THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOLOGY

SELECTIVE AND AGE-DEPENDENT CELLULAR DEGENERATION IN *DROSOPHILA*

ANNIE LIN
SPRING 2015

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Biology
with honors in Biology

Reviewed and approved* by the following:

Richard Ordway
Professor of Molecular Neuroscience and Genetics
Thesis Supervisor

Katriona Shea
Alumni Professor in the Biological Sciences
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Stress-induced cell degeneration is often associated with protein misfolding and aggregation. As a protection mechanism against the cellular toxicity of misfolded proteins, protein aggregates trigger a universal heat shock response pathway which mediates expression of molecular chaperones. Insufficient protection may contribute to degenerative disorders such as Parkinson's and Alzheimer's disease, hallmarks which include susceptibility of certain cell types to degeneration and onset of aging. Here we report that heat shock of wild-type *Drosophila* induces loss of flight ability along with selective degeneration of neurons, glia and muscle cells comprising the flight motor. The susceptibility of these cell types is dependent upon age, being observed in older but not younger flies, and is suppressed by overexpression of molecular chaperones. These findings, together with further genetic analysis in *Drosophila*, may advance our understanding of cell type-selective and age-dependent degeneration in disease as well as potential therapeutic applications for molecular chaperones.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank all present and past members of Ordway/Kawasaki Lab for providing the amazing opportunity for independent research and for creating an environment that has come to feel like home. Thank you to Dr. Richard Ordway and Dr. Fumiko Kawasaki especially for all the mentoring and support they have given me over the last three years. Their never-ending patience and guidance have taught me the enthusiasm, diligence, and perseverance needed to grow as a researcher and as a student. I would also like to thank Yunzhen Zheng for showing me what it means to have a feel for research and a forever upbeat attitude. I would like to thank Alexandra Strauss for taking me under her wing and being an incredible teacher and friend. I would also like to thank my former and current student companions in the lab for all the laughter, comradery, and support throughout our time together.

Chapter 1

Introduction

Degenerative Disease

Degenerative diseases are currently one of the most pervasive challenges in biomedical research and medicine. Incurable neurodegenerative disorders such as Alzheimer's or Parkinson's disease are characterized by the progressive deterioration of nerve cells, resulting in dementia and impaired balance and movement. Development of these disorders results from an amalgam of genetic and environmental factors, but one particular class of degenerative diseases called proteopathies is associated with protein misfolding and aggregation. Under normal conditions, the body regulates the presence of misfolded proteins in order to prevent their accumulation, a process called proteostasis. When proteostasis is disrupted or functions inefficiently, misfolded proteins increase, aggregate, and cause cellular toxicity (proteotoxicity) and degeneration. Thus, finding preventative measures of therapeutic treatments against degenerative disorders relies heavily on a mechanistic understanding of proteostasis.

Genetic and Environmental Factors

A combination of genetic predispositions and environmental factors may cause mature proteins to fold improperly and adopt aberrant conformations. If unattended, these misfolded proteins aggregate and are toxic to the cell. Protein misfolding and aggregation occur when exposed hydrophobic segments of polypeptide chains mediate aberrant interprotein interactions, leading to aggregation (Bucciantini 2002). Although mutations in a handful of genes have been strongly associated with Parkinson's and Alzheimer's diseases, familial cases make up a very

small proportion of total cases (Nussbaum 1997, Warner 2003). In Parkinson's disease in particular, genetic factors have a significant influence in early-onset cases, but environmental factors play the bigger role in age-associated cases. Studies comparing monozygotic and dizygotic twins were used to compare the incidence of Parkinson's or Alzheimer's in genetically identical and non-identical individuals raised under equalized environmental conditions. The results of these studies show that genetically identical individuals did not show a higher concordance of degenerative disorders, indicating that although there may be genetic components influencing the etiology of Parkinson's or Alzheimer's diseases, they are relatively insignificant compared to the role of environmental influence (Breitner 1990, Raiha 1997, Tanner 1999, Warner 2003).

