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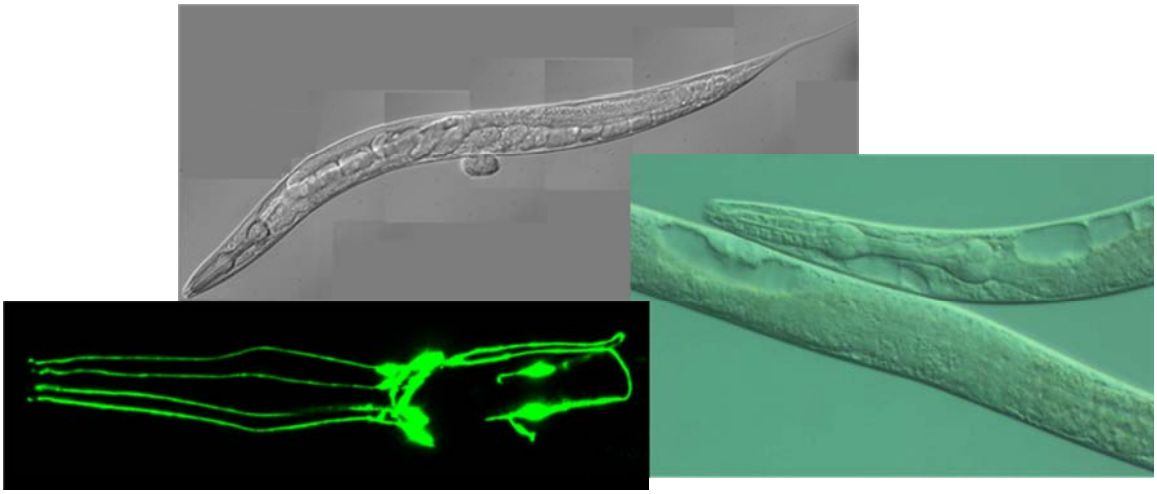
GRADUATE PROGRAM: "MOLECULAR BASIS OF HUMAN DISEASE"

MASTER THESIS

I. THE IMPLICATION OF PROTEASOMAL DEGRADATION IN AGING AND NEURODEGENERATION

II. INVESTIGATION OF THE PROTECTIVE ROLE OF THE HEAT SHOCK RESPONSE AGAINST NECROTIC CELL DEATH



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Part I

The role of proteasome degradation in aging and neurodegeneration

CONTENTS

ΠΕΡΙΛΗΨΗ

Η διατήρηση της ομοιόστασης των πρωτεϊνών είναι ζωτικής σημασίας για τη φυσιολογική λειτουργία του κυττάρου. Το πρωτεόσωμα αποτελεί την κύρια μηχανή αποικοδόμησης μακρόβιων

πρωτεϊνών. Τα βασικά υποστρώματα του πρωτεοσώματος είναι οξειδωμένες, μετουσιωμένες, πρόωρα τερματισμένες και γενικότερα βλαβερές για το κύτταρο πρωτεΐνες. Στη παρούσα μελέτη, χρησιμοποιήσαμε ως μοντέλο οργανισμό, το νηματώδη *Caenorhabditis elegans* προκειμένου να διερευνήσουμε εάν η ενίσχυση της πρωτεοσωμικής λειτουργίας, μέσω υπερέκφρασης της κεντρική περιοριστικής υπομονάδας του πρωτεοσώματος, PBS-5 οδηγεί σε αυξημένο προσδόκιμο ζωής. Τα μοριακά μονοπάτια που αφορούν στην γήρανση είναι συντηρημένα και πολύ καλά χαρακτηρισμένα στον νηματώδη. Στοχεύουμε να εξετάσουμε με γενετική ανάλυση εάν η αυξημένη πρωτεϊνική ανακύκλωση επεκτείνει το προσδόκιμο ζωής μέσω ή ανεξάρτητα από τα ήδη γνωστά μοριακά μονοπάτια που διέπουν τη γήρανση. Ακόμη, διερευνήσαμε την επίπτωση της υπερέκφρασης της περιοριστικής υπομονάδας *pbs-5* στην αντίσταση των σκουληκιών στο οξειδωτικό στρες. Επιπλέον, μελετήσαμε την επίδραση της αυξημένης πρωτεοσωμικής λειτουργίας σε μοντέλα νευροεκφυλιστικών ασθενειών στον νηματώδη. Η κατανόηση των μηχανισμών ενίσχυσης του πρωτεοσώματος που συμβάλουν στην αύξηση του προσδόκιμου ζωής, στην αντίσταση στο οξειδωτικό στρες και στη καταστολή του νευροεκφυλισμού μπορεί να οδηγήσει στη ανάπτυξη νέων αντιγηραντικών και θεραπευτικών παρεμβάσεων.

SUMMARY

Preservation of cellular protein homeostasis (proteostasis) is of critical importance. The cell employs various mechanisms to achieve this. The main machinery for the degradation of long-lived

or damaged proteins is the proteasome. We exploited the simple model organism *Caenorhabditis elegans* to examine whether enhancement of the proteasome activity, via overexpression of the core, rate-limiting proteasomal subunit, *pbs-5* can act beneficially on the lifespan of the nematode. *C. elegans'* lifespan is regulated by several conserved and well characterized pathways. We aim to investigate, by genetic analysis, whether increased protein turnover confers lifespan extension by acting through those pathways. In addition, we examined whether *pbs-5* overexpression and consequently increased proteasomal degradation confers oxidative stress resistance. Finally, there is a striking connection between aging and the onset of many neurodegenerative disorders. We took advantage of an established model of neurodegeneration in *C. elegans*. We examined whether enhancement of the main cellular degradation machinery may lead to suppression of neurodegeneration. Understanding the mechanism by which preservation of cellular proteostasis, via enhancement of the proteasomal activity, extends lifespan, confers oxidative stress resistance and suppresses neurodegeneration may lead to new anti-aging strategies and therapeutic interventions.

1. INTRODUCTION

1.1. The model organism *Caenorhabditis elegans*

Caenorhabditis elegans (*C. elegans*) is a small (about 1.3mm in length and 100μm in diameter), free-living, soil nematode worm. *C.elegans* possesses multiple exceptional characteristics which make is an ideal model for the study of aging and neurodegeneration.

The worm completes a reproductive life cycle in 2.5 days at 25°C, progressing from a fertilized embryo through four larval stages (L1 to L4), to become an egg-laying adult which consists of 959 cells and lives for about 2–3 weeks (Fig. 1.1). Under non-favorable conditions, such as starvation or stress, larvae may enter an alternative life stage, called dauer larva, during which animals move but do not feed. Dauers survive for weeks or even months (Klass and Hirsh, 1976). When a dauer encounters favorable environmental conditions, it re-enters the life cycle at the fourth larval stage (L4) and completes the final week or so of its lifespan. The last two weeks of its lifespan are characterized by a decline in locomotion, food pumping and recognizable tissue degeneration, revealing typical symptoms of aging. Due to its short life span, the nematode is particularly popular for studying the mechanisms of aging and aging related pathologies, like neurodegeneration.

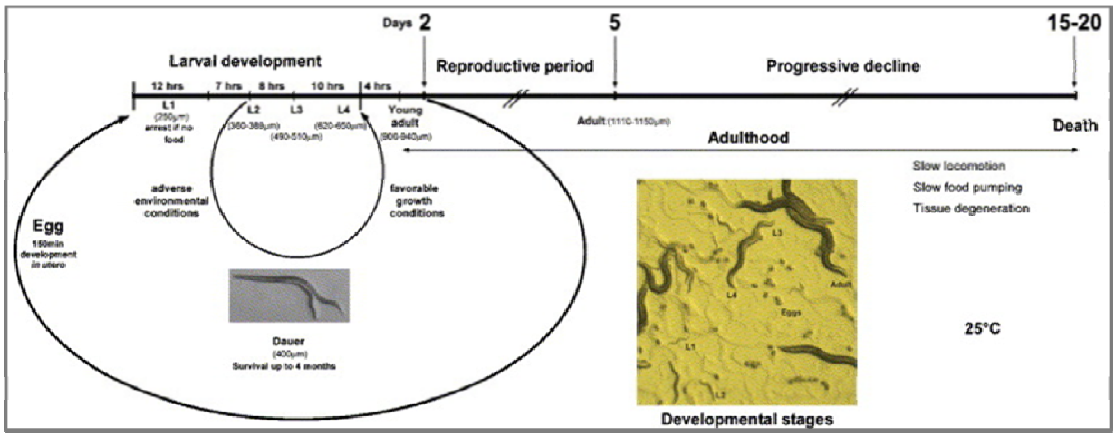

Figure 1.1. Life cycle of *Caenorhabditis elegans* at 25 ◦C. Following hatching, worms progress through four larval stages before reaching adulthood and complete their lifecycle in about 2 days, with a lifespan of between 15 and 20 days. If L2 larvae encounter stress conditions such as high temperature, lack of food and overcrowding, they enter a different developmental stage, the dauer larva. When the conditions become favorable, worms re-enter the normal lifecycle at the L4 stage and resume the rest of their development. As worms age, metabolic rhythms are slowed down and tissues deteriorate. The duration of each stage is shown in hours. The approximate animal length at each developmental stage is given in parentheses.

The ability of *C. elegans* to reproduce by self-fertilization leads to genotypically homogeneous populations and renders the production and recovery of mutants straightforward. Cross-fertilization with males (arising at a frequency of about 0.1%) is also possible, facilitating genetic analysis.

Its small size and simple dietary demands permit easy and cheap cultivation in the laboratory.*C. elegans* feeds on bacteria (usually *Escherichia coli* strain OP50), which are grown either on solid agar plates or in liquid cultures medium, and grows best at a temperature of 20°C (Brenner, 1974).

Another important advantage of the nematode is its transparency of the body which permits easy visualization of specific cells and subcellular structures and allow monitoring of cellular processes. The simple body plan, the transparent egg and cuticle, and the nearly invariant developmental program of this nematode have facilitated the detailed developmental and anatomical characterization of the animal (Wormatlas: http://www.wormatlas.org). These attributes have enabled the complete sequence of somatic cell divisions, from the fertilized egg to the 959- cell adult hermaphrodite, to be determined (Sulston, 1983; Sulston et al., 1983).

C. elegans molecular biology enables a considerable amount of information on *in vivo* activities of genes of interest to be determined rapidly. A physical map of the *C. elegans* genome, consisting of overlapping cosmid and YAC clones covering most of the six chromosomes, has been constructed

to facilitate cloning of genes that have been positioned on the genetic map (Waterston and Sulston, 1995). Sequencing and high quality annotation of the complete *C. elegans* genome organized in six chromosomes (5 autosomes and the sex chromosome X) has been accomplished (http://www.wormbase.org). Approximately 20,000 predicted open reading frames (ORFs) have been subjected to expression profiling under numerous conditions using microarray technology (Blumenthal et al., 2002; Kim et al., 2001). Detailed gene expression profiles and protein–protein interaction maps have been developed and are publicly available (Boulton et al., 2002; Matthews et al., 2001; Walhout et al., 2000).

C. elegans is also particularly amenable to reverse genetics studies. Investigators can take advantage of the wealth of genome data available to perform 'reverse genetics', directly knocking out genes (Edgley et al., 2002). Double-stranded RNA mediated interference (dsRNAi), a method of generating mutant phenocopies, enables probable loss-of-function phenotypes to be rapidly evaluated (Fire et al., 1998; Tavernarakis et al., 2000). Comprehensive RNAi approaches to knock down expression of each of the 20,000 ORFs have already been published (Kamath and Ahringer, 2003; Simmer et al., 2003). Transgenic nematodes for functional and genetic assays can be readily generated by microinjecting DNA manipulated *in vitro* into the syncytium gonad of hermaphrodite adults, where they generate inherited extrachromosomal arrays. This extrachromosomal array can further be integrated and stabilized in the genome through mutagenesis-induced integration (Mello and Fire, 1995; Rieckher et al., 2009). Vectors are available for identification of transformants, cell-specific expression, and generation of fusions to marker genes such as Green Fluorescent Protein (GFP) so that individual cells can be visualized in stained or living animals (Chalfie et al., 1994; Fire et al., 1990; Miller et al., 1999).

C.elegans has been widely used in the study of neurodegenerative diseases. The simple nervous system of the animal consists of only 302 neurons. The pattern of synaptic connections made by each of the neurons has been described, so that the full 'wiring diagram' of the nervous system is known (White et al., 1976; White and Rainbow, 1986). Neuronal cell death and protein inclusions can easily be detected and quantified using optical techniques. With the appropriate transgenic manipulations, the nervous system of the worm is readily accessible for fluorescence imaging, thus permitting phenotyping and visual screening as well as precise targeting and ablation of axons. Specific behaviors, such as locomotion, chemo- or thermotaxis, as well as learning and memory, can be experimentally associated with the relevant neuron(s). Major neurotransmitter systems are conserved in *C. elegans* contributing to the appeal of the nematode for the study of neurobiology. A large number human disease related genes have a *C. elegans* ortholog (Culetto and Sattelle, 2000), suggesting that most biochemical pathways are conserved across evolution. Arguably, the most attractive characteristic of the nematode is its suitability for experimental approaches that are not possible in mammalian models. For example, unbiased forward genetic screens using chemical mutagenesis can reveal genes integral to the biological process of interest. This approach has been used to elucidate the genetic and molecular interactions of various genes/pathways including synaptic vesicle trafficking, apoptosis, and mRNA translational regulation. In addition, reverse genetic analyses or screens can be undertaken by feeding *C. elegans* bacteria expressing double-stranded RNA (RNA interference, RNAi), which leads to almost global knockdown of target gene expression. While neurons are less sensitive to RNAi by bacterial feeding than some other tissues, this powerful strategy nevertheless has been used in numerous screens described below to identify modifier genes. For these reasons, *C. elegans* has emerged as an attractive and powerful *in vivo* model system for studying pathological mechanisms in several major neurodegenerative disorders.

