forms (isoforms #2 and #3) (Choi et al, 2004). Full length isoform #1 of UCH-L1, but not isoforms #2 and #3, was shown to be significantly oxidized in idiopathic PD brains compared to age-matched controls, providing the

first evidence linking carbonyl modified UCH-L1 to sporadic PD (Choi et al., 2004). More specifically, five oxidatively-modified methionine residues were detected in isoform #1 of UCH-L1 in PD brains. These oxidative modifications can be reversed by the peptide methionine sulfoxide reductase (MsrA), an enzyme that catalyzes the thioredoxin dependent reduction of methionine sulfoxide to methionine. The reversible methionine oxidation/reduction has been suggested to act in a manner analogous to phosphorylation/ dephosphorylation for regulating protein function and cellular processes (Hoshi, T., and Heinemann, S., 2001). Thus, methionine oxidation of UCH-L1 may serve as a specific, reversible mechanism for regulation of UCH-L1 activity according to intracellular redox status or as an important defense mechanism for scavenging ROS (Levine et al., 1996). Such a mechanism would suggest a novel antioxidant function for UCH-L1 that could lead to protection of neurons from oxidative stress.

4. N-terminal truncation

In a recent study, a new post-translational modification of UCH-L1 was discovered, a N-terminal truncation, which was found to render nigrostriatal dopaminergic neurons less vulnerable to degeneration in the MPTP mouse model of PD (Kim et al., 2014). Mitochondria are organelles characterized by high ROS generation and the expression of N-truncated UCH-L1 was shown to provide protection against various models of oxidative stress through a decrease in ROS production. Thus, although the physiological role of N-truncated UCH-L1 remains unclear, it is possible that N-truncated UCH-L1 might exert its neuroprotective effects in part via localization to the mitochondria.

5. C-terminal farnesylation

A subpopulation of UCH-L1 is farnesylated at the C-terminus (at position 220) and is tightly bound to membranes (Liu et al., 2009). Overexpression of WT UCH-L1 has been reported to increase the levels of membrane bound UCH-L1 (UCH-L1M) that sequentially led to accumulation of α-synuclein and neurotoxicity *in vitro* (Liu, 2009). A mutation in the farnesylation sequence (CKAARSKAA) of UCHL1, resulting in the C220S mutant, eliminated the membrane-associated species of UCH-L1, but had no effect on α-synuclein levels (Liu et al,

2009). On the other hand, pharmacological inhibition of UCH-L1 farnesylation reduced α-synuclein levels, possibly by promoting its degradation through lysosomal pathway, and increased cell viability. In the same study, cortical tissues of normal human brains and brains from AD and PD patients were analyzed; approximately 30% of UCH-L1 was found to be membrane-associated but no association between UCH-L1M/UCH-L1S (soluble UCH-L1) ratio and disease was detected (Liu et al, 2009).Interestingly, Kabuta et al. did not report an increase in α-synuclein expression levels due to WT UCH-L1 over expression using the same cell culture model system (Kabuta et al 2008).

A recent study suggested that C-terminal farnesylation is not required for UCH-L1 membrane localization (Bishop et al., 2014). These controversial results may be due to a differential regulation of UCH-L1 membrane association, depending on the cell types studied, suggesting that C terminal farnesylation and its physiological role in UCH-L1 function needs further examination.

B) Autosomal recessive inheritance genes

PARKIN (PARK-2)

Loss of function mutations in the Parkin gene in gene locus PARK2 cause recessively inherited parkinsonism, which is characterized by loss of SNpc dopaminergic neurons, generally without LBs. Parkin is an E3 ubiquitin ligase, a component of the UPS that targets misfolded proteins for proteasomal degradation. This E3 ligase activity is abolished in many Parkin mutations, resulting in accumulation of misfolded protein aggregates, characteristic of PD (Hattori et al., 2004). Indeed, increased levels of p38/JTV-1, a target protein of Parkin which is toxic for dopaminergic neurons *in vitro* and *in vivo,* have been found in brains of sporadic PD patients (Ko et al., 2005), while accumulation of other substrates has also been reported.

PINK-1 (PARK-6)

PINK-1 (PTEN-induced putative kinase 1) gene is also associated with an autosomal recessive form of PD and early onset Parkinsonism. PINK-1 is a mitochondrial serine-threonine kinase that is transcriptionally activated by PTEN and negatively regulates cell death through apoptosis (i.e cytochrome C release, caspase activation). Thus, mutant forms of PINK-1 may result in mitochondrial damage, a pathogenic feature of PD (Valente et al., 2004).

DJ-1 (PARK-7)

DJ-1 protein is a cytoplasmic protein with a yet unidentified function, coded by the genetic locus PARK7 (Bonifati et al. 2003). Experimental evidence suggests that, upon exposure to oxidative stress, DJ-1 increases and is translocated to the mitochondria, presumably to exert a protective role (Canet-Aviles et al., 2004). On the contrary, there is an increased vulnerability to oxidative stress in cases of loss of DJ-1 function (Martinat et al., 2004). Thus, DJ-1 is proposed to be significant in the context of PD pathogenesis as a cellular monitor of oxidative stress damage.

ATP13A2 (PARK-9)

Kufor-Rakeb syndrome represents another type of early onset Parkinsonism associated to the gene ATP13A2 (Najim al-Din et al. 1994). ATP13A2 is a membrane lysosomal protein with functional ATPase activity. Mutations in membrane lysosomal proteins cause failure of lysosomal-autophagic pathways and thus, although ATP13A2 linkage to PD still remains unclear, it may contribute to neurodegeneration through disruption of autophagy-lysosome machinery (Ramirez et al., 2006).

Sporadic PD: Proposed pathogenetic mechanisms

1. Mitochondrial damage

Mitochondria are responsible for ATP production through oxidative phosphorylation. They also

participate in the regulation of cell cycle, cell death through apoptosis and calcium homeostasis. The first discovery supporting mitochondria dysregulation to PD pathogenesis, was made using the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), that blocks the mitochondrial electron transport chain by inhibiting complex 1. Mice treated with MPTP represent one of the most trustworthy animal models of PD, as its systemic administration causes selective degeneration of the nigral dopaminergic neurons, both in animal models and humans (Davis et al. 1979; Burns et al. 1983; Langston et al. 1983). Those neurons are vulnerable to the toxin MPTP as they express the dopamine transporter DAT. MPTP crosses the blood brain barrier and is transported to astrocytes, where it is metabolized to MPP^+ (1-methyl-4-phenylpyridinium) by the enzyme MAO-B (monoamine oxidase B) (Chiba et al. 1984). MPP^+ enters dopaminergic neurons through the DAT transporter and inhibits complex I by evoking the release of cytochrome c, leading to neurodegeneration (Chiba et al. 1985; Javitch et al. 1985). Inhibition of complex I disrupts electron transport and ATP synthesis, causing oxidative damage through production of Reactive Oxygen Species (ROS) (Chiba et al., 1985; Javitch et al., 1985; Fornai et al., 2005). Decreased complex I activity was found in the SNpc of PD patients (Schapira et al. 1989; Keeney et al., 2006), probably resulting from oxidative modification of the enzyme (Shults et al., 1997). In addition, those patients manifested decreased levels of glutathione, an antioxidant protein (Jenner et al., 1992) and increased levels of the mitochondrial enzyme MnSOD (Mn Superoxide Dismutase), which normally absorbs hyperoxide in mitochondria (Yoritaka et al., 1997). The removal of damaged mitochondria is achieved through their selective autophagic degradation (mitophagy). Mitophagy involves the sequestration of targeted mitochondria into autophagosomes. Subsequently, autophagosomes fuse with lysosomes, in which sequestered mitochondria are degraded. In experimental PD models and postmortem PD brain samples, abnormal mitochondria readily accumulate in the cytosol of affected neurons (Dehay et al. 2010; Vila et al. 2011), indicating mitophagy impairment. In addition, PINK 1 protein, which is suggested to protect against mitochondrial damage, has been reported to be downregulated in several familiar forms of PD (Valente et al. 2001; Valente et al. 2004a, 2004b). Accumulation of dysfunctional mitochondria may contribute to dopaminergic cell death by an increased production of ROS and an enhanced release of mitochondrial pro-apoptotic factors (Vila et al. 2001; Vila and Przedborski 2003; Perier et al. 2005, 2007).

2. Oxidative stress

Redox homeostasis is the equilibration between oxidant and antioxidant levels. Imbalance between those levels, in favor of the former, results in oxidative stress, caused by increased production of ROS and Reactive Nitrogen Species (RNS). Oxidative stress causes modification of biomolecules such as proteins, lipids, DNA and sugars. High consumption of oxygen renders neurons more vulnerable to oxidative stress comparing to other cell types. Phospholipids in the neuronal plasma membrane contain many highly unsaturated fatty acids, which are important in the synaptic membrane fluidity, but are very fragile due to their high sensitivity to lipid peroxidation (Imre et al., 1994). In PD, dopamine and its metabolites are responsible for the selective vulnerability of dopaminergic neurons to oxidative stress. Dopamine metabolism by MAO generates hydrogen peroxide, while auto-oxidation of dopamine generates superoxide, both of which are toxic to cells. Dopamine and its metabolism products can affect mitochondria function through aberrant effects on electron transport chain (Brenner-Lavie et al., 2009).

Lysosomes are also implicated in oxidative stress conditions. They possess a unique function, the regulation of iron metabolism (a potentially hazardous metal) through autophagic degradation of iron-rich compounds by lysosomal enzymes (e.g metallothioneins, heat shock proteins, apo-ferritin). Under pathological conditions, increased amounts of iron can accumulate in lysosomes. Increased iron deposition may also occur in mitochondria due to accumulation of iron-bound transferrin (Mastroberardino et al., 2009). As these organelles are degraded by lysosomes, they might also result in intralysosomal iron-loading. Under normal conditions, hydrogen peroxide is formed continuously inside the cells, before it is degraded by catalase and glutathione peroxidase. However, some hydrogen peroxide might escape degradation and diffuse into lysosomes, which do not contain these enzymes. Inside the lysosomes, hydrogen peroxide reacts with iron, resulting in the formation of the extremely reactive hydroxyl radical (Fenton reaction).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^{-}$$

Physiologically, lysosomes contain iron chelators, such as desferrioxamine (DFO) (Yu et al., 2003), in order to control ROS production and to protect cells against oxidative insults.

Mitochondria play a role in redox homeostasis too, as they contain in abundance DJ-1 protein which is suggested to exert an anti-oxidant effect (Canet-Aviles et al., 2004). Under abnormal conditions, increased levels of iron together with hydrogen peroxide inside the lysosomes cause enhanced oxidative stress, which can lead to lysosomal and mitochondria damage and finally to cell death (reviewed by Kurz et al., 2008; Kubota et. al., 2009) (**Figure 2**).

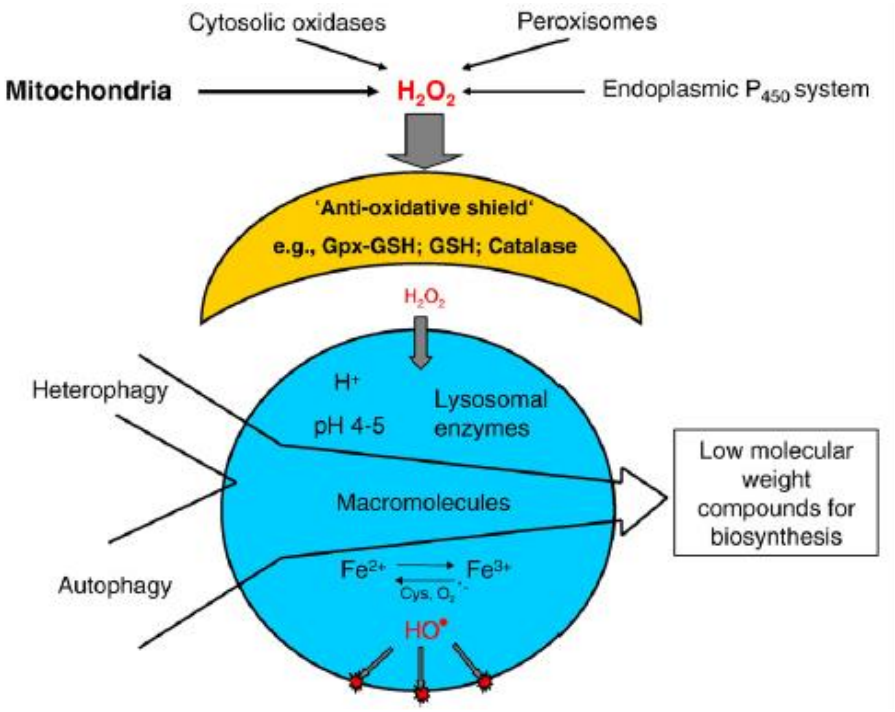

Figure 5: Consequences of intralysosomal formation of hydroxyl radicals under oxidative stress conditions. Hydrogen peroxide is produced continuously inside the cells by a variety of oxido-reductases and mainly by the mitochondria. Under abnormal conditions it is accumulated inside the lysosomes together with iron, which would normally be degraded by lysosomal enzymes. Long-term reaction of these two molecules results in formation of hydroxyl radicals or reactive ferryl and perferryl compounds. This enhanced oxidative stress condition can lead to lysosomal damage and subsequent release of lysosomal enzymes, harmful for the mitochondria. This can lead to cytochrome c release and activation of apoptotic pathways resulting in cell death (Kurz et al., 2008).