The exact etiology of protein misfolding is uncertain, but proteins are more prone to misfolding after exposure to environmental changes such as increased temperature, variation in pH, agitation, elevated glucose, or oxidative agents (Herczenik 2008). Misfolded proteins result in cell death, possibly via stimulation of apoptosis (Herczenik 2008). Because small increases in temperature may result in aberrant protein conformations and nonspecific aggregation, heat shock may be used as a model for the environmental stress that induces protein aggregation and cellular degeneration (Richter 2010).

The Heat Shock Response

The mechanism responsible for counteracting the accumulation of misfolded proteins in the cytoplasm is the heat shock response (Figure 1). Although the name refers to HS stress, this universal response reacts to a variety of nonspecific oxidative stressors. it acts through chaperone proteins, which recognize hydrophobic amino acid residues on proteins present in aberrant conformations (Kim 2013).

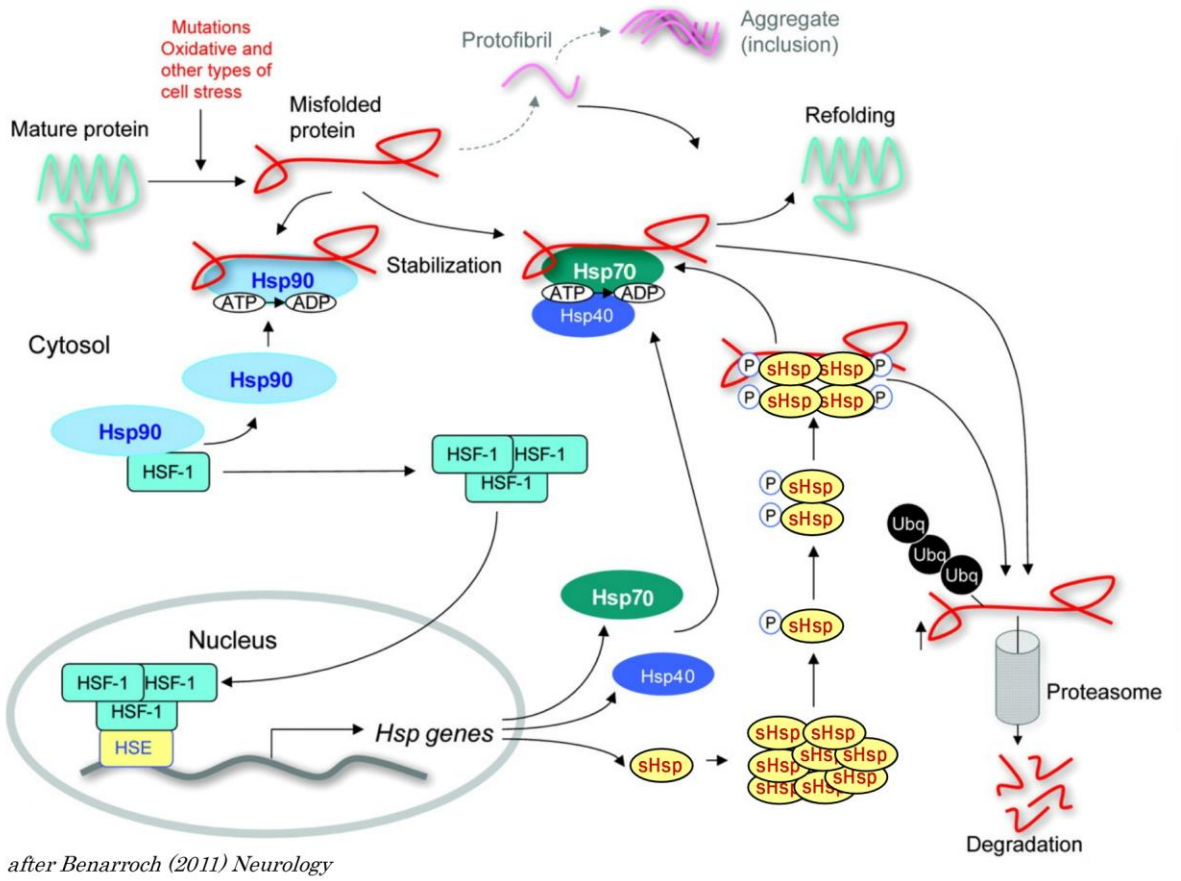

Figure 1. Heat shock response (HSR) pathway. Heat shock response pathway. HSP = heat shock protein, HSF = heat shock factor, HSE = heat shock element, UBq = ubiquitin