Nematode genes and major signaling pathways show a significant conservation during evolution and more than 50% of the *C. elegans* genes have counterparts in humans. In addition to its contribution in elucidating developmental processes, the worm has also served as a platform to

model many human pathological conditions such as neurodegenerative disorders, cancer, aging and associated diseases (Bussey et al., 2006; Kaletta and Hengartner, 2006; Kenyon, 2005). Systematic mapping of gene interactions and signaling pathways implicated in human disease using *C. elegans* has provided better understanding of complex pathologies (Bussey et al., 2006) . The ability to produce 'humanized' worms, which express human genes not present in the *C. elegans* genome, has further enhanced the experimental value of the nematode by allowing the dissection of molecular mechanisms relevant to human disorders. In addition, the ease of drug testing coupled with the efficiency of genetic screens in worms, has made *C. elegans* a favorable tool for the identification and validation of novel drugs and drug targets, aiming to battle human pathological conditions (Kaletta and Hengartner, 2006). These exceptional characteristics of the nematode model have allowed the study of aging process and age-related pathologies, like neurodegenerative diseases.

1.2. Proteasome and aging

The aging process is characterized by a progressive deterioration of the cellular homeostasis leading to perturbations at the organismal level and eventually to death. This progressive and irreversible phenomenon is controlled by genetic and environmental factors. A hallmark of the aging process is the accumulation of modified proteins which may, eventually, be toxic for the cell. Aging and the progression of various age-related degenerative diseases is accompanied by increase in free radical production and the relative levels of oxidatively modified proteins (Berlett and Stadtman, 1997). Therefore, mechanisms that efficiently remove those harmful proteins are vital. Given the fact that the proteasome is known to be the major proteolytic system involved in the removal of misfolded, damaged and oxidized proteins (Ciechanover and Schwartz, 1998; Grune et al., 1997), it is tempting to speculate that proteosomal dysfunction during aging represents a causative factor for the decline of cellular homeostasis and consequently for the aging process.

The proteasome is a multicatalytic enzymatic complex conserved in prokaryotes and eukaryotes (Wolf and Hilt, 2004). Different combinations of a catalytic core (the 20S proteasome) and several types of regulatory subunits (the 19S and the 11S) give rise to the various types of proteasomes. The minimal functional proteasome, composed of the catalytic core alone (20S), first identified and isolated by Harries, has been proposed to degrade untagged, abnormal and oxidatively damaged proteins in an ATP-independent manner. This catalytic core is a barrel shaped compartment composed of four stacked rings of seven different α_{1-7} (the outer two rings) or β_{1-7}(the inner two rings) subunits that have a molecular mass of 18±35 kDa, forming a complex particle of about 700 kDa (Fig.1.2.). The outer rings, formed by α-subunits, shield the internal active centre, formed by β subunits, from the external cytosolic environment, provide binding sites for regulatory particles and form a gated channel leading to the inner proteolytic chamber. The catalytic centers of the proteasome are located within the opening of the hollow cylinder in the middle, between the two β subunits rings. Three of the β-subunits are proteolytically active in the mature constitutive 20S proteasome, namely, β_1, β_2 and β_5 subunits. These subunits are responsible for the ability of the proteasome to hydrolyze peptide bonds on the carboxyl site of acidic (peptidylglutamylpeptide hydrolyzing activity, PGPH), basic (trypsin-like activity, T-L) and hydrophobic (chymotrypsin-like activity, CT-L) amino acids, respectively (Jung et al., 2009; Voges et al., 1999).

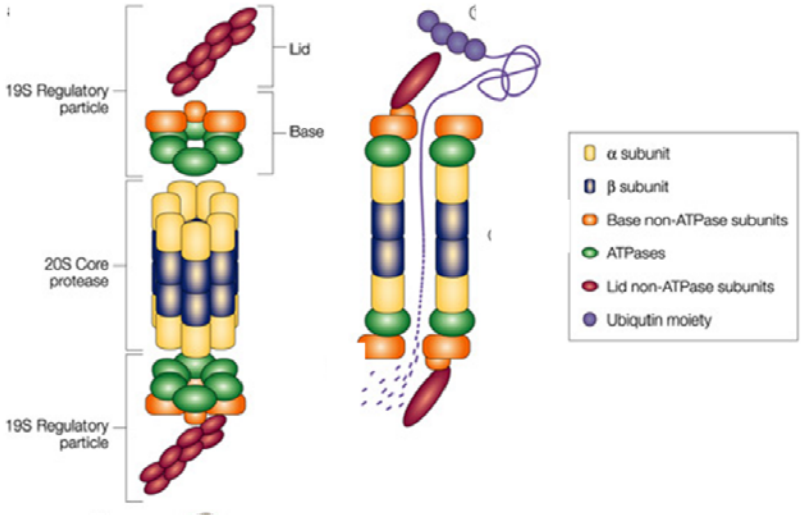

Figure 1.2. Schematic presentation of the 20S and the 26S proteasome. Adapted from (Sullivan et al., 2003)

20S proteasome is also central to the ATP/ubiquitin dependent intracellular protein degradation pathway where it represents the proteolytic core of the 26S complex. The 26S proteasome is activated by ATP and recognizes specifically poly-ubiquitinated substrates and degrades them rapidly (Ferrell et al., 1996). This complex is composed of 20S core, capped on each side by 19S regulatory complexes (Fig.1). The 19S particle is a multicomponent complex itself containing at least 17 subunits and can be further divided into two subcomplexes: the base and the lid. The base that consists of six AAA ATPase subunits (Rpt1–Rpt6) and two non-ATPase subunits (Rpn1 and Rpn2) is involved in the substrate unfolding and translocation-step of the degradation pathway (Braun et al., 1999). The lid composed by eight non-ATPase subunits (Rpn3, Rpn5–9, Rpn11 and Rpn 12) covers the base and it is involved in the recognition and ubiquitin chain processing of substrates before their translocation and subsequent degradation (Strickland et al., 2000). Rpn10 helps to tether the lid and the base and is a receptor for poly-ubiquitinated proteins (Glickman et al., 1998).

26S complex is directly related to degradation by the ubiquitin system in a two-step procedure. The target protein is first labeled via covalent attachment of multiple ubiquitin molecules and then the degradation of the tagged protein by the 26S complex occurs. Conjugation of ubiquitin to the substrate proceeds in general, via three steps. Ubiquitin is activated in its C-terminal Gly by E1, the ubiquitin-activating enzyme. Once activated, one of several E2 enzymes, ubiquitin-carrier proteins or ubiquitin-conjugating enzymes, transfers ubiquitin from E1 to a member of the ubiquitin-protein ligase family, E3, to which the target protein is specifically bound. This enzyme catalyzes the conjugation of ubiquitin to the substrate prior of the action of the 26S complex (Ciechanover and Schwartz, 1998).

Other proteasome variants, the 22S proteasomes or ‘immunoproteasomes’, have acquired particular relevance for their role in the processing of antigens for presentation by the major histocompatibility complex (MHC) class I pathways (Kloetzel, 2001). In these specialized proteasomes, the catalytic core, in which three of the seven β subunits are replaced by other β (β_i) subunits, is flanked by two 11S regulatory subunits. Immunoproteasome assembly and synthesis of the 11S regulator are strongly dependent on interferon-γ. The distinct subcellular distribution of the immunoproteasomes (mostly enriched at the ER surface) and subtle differences in cleavage specificity determine the efficiency of production of MHC class I binding peptides.

The 20S and 26S proteasomes play a crucial role in the turnover of cytosolic proteins and participate in the selective degradation of altered polypeptide chains (Carrard et al., 2003).

Degradation of oxidatively damaged proteins is carried out by the 20S proteasomes without prior ubiquitinization of the substrate polypeptide chains. Other forms of aberrant proteins may be selectively ubiquitinated prior to their ATP-dependent degradation by 26S proteasomes (Ciechanover, 2005). In fact, besides eliminating abnormal proteins that are either misfolded or altered, proteasome also participates to the activation of essential functions of the cell. Indeed, the proteasome is implicated in a broad range of cellular pathways such as turnover of normal proteins (Hilt and Wolf, 1995) apoptosis (Dubiel et al., 1995), cell cycle progression (King et al., 1996), cell differentiation (Bowerman and Kurz, 2006), control of gene expression (Palombella et al., 1994), and degradation of many important rate-limiting enzymes in metabolic pathways (Asher et al., 2005).

One of the most accepted theories of aging is the loss of quality control in protein turnover with the concomitant build-up of oxidatively modified and damaged proteins. As proteasomes selectively degrade oxidatively damaged as well as ubiquitinated proteins it is postulated that proteasome activity declines with aging. Several experimental data in various tissues and organisms support the impairment of protein function and the decline of proteasomal activity during aging. Failure of proteasome function with age could be due to decreased overall proteasome content (decreased proteasome expression; alterations of proteasome subunits), occurrence of posttranslational modification and formation of inhibitory proteasome substrates.

The proteasomal activity decreases in aging cells and in reverse, proteasomal inhibition in young cells is able to enhance the formation of poly-ubiquitinated protein-aggregates (Powell et al., 2005; Terman and Sandberg, 2002). Proteasome activities have been mainly reported to decline with advanced age both *in vivo* and *in vitro* (Gaczynska et al., 2001), although there are few reports showing no change or upregulation of certain activities in these conditions (Conconi et al., 1996; Shibatani and Ward, 1996). A decrease in the proteasomal activity has been shown in human tissue and specifically in muscle (Ferrington et al., 2005; Husom et al., 2004), lens (Viteri et al., 2004), lymphocytes (Carrard et al., 2003; Ponnappan et al., 1999), epidermal cells like keratinocytes (Petropoulos et al., 2000) and fibroblasts (Chondrogianni et al., 2000; Hwang et al., 2007; Sitte et al., 2000). An intracellular increase of oxidatively modified but not degraded proteins was found, too (Stadtman, 1992). Decrease in the proteasomal activity has been found in aged tissues of other mammals (mice, rats and bovines) like liver (Breusing et al., 2009; Shibatani and Ward, 1996), heart (Bulteau et al., 2002), lung (Breusing et al., 2009), kidney (Keller et al., 2000) lens (Shang et al., 1997), retina (Louie et al., 2002), spinal cord (Keller et al., 2000) and brain (Zeng et al., 2005). These findings argue for a direct implication of the observed age-related alterations of proteasome function in the decline of protein turnover with age.

To investigate endogenous factors, both expression and functionality of the single proteasomal subunits have been investigated, and the results revealed that the subunits were differently affected (Grune et al., 1997). Age-related variations of gene expression patterns have been reported for both mitotic (human fibroblasts) and post-mitotic (rat skeletal myocytes) cells using the microarray technology (Lee et al., 1999; Ly et al., 2000). The transcription of several genes encoding either 20S or 26S proteasome subunits were found to decline with age (Lee et al., 1999; Ly et al., 2000). Some but not all proteasome subunits have been found to be down-regulated in aged tissues like spinal cord (Keller et al., 2000), heart (Bulteau et al., 2000) and epidermis (e.g. keratinocytes; (Petropoulos et al., 2000). Studies in human epidermis and in cultured keratinocytes have shown that the age-related decline of proteasome peptidase activities can be explained by decreased proteasome content. In keratinocyte cultures, as well as in epidermis, there is an inverse relationship between the aging marker β-galactosidase and proteasome content (Petropoulos et al., 2000). The age-related decrease in proteasome activity in cellular rat extracts from cardiac myocytes were also associated with a decreased 20S proteasome content and loss of specific

activities suggesting that alterations in proteasome subunit composition and/or structure (Bulteau et al., 2002).

A decline of content has also been reported in the model of senescent fibrolasts (Chondrogianni et al., 2003; Merker and Grune, 2000). The expression of the α-subunits was not down-regulated, and the loss of function seems to be due to a reduced availability of the β-subunits (Chondrogianni et al., 2003). To overcome the decrease in proteasome activity observed in senescent fibroblasts, the β5 subunit was stably transfected into fibroblasts. Overexpression of the β5 subunit resulted in higher levels of the β1 and β2 subunits as well as in increases in all three proteasome activities and exhibited higher survival rates when treated with the proteasome inhibitors or with various oxidants and stressors (Chondrogianni et al., 2005).

1.3.1. The involvement of proteasome degradation in Parkinson's disease

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder that impacts motor skills and speech, usually in the elderly. Patients suffer from involuntary movements including resting tremor, muscle rigidity, slowed movement and difficulty in balance, symptoms associated with loss of neurons that produce dopamine in specific parts of the brain. The two most prevalent PD pathological hallmarks are the formation of the intracytoplasmic inclusions called Lewy bodies (LB) in PD patient brains and the selective loss of dopamine (DA) neurons in the substantia nigra pars compacta. The main component of LBs is α-synuclein (Kawahara et al., 2008; Lee, 2003), and is usually found in a polyubiquitinated state. A missense mutation of α-synuclein is responsible for some early-onset forms of familial PD.

Connections between UPS aberration and sporadic PD were reinforced by the observation that administration of proteasomal inhibitors to rodents recapitulated the symptoms of PD, including selective nigral cell loss, Lewy body-like inclusions, and clinical signs of bradykinesia, rigidity, and tremors (McNaught et al., 2004). Other experiments pointing into the direction of a proteasome/UPS failure in PD have been performed by treating rats with the proteasome inhibitor lactacystin. The result was a quick progression of the somatic symptoms in PD and an increased loss of dopaminergic neurons, while the formation of α-synuclein aggregates was also induced (Lev et al., 2006). Quite recently, it has been suggested that inhibition of the proteasome activity in *C. elegans* resulted in neurodegeneration of dopaminergic neurons (Caldwell et al., 2009a) establishing a compelling link between proteasome dysfunction and the development of neuronal cell death.