Clinical evidence from PD patients further supports the importance of oxidative stress in the development of the disease. Markers of oxidative stress, such as intracellular Fe (Youdim et al. 1989), lipid peroxides (Dexter et al. 1989) and oxidized proteins (Yoritaka et al. 1996) have been

shown to increase in the SNpc of PD patients. In addition, decreased activity of antioxidant glutathione and loss of function of the antioxidant protein DJ-1 in PD patients, also point to an implication of oxidative stress in PD (Sofic et al., 1992; Bonifati et al., 2003). As already mentioned above, the S18Y polymorphism of the UCH-L1 protein is found to be protective in disease manifestation in several populations (Maraganore et al., 1999; Wintermeyer et al., 2000; Satoh et al. 2001a; Momose et al., 2002) and to exert anti-oxidant properties (Kyratzi et al., 2008), further supporting an implication of oxidative stress in PD pathogenesis.

3. Dysregulation of Protein Degradation Systems

Protein homeostasis is fundamental for normal cell function, especially for neurons, which are post-mitotic and rely a lot on proper protein quality control for the removal of misfolded proteins. Three main intracellular systems are involved in protein degradation: the Ubiquitin Proteasome System (UPS), the Ubiquitin independent system and the Autophagy-Lysosome Pathway (ALP). The two main protein degradation systems, UPS and ALP, will be briefly discussed below.

3a) Ubiquitin Proteasome System (UPS)

The Ubiquitin Proteasome System (UPS) consists of two components: the ubiquitination system, which targets proteins for degradation by the addition of ubiquitin molecules, and the proteasome, a multimeric protein complex that performs the degradation. Binding of ubiquitin to the target protein takes place in a three-step reaction. First, ubiquitin is linked to an ubiquitin-activating enzyme (E1) in an ATP-dependent manner. Subsequently, the activated ubiquitin is transferred to an E2 conjugating enzyme, followed by attachment of E2 to a specific E3 ubiquitin ligase enzyme that binds the target protein. Ubiquitin is then transferred by the E2 enzyme to the target protein (**Figure 2**). Mono-ubiquitination at one or more lysines triggers a variety of effects depending on the substrate protein, including endocytosis, gene silencing, DNA repair and degradation through the endosome–lysosomal pathway. In poly-ubiquitination, a lysine side chain of the first ubiquitin is ligated to another ubiquitin, a process that is repeated

to build a chain of poly-ubiquitin from the target protein (Pickart, 1997). Poly-ubiquitinated proteins (covalently linked to Lys48 of ubiquitin) are targeted to the 26S proteasome for degradation. On the other hand, K63-linked poly-ubiquitination plays a signaling role in the regulation of various proteasome-independent cellular processes, including endocytosis, DNA repair and protein trafficking for lysosomal degradation. Regulation of protein degradation is also mediated by deubiquitinating enzymes (DUBs) -including UCH-L1- that can reverse ubiquitination by removing ubiquitin residues of mono- or poly-ubiquitinated proteins.

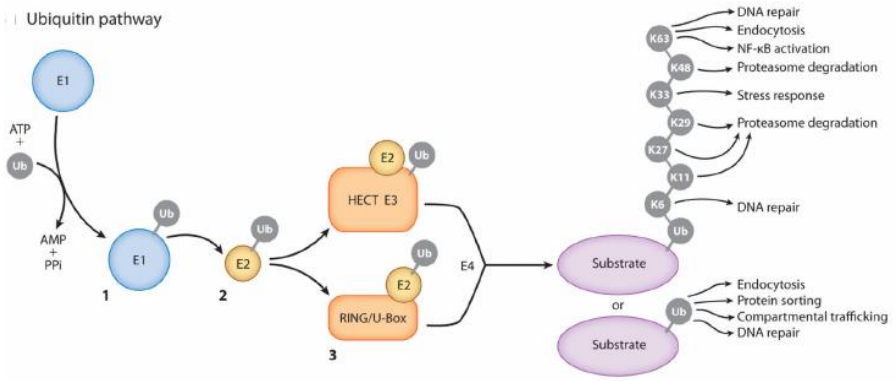

Figure 6: The Ubiquitin pathway (Mabb A.M. and Ehlers M.D., 2010)

UPS dysfunction occurs at early stages of PD (Tofaris et al, 2003). A role for this machinery in PD manifestation was firstly suggested when mutations in the PARKIN protein associated with the disease were discovered. PARKIN functions as an ubiquitin ligase leading proteins to proteasomal degradation (Imai et al., 2000). Post translational modifications and mutations of this protein cause a loss of function of E3 ligase activity and have been associated with UPS impairment and disruption of the neuroprotective effects of PARKIN (Ali et al., 2011). Indeed, loss of PARKIN activity has been shown to cause accumulation of α-synuclein through the formation of pathogenic aggregates (protofibrils) (Lansbury and Brice, 2002). Mutant α-synuclein has been also shown to decrease proteasome activity *in vitro* (Emmanouilidou et al 2010, Liu et al 2005), an effect that has been reported in the SNpc of PD patients (McNaught et al., 2003). Further confirming the implication of the UPS in PD pathology, formation of LB-like structures positive for ubiquitin has been reported in cell lines with impaired proteasomal activity (Lee et al., 2001; Rideout et al., 2001).

3b) Autophagy-Lysosome Pathway

Autophagy Lysosome Pathway (ALP) is different from the UPS in that the ALP is responsible for the degradation of long-lived proteins, and that, it can also degrade cellular organelles through the process of macroautophagy. ALP degrades cytoplasmic components through three distinct pathways: microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA) (Figure 3). Microautophagy includes invaginations at the level of the lysosomal membrane, that engulf neighboring cellular constituents (ex. organelles, lipids, or proteins) (Muller O., et al. 2000). In macroautophagy, double-membrane structures called phagophores are formed and engulf organelles and/or other intracellular compartments, generating the autophagic vacuoles (AVs). The AVs then fuse with the lysosome creating the autophagolysosome, where the vesicular components are being degraded (Xilouri M. and Stefanis L., 2010).

Chaperone Mediated Autophagy (CMA)

CMA, in contrast to the other two lysosomal pathways, does not involve vesicle formation; the substrate proteins seem to cross the lysosomal membrane directly to reach the lysosomal lumen. CMA is a highly selective mechanism as only cytosolic proteins bearing the pentapeptide motif KFERQ or a biochemically related sequence (KFERQ-like) are recognized by a complex of chaperones / co-chaperones in the cytosol. Sequentially, they are translocated one by one to the lysosomal membrane, where they bind to another complex and through this binding they are finally threaded into the lysosomes and degraded. The CMA machinery comprises of the lysosome-associated membrane protein type 2a (LAMP-2a), the heat shock cognate protein of 70 kDa (Hsc70) and Hsc70 co-chaperones. CMA and macroautophagy are interconnected; experimental blockage of one up-regulates the other, revealing a close cross-talk between the two major mammalian autophagic pathways (reviewed by Xilouri M., Brekk O.R., Stefanis L., 2013).

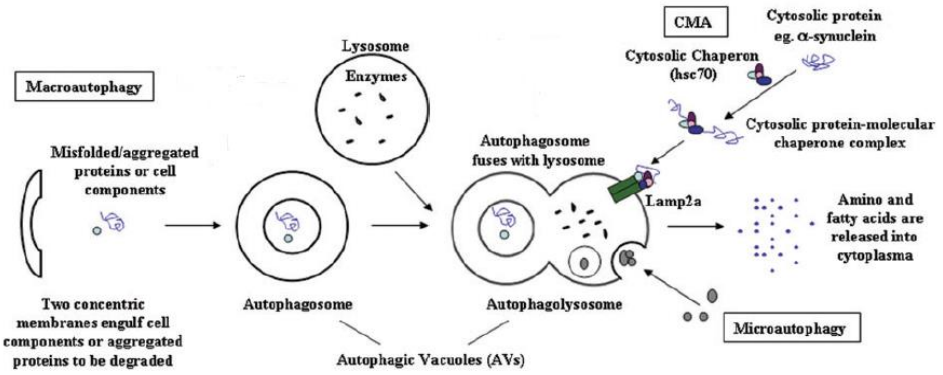

Figure 7 Autophagy-lysosome pathway (ALP) in mammalian cells (adapted from Jankovic J. et al., 2008)

Increasing evidence suggests that ALP dysregulation causes accumulation of abnormal proteins or damaged organelles, characteristic of neurodegenerative diseases. Evidence from post-mortem material, transgenic mice, and animal and cellular models of PD suggest that both macroautophagy and CMA are malfunctioning. Numerous studies propose the implication of several proteins genetically-linked to autosomal dominant PD, in particular α-synuclein (Cuervo et al., 2004; Vogiatzi et al., 2008), LRRK2 (Orenstein et al. 2013) and UCH-L1 (Kabuta et al., 2008), in autophagic pathways. Proteins involved in recessive PD, also contribute to autophagy malfunction. PINK1 and PARKIN are important for the physiological function of mitophagy (engulfment of damaged mitochondria by macroautophagy) and accumulating evidence suggest that inability to degrade damaged mitochondria through mitophagy might be a factor leading to autosomal recessive PD (Narendra, D. et al., 2008; Yao, Z. and Wood, N.W., 2009).

4) Inflammation

There is growing evidence supporting the implication of inflammatory mechanisms in PD pathogenesis. Dysfunction of the innate and adaptive immune response system has been reported in PD patients. Activated microglia and increased cytokine release in the SNpc of sporadic PD patients suggest the manifestation of inflammatory responses (Boka et al., 1994; McGeer et al., 1988). Genes associated to PD may also play a role in immune responses; DJ-1 has been reported to regulate mast cell activation and IgE-mediated allergic responses (Kim et al., 2013) and PARKIN-deficient mice and flies are sensitive to various intracellular bacterial infections (Maznanillo P.S., et al., 2013).

AIM OF THE STUDY

Even though the S18Y UCH-L1 polymorphism was found to exert a neuroprotective role against well-established pro-oxidants, such as MPP^+ and H_20_2, the precise mechanism via which this is accomplished, remains unclear. Moreover, it would be of interest to investigate whether such protective properties might be observed against other neurotoxic stimuli, such as the excitotoxic cell death induced by L-Glutamate, or the neurotoxicity mediated by α-synuclein overexpression. Thus, the aim of the present study was to examine the mechanisms underlying the potential anti-oxidant protective effects of the S18Y polymorphism of UCH-L1 protein in neuronal cells. For this purpose, the following objectives were examined:

A. Identification of the intracellular localization of WT- and S18Y- UCH-L1 protein in human neuroblastoma SH-SY5Y cells

I. Study of the subcellular localization of WT UCH-L1 protein and the S18Y polymorphism under normal conditions

The discovery of the potential differential localization between WT- and S18Y UCH-L1 protein may shed light on the mechanisms via which the S18Y polymorphism exerts its protective role. Human neuroblastoma SH-SY5Y cells that overexpressed WT- or S18Y UCH-L1 were subjected to sequential centrifugations and the presence of WT- and S18Y UCH-L1 proteins in the resulting subcellular fractions (cytosol, nuclei, mitochondria, lysosomes) was examined. Overexpression was accomplished with transient transfection with plasmids that express the various UCH-L1 forms.

II. Study of the cytosolic and membrane localization of S18Y UCH-L1 polymorphism and the double mutant S18Y-C220S UCH-L1, under normal conditions.

In an attempt to investigate whether post-translational modifications of the S18Y UCH-L1 polymorphic variant, such as farnesylation that has been previously shown to be dispensable for the association of WT UCH-L1 to membranes, alter the protective effects of S18Y-UCH-L1, the S18Y-C220S UCH-L1 double farnesylation mutant was used. For the purpose of these experiments, S18Y-C220S UCH-L1 and S18Y UCH-L1 proteins were overexpressed in human

neuroblastoma SH-SY5Y cells, through transient transfection with the corresponding plasmids and their localization in cytosolic and membrane fractions was examined.

III. Assessment of the effects of the double mutant S18Y-C220S UCH-L1 protein on cell survival, under oxidative stress conditions induced by mitochondrial toxin MPP^+.

Human neuroblastoma SH-SY5Y cells were transiently co-transfected with the plasmids coding for GFP protein and proteins of interest (S18Y UCH-L1 or S18Y-C220S UCH-L1) or control protein (PAI-2). Next, the effect of double mutant S18Y-C220S UCH-L1 protein on cell survival after MMP^+ exposure was assessed.