When an organism is exposed to environmental stress, the heat shock response is induced instantaneously (Abravaya 1991). In the heat shock response, HSP90 in the cytosol binds to transcription factor HSF under normal conditions. In the presence of misfolded proteins, HSF uncouples with HSP90 and assembles into its active trimeric form (Morimoto 2011). The transcription factor permeates the nuclear membrane and binds to heat shock elements (HSE) in the promoters of heat shock protein genes, thus elevating expression of genes such as HSP70, HSP40, and small HSPs. HSP40 is a cofactor for HSP70; the two work co-and post-translationally to identify and re-fold misfolded proteins, or mark them for degradation by the

proteasome (Richter 2010). Small HSPs are multimeric proteins which perform similar functions in proteostasis. Our investigations aim to determine how these heat shock response mechanisms interact to protect the organism from protein misfolding and aggregation when exposed to the heat shock stress model.

Age Dependence

Onset of degenerative disease is known to be positively associated with increase in age (Beal 1995). One contributor to the effect of age on neurodegeneration may be reduced molecular chaperone efficacy. The role of the heat shock response in protecting against cell degeneration is mediated by the activity of molecular chaperones such as the HSPs. Molecular chaperones are the main defense against protein misfolding and toxicity, yet they are subject to significant decline in efficacy with aging (Balch 2008, Sherman 2001). As effectiveness of molecular chaperones diminishes, proteins are more likely to adopt aberrant tertiary conformations, which cause the formation of higher-order aggregations and more pervasive cellular dysfunction and neuronal death (Douglas 2010). Thus, increasing age is correlated with diminished heat shock response and greater susceptibility to stress-induced cellular degeneration, which may contribute to the onset of neurodegenerative disease. As discussed in a later section of the Introduction (The Experimental Model), our model involving HS stress-induced cell degeneration also exhibits age-dependent changes in the susceptibility of cells to degenerative mechanisms.

In addition to impairment of molecular chaperones and the HSR pathway, increasing age also decreases the efficiency of general proteostasis. Cells can maintain misfolded proteins in benign states for extended periods of time until age-associated factors cause a change in proteostasis and aggregates become toxic (Douglas 2010). These factors may include genetic

predisposition or epigenetic changes to the cell's function over time. Depending on the type of cell, the influence of age on stasis can manifest at different stages, thus contributing to varied predisposition to and cell susceptibility to proteotoxicity (Saxena 2011).

Cell Type Susceptibility

Certain neurons are more prone to accumulation of misfolded proteins and degeneration. This hypothesis has been studied extensively in *Caenorhabditis elegans* on proteins conserved between the model organism and humans. Results have shown that misfolding proteins disrupt cellular protein networks differently in various types of neurons, thus contributing to selective susceptibility (Saxena 2011). This effect comes into play particularly in neurodegenerative diseases; cellular degeneration often begins in one region of the brain and "spreads" to others as the disease progresses. One example of this is Parkinson's disease. Cellular degeneration begins in the dopaminergic neurons of the substantia nigra pars compacta (Moussa 2004). The neurons in this structure of the brain are especially vulnerable to protein misfolding and aggregation due to characteristic mitochondrial deficits (Chan 2007, Sherer 2003). High levels of oxidative damage in mitochondria-rich cells are a particularly strong determinant of susceptibility to stress-induced degeneration (Beal 1995, Mecocci 1994). This agrees with the result that HS stress in *Drosophila* most strongly affected cells in the flight motor, which contain a relatively large proportion of mitochondria. These findings illustrate the hypothesis that unique characteristics of certain cell types may result in increased susceptibility to protein aggregation and cellular degeneration. As discussed in a later section of the Introduction (The Experimental Model), our model involving HS stress-induced cell degeneration also exhibits cell type-specific susceptibility to degeneration and provides an opportunity for genetic analysis of this phenomenon.