The tendency of α-synuclein, a major component of the LB, to form protofibrils has been suggested as inhibitory for proteasomal functioning (Bennett, 2005). Although the mechanisms behind proteasome inhibition by protofibrils requires further investigation, both steric blockage of the catalytic core of the proteasome by the fibrils and alterations of particular subunits or interacting components have been proposed. For example, point mutations in synphilin-1, an α-synuclein interacting protein known to interact with the regulatory proteasomal unit S6 ATPase, have been reported in some individuals with sporadic PD. Furthermore in human SH-SY5Y neuroblastoma cells treated with the PD mimetics neurotoxin 1-methyl-4-phenylpyridinium (MPP+), the result was a change in proteasomal activity after 24 h of MPP+ incubation, shown by a decrease of the β5- and β1-activity (Caneda-Ferron et al., 2008).

The more direct association of UPS with PD pathogenesis originated from the identification of *parkin* and *UCH-L1* as familial PD-linked genes due to the fact that both gene products perform specific functions in the proteasomal degradation pathway (Leroy et al., 1998). Parkin functions as an E3 ligase, an enzyme that catalyzes the addition of ubiquitin chains to target proteins marked for destruction by the proteasome. Mutations in *parkin* gene are common and underscore the cause of almost half of early onset autosomal recessive parkinsonism. Mutations in *UCH-L1*, a ubiquitin C-

terminal hydrolase L1, which usually cleaves polyubiquitin chains into monomeric ubiquitin, have also been identified in some PD patients. Proteasomal-independent activities mediated by parkin and UCH-L1 should be taken into account when investigating their role in PD pathogenesis. Both parkin and UCH-L1 mediate a different assembly of ubiquitin chains onto proteins (K63 linkages), for purposes other than degradation *via* UPS, such as to promote inclusion formation and facilitate preferential clearance of inclusions *via* autophagy (Tan et al., 2008). This highlights a potential way of modulating the degradative fate of protein substrates *via* tagging with different ubiquitin chains. Indeed, parkin-mediated K63 ubiquitination enhanced LB-like inclusion formation by coexpression of synphilin-1 and α-synuclein (Lim et al., 2005). As for UCH-L1, its K63-ubiquitination specific ligase activity has been shown to promote α-synuclein accumulation within cells (Liu et al., 2002). Although the roles of both enzymes in the autophagic pathway remain to be elucidated, conceivably, PD-linked mutations may potentially affect their roles in both the UPS and lysosomal degradation pathways.

1.3.2. Models of Parkinson disease in *C.elegans*

In rodent and primate models of Parkinson disease, neuroroxicity is induced by the neurotoxins 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and more recently the pesticide rotenone. The pathway through which neurons degenerate depends on the neurotoxin and the conditions used (Choi and Cheon, 1999). Presynaptic dopamine transporters (DATs) are necessary for the accumulation of these toxins to dopamine neurons, resulting in an increase in reactive oxygen species production and/or mitochondrial dysfunction. In *C. elegans*, dopamine neurons degenerate after exposure to 6-OHDA.

PD has also been modeled in *C. elegans* by overexpression of wild and mutant types of α-synuclein, the main component of LB. Overexpression throughout the nervous system or specifically in motor neurons caused motor deficits in transgenic worms. In addition, loss of a number of cell bodies and dendrites of dopaminergic neurons was observed when human α-synuclein was overexpressed under the control of a dopaminergic or pan-neuronal promoter (Lakso et al., 2003).

An MPTP-based nematode model of Parkinson's disease, targeting specifically the dopaminergic neurons has also been established and used as a platform for drug testing in living animals. Treatment of *C. elegans* with MPTP or its active metabolite MPP+ (1-methyl-4-phenylpyridinium) resulted in significantly reduced mobility and increased lethality, which was accompanied by specific degeneration of the dopaminergic neurons (Braungart et al., 2004). Taking advantage of the PD models by implementation of large-scale genetic and pharmacological screenings designed will elucidate the molecular mechanisms underlying neuronal death and identify targets and drugs for therapeutic intervention.

1.4. Aim of the study

We examined the role of proteasomal degradation in aging and neurodegeneration *in vivo*. We explored the effect of increased proteasomal activity via overexpression of the core, rate-limiting proteasomal subunit, PBS-5 on lifespan of *C. elegans* and we aim to reveal the interplay with known lifespan extending pathways. We investigated whether increased protein turnover caused by the enhancement of proteasome activity confers oxidative stress resistance. Finally, we examined the impact of *pbs-5* overexpression on established models of neurodegeneration. Understanding the mechanism by which preservation of cellular proteostasis, via enhancement of the proteasomal activity, extends lifespan, confers oxidative stress resistance and suppresses neurodegeneration may lead to new anti-aging strategies and therapeutic interventions.

2. MATERIALS AND METHODS

2.1. *C.elegans* maintenance

2.1.1 Preparation of Nematode Growth Media petri plates

C. elegans is maintained in the laboratory on Nematode Growth Medium (NGM) agar which has been aseptically poured into petri plates. Medium size plates (5cm diameter) are useful for general strain maintenance. The NGM agar medium can be poured into petri plates easily and aseptically using a peristaltic pump.

Preparation of NGM plates (2L):

1. Add 1500ml H_2O into a 2 liter Erlenmeyer flask. Mix 6 g NaCl, 5 g bactopeptone, 34 g agar, and 0,4g streptomycin. Cover mouth of flask with aluminium foil. Autoclave for 20 min.
2. Cool flask.
3. Add 1 ml 1 M $CaCl_2$,1 ml of nystatin 100mg/ml in 70% ethanol, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M $MgSO_4$ and 50 ml 1 M KPO_4 buffer. Swirl to mix well.
4. Using sterile procedures to dispense the NGM solution into petri plates using a peristaltic pump. Fill 5cm plates with 11,5ml of NGM.
5. Leave plates at room temperature O/N before use to allow for detection of contaminants, and to allow excess moisture to evaporate.

Plates stored in an air-tight container at 4^{o}C temperature will be usable for several weeks.

2.1.2. Preparation of bacterial food source

C. elegans is usually grown monoxenically in the laboratory using *Escherichia coli* (*E. coli*) strain OP50 as a food source. *E. coli* OP50 is a uracil auxotroph whose growth is limited on NGM plates. Starter culture is used to isolate single colonies on a streak plate of a rich medium such as LB agar. Inoculate of about 50 ml LB with a single OP50 colony and incubate at 37˚C, shaking for 5hours. The *E. coli* OP50 solution is then ready for use in seeding NGM plates. The *E. coli* OP50 streak plate and liquid culture should be stored at 4°C and will remain usable for several weeks.

2.1.3. Seeding NGM plates

Using sterile technique, approximately 150µl of *E. coli* OP50 liquid culture is applied to medium NGM plates using a pipette. The drop can be spread using the pipette tip or a glass rod. The lawn is not spreaded all the way to the edges of the plate, only in the center. *E. coli* OP50 lawn is grown overnight at room temperature (cool plates to room temperature before adding worms). Seeded plates can be stored stored in an air-tight container for 2-3 weeks.

2.1.4. Solutions

2.1.4.1. M9 Buffer

For 1 L:	KH_2PO_4	3 g
	Na_2HPO_4	6 g
	NaCl	5 g
	$MgSO_4$ (1M)	1 ml
	dH_2O	1 L

Combine above ingredients in large beaker or graduated cylinder using magnetic stir bar. Aliquot into appropriate sized bottles and autoclave for 20 minites.

2.1.4.2. Freezing Solution

For 1L:	NaCl	5,85g
	KPO4 1M, pH 6	50ml
	Glycerol	300ml
	X ml dH_2O to 1L	

Autoclave for 20 min. Add 3ml of MgSO4 1M

2.1.4.3. Bleaching Solution

1ml NaOH 5N
2ml NaOCl 5%
7ml ddH_2O

2.1.5. Freezing protocol

Pick 10 young adult worms onto each of 5-6 5 cm plates that have been seeded with OP50.
When the plates are ready to freeze (just starved with plenty of L1s):

1. Add about 9 ml of M9 to all the plates.
2. Give each plate a swirl to loosen worms still stuck to the agar and then tilt plates on their lids so the liquid drains to one side of the plate.
3. Using a glass pipette, collect the liquid (worms and M9) in a 15 ml conical tube (about 7 mL total).
4. Add an equal volume of freezing solution.
5. Briefly agitate the vial to suspend the worms and aliquot the worm suspension into 10 cryovials clearly labeled with the strain name, code number and the date.
6. Store in –80°C freezer space.
7. Test that the worms about 1 month later to ensure a successful freeze. A good freeze is when at least more than 10 worms survive. Pick several survivors on to a fresh plate to make sure they can produce progeny of the correct phenotype.

Work on ice and keep chilled all reagents.

2.1.6. Bleaching protocol

Cleaning contaminated *C. elegans* plates

Generation of synchronized populations

1. Use *C. elegans* stock plates that have many gravid hermaphrodites. Wash the plates with 2ml of M9. Pour the M9 across the plate several times to loosen worms and eggs that are stuck in the bacteria.
2. Collect the liquid in a sterile eppendorf tube. Centrifuge and discard the liquid.
3. Add 500μl of freshly made bleaching solution
4. Shake well or vortex the tube for a few seconds. Repeat shaking/vortexing every 2 minutes for a total of 4 minutes.
5. Spin the tube in a table top centrifuge for 27 seconds at 1300 x g to pellet released eggs.
6. Aspirate to 0,1 ml/
7. Wash with 1ml of M9. Shake well or vortex for a few seconds.
8. Repeat steps 5 and 6.
9. Use a Pasteur pipette to transfer the eggs in the remaining 0.1 ml of liquid to the edge of a clean NGM plate seeded with an *E. coli* OP50 lawn.

2.1.6. Generation of synchronized population (Egg laying)

Synchronous populations of nematodes were established by allowing 20 adult hermaphrodites to lay eggs for a limited time interval (3–4 h) on NGM plates seeded with *E. coli* OP50.

2.2. Strains and genetics

We used the following strains: Wild-type: N2 Bristol isolate, mec-4(u231)X, referred to in the text as mec-4(d), DR26: *daf-16(m26)I*, TK22:*mev-1(kn1)III*, UA44: [*baInl1*; p*dat-1*::α-*syn, Pdat-1*::*gfp*, UA49: [*baInl2*; p*unc*-54::α-*syn*::*gfp*, *rol-6 (su1006)*], N2*Ex*[$p_{let-858}$PBS-5 pRF4] line 1, N2*Ex*[$p_{let-858}$PBS-5 pRF4] line 2, N2*Ex*[$p_{let-858}$PBS-5 pCOEL] line 1, N2*Ex*[$p_{let-858}$PBS-5 pCOEL] line 2, *mec-4(d)Ex*[$p_{let-858}$PBS-5], P_{dat-1}::α-syn P_{dat-1}::GFP *Ex*[$p_{let-858}$PBS-5], p_{unc-54}::α-syn::GFP*Ex*[$p_{let-858}$PBS-5].

2.3. *C. elegans* transformation- DNA Microinjection

We generated by injecting 15 ng/μl of expression plasmid containing the *pbs-5* cDNA and 50 μg/ml of *rol-6* into N2, an integrated line of UA44 [*baInl1*; *Pdat-1*::α- *syn, Pdat-1*::*gfp*] and an integrated line of UA49: [*baInl2*; p*unc*-54::α-*syn*::*gfp*, *rol-6 (su1006)*]. Two stable lines were generated for each strain. In addition, we generated by injecting 15 ng/μl of expression plasmid containing the *pbs-5* cDNA and 50 μg/ml of p*coel* into N2 and *mec-4(d)* mutant animals. Two stable lines were generated for each strain. The DNA microinjection protocol we followed is described by Rieckher et. al. (Rieckher et al., 2009)

2.4. Lifespan assays

Lifespan assays were performed at 20^{o}C. Synchronous populations of nematodes were established by allowing 20 adult hermaphrodites to lay eggs for a limited time interval (4–5 h) on NGM plates seeded with *E. coli* OP50. Progeny were grown on OP50-seeded plates at 20^{o}C, through the L4 larval stage, and then transferred to fresh OP50-seeded plates at groups of 10–20 nematodes per plate for a total of 100–150 individuals per experiment. The first day of adulthood was used as t=0. Animals were transferred to fresh plates every 2–4 days thereafter and were examined every day for touch-provoked movement and pharyngeal pumping, until death.

2.5. Statistical analysis of lifespan data

The log-rank (Mantel-Cox) test was used to evaluate differences between survivals and determine P values. We used the Prism software package (GraphPad Software Inc., San Diego, USA), to carry out statistical analysis and to determine lifespan values. Worms that died due to internally hatched eggs, an extruded gonad or desiccation due to crawling on the edge of the plates, were censored and incorporated into the data sets.

2.6. Oxidative stress resistance assays.

For paraquat resistance assays synchronously cultured animals were kept on NGM plates at 20°C until the L4 stage. For each strain, 4 × 25 L4s were transferred on paraquat (Sigma) containing plates (2mM)-paraquat added on top of NGM plates already seeded with OP-50. Animals were incubated on these plates at 20°C scored for survival at the 5th day of exposure. Each paraquat resistance assay was repeated at three times. For NaN_3 resistance, nematodes at the L4 stage of development were selected, washed with M9 and incubated for 30 min at 20°C with 0.5 M freshly made NaN_3 (Sigma, Munich Germany) in M9. Worms were washed with M9 and placed into NGM plates to recover. The percentage of living worms was calculated after 24 hours of recovery

2.7. Neurodegeneration assays

2.7.1. *mec-4(d)* neurodegeneration assays

Neurodegeneration was scored during the mid-L1 larval stage. Synchronous animal populations were generated by hypochlorite treatment of gravid adults to obtain tightly synchronized embryos that were allowed to develop into L1 larvae for 4-5 hours. For the strains expressing *mec-4(d)* we scored neurodegeneration by the characteristic vacuolated appearance of the six touch receptor neurons using differential interference contrast (Nomarski) microscopy.