IV. Study of the protective role of the S18Y UCH-L1 protein against L-Glutamic acid (L-Glu)-induced toxicity in neuronally differentiated SH-SY5Y cells

Effects on cell survival

Neuronally differentiated SH-SY5Y cells were transduced with recombinant adenoviruses (rAd) expressing WT UCH-L1 protein, S18Y UCH-L1 protein or control protein (GFP) and cell survival was assessed in the presence/absence of L-Glutamate.

Effects on ROS generation

As the neuroprotective effects of the S18Y polymorphism of UCH-L1 protein are suggested to be due to its anti-oxidant function, it was important to examine whether L-Glu treatment was accompanied by ROS generation, and respectively whether the potential neuroprotective effect of S18Y UCH-L1 was being mediated via its anti-oxidant properties. Neuronally differentiated SH-SY5Y cells were treated with L-Glu and then subjected to DCF staining, a dye which detects ROS generation.

B. Study of the protective role of the S18Y UCH-L1 protein against L-Glu-induced toxicity in rat primary cortical neurons.

In order to verify the protective effect of S18Y UCH-L1 in primary neuronal cultures, rat primary cortical neurons were transduced with rAd- WT UCH-L1, rAd-S18Y UCH-L1 or rAd-GFP and cell survival was assessed upon treatment with L-Glu.

C. Study of the protective role of the S18Y UCH-L1 protein on human SH-SY5Y neuroblastoma cells inducibly overexpressing wild-type (WT) α-synuclein.

To investigate whether the S18Y UCH-L1 polymorphism may exert a protective role against other neurotoxic insults such as α-synuclein-related toxicity, cultures of neuronally differentiated cells that overexpressed WT α-synuclein were transduced with rAd-WT UCH-L1, rAd-S18Y UCH-L1 or rAd-GFP and assessment of cell survival was achieved by measurement of intact nuclei.

D. Degradation of WT-, WT-C220S-, S18Y-, S18Y-C220S- UCH-L1 proteins under normal conditions

WT UCH-L1 is a long-lived protein with a half-life greater than 48H and is mainly turned over by macroautophagy (Kabuta et al., 2008), while other reports suggest that the proteasome is also involved in the protein turnover (Ardley et al., 2004). We wanted to examine the manner of S18Y UCH-L1 degradation under normal conditions and whether farnesylation mutants (WT-C220S, S18Y-C220S) alter the degradation of WT-, and S18Y UCH-L1 proteins. For this purpose, human neuroblastoma cells overexpressing WT-, WT-C220S-, S18Y- or S18Y-C220S UCH-L1 forms were treated with a mixture of lysosomal inhibitors (NH_4CL and Leupeptin), or with a potent proteasome inhibitor (epoxomicin) and protein expression levels were assessed by western blot.

MATERIALS AND METHODS

Cell cultures

Human neuroblastoma SH-SY5Y cell line

Cells were cultured in the DMEM-D6429 (Invitrogen, Carlsbad, CA, USA) culture medium containing 10% heat inactivated fetal bovine serum (10% FBS) and 1% solution of Penicillin-Streptomycin (1% P/S, Invitrogen). Cell cultures were kept in incubator with 5% CO_2 at 37˚C.

Differentiation of human neuroblastoma SH-SY5Y cell line

Human neuroblastoma SY-SY5Y cells were differentiated in culture medium [DMEM (10% FBS, 1%P/S) containing all-trans Retinoic Acid (RA) 20μM. RA is a ligand for the retinoic acid receptor and the retinoid X receptor that both transcriptionally regulate the differentiation of cells.

Inducible WT α-synuclein-expressing SH-SY5Y cell line

This cell line was cultured in the RPMI-R8758 (Invitrogen, Carlsbad, CA, USA) medium containing 10% FBS and 1%P/S. Cell cultures were kept in incubator with 5% CO2 at 37˚C. In the presence of the antibiotic doxycycline [(dox), 1mg/ml], these cells express low levels of α-synuclein, whereas in the absence of dox, cells robustly express the protein (tet-off system) (Vekrellis et al., 2009). Cycling cells in which α-synuclein is induced have no obvious signs of cell death.

Differentiation of inducible WT α-synuclein-expressing SH-SY5Y cells

Induction of human WT α-synuclein in differentiated SHSY-5Y cells leads to accumulation of soluble α-synuclein oligomers and cellular degeneration (Vekrellis et al., 2009). For the purposes of these experiments, cells cultured in the presence of dox (WT^+) or in the absence of dox (WT^-) for at least 7 days (in order for α-synuclein overexpression to be achieved), were used for neuronal differentiation with 20μM RA.

Primary rat cortical neurons

Cultures of primary rat [embryonic day 18(E18)] cortical neurons were prepared as described previously (Vogiatzi et al., 2008; Stefanis et al., 1999). Cells were maintained in Neurobasal medium, with B27 supplement (Invitrogen), L-glutamine (0.5 mM) and penicillin/ streptomycin (1%) and were kept in incubator with 5% CO2 at 37˚C. More than 98% of the cells cultured under these conditions represent post-mitotic neurons (Rideout and Stefanis, 2002).

cDNAs

For the purposes of the present study, cells were transfected with plasmids expressing cDNAs of interest (UCH-L1 WT, UCH-L1 WT-C220S, UCH-L1 S18Y, UCH-L1 S18Y-C220S) and/or reporter cDNA (GFP). The double mutant constructs UCH-L1 S18Y-C220S and UCH-L1 WT-C220S had been previously constructed in the laboratory with the method of PCR-mediated site directed mutagenesis, according to Stefanis et al., 2001. The cDNA sequences coding for UCH-L1-WT and UCH-L1-WT-C220S were provided by Dr K.Wada (National Institute of Neuroscience, Tokyo, Japan).

Transient transfections

For the cell survival experiments (strip counts), SH-SY5Y cells were co-transfected with cDNAs of interest (UCH-L1-S18Y plasmid, UCH-L1 S18Y-C220S plasmid, PAI-2 plasmid) and the green fluorescent protein (GFP) cDNA, in a ratio 3:1, using the Polyethylamine (PEI) reagent [Polysciences.com #23966]. For the membrane/cytosol and subcellular fractionations, SH-SY5Y cells were transfected with cDNAs of interest (UCH-L1 S18Y, UCH-L1 S18Y-C220S, UCH-L1 WT, UCH-L1 WT-C220S) and control cDNA (GFP), using the PEI reagent.

Viral infections

The cDNAs for WT UCH-L1, S18Y UCH-L1 or GFP, were cloned into a modified version of the PENTR.GD entry vector and introduced into the pAd/ PL-DEST Gateway vector (Invitrogen). Second-generation E1, E3, E2a-deleted recombinant human serotype 5 adenoviruses (rAd) were generated, as described previously (He et al, 1998). Viral vector stocks were amplified from plaque isolates in order to guarantee homogeneity of the production. Final vector stocks were purified and concentrated using double discontinuous and continuous cesium chloride (CsCl) gradients. Viral titers of purified vector stocks were determined by Adeno-X Rapid Titer kit (Clontech) and (optical density) OD260 measurements. The following titers were obtained, expressed as viral particles (vp)/**m**l: $2.5x10^{11}$ vp/ml for rAd-WT UCH-L1, $1.8x10^{12}$ vp/ml for rAd-S18Y UCH-L1 and $1.54x10^{12}$ vp/ml for rAd-GFP.

Transduction of neuronally differentiated SH-SY5Y cells and α-synuclein-expressing cells

At day 3 of differentiation, cells were transduced with rAd- WT-UCH-L1, rAd-S18Y-UCH-L1 or rAd-GFP [Multiplicity of Infection (MOI) 50 was used for each virus]. 24H later, cells were washed once in culture medium and then incubated in fresh culture medium, for the time points indicated. For α-synuclein-expressing cells, transduction with the viruses was performed at day 5 of differentiation.

Transduction of primary rat cortical neurons

Five-day-old E18 primary rat cortical neurons were transduced with rAd-WT or rAd-S18Y UCH-L1 or the control virus rAd-GFP (MOI 250 for each virus), for 24H. Cells were then washed with fresh HBSS and cultured in fresh culture medium, for the time points indicated.

Cell survival - Measurement of intact nuclei

Neuronally differentiated SH-SY5Y cells and α-synuclein-expressing cells

Neuronal differentiated SH-SY5Y cells were treated at day 7 with L-Glu [20mM (Sigma Aldrich)]. 24 H later, cells were lysed in a detergent-containing solution, which enables the quantification of viable cells by counting the number of intact nuclei in a hemocytometer (Farinelli et al. , 1998; Rukenstein et al. , 1991). Cell counts were performed in triplicate and are reported as means ± SEM. For α-synuclein-expressing cells, cell survival was assessed on the 7^{th} day of differentiation.

Primary rat cortical neurons

At day 8 after plating, rat primary cortical neurons were treated with L-Glu (20mM). 16H later cells were lysed in the aforementioned lysis buffer (nuclear counts buffer) and survival was assessed with measurement of intact nuclei.

Nuclear counts buffer:
lysis buffer 10% (for 50ml: 2,5gr EHDA, 1,4ml glacial acetic acid, 0,282ml NaCl 5M in ddH_2O), 0.5% Triton, MgCl2 2mM in ddH_2O.

Cell survival - MPP^+ treatment

Survival of cycling human neuroblastoma SH-SY5Y cells was assessed by an investigator blinded to the experimental conditions, by measurement of GFP positive cells across the same two fields of view (strips) 0 and 16 hours upon treatment with 2mM MPP^+ (Sigma) or with culture medium (control samples). Survival was estimated as a percentage of transfected cells that survived after 16H comparing to their initial number at 0 hours.

Detection of Reactive Oxygen Species (ROS) production

For the detection of ROS, the Cellular Reactive Oxygen Species Detection Assay Kit ''DCFDA'' (Abcam) was used. This kit contains the cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and ROS activity within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2', 7' –dichlorofluorescein (DCF). DCF is a highly fluorescent compound, which can be detected by fluorescence microscopy.

For the detection of DCF, neuronally differentiated SH-SY5Y cells were washed with fresh medium and then treated with 5μM DCFDA in culture medium for 20 minutes at 37˚C. Cells were then washed three times with culture medium, followed by addition of fresh culture medium. Then, cells returned in the incubator for additional 30 minutes, before the observation under an inverted fluorescent microscope Leica DMIRB (Leica Microsystems, Bannockburn, IL, USA).

Subcellular fractionation

Cycling SH-SY5Y cells were transiently transfected with cDNAs of interest (WT UCH-L1, S18Y UCH-L1) or control cDNA (GFP). They were collected 48H later for the isolation of cytosolic, nuclei, mitochondria and endosomal fractions through sequential centrifugations. In more detail, cells were first homogenized with homogenization buffer and subjected to 4 cycles of snap-freeze (dry ice and methanol) and thaw (37 ˚C incubation). The lysate was then passed through an insulin syringe three times and then through a homogenizer (3 sets of 6 bounces). Homogenate was then centrifuged at 2000 X g for 15 min at 4˚C and a nuclei enriched pellet was collected. Supernatant no.1 was centrifuged again at 9000 X g for 15min at 4˚C and mitochondria enriched pellet together with supernatant no.2 were collected. Supernatant no.2 was centrifuged at 100.000 X g for 3H at 4˚C in order for endosomal-lysosomal enriched fraction and cytosolic fraction (supernatant no.3) to be separated. Thus, this subcellular fractionation protocol results in three pellets that are then lysed in RIPA buffer (nuclei enriched-, mitochondria enriched- and endosome- enriched pellets) and in one supernatant , which is the cytosolic fraction.

Homogenization buffer: 0,25M Sucrose, 10mM Sodium Hepes (pH=7.4), 1mM EDTA (pH=8), 5Mm $MgCl2{\cdot}6H_2O$, protease inhibitors in ddH_2O

RIPA buffer: 50mM Tris-HCL pH=8.0, 150mM NaCl, 1%Nonidet P40, 0.1% SDS, 2mM

Cytosol-Membrane fractionation

Cycling SH-SY5Y cells were transiently transfected with cDNAs of interest (WT UCH-L1, WT-C220S UCH-L1, S18Y UCH-L1, S18Y-C220S UCH-L1) and control cDNA (GFP) and collected 48H later. Isolation of membrane and cytosolic fractions was accomplished according to Liu et al., 2009. In more detail, cells were lysed in 50mM Tris HCL (pH=7.6) supplemented with 1mM EDTA (pH=7.4) and protease inhibitor mixture and were homogenized by passing through insulin syringe 10 times. The lysate was clarified by spinning at 600 X g for 5min, 4˚C, and supernatant was subjected to ultracentrifugation at 100,000 X g for 2H to separate into membrane and cytosolic fractions. The resultant supernatant was kept as the cytosolic fraction. The pellet was washed and lysed in RIPA buffer (supplemented with 1% SDS and protease inhibitor mixture) and SDS soluble fraction (membrane fraction) was collected after centrifugation at 13,800 X g for 15min at 4˚C. Thus, this protocol of cytosol-membrane fractionation yields two fractions: one cytosolic fraction and one membrane fraction (SDS soluble).