Cell Non-autonomous Mechanisms

Neurodegenerative diseases are classified as "progressive" because cell death "spreads" to adjacent areas of the brain. One mechanism by which this phenomenon occurs may be due to differential cell-type susceptibility. Although misfolded proteins may be present in all cells, they lay in a dormant, benign state until age-associated factors trigger proteotoxicity. Differential cell-type susceptibility creates a staggered initiation of degeneration in various areas of the brain. Another mechanism by which spreading of degeneration can occur is through spreading of misfolded proteins themselves (Decarli 2004). This can occur through extracellular space, systemically, or through axonal projections (Aguzzi 2009, Frost 2010, Saxena 2011). However, studies in overexpression of molecular chaperones do not support the spread of misfolded proteins with regard to the heat shock response. As described later under Investigation of the Heat Shock Response (Chapter 4), HSP23 overexpression in certain targeted cell types does not protect against degeneration. This indicates that solely combatting the presence of misfolded proteins does not stop damage to cells, so disease spread due to the movement of misfolded proteins appears improbable. One possible mechanism is the spread of degeneration via induced toxicity around the original damaged cell. Our work with HSP23 investigates these cell non-autonomous mechanisms.

The Experimental Model

Our laboratory has developed a new model for genetic analysis of HS stress-induced degeneration. This model builds on our observation that heat shock induces loss of flight ability in *Drosophila* and selective degeneration of neurons, glia, and muscle cells in the flight motor. Advantages of this model include that it exhibits features characteristic of degenerative disease, including age-dependence and cell type-specific susceptibility, as well as cell non-autonomous

signaling mechanisms. Importantly, this model also permits acute induction of degenerative mechanisms and temporal resolution of subsequent steps in the process. In combination with powerful genetic approaches in *Drosophila*, the preceding features make this a highly attractive model for genetic analysis of degenerative and protective mechanisms.

Chapter 2

Materials and Methods

Genetics

Transgenic lines were generated for expression of the *Drosophila* HSP90-type protein, HSP83, under the control of the GAL4-UAS system. This method uses the GAL4 transcription activator protein from yeast, combined with the Upstream Activation Sequence (UAS) enhancer. GAL4 binds to the UAS enhancer in order to activate gene transcription (Brand 1993). The study of transgene expression levels involved generating a molecular construct which included the HSP83 open reading frame (ORF) under the control of GAL4-responsive UAS gene regulatory elements (UAS-hsp83). This construct was generated in a P element-based vector (pUAST) permitting its integration into the *Drosophila* genome and the generation of stable transgenic fly lines. An hsp83 cDNA clone was acquired from the Drosophila Genomics Resource Center in a pOTB7 vector. The cDNA was amplified using a polymerase chain reaction (PCR) with primers designed by Integrated DNA Technologies with standard desalting, diluted to a working concentration of 5μM.

Once the PCR product was confirmed through sequencing by the Pennsylvania State University nucleic acid facility, the hsp83 ORF was cloned using PFU polymerase. The first half was digested using restriction enzymes Xba I and Sal I, and ligated into a pBlueScript SK- vector. Another PCR reaction amplified the hsp83 ORF so that the second half could be digested with enzymes Sal I and Kpn I, then ligated into the pBluescript SK- vector containing the first

half of the hsp83 ORF. Using Not1 and Kpn1, the entirety of the hsp83 ORF was digested and shuttled into the fly transformation vector, pUAST. The completed construct was then transformed into JM109 competent cells. Plasmid preparation, diagnostic digest, and gel electrophoresis confirmed the successful ligation and transformation.

Additional transgenic lines were generated for expression of a *Drosophila* small HSP, HSP23, fused with the Green Fluorescent Protein (GFP). This fusion protein will permit fluorescence imaging of HSP23 in living cells and the GFP tag may also be used for biochemical analysis of HSP23 using an anti-GFP antibody. The hsp23 ORF was amplified with PCR using primers that integrated Xba 1 and EcoRI restriction enzyme cut sites on either end of the segment. The segment was digested and ligated into a pBluescriptII SK+ vector already containing the gfp ORF. Using Not I and Kpn I, the compound segment was digested and ligated into the fly transformation vector pUAST.