2.7.2. α-synuclein-induced neurodegeneration assays

Synchronous populations of nematodes were established by allowing 20 adult hermaphrodites to lay eggs for a limited time interval (4h) on nematode growth medium (NGM) plates seeded with *E. coli* OP50. Progeny were grown on OP50-seeded plates at 20^{o}C, through the L4 larval stage, selected for the presence of the transgene and transferred to fresh OP50-seeded plates at groups of 10–20 nematodes per plate for a total of 100 individuals per experiment. Animals were transferred to fresh plates every 2 days until the 7th day of adulthood. Worms were immobilized by a mix of 5μl of M9 and 9μl of sodium azide (20mM) applied on glass slide, a coverslip was placed on the slide and sealed using nail polish. The 6 anterior DA neurons (4 CEP and 2 ADE neurons) of 60 animals/ trial were examined for neurodegeneration under a Zeiss Axio Imager Z2 Epifluorescence/DIC Microscope. Each α-Synuclein-induced neurodegeneration assay was repeated at least three times.

2.8. Confocal sample preparation

Wash the worm plates with 2 ml of M9-centrifuge and remove the liquid. Add 1ml of M9, vortex, centrifuge and remove the liquid. Add 70μl of sodium azide (20mM), centrifuge and remove the liquid. Mount the remaining worms on a glass slide, place a coverslip on the slide and seal using nail polish.

3. RESULTS

3.1. *pbs-5* overexpression extends *C. elegans*' lifespan

In order to examine the impact of enhanced proteasomal activity on lifespan, we overexpressed the core, rate-limiting proteasomal subunit *pbs-5* in wild type worms (N2) and assessed their lifespan. For the genetic transformation of the nematode we used DNA microinjection technique. Transgenic animals are generated by injecting appropriate DNA fragments or plasmids. DNA is injected into the distal gonad syncytium. Injected DNA forms large extra-chromosomal arrays incorporating between 50 and 300 copies, which are inherited by the progeny of injected animal. Identification of transgenic individuals is facilitated by the use of several available transformation markers, which are co-injected along with the DNA of interest. We injected wild type worms (N2) with a plasmid containing the coding region of *pbs-5* under a ubiquitous promoter (*let-858*) (created by Nikos Kourtis). We used two different co-injection markers, *rol-6(su1006)* allele which induces a distinctive rolling phenotype in transgenic progeny and p_{coel},an endocytic marker which drives Green Fluorescent Protein (GFP) expression to coelomocytes in transgenic progenies. We found that *pbs-5* overexpression extends the lifespan of wild type worms as it is documented by fig. 3.1.1 (a and b) and table 3.1.1 (a and b).

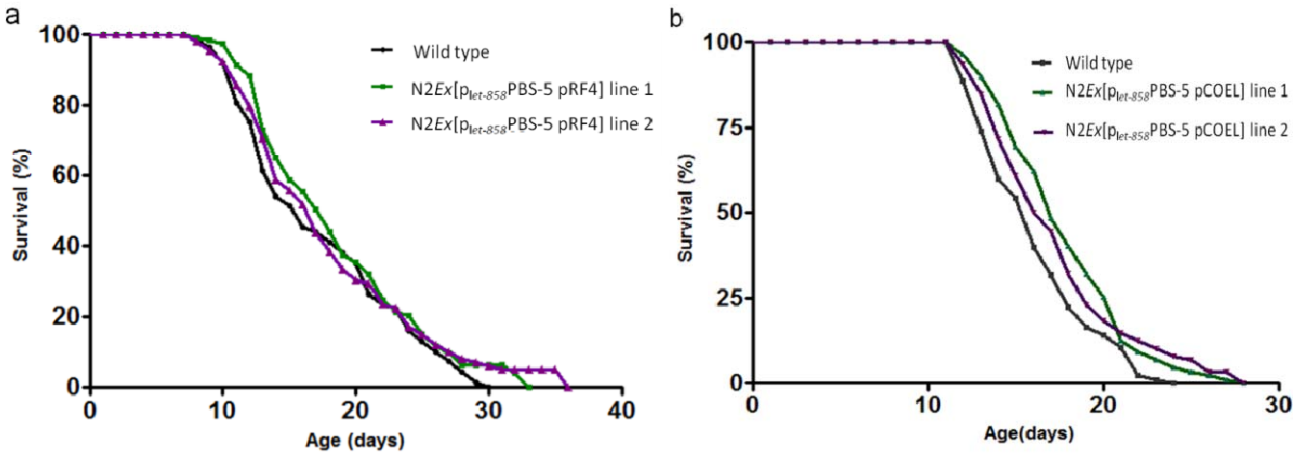

Figure 3.1.1 *pbs-5* overexpression extends the lifespan of wild type worms. Survival curves of *pbs-5*-transgenic animals are shown. (a) N2*Ex*[$p_{let-858}$PBS-5 pRF4] line 1, N2*Ex*[$p_{let-858}$PBS-5 pRF4] line 2. (b) N2*Ex*[$p_{let-858}$PBS-5 pCOEL] line 1, 2*Ex*[$p_{let-858}$PBS-5 pCOEL] line 2. The percentage of animals remaining alive is plotted against animal age.

a

T=20°C	Median Lifespan (days)	Maximal Lifespan (days)
Wild type	16	30
N2Ex[$p_{let-858}$PBS-5 pRF4] ln 1	18	33
N2Ex[$p_{let-858}$PBS-5 pRF4] ln 2	17	36

b

T=20°C	Median Lifespan (days)	Maximal Lifespan (days)
Wild type (N2)	16	24
N2Ex[$p_{let-858}$PBS-5 pCOEL] ln 1	17	28
N2Ex[$p_{let-858}$PBS-5 pCOEL] ln 2	17	28

Table 3.1.1 Median and maximal lifespan of *pbs-5*-trangenic animals (a) N2*Ex*[$p_{let-858}$PBS-5 pRF4] line 1, N2*Ex*[$p_{let-858}$*PBS*-5 pRF4] line 2. (b) N2*Ex*[$p_{let-858}$PBS-5 p_{coel}] line 1, N2*Ex*[$p_{let-858}$PBS-5 p_{coel}] line 2.

3.2. *pbs-5* confers oxidative stress resistance

Oxidative stress is associated with aging and age-related diseases because the oxidized forms of many proteins, lipids and other molecules have been shown to increase during these processes (Shringarpure and Davies, 2002). In order to investigate whether the increased proteasomal activity, caused by the overexpession of the proteasomal subunit offers a protective effect against oxidative stress, we exposed *pbs-5*-transgenic worms in established oxidative stressors and document periodically their survival comparing to wild type worms. To assess stress resistance, we challenged pbs-5 overexpressing animals with paraquat (N,N9- dimethyl-4,49-bipyridinium dichloride) and sodium azide (NaN_3). The herbicide paraquat is a generator of superoxide anions (Lin and Culotta, 1995). Paraquat is readily reduced to radical ions, which generate superoxide radicals inducing oxidative stress in worms (Vanfleteren, 1993). NaN_3 binds to the heme cofactor of cytochrome c oxidase, similarly to carbon monoxide, and inhibits the mitochondrial electron transport chain complex IV, resulting in increased H_2O_2 production and acute oxidative stress NaN_3 is a potent and specific inhibitor of cytochrome c oxidase, which is part of the mitochondrial electron transport chain complex IV, resulting in increased H_2O_2 production and acute oxidative stress (Yoshikawa et al., 1998). *pbs-5* transgenic worms posessed increased survival after to both paraquat (Fig. 3.2a) and NaN_3 (Fig. 3.2b) treatment, which indicates that *pbs-5* overexpression confers oxidative stress resistance.

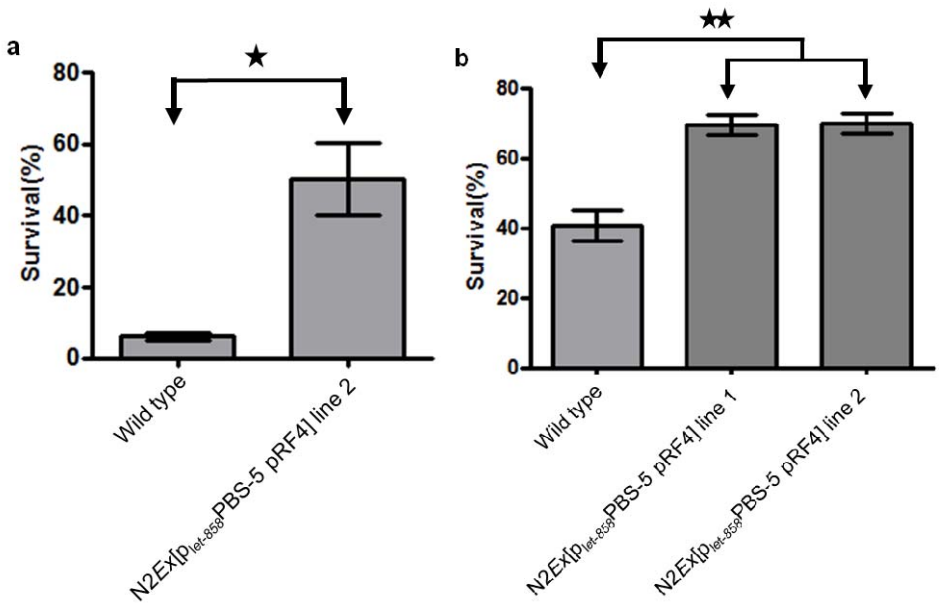

Figure 3.2 *pbs-5* overexpression protects against oxidative stress. Survival of synchronized wild type and *pbs-5* overexpressing populations under oxidative stress-induced by treatment with (a) paraquat (*n*=100-125 worms per line per independent experiment) or (b) sodium azide (*n*=70 worms per line per independent experiment). Statistical significance between control and transgenic line was determined by unpaired t-test *(p>0,05).* Error bars denote standard error of the mean between independent experiments.

In addition, we used the hypersensitive to oxidative damage and short lived mutant, *mev-1(kn1) (abnormal methyl viologen sensitivity-1)*, in order to explore if *pbs-5* overexpression restores lifespan and oxidative stress sensitivity. *mev-1* encodes for a subunit of the electron transport chain complex and mutants for this gene are hypersensitive to paraquat and elevated oxygen levels (Ishii, 2000). For this reason, we generated pbs-5 overexpressing worms in a *mev-1(kn1)* mutant background, using microinjection technique, aiming to correlate the presence of multiple copies of *pbs-5* with beneficial effect on lifespan and oxidative stress resistance of *mev-1(kn1)* mutants.

3.3. The interplay with other known longevity associated pathways- IGF pathway

Aging in *C. elegans* is mainly regulated by a conserved endocrine signaling pathway that involves the insulin/insulin-like growth factor (IGF) receptor DAF-2 (abnormal dauer formation-2) and the phosphatidylinositol-3-OH kinase (PI(3)K) AGE-1 (aging alteration-1). Mutations that reduce the activity of DAF-2 or AGE-1, result in lifespan extension. Longevity conferred by *daf-2* and *age-1* mutations requires the forkhead box, sub-group O (FOXO) transcription factor DAF-16 (Kenyon, 2005b). In order to investigate whether the lifespan extending effect of the *pbs-5* overexpression is mediated through the insulin pathway, we overexpessed this proteasomal subunit in *daf-16* defective worms, carrying the allele *m26*. If the longevity conferred by *pbs-5* overexpression can be suppressed by the absence of *daf-16*, this would indicate that the beneficial effect of proteasome activity enhancement requires a functional DAF-16 transcription factor.

3.4. The effect of increased proteasomal activity on neurodegeneration

3.4.1. The effect of *pbs-5* overexpression in a *C. elegans* model of Parkinson's disease (PD)

The proteasome degradation pathway plays a critical role in aging and the pathogenesis of most neurodegenerative diseases. High levels of oxidized proteins detected in the aging brain are an indication of proteasome impairment, as this proteolytic complex degrades the majority of oxidatively modified proteins (Grune et al., 2004). Moreover, the accumulation and aggregation of ubiquitinated proteins detected in most neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, is also a sign of proteasome degradation pathway dysfunction, as this pathway degrades ubiquitinated proteins (Hyun et al., 2004).

Parkinson's disease (PD) is a disabling neurodegenerative disorder marked by progressive motor dysfunction and characterized by the loss of nigrostriatal dopamine (DA) neurons and cytoplasmic inclusions termed Lewy bodies (Olanow, 1999). The protein alpha-synuclein (α-syn) appears to have a central role; mutations or overexpression of this protein leads to autosomal dominant PD (Singleton et al., 2003), and accumulation of α-syn is observed even in sporadic cases of PD (Kotzbauer et al., 2004), possibly as a result of impaired protein clearance. We exploited an established *C. elegans* model of PD aiming to correlate the appearance of protein aggregates with neuronal functionality, in the presence of multiple copies of proteasomal subunits, *in vivo*. We overexpressed the key proteasomal subunits in worms that overexpress human α-syn in DA neurons (p_{dat-1}::α-syn p_{dat-1}::GFP). The DA neurons are tagged with GFP in order to monitor neuronal loss caused by α-syn toxicity during aging using confocal microscopy. We measured the number of cephalic (CEP) and anterior dendritic (ADE) classes of DA neurons of *pbs-5*-overexpressing worms (p_{dat-1}:: α-syn p_{dat-1}::GFP *Ex*[$p_{let-858}$PBS-5]) and control (p_{dat-1}:: α-syn p_{dat-1}::GFP) animals on the 7th day of adulthood. We found that *pbs-5* overexpression attenuates α-syn-induced DA neurodegeneration (fig. 3.4.1.1 and fig. 3.4.1.2).