RIPA buffer: 50mM Tris-HCL pH=8.0, 150mM NaCl, 1%Nonidet P40, 0.1% SDS, 2mM EDTA: 0.5% deoxycholate, in ddH_2O

Wash buffer: 50 mM Tris-HCl (pH=7.6), 1mM EDTA (pH=7.4), 1M NaCl and protease inhibitors

Treatment with protein degradation inhibitors

SH-SY5Y cells were transiently transfected with plasmids coding for WT-, WT-C220S-, S18Y-, or S18Y-C220S UCH-L1 protein and 48H later were treated with a mixture of lysosomal inhibitors [NH_4CL (20mM, Sigma) + Leupeptin (10μM, Sigma)] or with the proteasome inhibitor epoxomicin (15nM). Protein levels were measured 16H later using the Bradford (BIORAD) method.

SDS Polyacrylamide Electrophoresis- Western Blotting

The separation of denatured proteins according to their molecular weight is achieved by the SDS electrophoresis technique. SDS denatures and binds the proteins through the addition of a negative charge. Western blot is an analytical technique that includes the transfer of those proteins from the SDS/Polyacrylamide Gel to a nitrocellulose membrane. Proteins of interest are then targeted using monoclonal or polyclonal antibodies specific to the target protein.

Preparation of samples for Western Blot

In order for the same amount of protein to be analyzed for all the samples, the concentration of the protein for each of the samples was measured using the Bradford method (Bio-Rad, Hercules, CA, USA). The samples to be analyzed include the desired amount of protein, water (if necessary) and sample buffer which contains a) mercaptoethanol (which provides denaturing conditions by reduction of the disulfide bonds), b) SDS (anionic detergent which gives the samples with a negative charge, c) glycerol, which enables the loading of samples to the gel and d) bromophenol blue which also enables the loading of the samples. Before loading on the SDS gel, complete denaturation of the samples is achieved with boiling at 95˚C for 10'.

Preparation of SDS gel

For the protein separation of the samples, a 12% (w/v) acrylamide separating gel was used. More specifically, the gel contained H_2O (33%), 12% (w/v) acrylamide, 375 mM Tris pH=8.8, 0,1% (w/v) sodium dodecyl sulfate SDS, 0,1% (w/v) ammonium persulfate (APS), 0,04% (v/v) tetramethylethylenediamine (TEMED). Above the separating gel, the stacking gel is created where the samples are loaded. Stacking gel is responsible for the simultaneous entrance of the samples to separating gel, and thus for their simultaneous separation. It contains H_2O (70%), 125mM Tris pH=6.8, 5% (w/v acrylamide), 0.1% (w/v) SDS, 0,1% (w/v) APS, 0,05% (v/v) TEMED.

Electrophoresis-SDS Polyacrylamide

Electrophoresis takes place in a specific buffer solution (running buffer) (Tris 25mM, Glycine 190mM, 0.1% SDS), under stable voltage 150V. A mix of proteins of known molecular weight (Page Ruler Prestained Protein Ladder, Fermentas) is also loaded in order for the identification of the target protein depending on its molecular weight.

Western Blot

With this method separated proteins are transferred from the electrophoresis gel on a nitrocellulose membrane (Protran, Schleider Schuler, 0,45μm). The protein transfer is accomplished in Transfer Buffer (Tris 25mM, Glycine 190mM, 20% methanol), at stable current 400mA, on ice for 2H. After the protein transfer, the nitrocellulose membrane is incubated with a buffer that blocks non-specific binding [5% low fat milk, Tris buffered saline (TBS), 0,05% Tween 20] for 1H, while shaking, at room temperature (RT). TBS buffer consists of: 50mM Tris-HCL, pH=7.2, 150mM NaCl, 0,05% (v/v) Tween-20. Detection of protein of interest on nitrocellulose membrane is accomplished with incubation with a primary antibody specific for the target protein for 16H at 4˚C, while shaking. For the purposes of this study the following primary antibodies were used: monoclonal antibody for flag [1:1000 (Sigma)], polyclonal antibody for UCH-L1 (PGP 9.5) [1:2000 (Affinity)], monoclonal antibody for actin [1:5000 (Sigma)], polyclonal antibody for E-Cadherin [1:1000 (Transduction Laboratories)] and polyclonal antibody for ERK [1:1000 (Sigma)]. The following antibodies were also used: monoclonal antibody for ubiquitin [1:1000 (Chemicon)], monoclonal antibody for SQSTM1/P62 (P62) [1:1000(Chemicon)], polyclonal antibody for microtubule-associated protein light chain 3 (LC3) [1:1000 (Chemicon)], polyclonal antibody for LAMP2-A [1:1000 (Santa Cruz)], monoclonal antibody for karyopherin [1:1000, (Santa Cruz)], polyclonal antibody for Lamin [1:1000 (Santa Cruz)], monoclonal antibody for cytochrome oxidase complex IV (Oxphos) [1:1000 (Molecular Probes)]. In all cases, antibodies were diluted in 5% low fat milk in TBS-0,05% Tween. Membrane was then washed twice in TBS-T for 15 minutes at room temperature (RT) and incubated for 1.5H with a secondary antibody (which binds specifically to the stable Fc region of the first antibody). Secondary antibodies used for the purposes of this study are the following:

anti-rabbit antibody [1:10.000 (Pierce)], anti-mouse antibody [1:10.000(Pierce)]. Secondary antibodies are also diluted in 5% low fat milk in TBS-0,05% Tween. After incubation with the secondary antibody, membrane was washed three times in TBS-T for 10 minutes, at RT.

Development of the protein of interest with the Enhanced ChemiLuminescence system (ECL)

ECL system is based on chemiluminescence; Luminescence is the emission of light in response to release of energy from a stimulated substrate. In the case of chemiluminescence, stimulation of the substrate is caused by a chemical reaction; Luminol diacylhydrazide is oxidized in the presence of HRP/H_2O_2 and chemical enhancers (phenols) and is stimulated. At resting state it produces light (wave length 428nm), which can be detected on light-sensitive autoradiography films after short exposure. ECL mixture consists of two reagents, A and B. Reagent A contains HRP/H_2O_2 and reagent B contains luminol and enhancers of chemiluminescence. The membrane is incubated with the mixture of the two reagents in ratio 1:1, for 5 minutes in dark. The duration of the exposure depends on the quantity of the protein of interest inside the sample and on its interaction with the primary antibody. The development of the membrane occurs inside a development machine (developer).

Immunofluorescence

For the purposes of the immunofluorescence experiments, cells are washed twice with Phosphate Buffered Saline (PBS), in order for the culture medium serum to be removed. Next, cells are fixed with 4% (w/v) Paraformaldehyde (PFA) in PBS for 20 minutes at RT. The cells are then washed three times with PBS and blocked [10% NGS (natal goat serum), 0,4% Triton-X in PBS] for 1H at RT. The primary antibodies are diluted in PBS 2(% NGS-0,1% Triton –X) and added for 16-18h at 4˚C, in dark. The cells are then washed three times and the secondary antibodies together with Hoechst which dies the nuclei are added for 1H at RT, in dark. Before observation in the microscope, cells are washed three times with PBS. Immunofluorescence photos were taken using inverted fluorescent microscope Leica DMIRB. The primary antibodies used were the following: monoclonal antibody for M_2 Flag [(1:400), Sigma], polyclonal antibody for rabbit Flag [(1:400), Sigma], polyclonal antibody for rabbit GFP [1:1000 (Abcam)] monoclonal antibody for mouse cadherin (1:1000), Transduction laboratories]. The secondary antibodies used were the following: cy3 anti-mouse IgG [Jackson Immunoresearch Laboratories (1:300)] and cy2 anti-

rabbit IgG [Jackson Immunoresearch Laboratories (1:250)]. Hoechst, which dyes the nuclei, is used at a dilution 1:000 (Sigma, St Louis, MO, USA)].

Statistics

All data are expressed as mean ± SEM. Statistical significance of differences was evaluated with one way ANOVA followed by the Tukey's Post hoc test or the Newman-Keuls test. Probability values <0,05 were considered significant.

RESULTS

A. Identification of the intracellular localization of WT- and S18Y- UCH-L1 protein in human neuroblastoma SH-SY5Y cells

i) Study of the subcellular localization of WT- and S18Y- UCH-L1 protein under normal conditions

As discussed above, mitochondria and lysosomes regulate ROS equilibrium and damage of those organelles is associated with oxidative stress. As mentioned above, UCH-L1 protein acts as a mono-ubiquitin stabilizer, *in vivo* (Osaka et al., 2003). This function is suggested to be achieved through the localization of the protein in the lysosomes. Thus, as lysosomes are a subcellular organelle where WT-UCH-L1 is normally found, it is reasonable to assume that S18Y UCH-L1 might further interact with this organelle, acting as a scavenger for ROS. In addition, as N-truncated UCH-L1 might exert its neuroprotective effects through localization in the mitochondria (Kim et al., 2014), it was also interesting to examine a potential differential localization of the S18Y UCH-L1 polymorphism, in comparison to the WT UCH-L1 protein, in this organelle.

In order to examine the potential differential subcellular localization of WT- and S18Y UCH-L1 proteins under normal conditions, human neuroblastoma SH-SY5Y cells were transiently transfected with plasmids coding for proteins of interest (WT UCH-L1 and S18Y UCH-L1, which are both tagged with the epitope FLAG at the N-terminus) or control protein (GFP). Subcellular fractionation experiments were performed 48 hours later in order to isolate a cytosolic fraction, a nuclei-enriched fraction, a mitochondria-enriched fraction and a microsome/endosome-enriched fraction. The resultant fractions were then analyzed with Western immunblot for the detection of the FLAG epitope (for the detection of the overexpressed UCH-L1 protein) and of the UCH-L1 protein (for the detection of both the endogenous and the overexpressed protein, detected at 25 and 27kDa, respectively). As a cytoplasmic marker an antibody against karyopherin protein was used, whereas an antibody against Lamin was used as a marker for the nuclei. In order to confirm the successful separation of mitochondria and endosome fractions antibodies against OXPHOS and LAMP-2A were used, respectively. With this approach we found that both WT- and S18Y- UCH-L1 proteins are detected in similar levels in all subcellular

compartments examined (**Figure 1C**). These data suggest that the previously described anti-oxidant effect of the S18Y UCH-L1 polymorphism does not seem to be attributed to a differential subcellular localization of the protein.

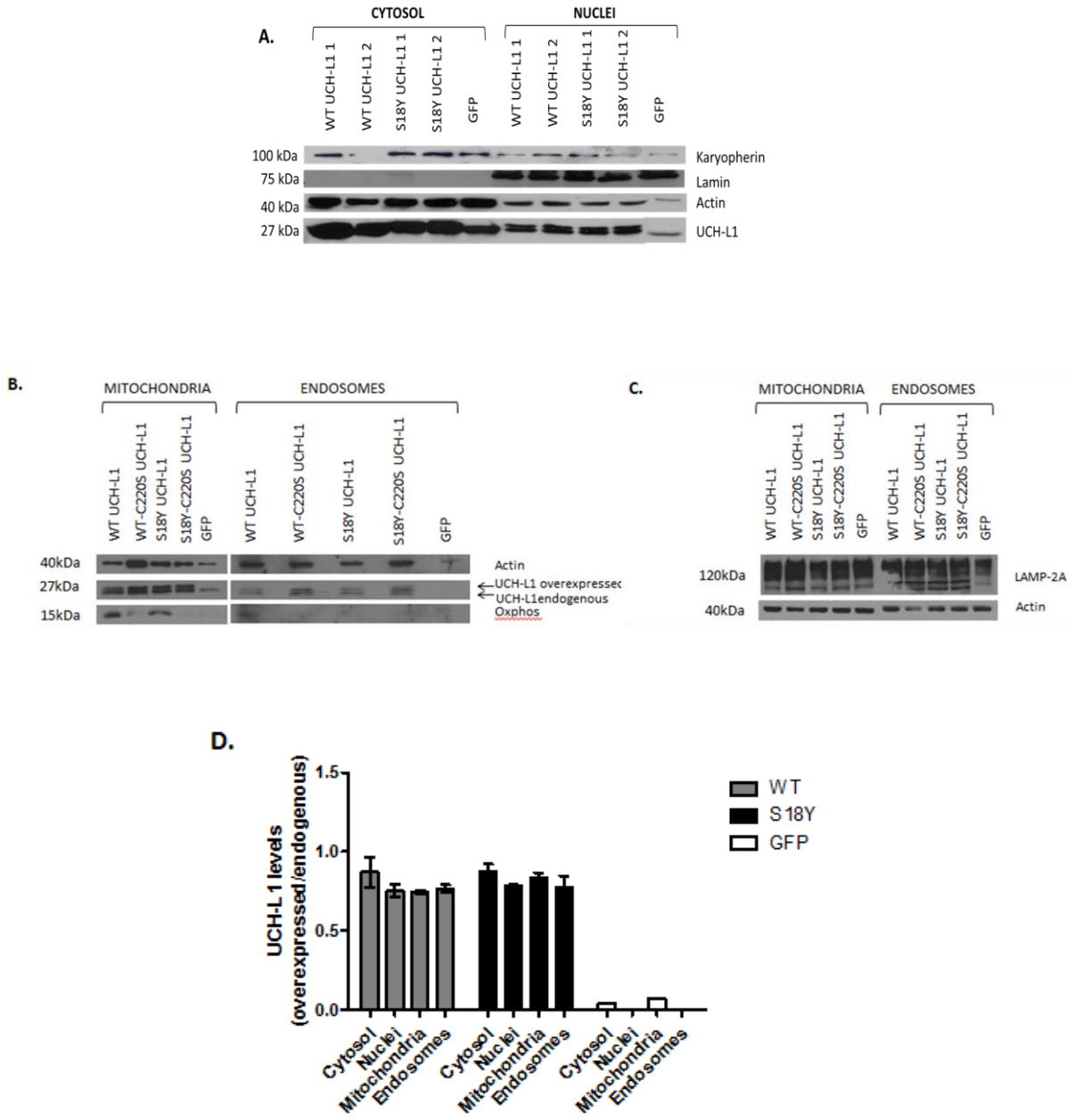

Figure 1: Overexpressed WT UCH-L1 and S18Y UCH-L1 are localized in similar levels in Cytosolic, Nuclear, Mitochondrial and Endosomal Fractions. (A-C) Human neuroblastoma SH-SY5Y cells were transiently transfected with plasmids coding for proteins of interest (WT UCH-L1 and S18Y UCH-L1 which are both tagged with the epitope FLAG at the N-terminus) and control protein (GFP). Subcellular fractionation