Once transgene constructs in pUAST were completed, a large scale purification kits (Qiagen) was used to purify the DNA before it was injected into the posterior end of fly embryos. Since development of germ cells in the fly occurs in the posterior region, injection of transgenic DNA constructs into this area increased the likelihood of transgene integration into the gametes, allowing the transgene to be expressed in future generations. A succession of genetic crosses was performed in order to isolate single transgene insertions, determine the chromosome location, and establish homozygous stocks (Figure 2).

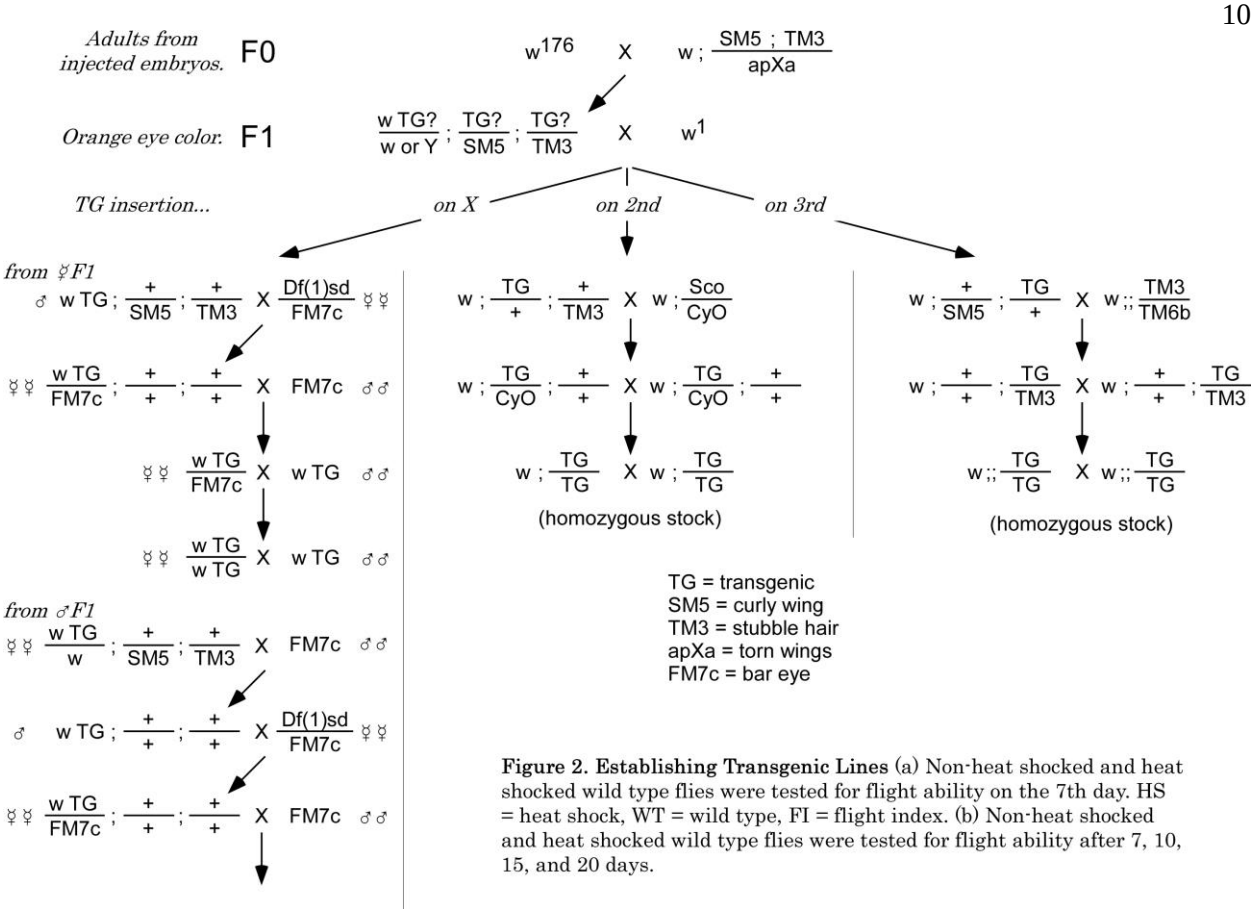

Figure 2. Establishing Transgenic Lines (a) Non-heat shocked and heat shocked wild type flies were tested for flight ability on the 7th day. HS = heat shock, WT = wild type, FI = flight index. (b) Non-heat shocked and heat shocked wild type flies were tested for flight ability after 7, 10, 15, and 20 days.