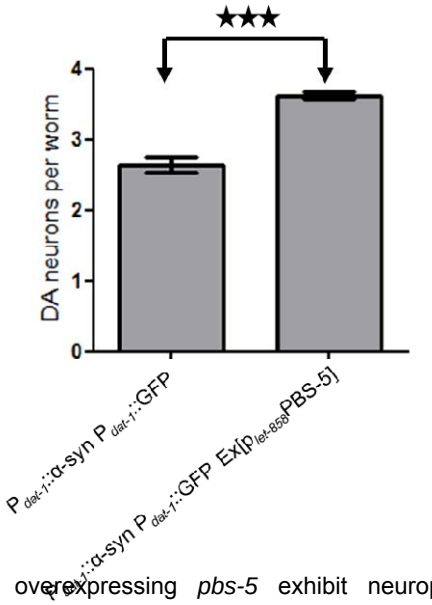

Figure 3.4.1.1 Transgenic animals overexpressing *pbs-5* exhibit neuroprotection from α-syn induced neurodegeneration. P_{dat-1}::α-syn P_{dat-1}::GFP control worms and *pbs-5* overexpressing worms in the P_{dat-1}::α-syn P_{dat-1}::GFP background were scored for degeneration of the 4 CEPs and 2 ADEs DA neuron (*n*=40-50 worms per line per independent experiment). Statistical significance between control and transgenic line was determined by unpaired t-test (*p<0,001)*. Error bars denote standard error of the mean between independent experiments.

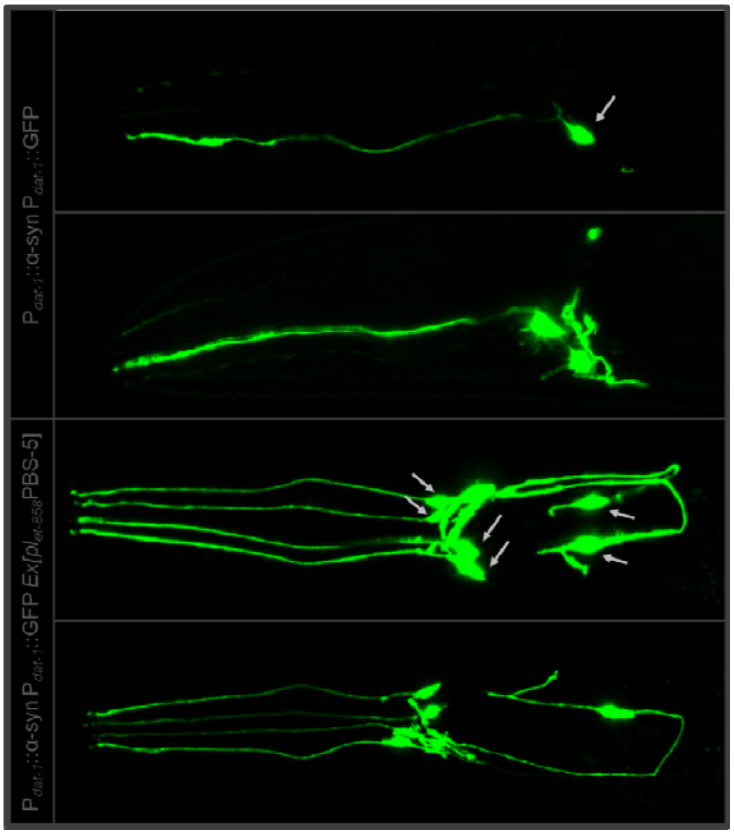

Figure 3.4.1.2 Overexpression of *pbs-5* protects DA neurons from α-syn-induced degeneration. Worm DA neurons degenerate as animals age. At the 7 day of adulthood, most worms are missing anterior DA neurons of the CEP (cephalic) and/or ADE (anterior deirid) classes. Note the presence of 1 of 4 CEP DA neurons (arrow) and the absence of the 2 ADE neurons. Overexpression of *pbs-5* protects worms from neurodegeneration whereby worms display all 4 CEP (arrows) and both ADE (arrows) neurons.

In addition, we overexpressed *pbs-5* in worms that express α-syn (fused to GFP) in body wall muscle cells (p_{unc-54}::α-syn::GFP). In these worms, human α-syn::GFP forms aggregates as worms develop and age. Muscle cells, due to their bigger size comparing to neurons, are more suitable for monitoring of the α-syn aggregation. We monitored α-syn aggregation at the 5th day of adulthood of *pbs-5* trangenic (p_{unc-54}::α-syn::GFP*Ex*[$p_{let-858}$PBS-5]) and control (p_{unc-54}::α-syn::GFP) worms by confocal microscopy, in order to investigate the contribution of increased proteasomal clearance in the formation of α-syn aggregates during the aging process. *pbs-5* overexpression ameliorates α-syn aggregate formation. We found that pbs-5 overexpression ameliorates α-syn aggregate formation (Fig 3.4.1.3)

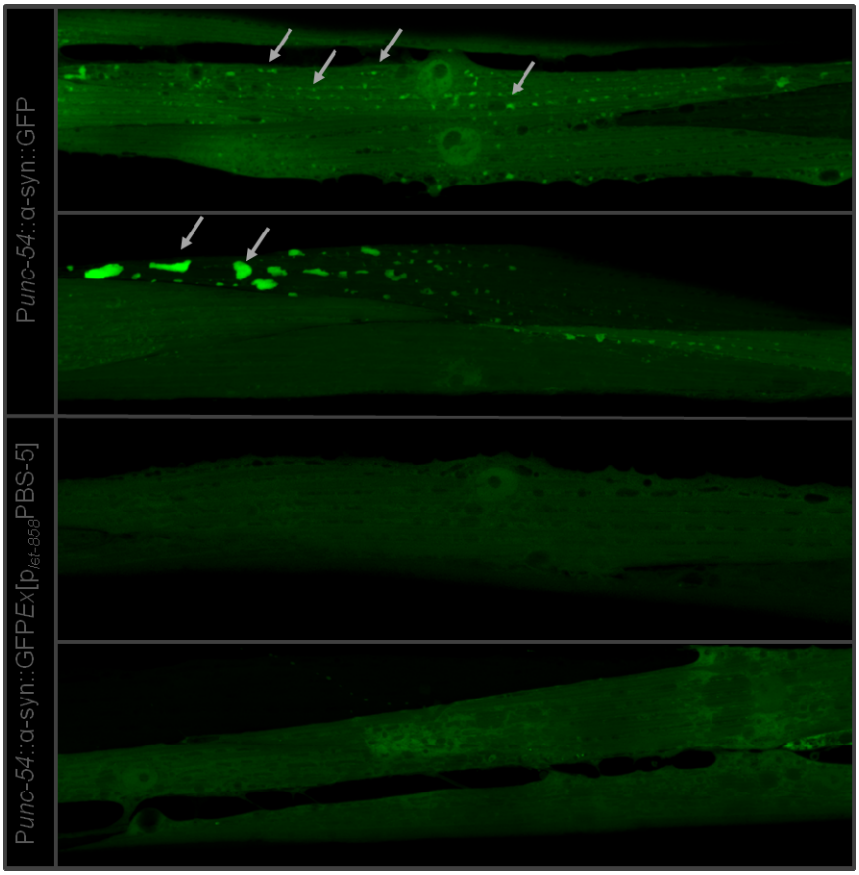

Figure 3.4.1.3 Overexpression of *pbs-5* protects against aggregation of human α-syn *in vivo*. Worms expressing α-syn::GFP alone display multiple α-syn aggregates in body wall muscle cells, whereas *pbs-5* overexpressing worms lack the toxic aggregates. The presence of *pbs-5* attenuates the misfolded α-syn protein.

3.4.2. The impact of *pbs-5* overexpression on necrotic cell death triggered by a neurotoxic channel

In order to further examine the effect of enhanced proteasomal clearance on neurodegeneration, we took advantage of a well established model of necrotic cell death caused by a neurotoxic channel allele. MEC-4 is the core of the mechanotransduction apparatus of the six touch receptor neurons (Driscoll and Chalfie, 1991). A single dominant mutation of the gene causes a substitution with a bulkier aminoacid that favors an open-channel conformation by causing steric hindrance, resulting in Na^+ influx that triggers the necrotic-like cell death of the neurons (Syntichaki and Tavernarakis, 2004) (Fig 3.4.2). We generated *mec-4(d)* transgenic animals overexpressing *pbs-5* using microinjection. Neurodegeneration was scored by monitoring the characteristic vacuolation of the

six touch receptor neurons using differential interference contrast microscopy (DIC) during the L1 larval. We found that *pbs-5* overexpression does not confer any protective effect on *mec-4(d)-induced* neurodegeneration.

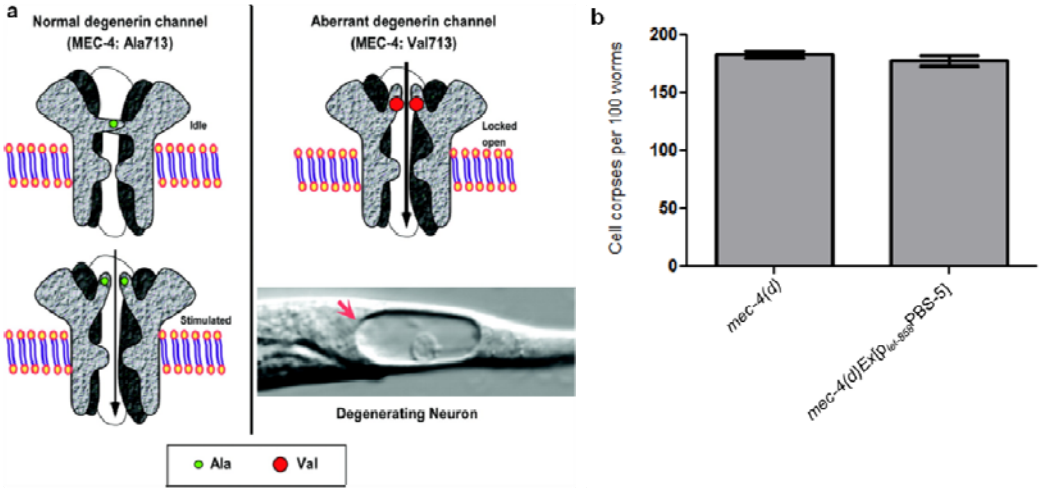

Figure 3.4.2 *mec-4(d)* induced neurodegeneration.(a) Gain-of-function mutations in the degenerin gene *mec-4* encode substitutions for a conserved alanine adjacent to MSDII and result in neuronal degeneration. Amino acids with bulkier side chains at this position are thought to favor an open-channel conformation by causing steric hindrance, resulting in Na^+ influx that triggers the necrotic-like cell death. Adapted from Syntichaki and Tavernarakis, 2004.(b) Number of touch receptor neuron corpses per 100 L1 progeny of *mec-4(d)* animals and mutants carrying extra copies of *pbs-5* transgene.

4. DISCUSSION

The proteasome system comprises the central degradation machinery for long lived and damaged proteins. Maintaining proteostasis is of critical importance for the cell. Previous studies with cell and tissues have declared the importance of proteasomal degradation during aging by implicate the malfunction of the proteasome with aging phenotype. For the first time, we enhanced the proteasomal degradation system *in vivo*. *C. elegans* has been instrumental for the study of aging and neurodegeneration. We upregulated protein turnover through overexpression of the core, rate-limiting subunit of the proteasome, PBS-5 and evaluated the effect on the lifespan of a multicellular organism. Interestingly, *pbs-5* overexpression resulted in lifespan extension of wild type worms, inferring that enhanced protein clearance plays an important role in preservation of a healthy and youth phenotype. The fact that *pbs-5* transgenic worms possess increased resistance to oxidative stress, which is a crucial factor implicated in aging process is in consensus with the extended lifespan. The genetic amenability of our model organism allows the analysis of the interplay between the enhanced protein degradation pathway and other well characterized pathways that affect aging in *C. elegans*. To this point, we overexpressed β5 subunit in mutant worms that lack genes responsible for lifespan extension, aiming to clarify the crosstalk of the pathways.

Other special features of *C. elegans* such as its transparency, allow us to monitor neurodegeneration and aggregation of pathological proteins during aging. A diverse group of neurodegenerative disorders that are characterized by an accumulation of ubiquitinated proteins, suggesting that proteasomal dysfunction is likely to play a prominent role in the pathogenesis of neurodegenerative diseases (Bedford et al., 2008). Quite recently, it has been suggested that inhibition of the proteasome resulted in neurodegeneration of dopaminergic neurons (Caldwell et al., 2009) establishing a compelling link between proteasome dysfunction and the development of neuronal cell death. By exploiting a well established model of PD in *C. elegans*, we concluded that *pbs-5* overexpression and consequently enhanced proteasomal clearance protects against neuronal loss and aggregation of toxic proteins. In addition, we examined the effect of *pbs-5* in a model of degeneration triggered by toxic channel allele, which causes necrotic cell death through ionic imbalance. Upregulation of proteasomal activity did not affect this type of neuronal cell death probably because the insult of degeneration was very severe and due to the fact that the neuronal death took place in early development stages in which preservation of cellular homeostasis could not exert its beneficial effects.

Further studies that focus on the *in vivo* monitoring of proteasomal activity during aging and neurodegeneration could elucidate the implication of the proteasome in these conditions. In addition, evaluation of the proteasomal activity in long-lived mutant animals could directly link the preservation of proteasome degradation with longevity. Future studies that explore the mechanisms by which proteosomal abnormalities contribute to the selective and progressive pattern of age-related neuronal loss are needed. Another principal question should be answered is whether proteasomal decline is a primary or secondary event in aging and neurodegeneration. Can interventions directed at this decline alter the progression of the disease?

Understanding the mechanism by which preservation of cellular proteostasis, via enhancement of the proteasomal activity, extends lifespan, confers oxidative stress resistance and suppresses neurodegeneration may lead to new anti-aging strategies and therapeutic interventions.