experiments were performed 48 hours later in order to isolate a cytosolic fraction and a nuclei-enriched fraction, a mitochondria-enriched fraction and an endosomes-enriched fraction. Representative western blots are shown. **(D)** Quantitative analysis of WT-, S18Y- UCH-L1 and GFP protein levels in the different subcellular fractions reveals that the WT UCH-L1 protein and the S18Y polymorphism are localized in similar levels in the different compartments (n=2). Data are expressed as levels of the overexpressed protein (FLAG positive) versus the endogenous UCH-L1 protein.

ii) Study of the cytosolic-membrane localization of S18Y polymorphism and the double mutant C220S-S18Y UCH-L1, under normal conditions.

Liu et al. reported recently that a subpopulation of UCH-L1 is bound to membranes and that this binding is dependent on farnesylation (at cysteine 220) of the protein (Liu et al., 2009). Mutation of the putative farnesyl ‘‘acceptor’’ Cysteine residue to Serine (C220S) essentially eliminated the membrane-associated WT-C220S UCH-L1 levels, compared to the WT-UCH-L1 (Liu et al., 2009). In an attempt to clarify whether the potential anti-oxidant role of S18Y UCH-L1 requires membrane association, we have generated farnesylation deficient WT and S18Y UCH-L1 proteins, the WT-C220S UCH-L1 and the S18Y-C220S UCH-L1, respectively. In more detail, the potential differential cytosolic and membrane localization of the S18Y UCH-L1 and the double mutant S18Y-C220S UCH-L1 proteins was assessed. Accordingly, the probability that the mutant WT-C220S UCH-L1 might also alter the cytosol-membrane localization of the WT UCH-L1 protein was also examined. For the purposes of this study, human neuroblastoma SH-SY5Y cells were transfected with plasmids coding for proteins of interest (WT-, WT-C220S-, S18Y-, S18Y-C220S UCH-L1) or control protein (GFP). 48H post transfection the cytosol-membrane fractionation was performed. The membrane fraction includes the plasma membrane together with the subcellular membranes (endosomes, lysosomes, mitochondria, nuclei, etc). Cytosolic and membrane fractions were analyzed with SDS electrophoresis and Western blot and the following antibodies were used; antibody against FLAG epitope for the identification of the overexpressed proteins, antibody against UCH-L1 for the identification of total UCH-L1 protein (overexpressed and endogenous), antibody against ERK (cytoplasmic marker), antibody against E-Cadherin (membrane marker). Our results show that all forms of UCH-L1 did not differ significantly in terms of their relative membrane localization (**Figure 2A, B**). This finding points to the conclusion that inhibition of farnesylation does not decrease membrane binding of both WT- and S18Y- UCH-L1 proteins under normal conditions.

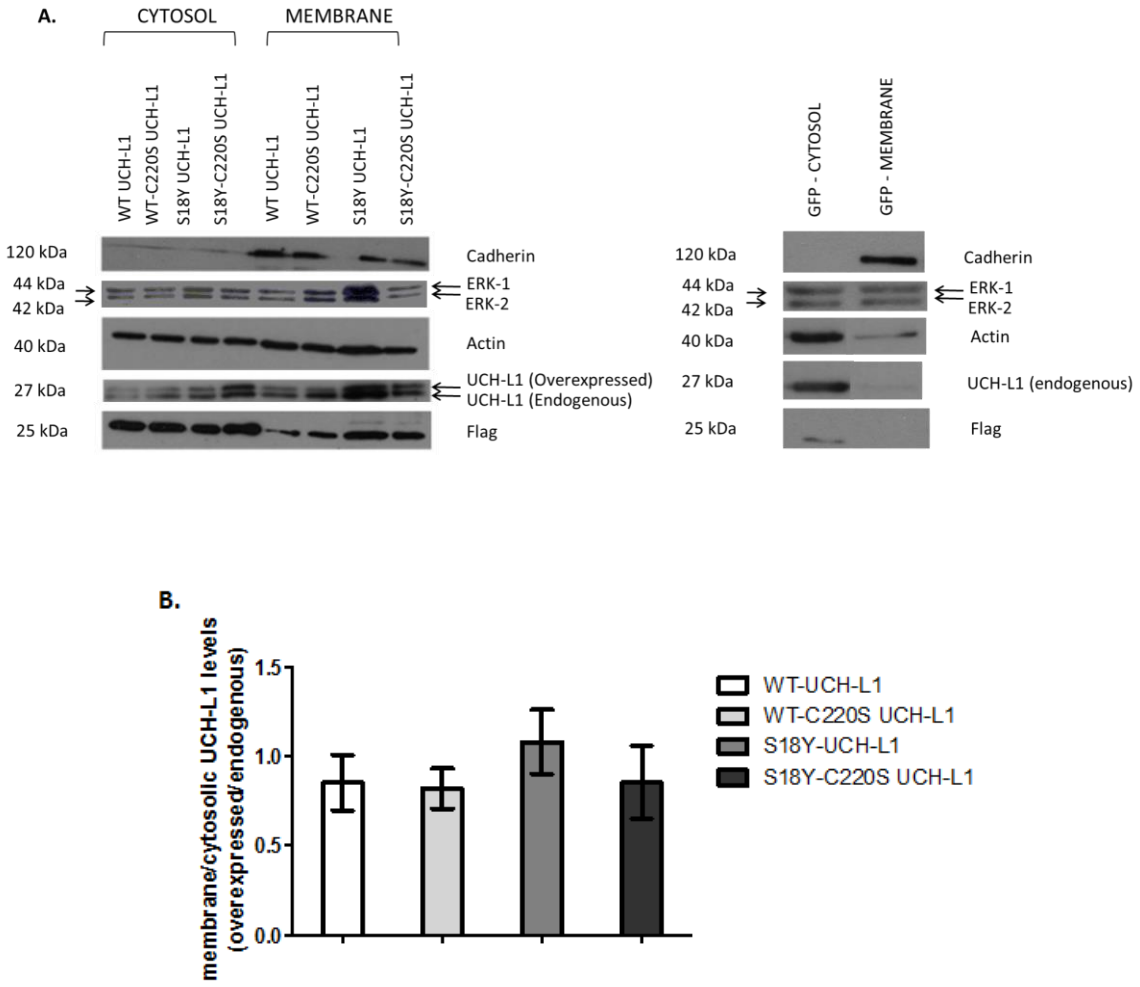

Figure 2: Overexpressed forms of UCH-L1 with or without farnesylation-disrupting mutations are localized in similar levels in membranes. (A) Human neuroblastoma SH-SY5Y cells were transfected with plasmids coding for proteins of interest (WT-, WT-C220S-, S18Y-, S18Y-C220S UCH-L1) or control protein (GFP). All forms of UCH-L1 protein are tagged with the epitope FLAG at the N-terminus. Cytosolic and membrane fractions were separated 48 hours post transfection and analyzed with SDS electrophoresis and Western Blot. Representative Western Blots are shown (n=4) **(B)** Quantitative analysis of WT-, WT-C220S-, S18Y-, S18Y-C220S- UCH-L1 and GFP overexpressed protein levels in membrane versus cytosolic fractions, compared to endogenous UCH-L1 levels. All UCH-L1 forms are localized in comparable levels between cytosolic and membrane fraction. Statistical significance of differences was evaluated with one way ANOVA followed by the Tukey's Post hoc test (n=4). Probability values <0,05 were considered significant.

In order to confirm the above results, the localization of the different forms of UCH-L1 (WT-, WT-C220S-, S18Y-, S18Y-C220S- UCH-L1) was also assessed with immunofluorescence in cells overexpressing WT-, WT-C220S-, S18Y- or S18Y-C220S UCH-L1. The epitope flag was shown to

co-localize with the plasma membrane marker E-Cadherin protein to the same extent among the different UCH-L1 forms, suggesting that farnesylation does not alter the membrane association neither of WT-, nor of S18Y UCH-L1 (**Figure 3**).

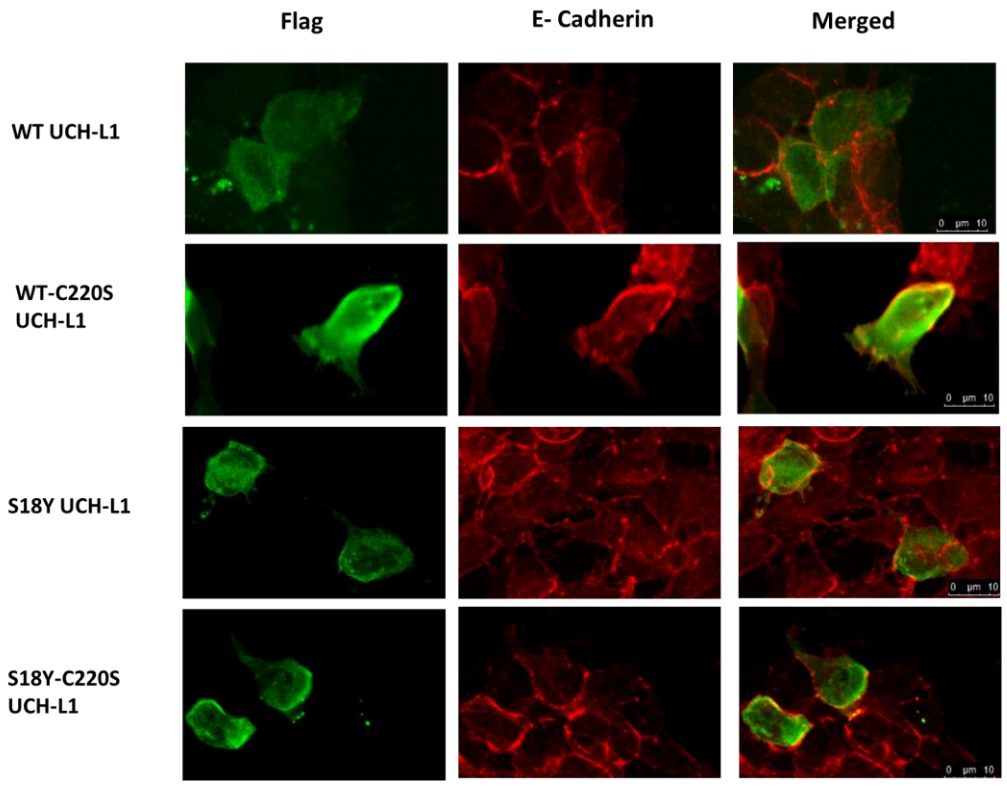

Figure 3: Membrane localization of WT, WT-C220S, S18Y and S18Y-C220S UCH-L1 proteins. Human neuroblastoma SH-SY5Y cells were transfected with plasmids coding for proteins of interest (WT, WT-C220S, S18Y, S18Y-C220S UCH-L1) and 48H post transfection, cells were fixed and stained for anti-Flag (green) and anti-E-Cadherin (red) antibodies. Cells were visualized by confocal microscopy. The different forms of UCH-L1 are detected at similar levels in the plasma membrane.

V. Assessment of the effect of double mutant protein C220S-S18Y UCH-L1 on cell survival, under oxidative stress conditions induced by the mitochondrial toxin MPP^+.