F0 generation flies that survived the injection process were crossed to a stock with balancer chromosomes for both the second and third chromosomes. Because injected embryos carry a mutation in the white gene (Figure 2), successful transgene insertions in the F1 generation were distinguished by orange eye color resulting from a w+ marker present in the injected constructs. A series of crosses with balancer chromosomes determined where the transgene insertion occurred (Figure 2), and these fly lines were eventually bred to generate homozygous lines.

Behavioral Testing

Transgenic flies were subjected to a HS paradigm and tested for behavioral abnormalities resulting from HS stress-induced cellular degeneration. HS stress involved exposing six flies of a

specific age to a series of three heat shocks at 36°C. Each heat shock was two hours in duration and occurred over a two day period such that they were initiated at 10:00 and 2:00 on day one and again at 10:00 on day two. Flies began heat shock at either four days old or seven days old, and were tested for behavior three days after the initial heat shock. Heat-shocked and control group flies were tested for climbing and flying ability. The climbing test evaluated muscle cell degeneration by measuring how quickly flies could climb to a standard height after being tapped down into the base of the chamber. The flying test was conducted using a closed glass cylinder in which the walls were marked at vertical intervals of 1cm. In order to perform the flight test, the inside walls of the apparatus were coated with oil and flies were dropped into the center. As flies gained usage of their flight motors, they flew and landed on the oil-coated cylinder wall perpendicular to the fall path. This measures the sensitivity and general functional capacity of the flight motor. If unable to fly, they dropped into the pool of oil at the bottom of the container. Each trial consisted of six flies and the average flight heights of multiple trials were combined to determine the flight index for a particular stress condition. Both climbing and flight ability were used to quantify the effects of cellular degeneration in the flight motor.

Western Blotting

Western blotting was used to analyze protein expression levels of HSP90 and HSP23-GFP. Fly samples were homogenized and lysed in SDS-Page, then run on 9% polyacrylamide gels. After transfer onto nitrocellulose membranes, the samples were incubated in primary antibody overnight. For HSP90, rabbit polyclonal α-HSP90 antibody (Cell Signaling) was used in a 1:2,000 dilution. For HSP23-GFP, a 1:500 dilution of mouse monoclonal α-GFP antibody (CLONTECH) was used trace the expression of the HSP23 fusion protein. Tubulin was the

sample loading control, detected by a 1:100,000 dilution of mouse monoclonal acetylated α-tubulin antibody (Sigma Technologies). After incubation with each respective fluorescent secondary antibody, the membranes were developed using the Licor Odyssey detection system.

Chapter 3

Heat Shock Stress-Induced Cell Degeneration in the Flight Motor

The studies and results discussed in the follow Chapters, 3 and 4, represent collaborative research efforts. Other members who contributed to this work include Dr. Fumiko Kawasaki and Yunzhen Zheng, as well as former and present undergraduate researchers Alexandra Strauss, Sharoz Fatima, and Pengshu Fang. I performed experiments in genetics, behavioral analysis, and Western blotting as described under Materials and Methods (Chapter 2).

This research builds on our initial discovery that heat shock, a model for environmental stress, induces degeneration of neuronal, glial and muscle cells comprising the flight motor. Initial experiments showed that wild type flies consistently lost the ability to fly after exposure to HS stress. This phenotype was quantified using a flight chamber (Figure 3a) which permitted the determination of a flight index (see Materials and Methods). Each group of heat-shocked flies, along with non-heat shocked control groups, were tested for flight ability.