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Part II

Investigation of the protective role of the heat shock response against necrotic cell death

CONTENTS

ΠΕΡΙΛΗΨΗ

Ο νηματώδης σκώληκας *Caenorhabditis elegans* (*C. elegans)* αποτελεί ιδανικό μοντέλο οργανισμό για τη μελέτη των κυτταροτοξικών συνεπειών των ακραία υψηλών θερμοκρασιών. Αναπτύξαμε ένα μοντέλο θερμικής αποπληξίας (heat stroke) στον *C. elegans* προκειμένου να μελετήσουμε τα κυτταρικά μονοπάτια θανάτου που το διέπουν. Χαρακτηρίσαμε τον κύριο μηχανισμό κυτταρικού θανάτου που προκύπτει από υποβολή σε ακραία υψηλές θερμοκρασίες. Ακόμη, μελετήσαμε την εμπλοκή βασικών μορίων τελεστών κυτταρικού θανάτου στην επιβίωση από θερμική αποπληξίας (heat stroke). Στη συνέχεια, αξιολογήσαμε την επίπτωση της επαγωγής του μονοπατιού απόκρισης σε θερμικό σοκ (heat shock response) στη αιφνίδια θερμική προσβολή. Λαμβάνοντας υπόψη ότι οι πρωτεΐνες απόκρισης σε θερμικό σοκ (Heat Shock Proteins (HSPs)) είναι οι κύριοι μεσολαβητές της απάντησης σε θερμική απορρύθμιση, θελήσαμε να μελετήσουμε την εμπλοκή τους στην θερμική αποπληξία. Για αυτό το λόγο υποβάλλαμε μεταλλαγμένα στελέχη των συγκεκριμένων πρωτεϊνών σε θερμικό σοκ. Επιπλέον, μας ενδιέφερε η συμμέτοχη του μονοπατιού απόκρισης σε θερμικό σοκ σε χρόνιο ερέθισμα που οδηγεί σε εκφυλισμό και για αυτό το σκοπό χρησιμοποιήσαμε ένα μοντέλο της νόσου του Parkinson στο *C. elegans*. Παρακολουθήσαμε τον εκφυλισμό των ντοπαμινεργικών νευρώνων σκουληκιών που υπερεκφράζουν την τοξική πρωτεΐνη α-συνουκλεινη (α-synuclein) και που έχουν υποβληθεί περιοδικές συνθήκες ήπιου θερμικής επώασης. Η παρούσα μελέτη στοχεύει στην διερεύνηση των μοριακών μηχανισμών που διέπουν την θερμική προσβολή και διασαφήνιση των ευεργετικών επιδράσεων του μονοπατιού απόκρισης σε θερμικό σοκ στο κυτταρικό εκφυλισμό.

SUMMARY

Caenorhabditis elegans (*C. elegans)* serves an ideal model system in the investigation of the cytotoxic effects of extreme temperature during heat stroke. We developed a *C. elegans* model of heat stroke in order to study the cell death pathways that are triggered under this condition. We characterized the main type of cell death that governs heat shock damage. We studied the implication of main executor molecules of cell death on heat stroke-induced cell death. In addition, we evaluated the impact of heat shock response on the acute death insult of heat stroke. Since Heat Shock proteins (HSPs) mediate the protective effect of heat shock response we evaluated the contribution of specific HSPs on survival after heat stroke. For this purpose, we measured the susceptibility of HSPs mutants on heat stroke induced death. Furthermore, we were interested to explore the effect of induction of heat shock response machinery on chronic degeneration insults. To this point we exploited a well established model of Parkinson's disease and evaluated the effect of mild heat shock on α-synuclein induced neurodegeneration. The present study aims to clarify the mechanism that underlie heat shock induce cell death and highlight the beneficial effects of heat shock response pathways on degeneration.

1. INTRODUCTION

1.1. Heat Shock Response

The heat-shock response enables the cell to elevate the expression of genes that function to protect against proteotoxic stress and to initiate a regulatory cascade for recovery and adaptation. Conditions of stress, including (but not limited to) temperature elevation, activate a cellular program known to trigger the heat shock response, which is characterized by a robust increase in the synthesis of a subset of heat shock proteins (HSPs) that are crucial for recovery from stress-induced protein damage (Lindquist, 1986). Almost all HSPs function as molecular chaperones, and they have been classified into six main families on the basis of their approximate molecular mass (in kDa): HSP100, HSP90, HSP70, HSP60, HSP40 and the small HSPs (sHSPs), which weigh less than 40 kDa. The heat shock response is conserved from archaebacteria to mammals. HSPs, as molecular chaperones, typically bind to nonnative conformations of proteins that persist upon cell stress and these interactions protect against misfolding, aggregation or premature clearance and enable refolding and the restoration of native conformations (Bukau et al., 2006; Hartl et al., 1994). Thus, the interaction of chaperones with diverse substrates in stressed cells or upon increased protein biogenesis enhances the stability of the proteome and restores the activities of signaling and growth regulatory molecules re-establishing cellular homeostasis (Morimoto, 1998).

The key regulator of HSP transcription in eukaryotes is heat-shock factor 1 (HSF1), which is highly conserved and ubiquitously expressed (Anckar and Sistonen, 2007; Morano and Thiele, 1999). In the absence of stress, the DNA-binding and transcriptional activities of HSF1 are inhibited by HSPs, which associate weakly to maintain a repressed state. The activity of HSF1 is induced by a variety of stress signals, including a wide range of acute and chronic perturbations of physiological states and disease (Morimoto, 1998). An increase in the level of intracellular misfolded proteins thought to trigger the stress response (Hightower, 1991) is proposed to titrate HSPs away from their association with HSF1, enabling HSF1 to trimerize and translocate into the nucleus and activate hsps gene transcription (Fig.1.1). The principal targets for HSF1 are heat-shock elements (HSEs) within promoter regions of HSPs genes (Williams and Morimoto, 1990; Xiao et al., 1991).

High rates of transcription are maintained only when HSF1 trimers remain bound to the HSEs. When either the stress signal is removed or damaged proteins are no longer generated, the heat shock response attenuates rapidly (Abravaya et al., 1991). Association of chaperones with HSF1 suppresses transcription (Abravaya et al., 1992; Shi et al., 1998). Stress-activated HSF1 is further modified posttranslationally by phosphorylation (Guettouche et al., 2005), sumoylation (Anckar et al., 2006), and acetylation. Modification of HSF1 at conserved residues has multiple regulatory consequences, to maintain HSF1 in a repressed state, to enhance transcriptional activity, or to signal attenuation. The combination of these post-translational modifications and chaperone interactions thus affords HSF1 with multiple forms and levels of control and feedback loops to precisely regulate chaperone levels in the cell.

Thus, it seems that the cell has evolved an elegant and efficient mechanism to autonomously deploy resources proportional to protein biogenesis needs, or in response to damage incurred by the environmental insult. The basal levels of HSPs set the threshold of the stress response, whereas the autoregulation of HSF1-dependent HSP transcription ensures the re-establishment and maintenance of proteostasis (Hartl et al., 1994; Morano and Thiele, 1999).

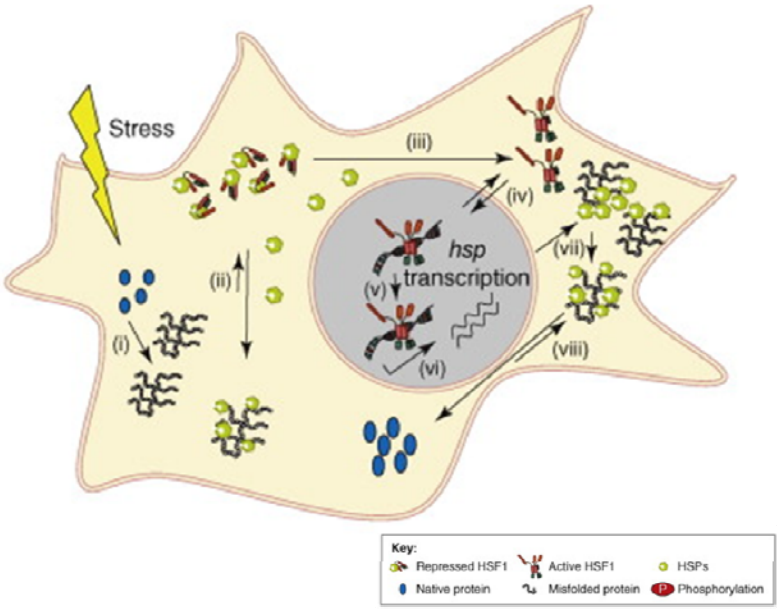

Figure 1.1 Heat shock response pathway (i) The increased flux of damaged or misfolded proteins in response to proteotoxic environmental conditions (stress) is the trigger for the induction of the cellular stress response. (ii) The damaged or misfolded proteins titrate away HSPs that are bound to HSF1 and maintain it in a repressed state before stress, resulting in its activation. (iii) Numerous post-translational modifications influence the ability of HSF1 to trimerize, (iv) translocate into the nucleus, and bind DNA. (v) The binding of HSF1 to DNA alone is insufficient to initiate HSP transcription and requires at least one additional signal. (vi) HSF1-dependent HSP transcription upregulates the cellular levels of HSPs (vii), enabling cells to re-establish cellular protein homeostasis by various processes such as selective degradation, or (viii) refolding the misfolded proteins. Adapted from Prahlad and Morimoto, 2009.

1.2.1. Necrotic cell death in *C. elegans*

Necrotic cell death is characterized by gross cellular swelling and distention of subcellular organelles such as mitochondria and endoplasmic reticulum. Necrotic cell death occurs in response to severe stress conditions such as lack of oxygen or essential nutrients, elevated temperature, contact with toxic compounds and excessive mechanical strain (such as trauma). Necrosis also underlies devastating pathological conditions in humans like stroke and neurodegenerative diseases (Martin, 1999). Necrosis was considered an inexorable, chaotic breakdown of cells under intolerable conditions; however, accumulating data dictate a conserved execution program, activated in response to injury, may exist. Necrotic cell death can be triggered by a wide variety of both extrinsic and intrinsic signals (Walker et al., 1988a). For example, hostile environmental conditions or mutated genes (Ferri and Kroemer, 2001; Walker et al., 1988b). Hypoxic conditions imposed either by shortage of oxygen or by chemical inhibitors of the respiratory chain (such as sodium azide) can induce cellular dysfunction and necrotic cell death in the nematode. The most extensively characterized paradigm of non-programmed cell death in *C. elegans* is the necrosis of cells expressing aberrant ion channels harboring unusual gain-of-function mutations (Syntichaki and Tavernarakis, 2003). A widely used model of necrotic degeneration is worms that carry dominant mutations in the *mec-4* gene (*mec*hanosensory; *mec-4*(*d*)) of six touch receptor neurons required for the sensation of gentle touch to the body (Syntichaki and Tavernarakis, 2004). MEC-4 functions as the core subunit of a multimeric, mechanically gated Na^+ channel complex. Large side chain amino acid substitutions near the MEC-4 pore enhance sodium and calcium conductivity and induce necrotic cell death. *mec-4* is a members of the *C. elegans* “degenerin” family, so named because several members can mutate to forms that induce cell degeneration (Chalfie et al., 1993).

Degenerins bear sequence similarity to mammalian epithelial sodium channels (ENaCs). The time of degeneration onset correlates with the initiation of degenerin gene expression and the severity of cell death is analogous to the dose of the toxic allele (Hall et al., 1997). Expression of mammalian homologous proteins, carrying amino-acid substitutions analogous to those of toxic degenerins, leads to degeneration of cells in a manner reminiscent of necrotic cell death in *C. elegans*.

Cell demise is accompanied by characteristic morphological features (Hall et al., 1997). During the early phase of death, the nucleus and cell body become distorted. Gradually, the cell swells to several times its normal diameter. A distinguishing feature of the necrotic process is the formation of vacuoles; small, tightly wrapped membrane whorls, originating from the plasma membrane. These whorls are internalized and seem to coalesce into large, electron dense membranous structures (Hall et al., 1997). It is striking that these membranous inclusions appear also in mammalian neurodegenerative disorders, such as neuronal ceroid lipofuscinosis (Batten's disease; the *mnd* mouse) and in *wobbler* mouse, the model of amyotrophic lateral sclerosis (ALS) (Blondet et al., 2002; Cooper et al., 1999). Furthermore, disrupted trafficking has been implicated in Alzheimer's (Katayama et al., 1999; Nixon et al., 2001; Paschen and Frandsen, 2001; Singleton et al., 2003) and Huntington's disease (Davies et al., 1997; DiFiglia et al., 1995).

Ionic imbalance and subsequent necrotic cell death induced by aberrant ion channel function in *C. elegans* is mechanistically and morphologically similar to excitotoxicity in mammals. Excitotoxic cell death is prevalent during stroke, where the energy required sustaining ionic gradients and the resting potential of neurons is lost. Because membrane potential collapses, massive amounts of the excitatory neurotransmitter glutamate are released at synaptic clefts (Kauppinen et al., 1988). Energy depletion also prevents re-uptake of glutamate by dedicated transporters leading to accumulation of glutamate at synapses, hyper-excitation and eventually necrotic death of downstream synaptic target neurons. Excitotoxicity is critically dependent on Ca^{2+} influx through glutamate-gated receptor ion channels.