As already mentioned, Kyratzi et al. reported that the S18Y polymorphism confers an anti-oxidant function against MPP^+ induced toxicity in SH-SY5Y cells by reducing ROS generation (Kyratzi et al., 2008). To this end, we have performed double transfections using the S18Y or the C220S S18Y UCH-L1 protein together with the GFP protein (ratio 3:1, favoring the UCH-L1

proteins) and assessed survival of the transfected cells in this model. Double transfection of the irrelevant PAI2 protein plus the GFP protein was used as control. Our results show that cells transfected with the S18Y or the double mutant C220S-S18Y UCH-L1 proteins were equally protected against MPP^+ induced toxicity, compared to cells transfected with the control protein PAI-2 (**Figure 4B**). This result is in accordance with the previous finding showing that inhibition of UCH-L1 farnesylation does not significantly alter the membrane association of the S18Y UCH-L1.

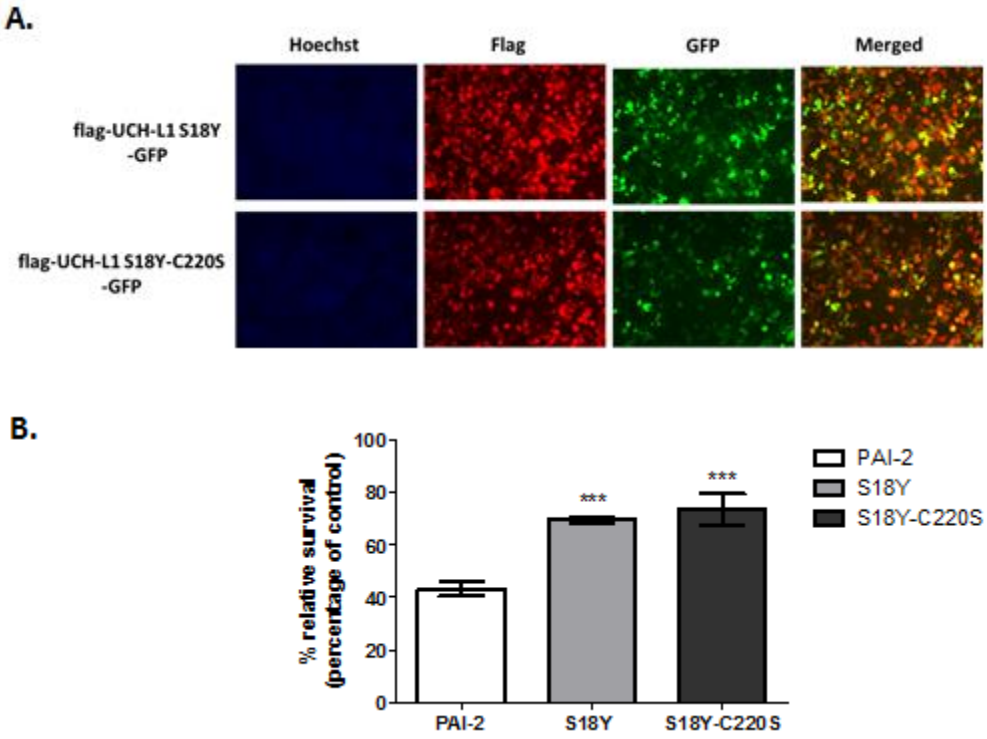

Figure 4: Transient transfection of SH-SY5Y cells with the double mutant S18Y-C220S UCH-L1 does not eliminate the protective effect of the S18Y UCH-L1 against MPP^+-induced toxicity. Proliferating SH-SY5Y cells were transiently co-transfected with plasmids coding for proteins of interest (S18Y UCH-L1, S18Y-C220S UCH-L1) or control protein (PAI-2) and GFP protein, in a ratio 3:1, respectively. **A**. In order to confirm that the majority of GFP-positive cells also express the protein of interest, cells were fixed 48H post transfection and stained for Flag (in red) and GFP (in green). Cells were also stained with Hoechst (blue). **B**. 48H post transfection cells were treated with 2mM MPP^+ or with culture medium (control cells) and cell survival was assessed by strip counts of $EGFP^+$ cells, 0 and 16 hours after treatment. Cells transfected with the S18Y UCH-L1 and the S18Y-C220S UCH-L1 proteins, were significantly protected against MPP^+, compared to cells expressing the PAI-2 protein. No statistical significant difference was observed between survival levels of cells expressing S18Y-UCH-L1 and S18Y-C220S UCH-L1. Statistical

significance of differences was evaluated with one way ANOVA followed by the Tukey's Post hoc test (n=3). Probability values <0,05 were considered significant.

B. Study of the protective role of the S18Y UCH-L1 protein against L-Glutamic acid (L-Glu)-induced toxicity in neuronally differentiated SH-SY5Y cells

Effects on cell survival

Glutamate is the major excitatory neurotransmitter in the central nervous system where it acts upon ionotropic [N-methyl-D-aspartate (NMDA) and α-amino-3-hyroxy-5-methylisoxazole proprionic acid (AMPA)] or metabotropic (mGlu1-mGlu8) receptors (Dingledine et al., 1999; Greenamyre and Porter, 1994). Although glutamate exerts a significant role in excitatory neurotransmission, an imbalance in glutamate homeostasis can evoke neurodegeneration through activation of neurotoxic or excitotoxic cascades (Olney et al., 1990). Indeed, in a recent study, Meredith et al. reported disruption of glutamate homeostasis in the MPTP mouse model of PD (Meredith et al., 2009). A significant increase in glutamate levels was observed in MPTP mice comparing to control animals, an effect that was alleviated in MPTP mice treated with L-DOPA, highlighting the association of glutamate homeostasis with the pathophysiology of PD.

In order to examine the potential neuroprotective effect of the S18Y UCH-L1 polymorphism against L-Glutamate (L-Glu) toxicity in neuronally differentiated cells, we have overexpressed the WT or the S18Y UCH-L1 protein, or the control GFP protein, via adenoviruses. In immunostaining experiments, efficient transduction of the majority of the cells was detected in all conditions (**Figure 5A**). Moreover, overexpression of all viruses did not affect neuronal survival per se (**Figure 5B**). Interestingly, adenoviral overexpression of the S18Y polymorphism of UCH-L1 protein did not confer protection against the excitotoxic L-Glu-induced cell death in neuronally differentiated cells, since no difference between the S18Y UCH-L1, the WT UCH-L1 and the GFP proteins was observed (**Figure 5C**). We also used the compound Trolox, an analogue of Vitamin E, which exhibits radical scavenging and anti-oxidant properties (Giulivi and Cadenas, 1993; Mazor, D., et al., 2006), in order to assess whether a known anti-oxidant protects this cellular system against L-Glu toxicity. Pre-treatment of cells with this compound did not improve L-Glu-induced toxicity, implying that L-Glu-induced cell death is not mediated through induction of oxidative stress (**Figure 5D**).

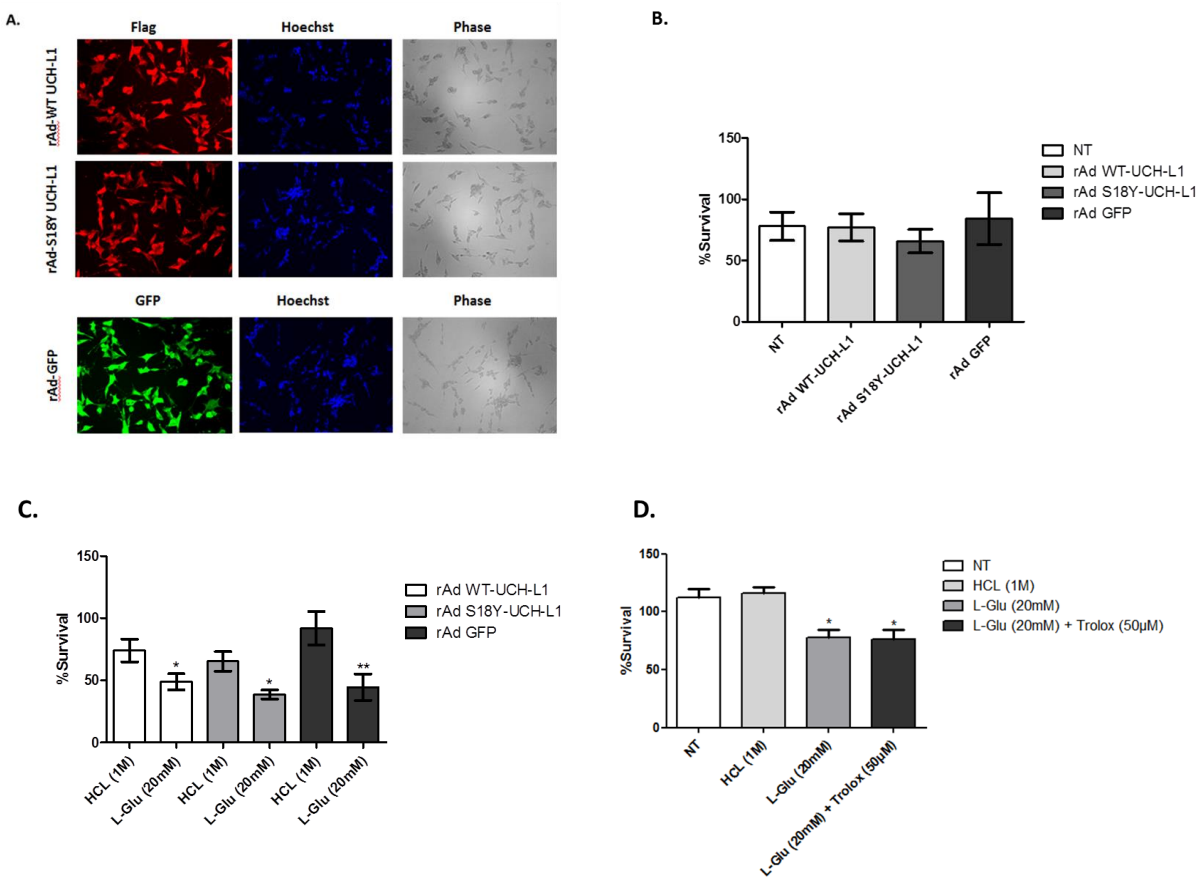

Figure 5: Expression of rAd viruses and study of the potential neuroprotective effects of the S18Y variant of UCH-L1 protein against L-Glu induced toxicity, in neuronally differentiated SH-SY5Y cells. A. Representative immunofluorescence photomicrographs of neuronally differentiated SH-SY5Y cells 48H post-transduction with the rAd-WT-UCH-L1 (flag tagged), rAd-S18Y UCH-L1 (flag tagged) or rAd-GFP (control virus) are shown. **B**. In order to assess whether viral transduction was toxic, SH-SY5Y cells were transduced with rAd-WT-UCH-L1, rAd-S18Y UCH-L1 or rAd-GFP and cell survival was assessed 5 days later with the measurement of intact nuclei. No significant toxicity was reported for any of the viruses used. **C.** Neuronally differentiated SH-SY5Y cells were transduced at day 3 of differentiation with rAd-WT UCH-L1, rAd-S18Y UCH-L1 or the control virus rAd-GFP (MOI 50for each virus). Four days later, cells were exposed to 20mM L-Glu and 24H later cell survival was assessed with measurement of intact nuclei. Neither WT-, nor S18Y UCH-L1 overexpression protected against L-Glu induced cell death, compared to untransduced cells (NT) or cells overexpressing GFP. **D.** Untransduced cells were pre-treated with the anti-oxidant Trolox (50µM) 24H before L-Glu treatment (20mM). Pre-treatment with this compound was not protective against L-Glu-induced toxicity (20mM). Statistical significance of differences was evaluated with one way ANOVA followed by the Newman Keuls test (n=3). Probability values <0,05 were considered significant.

Effects on ROS generation

The neuroprotective effects of the S18Y polymorphism are suggested to be due to its anti-oxidant function. Since the S18Y UCH-L1 did not protect neuronally differentiated SH-SY5Y cells from excitotoxic cell death evoked by L-Glu treatment, it was important to verify whether the causative effect to the cell death was induction of oxidative stress. As shown in **Figure 6A**, L-Glu treatment did not cause oxidative stress, in the concentration administered. The finding that the S18Y polymorphism was not protective against this insult further supports the theory that the protective effects of S18Y UCH-L1 are selective against oxidative-stress related insults. Interestingly, L-Glu treatment resulted in ROS production in neuronally differentiated SH-SY5Y cells when administered at a lower dose (10mM) (**Figure 6B**). However, this insult only had a slight impact on cell survival after a longer exposure (48H) (**Figure 6C**), suggesting that different doses of L-Glu can activate different cell death pathways resulting to an immediate (absence of ROS generation, probably necrosis) or a more slowly progressing mode of neuronal demise (ROS generation).