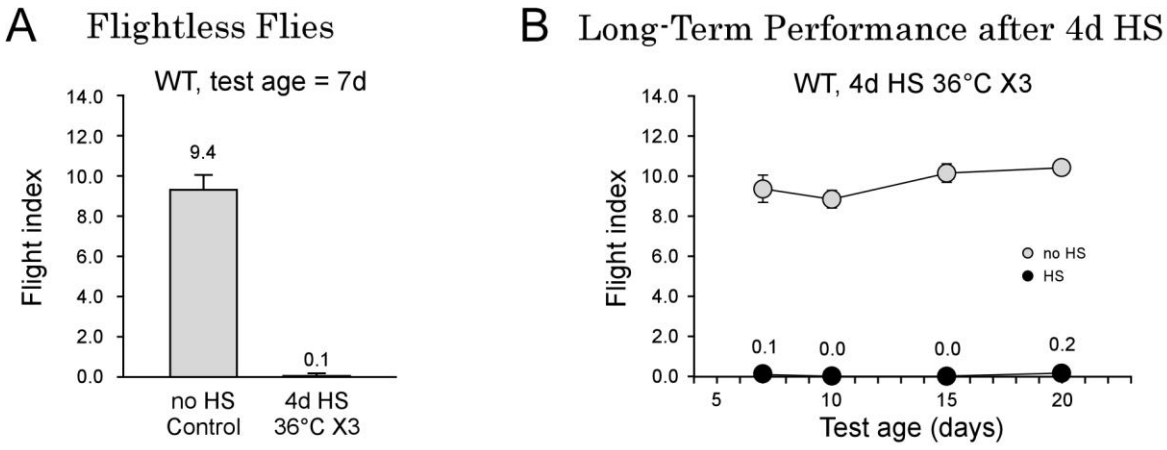

Figure 3. Loss of flight after heat shock. (a) Non-HS and HS wild typeflies were tested for flight ability on the seventh day. HS = heat shock, WT = wild type, FI = flight index (b) Non-HS and HS wild type flies were tested for flight ability after 7, 10, 15, and 20 days.

Seven day-old wild-type flies which had been stressed by heat shock starting at 4 days of age exhibited a dramatic loss of flight ability in comparison to seven day-old control flies which had not been exposed to HS (Figure 3a). These results demonstrate that HS stress disrupts the ability to fly. Comparisons of HS flies and non-HS controls were made at later time points after HS, up to the age of twenty days, and it was found that loss of flight ability after heat shock was irreparable (Figure 3b). In order to investigate the association between this behavioral deficit and cellular degeneration, confocal immunofluorescent microscopy studies performed by Dr. Fumiko Kawasaki examined the effect of HS on neurons, glia, and muscle cells known to critical for flight. These studies focused on three cell types: (1) A primary set of flight muscles, called the Dorsal Longitudinal Flight Muscles (DLMs), (2) the motor neurons that innervate them through neuromuscular synaptic connections and (3) associated glial cells which interact with neurons and muscle at synaptic contacts to form three-part or tripartite synapses (Figure 4) (Danjo 2010). These cells function together in flight and, along with analogous cell types of the complementary Dorsal Ventral Flight Muscles (DVMs), comprise the flight motor.