1.2.2. Molecular mechanisms of necrotic cell death

Excessive and prolonged activation of ion channels irreversibly compromises cellular ionic homeostasis (Fig.1.2;Kourtis and Tavernarakis, 2007). Intracellular calcium overload through different sources is considered as one of the initial steps in the necrotic pathway. Calcium may enter the cell through voltage-gated channels and the Na^{+}/Ca^{2+} exchanger. Mutations that increase sodium influx facilitate calcium entry through these paths. The main intracellular compartment for calcium storage is the endoplasmic reticulum (ER) (Paschen, 2001; Paschen and Frandsen, 2001). Calcium is sequestered into the ER by the sarco-endopasmic reticulum Ca^{2+}-ATPase (SERCA) and is released back to the cytoplasm by ryanodine (RyR) and inositol-1,4,5-triphosphate receptors (Ins(1,4,5)P3PR). Extensive genetic screens for suppressors of *mec-4*(*d*)-induced necrosis have identified genes required for the execution of necrotic cell death. Calreticulin and calnexin, which are calcium binding chaperones regulate intracellular calcium levels and are required for necrotic cell death (Xu et al., 2001). Treatment of animals with thapsigargin, which induces release of calcium from the ER to the cytoplasm, triggers necrotic cell death. In contrast, pharmacological treatments or genetic mutations that inhibit calcium release from the ER have a strong protective effect against necrotic cell death.

Genetic studies in *C. elegans* have also shown that intracellular pH is an important modulator of necrotic cell death. Cytoplasmic acidification occurs during necrosis, whereas the vacuolar H^{+}-ATPase, which is a pump that acidifies lysosomes and other intracellular organelles, is required downstream of cytoplasmic calcium overload to promote necrotic cell death (Syntichaki et al., 2005). Reduced vacuolar H^{+}-ATPase activity or alkalization of acidic endosomal/lysosomal compartments

by weak bases has a neuroprotective role against necrosis. Acidic conditions are required for full activity of cathepsins, aspartyl proteases that are primarily confined to lysosomes and other acidic endosomal compartments (Ishidoh and Kominami, 2002).

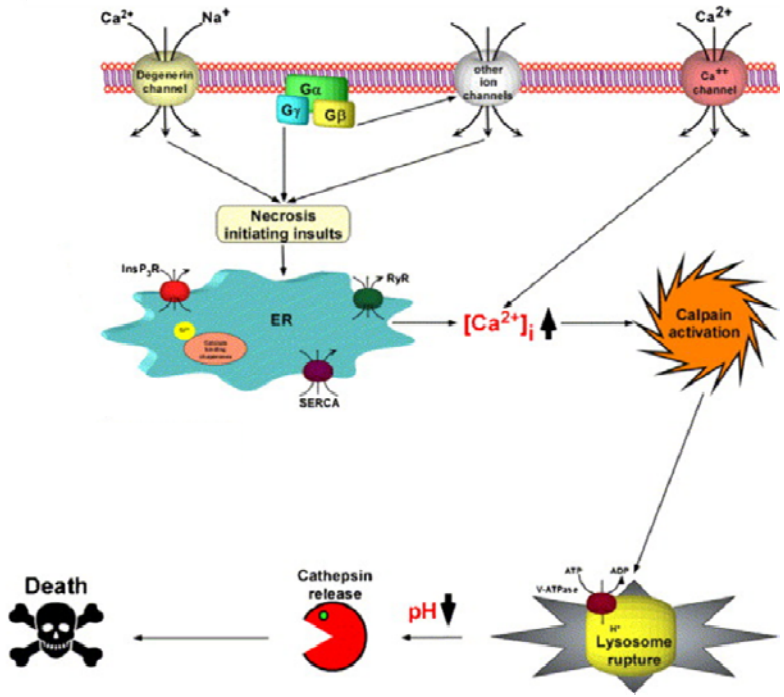

Figure 1.2. Necrotic cell death pathways in *Caenorhabditis elegans*. Various necrosis-initiating insults converge to elicit a sharp increase of cytoplasmic calcium concentration ($[Ca^{2+}]_i$), which is the principal death-signaling event. Intracellular calcium stores also contribute to the elevation of calcium concentration beyond tolerable levels. The channels and molecules involved in calcium homeostasis are shown. Increased calcium concentration activates cytoplasmic calpain proteases, which facilitate lysosomal rupture and release of acidic lysosomal contents into the cytoplasm, which consequently becomes acidified. The pump responsible for lysosomal acidification is depicted (V-ATPase). Low pH conditions favor activation of cathepsin proteases and contribute to cellular destruction. Expression of human disease proteins in worms also disturbs cellular homeostasis mechanisms and induces stress, which, beyond a certain threshold, becomes detrimental for the cell ($[Ca^{2+}]_i$, cytoplasmic calcium concentration; ER, endoplasmic reticulum; G_α, G_β, G_γ, G-protein subunits; $InsP_3R$, inositol triposphate receptor; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; V-ATPase, vacuolar H^+-ATPase). Adapted from (Kourtis and Tavernarakis, 2007).

Lysosomal as well as cytoplasmic, proteases have been implicated in cellular destruction following the onset of necrosis. Calpains are cytoplasmic, papain-like cystein proteases that depend on calcium for their activity. Under normal conditions, calpains function to mediate essential signaling and metabolic processes. However, during the course of necrotic cell death these proteases localize onto lysosomal membranes and may compromise lysosomal integrity, thereby causing leakage of their acidic contents, including lysosomal proteases, into the cytoplasm (Yamashima, 2004). In primates, calpains rapidly localize to lysosomal membranes after the onset of ischaemic episodes (Yamashima, 2000). It is likely that ensuing cytoplasmic acidification activates the lysosomal, low-pH dependent cathepsins and hydrolases that contribute to cell demise. Mutations that interfere with lysosomal biogenesis and function influence necrotic cell death. Interestingly, lysosomes appear to coalesce around the nucleus and dramatically enlarge

during early and intermediate stages of necrosis. In advanced stages of cell death, GFP-labeled lysosomal membranes fade, indicating lysosomal rupture.

1.3. Aim of the study

We developed a *C. elegans* model of heat stroke in order to study the cell death pathways that are triggered under this condition. In addition, we wish to evaluate the impact of heat shock response induction on the acute death insult of heat stroke. Since Heat Shock proteins (HSPs) mediate the protective effect of heat shock response, we aim to evaluate the contribution of specific HSPs on survival after heat stroke. Furthermore, we were interested to explore the effect of induction of heat shock response machinery on chronic degeneration insults. To this point we exploited a well established model of Parkinson's disease and evaluated the effect of mild heat shock on α-synuclein induced neurodegeneration. The present study aims to clarify the mechanism that underlies heat stroke induce cell death and highlight the beneficial effects of heat shock response pathways on necrotic cell death.

2. MATERIALS AND METHODS

2.1. *C. elegans* Strains and Genetics

Nematode rearing temperature was kept at 20°C unless noted otherwise. The following strains were used in this study: Wild-type: N2 (Bristol isolate), KJ216: *crt-1(jh101)*, RB791: *hsp-16.1(ok577)*, VC475: *hsp-16.2(gk249)*, VC1099: *hsp-4(gk514)*, RB1104: *hsp-3(ok1083)*, *hsp-6(tm515)*, *hsp-16.41(tm1093)*, *hsp-70(tm2318)*, *N2;Ex*[p_{asp-4}ASP-4::GFP] .

2.2. Heat Shock Treatment

To induce hyperthermia, animals were incubated in 200μl M9 buffer, at 39°C for 15 minutes. Untreated worms were kept at 20°C for the same time period. To test whether prior activation of the heat shock response leads to increased survival against hyperthermia, animals were incubated at 34°C for 30 minutes, following 20 minutes incubation at 20°C and then hyperthermia.

2.3. EGTA assays

For EGTA worms were grown from the egg stage on OP50 seeded NGM plates containing 10mM EGTA, and survival after heat stroke was scored at the young adult stage. Control animal cohorts were grown on NGM plates seeded with *E. coli* OP50. In each trial 300-500 worms were scored for survival for each condition. Each EGTA assay was repeated at least three times.

2.4. Dantrolene assays

For dantrolene assays, worms were grown from the egg stage on OP50 seeded NGM plates containing 10μM dantrolene, and survival after heat stroke was scored at the young adult stage. Control animal cohorts were grown on NGM plates seeded with *E. coli* OP50. In each trial 300-500 worms were scored for survival for each condition. Each EGTA assay was repeated at least three times.

2.5. α-synuclein-induced neurodegeneration assays

Synchronous populations of nematodes were established by allowing 20 adult hermaphrodites to lay eggs for a limited time interval (4h) on NGM plates seeded with *E. coli* OP50. Progeny were grown on OP50-seeded plates at 20°C, through the L4 larval stage and transferred to fresh OP50-seeded plates at groups of 25 nematodes per plate for a total of 100 individuals per experiment. The first group of animals was incubated at 34°C twice a day for 30 minutes from L4 larval stage. The second group of animals was incubated daily at 34°C for 1 hour. Control worms remained at 20°C through the experiment. Animals were transferred to fresh plates every 2 days until the 7th day of adulthood. Worms were immobilized by a mix of 5μl of M9 and 9μl of sodium azide (20mM) applied on glass slide, a coverslip was placed on the slide and sealed using nail polish. The 6 anterior DA neurons (4 CEP and 2 ADE neurons) of 60 animals/ trial were examined for neurodegeneration under a Zeiss Axio Imager Z2 Epifluorescence/DIC Microscope. Each α-synuclein-induced neurodegeneration assay was repeated at least three times.

2.6. RNA interference

For RNAi experiments, we constructed plasmids that direct the synthesis of dsRNA corresponding to the genes of interest, in *E. coli* bacteria, which were then fed to animals. Gene-specific fragments were obtained by PCR amplification directly from *C. elegans* genomic DNA. PCR-generated fragments were sub-cloned into the pL4440 plasmid vector and resulting constructs were transformed into HT115(DE3) *E. coli* bacteria as described previously (Kamath et al., 2003). In all RNAi experiments, control animal cohorts were grown on HT115 bacteria transformed with empty pL4440 plasmid vector.

3. RESULTS

3.1. Modeling of heat stroke in *C. elegans*-Activation of the heat shock response pathway protects against death induced by heat stroke

In order to replicate heat stroke model in *C. elegans*, we tested various heat shock conditions that could cause extensive damage and could be critical for the survival of the nematode. Incubation of young adult, wild type animals at 39^{o}C for 15 minutes resulted in severe attenuation of survival. Animals exposed to these hyperthermic conditions were characterised by rod-like phenotype and the presence of multiple vacuole-like cells throughout the body of the animal, indicating global damage (Fig 3.1).

Previous studies in mammalian animal models of heat stroke suggest that that heat shock preconditioning upregulates major Heat Shock Proteins' (HSPs) expression in multiple organs and protects against detrimental heat induced pathophysiologies like cerebral ischemia, neuronal damage, oxidative stress and overall animal death during heat stroke (Lee et al., 2006; Wang et al., 2005; Yang and Lin, 1999).

To test whether in *C.elegans* model of heat stroke, heat shock response had the same protective effect, we pre-incubated worms at 34^{o}C for 30 minutes in order to activate the pathway. We allowed a 20 minutes recovery period at 20^{o}C, worms were exposed to heat stroke conditions (39^{o}C-15 minutes). Preconditioned animals showed a great increase in survival and attenuation of the vacuolation throughout the body (Fig.3.1 c and d).

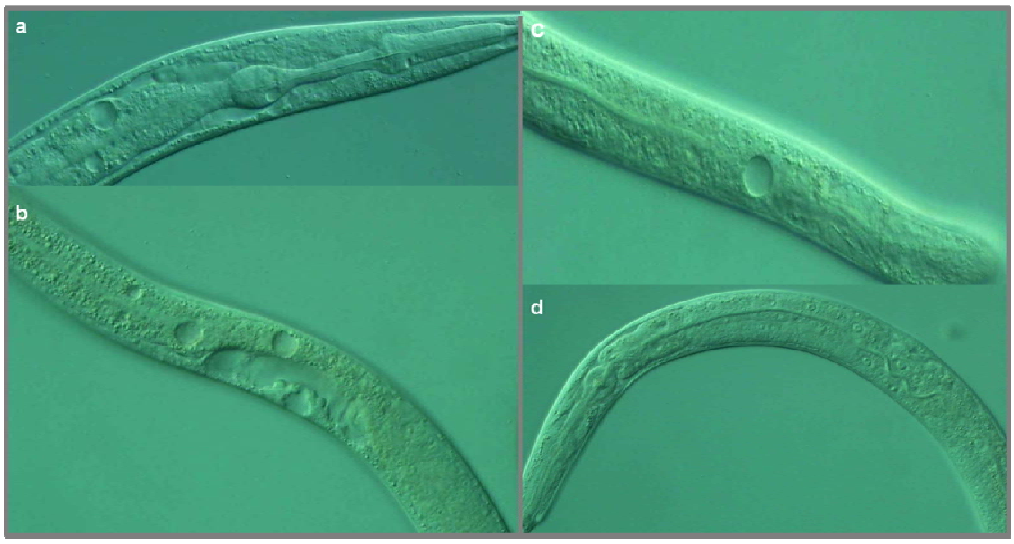

Figure 3.1 Activation of the heat shock response pathway protects animals from hyperthermia Morphology of wild type animals after incubation at 39^{o}C for 15min (a and b). Vacuoles can be observed within intestinal cells of young adult animals after hyperthermia. In contrast, no vacuolization was seen in the intestines of pre-conditioned worms (c and d).

3.2. Heat stroke induced death is mediated by necrotic cell death pathway

3.2.1. Attenuation of the autophagy does not affect survival after heat stroke

In order to investigate the nature of cell death that is induced after heat stroke, we suppressed essential components of death pathways and evaluated the impact of this elimination on animals that have been subjected to heat shock. To examine whether the autophagic cell death is implicated in heat stroke induced cell death, we downregulated *beclin 1(bec-1)*, a key gene involved in

autophagosome nucleation. Animals exposed to *bec-1RNAi* showed no increase in survival after heat stroke (Fig. 3.2.1)

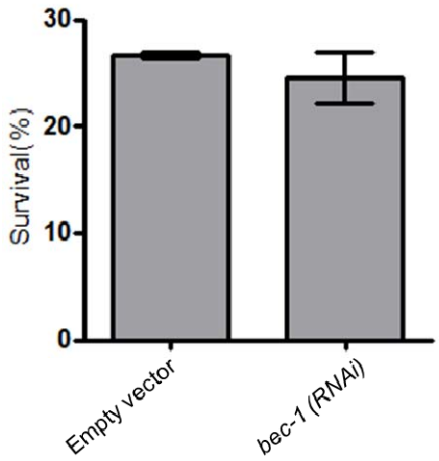

Figure 3.2.1 Downregulation of the autophagic pathway does not confer any protective effect against heat stroke induced cell death. Survival of worms subjected to *bec-1* RNAi and control animal that were fed with the empty vector against hyperthermia (*n*=200 worms per treatment per independent experiment). No statistical significance between control and *bec-1* RNAi cohorts was determined by unpaired t-test *(p>0,05).* Error bars denote standard error of the mean between independent experiments.