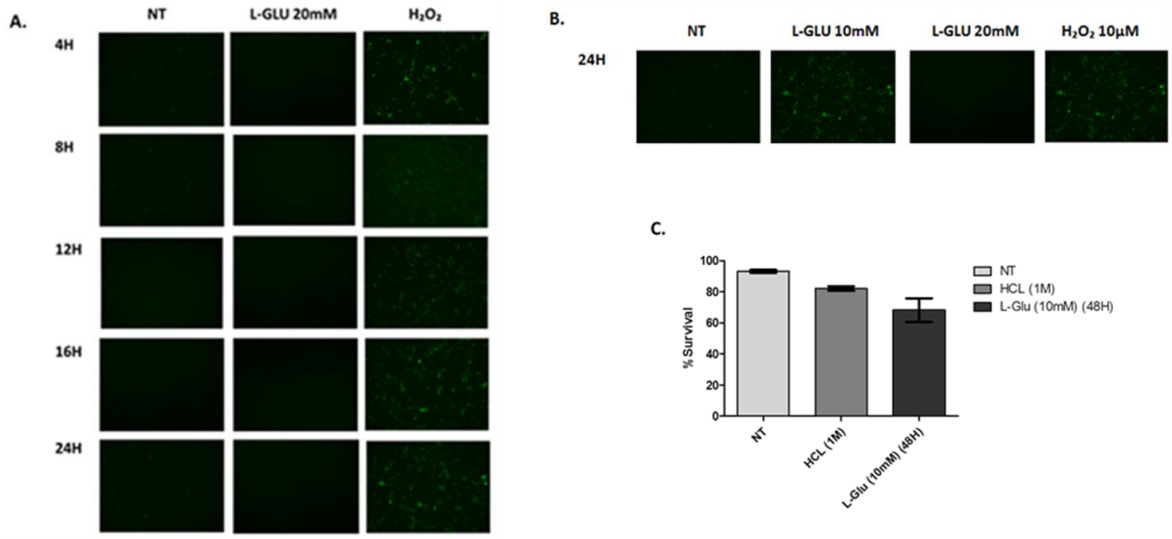

Figure 6: L-Glu-evoked death (20 mM) of neuronally differentiated SH-SY5Y cells is not being mediated via induction of oxidative stress A. Neuronally differentiated SH-SY5Y cells were treated with 20mM L-Glu at day 7 of differentiation and DCF detection was performed at different time points (4H, 8H, 12H, 16H, 24H). As a positive control for ROS generation, cells treated with H_2O_2 [40µM (4H and 8H), 20µM (12H), 15µM (16H), 10µM (24H)] were used. **B.** Administration of L-Glu at a lower dose (10mM) induced oxidative stress in neuronal differentiated SH-SY5Y cells, an insult that slightly compromised cell survival,

only after prolonged exposure (48H). Statistical significance of differences was evaluated with one way ANOVA followed by the Newman Keuls test (n=3). Probability values <0,05 were considered significant.

C. Study of the protective role of the S18Y UCH-L1 protein against L-Glu-induced toxicity in rat primary cortical neurons.

The potential protective role of the S18Y UCH-L1 protein against L-Glu toxicity was also assessed in primary rat cortical neurons. Towards this direction, we have overexpressed the WT UCH-L1, the S18Y UCH-L1 and the GFP proteins with adenoviruses as above. Efficient transduction of the majority of the neurons was achieved with all the viruses used (**Figure 7A**), without affecting neuronal survival **(Figure 7B).** In agreement with the experiments in neuronally differentiated SH-SY5Y cells, neurons transduced with the S18Y UCH-L1-expressing virus or the WT UCH-L1 virus were not protected against L-Glu-induced neurotoxicity, compared to untransduced neurons or neurons overexpressing the GFP virus **(Figure 7C).** Furthmerore, as above, the antioxidant Trolox did not confer protection either.

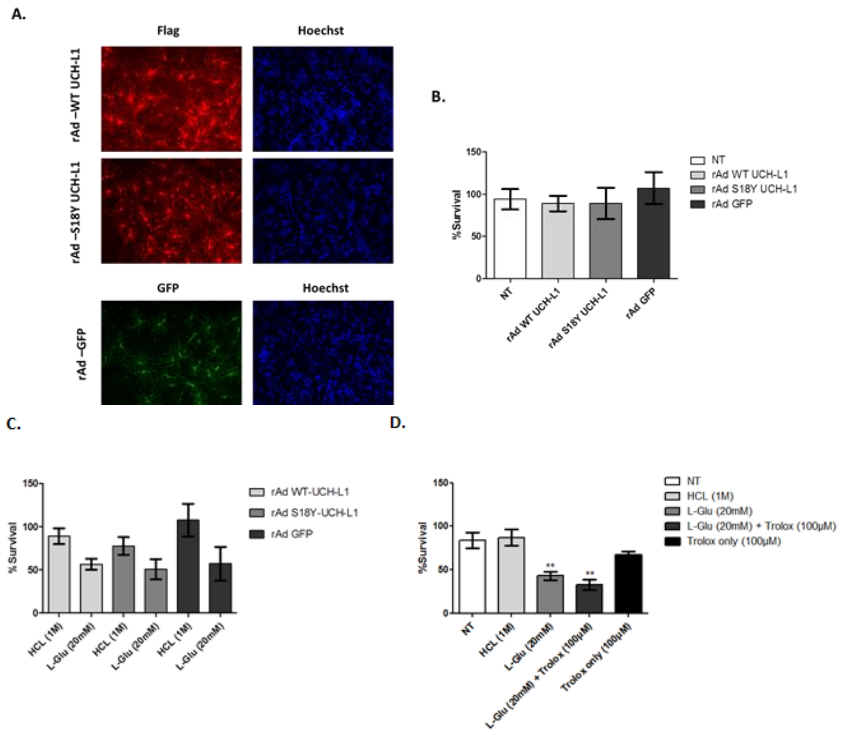

Figure 7: Expression of rAds and study of the potential neuroprotective effects of the S18Y variant of UCH-L1 protein in primary rat cortical neurons. A. Representative immunofluorescence

photomicrographs of primary rat cortical neurons 48 hours post-transduction with rAd-WT-, rAd-S18Y UCH-L1 (flag tagged) or rAd-GFP (control virus) are shown. **B.** Rat primary cortical neurons cultured for 5 days were transduced with rAd-WT-, rAd-S18Y UCH-L1 or rAd GFP (control virus) and cell survival was assessed 4 days later with measurement of intact nuclei. Transduction with the above viruses did not confer toxicity to primary rat cortical neurons. **C.** Primary rat cortical neurons cultured for 5 days were transduced with rAd-WT-, rAd-S18Y UCH-L1 or rAd GFP (control virus). Exposure to L-Glu 20mM was performed 4 days after transduction and cell survival was assessed 16H later with the measurement of intact nuclei. S18Y UCH-L1 protein, together with WT UCH-L1 protein did not confer a neuroprotective effect against L-Glu toxicity, comparing to GFP. **D.** The potential protective effects of Trolox were examined in the same conditions as **(C)** and no alleviation of neurotoxicity was found. Statistical significance of differences was evaluated with one way ANOVA followed by the Newman Keuls test (n=3). Probability values <0,05 were considered significant.

D. Study of the protective role of the S18Y UCH-L1 protein on human SH-SY5Y neuroblastoma cells inducibly overexpressing wild-type (WT) α-synuclein.

Accumulating evidence suggests an interrelationship of WT α-synuclein with oxidative stress (reviewed by Mouradian et al., 2013; Aguayo G.L., et al., 2015). In a recent study, Vekrellis et al., generated a tet-off inducible cell line conditionally overexpressing WT α-synuclein and reported that upon neuronal differentiation, α-synuclein-expressing cells degenerate through a caspase-dependent non-apoptotic manner (Vekrellis et al., 2009). Neuronally differentiated cells overexpressing α-synuclein (WT–dox) start to degenerate at day 6 of differentiation, in contrast to cells expressing basal, low levels of α-synuclein (WT+dox) (**Figure 8A**). The S18Y polymorphism of UCH-L1 protein did not confer protection against α-synuclein induced toxicity, implying that this model of synucleinopathy may not be associated with induction of oxidative stress (**Figure 8B**).

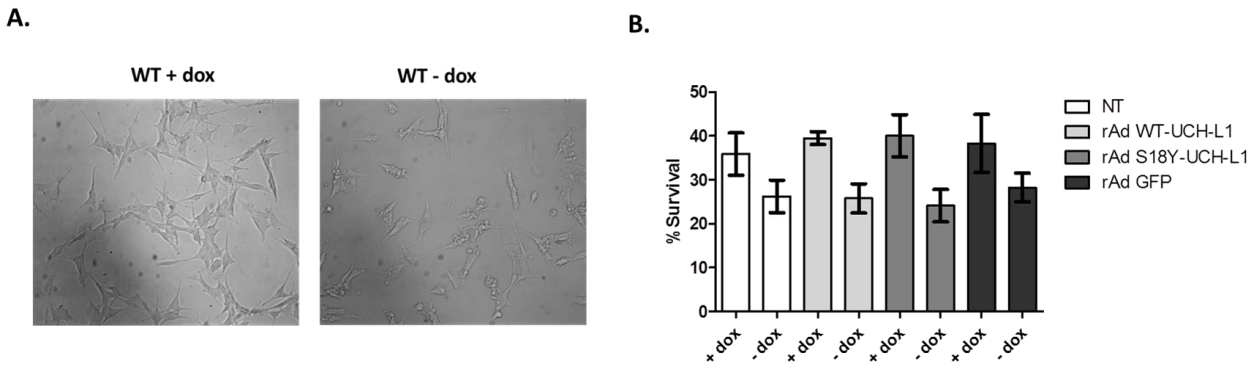

Figure 8: S18Y polymorphism of UCH-L1 protein did not alleviate α-synuclein-induced toxicity in neuronally differentiated SH-SY5Y cells. A. Differentiated SH-SY5Y cells overexpressing WT α-synuclein (WT-dox) exhibit retraction of processes with blebbing of their plasma membranes and eventually start to die at day 6 of differentiation. Uninduced cells (WT+dox) do not display any such abnormalities. **B.** Differentiated SH-SY5Y cells that inducibly overexpress WT α-synuclein (+dox 1mg/ml), manifest approximately 70% survival at day 7 of differentiation, comparing to uninduced cells (-dox). Differentiated cells, in the presence or absence of dox, were transduced with rAds expressing WT-, S18Y UCH-L1 or GFP (control virus) at day 5 of differentiation and cell survival was assessed 48H later with the measurement of intact nuclei. Overexpression of the S18Y polymorphism of UCH-L1 protein did not alleviate WT α-synuclein-induced neurotoxicity, since similar survival was observed between differentiated α-synuclein-expressing cells (-dox) transduced with the WT- or the S18Y-UCH-L1 or the GFP virus. Statistical significance of differences was evaluated with one way ANOVA followed by the Newman Keuls test (n=3). Probability values <0,05 were considered significant.

E. Degradation of the WT- and S18Y UCH-L1, as well as of the farnesylation mutants WT-C220S-, S18Y-C220S- UCH-L1 ,under normal conditions

SH-SY5Y cells overexpressing WT-, WT-C220S, S18Y- or S18Y-C220S UCH-L1 were treated with a mixture of lysosomal inhibitors [NH_4CL (20mM) and Leupeptin (10μM)] or the proteasome inhibitor epoxomicin (15nM) and 16H later, protein levels were assessed. As a marker for autophagy impairment, antibodies against LC3 and p62 protein were used, while the polyubiquitin antibody was used to verify the proteasomal impairment upon epoxomicin treatment. During autophagy, cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is recruited to autophagosomal membranes. Autophagosomes then fuse with lysosomes to form autolysosomes, and intra-autophagosomal components together with LC3-II are degraded by lysosomal hydrolases. LC3-II can be accumulated either due to inhibition of fusion of

autophagosomes with lysosomes, thus preventing autophagy, or due to hyperactivation of the early stages of autophagy (Tanida et al., 2008). p62 binds to ubiquitynated substrates and is itself degraded by autophagy (Pankiv et al., 2007; Ichimura et al., 2008). It is suggested to mediate the targeting of ubiquitynated proteins for degradation by selective autophagy (Ichimura et al., 2008). Under conditions of dysfunctional autophagy ubiquitynated substrates bound to p62 accumulate, rendering this protein suitable for detecting autophagy dysfunction.

WT UCH-L1 and its farnesylation mutant WT-C220S UCH-L1 manifested a very slight trend for accumulation following epoxomicin treatment, suggesting possible preferential degradation from the proteasome. The same trend was present for endogenous UCH-L1 (**Figure 8C**). S18Y UCH-L1 and its farnesylation mutant S18Y-C220S showed a trend for accumulation in NH_4CL+Leupeptin treated cells, implying degradation mainly through autophagic machinery (**Figure 8C**). It should be noted that in these experiments proteasomal inhibition was achieved robustly with epoxomicin treatment, as indicated by the accumulation of polyubiquitinated proteins and p62 accumulation (p62 accumulates under conditions of proteasomal inhibition as well). However, p62 did not accumulate and LC3-II only showed slight accumulation following NH_4CL+Leupeptin treatment, suggesting that the autophagic/lysosomal inhibition achieved was not robust. Further experiments will be needed to achieve more robust autophagic induction and evaluate whether the possible dissociation between WT and S18Y degradation holds up.

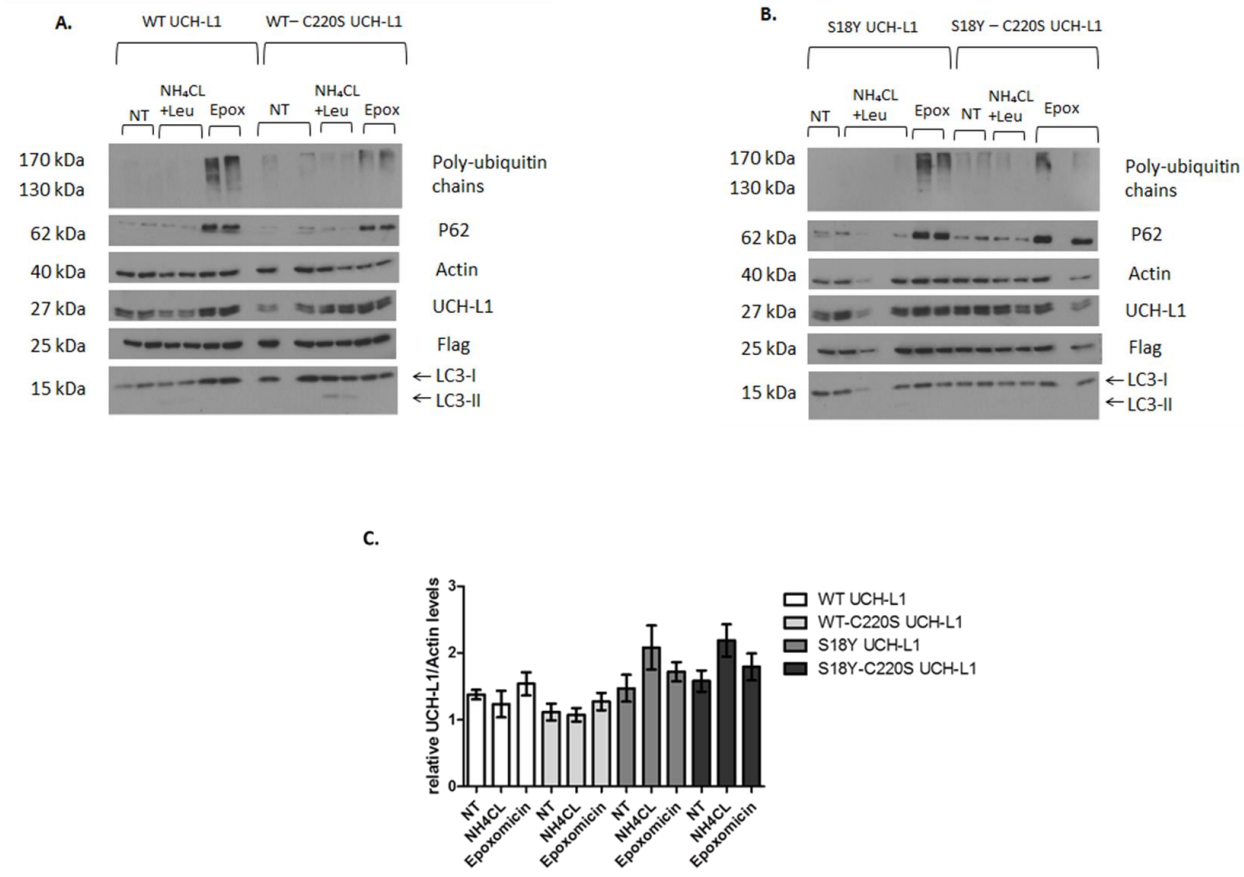

Figure 8: Degradation of WT- and S18Y UCH-L1 and farnesylation mutants WT-C220S-, S18Y-C220S UCH-L1. SH-SY5Y cells were transfected with plasmids coding for proteins of interest (WT-, WT-C220S, S18Y-, S18Y-C220S UCH-L1) and 48H later cells were treated with the lysosomal inhibitors, NH_4CL (20mM) + Leupeptin (10μM), or the proteasome inhibitor, epoxomicin (15nM) or culture medium (control samples). Protein expression levels were measured 16H later. Statistical significance of differences was evaluated with one way ANOVA followed by the Tukey's Post hoc test (n=2). Probability values <0,05 were considered significant.

Discussion

S18Y polymorphism of UCH-L1 protein has been linked with decreased susceptibility to sporadic forms of PD (Maraganore et al., 1999; Wintermeyer et al., 2000; Satoh et al. 2001a; Momose et al., 2002). However, association of S18Y UCH-L1 with PD has been questioned by many studies (Mellick et al. 2000; Levecque et al. 2001; Healy et al., 2006; Zhang et al., 2008; Wang et al 2011, Liu et al., 2015), while others suggest an age dependent protective effect (Elbaz et al. 2003; Xiromerisiou et al., 2011). The elucidation of the precise mechanism(s) via which the S18Y polymorphic variant of UCH-L1 protein exerts its neuroprotective role may shed light on the above discrepancies.

A previous study from our lab reported that the S18Y UCH-L1 selectively protected human neuroblastoma cell lines and primary cortical neurons against oxidative insults, such as H_2O_2 and MPP^+, by reducing ROS generation, but not against other insults (Kyratzi et al., 2008). This anti-oxidant effect was recapitulated *in vivo* in a subsequent study, where nigral overexpression of S18Y UCH-L1 protected wild-type mice against MPTP induced toxicity (Xilouri et al., 2012). As genetic factors may act synergistically with sporadic, resulting in differential propensity to PD manifestation, S18Y polymorphism might provide a protective effect for UCH-L1 protein only in PD cases associated with oxidative stress production. Recent genome-wide association studies (GWAS) have identified a number of genetic loci associated with sporadic PD and none of them encode genes relevant to oxidative stress pathways (Simon-Sanchez, J. and Singleton, A., 2008; Simon-Sanchez, J., et al., 2009; Gasser, T., 2009), implying that oxidative stress exerts a minor role in PD pathogenesis. Thus, it is reasonable to assume that S18Y UCH-L1 might be implicated in PD progression rather than in the initial stages of the disease.

Mechanism(s) of anti-oxidant activity of the S18Y polymorphism of UCH-L1 protein

The aim of the present study was to examine the mechanism via which the S18Y polymorphism of UCH-L1 protein exerts a neuroprotective role. As discussed above, experimental evidence suggests that the known molecular functions of ubiquitin-binding and hydrolase activities of UCH-L1 protein are not dispensable for the protective effects of S18Y polymorphism (Hegde et

al., 1997; Johnston et al., 1997, 1999; Kyratzi et al., 2008). Thus, the mechanism of the neuroprotective effect of this variant remains elusive.

As discussed above, lysosomes and mitochondria are suggested to play a role in redox homeostasis. S18Y UCH-L1 might exert its anti-oxidant role through preferential localization in one or both of these organelles, acting as a ROS scavenger, or promoting anti-oxidant endogenous responses. In the present study, we examined the potential differential intracellular localization of the S18Y polymorphism, which was not shown to differ compared to the WT UCH-L1 protein, as similar levels of both S18Y- and WT UCH-L1 were found in nuclei, cytosol, mitochondria, and endosomes. However, the presence of the S18Y polymorphism in all isolated fractions, suggests that it acts via localization in one or more of these compartments. As other anti-oxidant proteins, selective subcellular localization of S18Y UCH-L1 and increased expression levels might only be dispensable under oxidative stress insults. Thus, it will be interesting to examine the potential differential localization of this protein under oxidative stress insults, such as MPP^+.

A post translational modification of UCH-L1 protein (farnesylation at the C-terminus) was reported to enhance association with cellular membranes and disruption of this modification inhibited membrane binding (Liu et al., 2009). In this study, mutation at the farnesylation site (substitution of cysteine at position 220 to serine) decreased only slightly the membrane binding of S18Y-, but not of the WT -UCH-L1, consistent with data from Bishop et al., suggesting that under normal conditions, farnesylation is not dispensable for membrane binding of the S18Y UCH-L1. In addition, S18Y UCH-L1 was shown to protect against oxidative stress-dependent cell death induced by MPP^+, an effect that was not eliminated by the double mutant C220S-S18Y UCH-L1. These findings together propose that farnesylation of the S18Y UCH-L1 does not affect localization to the membranes under normal conditions and neither determines the anti-oxidant effects of the protein under oxidative stress conditions.

In order to examine whether the protective effects of S18Y UCH-L1 are selective solely for oxidative stress insults or are recapitulated in other insults associated to PD pathogenesis, the role of S18Y UCH-L1 protein against L-Glutamic acid-induced toxicity was examined. S18Y UCH-L1 polymorphism did not alleviate L-Glu-induced neurotoxicity and neither did WT UCH-L1

protein, both in neuronally differentiated cells and in rat primary cortical neurons. Moreover, L-Glu did not induce ROS production (in the dose administered) in neuronally differentiated cells, suggesting that the S18Y UCH-L1 may indeed act selectively against oxidative stress insults. As L-Glu was shown to induce neurotoxicity through ROS production in a lower dose, in future experiments, the effects of S18Y polymorphism will be examined under these conditions. In an attempt to examine the role of S18Y UCH-L1 in an *in vitro* model of synucleinopathy, it was found that the S18Y UCH-L1 polymorphism was not protective against α-synuclein-induced neurodegeneration. This finding was in accordance with the fact that the cell death induced by α-synuclein in this model is not accompanied by ROS generation (data not shown), further supporting the notion that the protective effect of the S18Y UCH-L1 protein is selective against oxidative stress-related insults. Taking into account that the involvement of oxidative stress in WT α-synuclein induced toxicity remains unclear, this finding further supports the selective effects of S18Y polymorphism on oxidative stress insults. This is consistent with the idea that the process of oxidative stress may not play a major role in synucleinopathies.

Current knowledge suggests that WT UCH-L1 is degraded by both the UPS and macroautophagy (Ardley et al., 2004; Kabuta et al., 2008). Dysregulation of the UPS led to inclusion formation in cells overexpressing WT-, I93M- or S18Y UCH-L1 (Ardley et al., 2004). Interestingly, the S18Y polymorphism appeared to be capable of reducing inclusion formation in cells co-transfected with the PD mutant I93M, suggesting that S18Y may reduce aggregation proneness of those cells (Ardley et al., 2004). In the present study, WT UCH-L1 manifested a trend for accumulation upon proteasome inhibition, whereas S18Y UCH-L1 slightly accumulated following lysosomal inhibition. The above results were not statistically significant, suggesting that more experiments need to be conducted. The emerging dissociation hoever between the degradation of WT and S18Y UCH-L1, if it holds up, suggests that S18Y may indeed, as part of its cycling in the cells, associate more cosely with autohagic/lysosomal components compared to the WT, something that cannot be discerned with static assessments of subcellular localization. As a potential anti-oxidant, S18Y UCH-L1 might be specifically localized upon an oxidative insult. Oxidative stress can cause dysfunction of protein degradation systems (for example through damage to lysosomes and mitochondria). Thus, it would be interesting to examine whether S18Y localizes in

and accumulates to a specific compartment, alleviating the effects of a dysfunctional proteolytic system.

In recent study, the cerebrospinal fluid (CSF) UCH-L1 levels were examined and found to be significantly decreased in patients with PD, Multiple System Atrophy (MSA) as well as Progressive supranuclear palsy (PSP) compared with controls, and in PD patients compared with Atypical Parkinsonian Disorder (APD) patients (Mondello et al., 2014). Indeed, Kabuta et al., recently reported that a portion of WT UCH-L1 is secreted from cultured cells and that secretion depends on the ubiquitin binding activity, but not on the hydrolase activity of the enzyme (Kabuta et al., 2011). In an attempt to examine the mechanism via which UCH-L1 secretion is mediated, the same group found that I93M UCH-L1 and mutant, but not WT forms of α-synuclein, inhibited UCH-L1 secretion. UCH-L1 can be modified by 4- hydroxynonenal (HNE) (Nishikawa et al., 2003), which is a product and mediator of oxidative stress. I93M UCH-L1 and HNE-modified UCH-L1 were reported to share common abnormal molecular properties as they both increased the insolubility of UCH-L1 (Kabuta et al., 2008b). Thus, the secretion of UCH-L1 protein might also be affected by oxidative modification. Considering the above, it would be interesting to investigate whether the mechanism(s) underlying the neuroprotective effects of S18Y UCH-L1 polymorphism associate with the ability of this protein to secrete.

The elucidation of the mechanism of the anti-oxidant activity of S18Y UCH-L1 protein is crucial as it will shed light on the mechanism underlying decreased susceptibility to PD in certain populations. In neurodegenerative conditions, such as PD, there is interplay between genetic and environmental factors, through common molecular pathways. Thus, the discovery of the molecular pathways with which S18Y polymorphism interacts will help to unravel new therapeutic targets in PD and related neurodegenerative conditions.

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