3.2.2. Suppression of necrotic pathway leads to increased survival after heat stroke

To verify the necrotic nature of cell death induced after heat stroke, we subjected *crt-1(jh101)* mutants to heat stroke and evaluated their survival. Calreticulin (*crt-1*) is Ca^{2+}-binding chaperone of the ER and *crt-1* null mutant alleles have been identified as suppressors of necrotic cell death (Xu et al., 2001). We found that *crt-1(jh101)* mutation suppressed heat stroke induced cell death (Fig. 3.2.2).

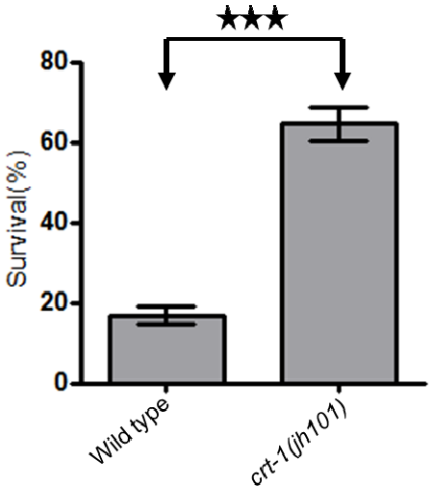

Figure 3.2.2 Suppression of essential component of the necrotic pathway leads to increased survival after heat stroke. Survival of wild type and *crt-1(jh101)* mutant animals against hyperthermia is depicted (*n*=150-200 worms per cohort per independent experiment). Statistical significance between wild type and *crt-1(jh101)* mutant cohorts was determined by unpaired t-test *(p<0,0001).* Error bars denote standard error of the means between independent experiments.

3.3. Ca^{2+} levels are critical in heat stroke induced cell death

To confirm that Ca^{2+} levels deregulation has a key role in cell death induced by heat stroke, we treated animals with EGTA, a Ca^{2+} specific chelator. EGTA-treated animals were protected from heat stroke induced death (Fig.3.3).

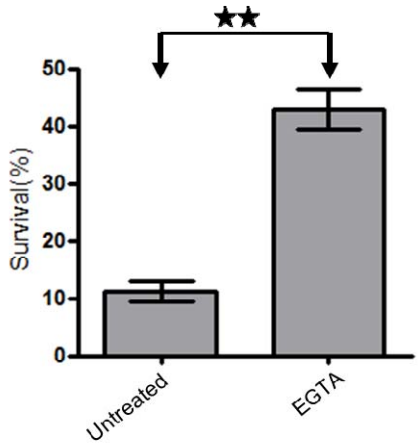

Figure 3.3 Downregulation of intracellular Ca^{2+} levels protects against heat stroke. Survival of untreated wild type worms and worms subjected to EGTA treatment against hyperthermia (*n*=100-150 worms per treatment per independent experiment). Statistical significance between untreated and EGTA-treated cohorts was determined by unpaired t-test *(p<0,01).* Error bars denote standard error of the mean between independent experiments.

3.4. Preclusion of Ca^{2+} release from the intracellular stores protects from heat stroke induced death

In order to further investigate the implication of Ca^{2+} homeostasis in heat stroke induced cell death, we used an established compound that regulates intracellular Ca^{2+} concentration. Dantrolene specifically decreases Ca^{2+} release by binding to ryanodine receptors (RyRs). RyRs mediate the release of Ca^{2+} from intracellular stores to the cytosol. In addition to endoplasmic and sarcoplasmic reticulum, RyRs have been also found localized to the Golgi complex (Cifuentes et al., 2001; George et al., 2007). Wild type animals grown on plates containing dantrolene were subjected to heat stroke conditions. Interestingly, in dantrolene treated animals, survival was markedly increased (Fig.3.4).

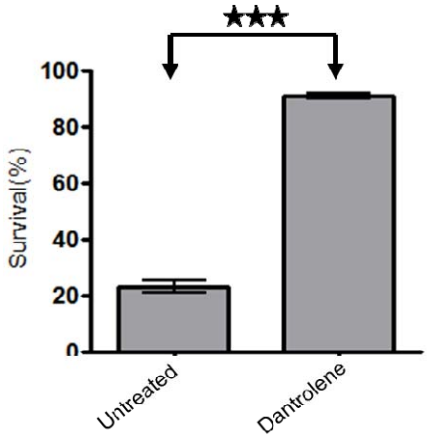

Figure 3.4 Preclusion of Ca^{2+} release from the intracellular stores attenuates heat stroke induced cell death. Survival of untreated wild type worms and worms subjected to dantrolene treatment against hyperthermia (*n*=150-200 worms per treatment per independent experiment). Statistical significance between untreated and dantrolene-treated cohorts was determined by unpaired t-test *(p<0,001).* Error bars denote standard error of the means between independent experiments.

3.5. Screening for HSP mutations that affect heat stroke induced cell death

A functional heat shock response is of critical importance to defend against necrotic insults derived from hyperthermia, as it is supported by the increased survival of heat shock preconditioned animals. To evaluate the contribution of specific HSPs in heat shock response during heat stroke, we assessed the survival of deletion mutants after incubation in high temperature (Fig.3.5).

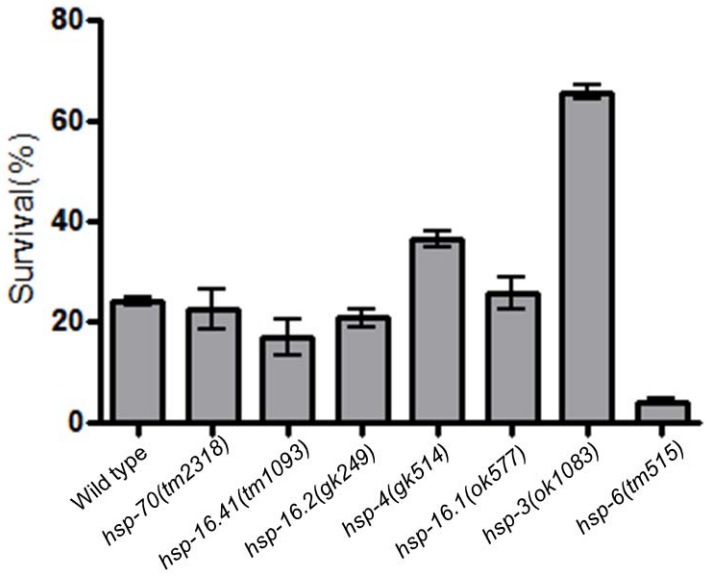

Figure 3.5 Screening for HSP mutations that affect heat stroke induced cell death. Survival of untreated wild type animals and hsp mutants against hyperthermia is depicted (*n*=150-200 worms per cohord per independent experiment). Error bars denote standard error of the mean between independent experiments.

3.6. Repeatedly mild heat shock protects from α-synuclein induced neurodegeneration

Previous studies in our lab have demonstrated that activation of heat shock response pathway is a potent suppressor of neurodegeneration triggered by toxic channel alleles (Kourtis, unpublished). We were interested to evaluate the impact of induction of heat shock response pathway on a model of α-synuclein-induced degeneration. In this model human α-synuclein (α-syn) is expressed in the dopaminergic (DA) neurons and Green Fluorescent Protein (GFP) is expressed under a DA specific promoter. We repeatedly subjected α-syn overexpressing worms on two different conditions of mild heat shock and assessed the 6 anterior DA neurons for degeneration at the 7th day of adulthood. We concluded that activation of the heat shock pathway by mild heat shock has beneficial effects on α-synuclein induced neurodegeneration (Fig 3.6).

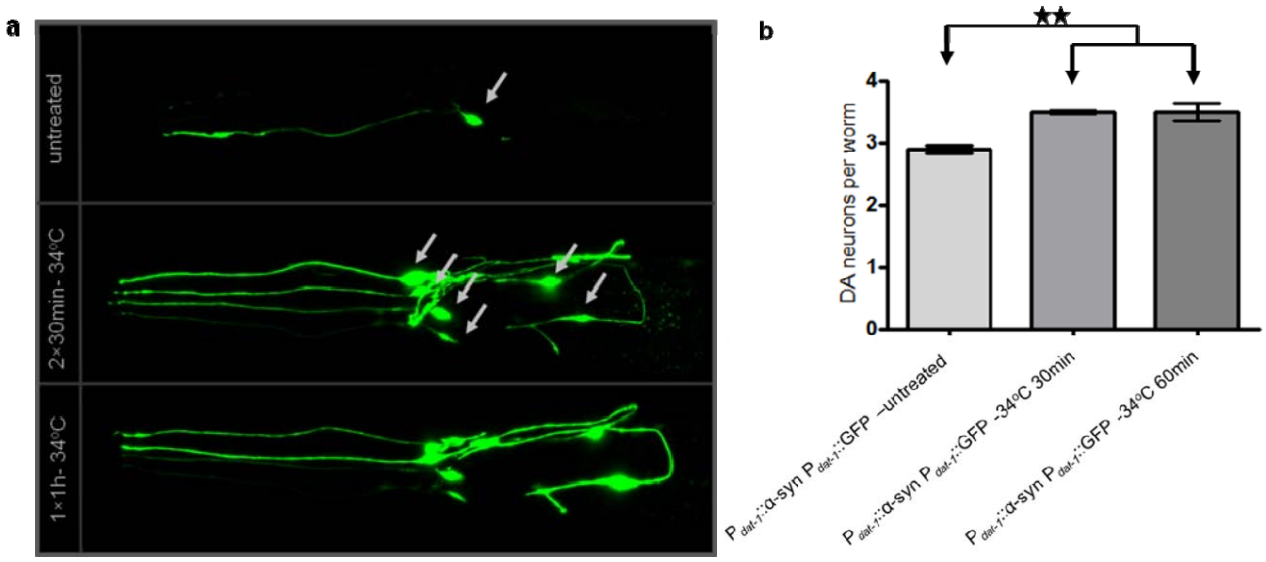

Figure 3.6 Repeatedly mild heat shock attenuates α-synuclein induced neurotoxicity. The effect of heat shock pathway on neurodegeneration was tested in two different conditions; a cohort of α-syn overexpressing worms was subjected twice a day on incubation at 34^{o}C for 30 minutes. The other cohort of α-syn overexpressing worms was subjected daily on incubation at 34^{o}C for 1 hour. Control, α-syn overexpressing worms remained at 20^{o}C throughout the experiment. (a) Representative confocal pictures of control α-syn overexpressing untreated worms and α-syn overexpressing worms treated repeatedly with mild heat shock. At the 7 day of adulthood, most worms are missing anterior DA neurons of the CEP (cephalic) and/or ADE (anterior dendritic) classes. Note the presence of 1 of 4 CEP DA neurons (arrow) and the absence of the 2 ADE neurons in control animal. Overexpression of repeatedly mild heat shock protects worms from neurodegeneration whereby worms display all 4 CEP (arrows) and both ADE (arrows) neurons. (b) Number of anterior DA neurons per worm. α- syn overexpressing untreated worms and heat shock treated worms were scored for degeneration of the 4 CEPs and 2 ADEs DA neuron (n=40-50 worms per condition per independent experiment). Statistical significance between control and transgenic line was determined by unpaired t-test (*p<0,01)*. Error bars error bars denote standard error of the mean between independent experiments.

4. DISCUSSION

We developed a heat stroke model in *C. elegans* aiming to identify and characterize the cell death and the mechanisms underlying this phenomenon. We observed that exposure of wild type animals to extreme temperature (39^{o}C, 15min) resulted in detrimental lesion in the animals. The vacuolation that was observed in the heat shocked animals inferred that the nature of the cell death is necrotic. By reverse genetic and pharmacological treatments that block effector molecules of cell death pathways we concluded that heat stroke induced cell death is primarily caused by extensive necrotic cell death.

Furthermore, the fact that pretreatment of the heat shocked animals with dantrolene protected against heat stroke induced cell death, suggest that Ca^{2+} release from the intracellular stores is a key step in this type of cell death. In addition, our data indicate that *C. elegans* serves as a genetic screening platform for understanding the impact of heat shock proteins on the progression of heat stroke.

Our finding that heat preconditioning ameliorates necrotic cell death caused by heat stroke and genetic and pharmacological means, establishes the heat shock response as a powerful suppressor of necrosis. This is consistent with evidence that prior heat shock protects against heat stroke induced cerebral ischemia, neuronal damage and fatal arterial hypotension, in a rat heat stroke model (Yang et al., 1998). The beneficial effects of activation of heat shock pathway on neuronal death are in consensus with the fact that repeatedly mild heat shock conferred protection against α-sun induced neurodegeneration.

In summary, our data demonstrate that *C. elegans* serves as an ideal model system for the dissection of mechanisms of extreme heat-induced cytotoxicity, simulating severe cases of heat stroke. Activation of heat shock pathway protects against the necrotic cell death induced by heat stroke and attenuates neurodegeneration in a model of Parkinson's disease. The implication of specific hsps in the heat stroke model needs further study, in order to identify the crucial mediators of the pathway.

Our results confer a deeper understanding of the homeostatic mechanisms that govern during heat stress conditions. Clarification of the mechanisms of heat-induced cell damage will help combating the rising incidences of heat stroke.

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