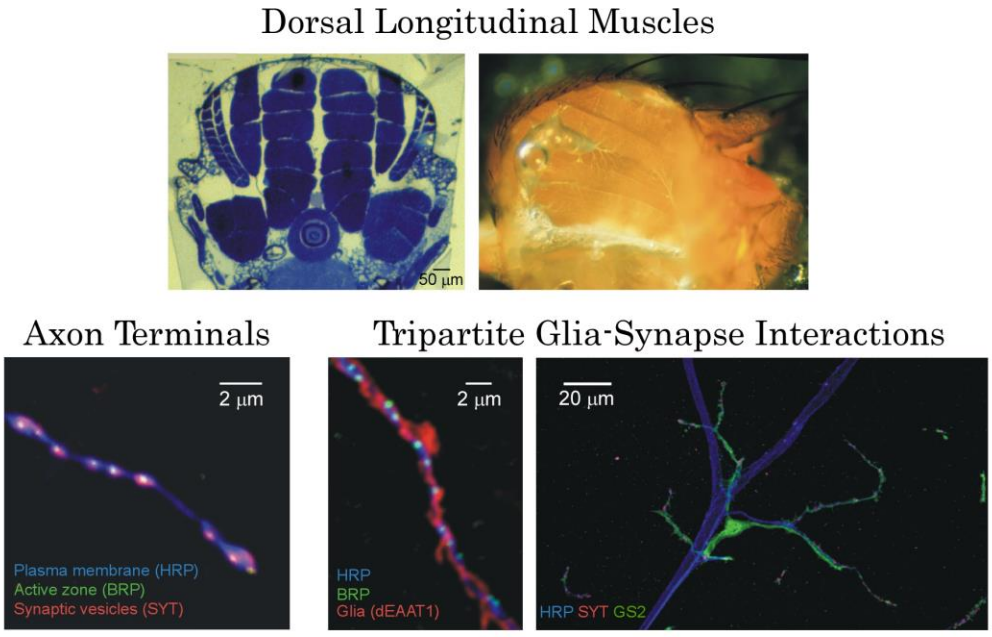

Figure 4. Elements of the flight motor. Top: Morphology of Dorsal Longitudinal Muscle (DLM) neuromuscular synapses. Six DLM fibers in a cross section and a lateral view. Bottom: Confocal immunofluorescence images of DLM neuro-muscular synapses: neuronal plasma membrane (HRP: blue), active zones (BRP: green), synaptic vesicles (SYT: red), glia (dEAAT1: red in center panel).

As expected, the neuronal and glial processes of a non-HS wild type fly are intact when the fly is ten days old (Figure 5a). However, HS stress induced degeneration of these processes, as indicated by their severe fragmentation. In addition, the DLM muscle exhibits severe degeneration as indicated by its loss of membrane potential (see Figure 7a). These results indicate that degeneration occurs at the cellular level after exposure to HS, including the three primary cell types of the flight motor. There is a consistent association between heat shock, loss of flight ability and degeneration of essential cell types in the flight motor. These findings indicate that HS-induced cell degeneration in the flight motor results in loss of flight ability.

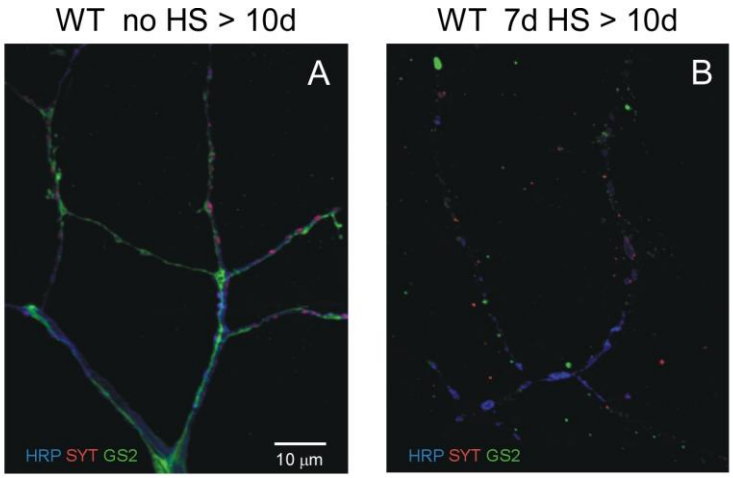

Figure 5. Cell degeneration in the flight motor. Confocal immunofluorescence images of DLM neuromuscular synapses including the glial marker GS2 (green). Shows cell degeneration in the neurons, glia, and muscle fibers of wild type flies (a) without and (b) with heat shock.

HS-induced flightlessness and cell degeneration were found to be dependent on age. This is evident from comparison of flies exposed to the HS paradigm starting at one day old with those first exposed at seven days old (Figure 6). All flies were tested for flight ability three days after HS was initiated. As observed for flies exposed to the HS paradigm at 4 days old (Figure 6), HS of 7 day old flies resulted in their loss of flight ability (Figure 6). In contrast, flies exposed to

HS stress at 1 day of age were highly resistant to both loss of flight ability and cell degeneration in the flight motor. More generally, these younger flies showed resistance to lethality induced by exposure to a more severe HS at 38°C (Figure 6). When heat shocked at 38°C, all flies in the older age group died while there was a high rate of survival among flies in the younger age group. The results from these experiments indicate that older flies are more severely affected by heat shock stress.

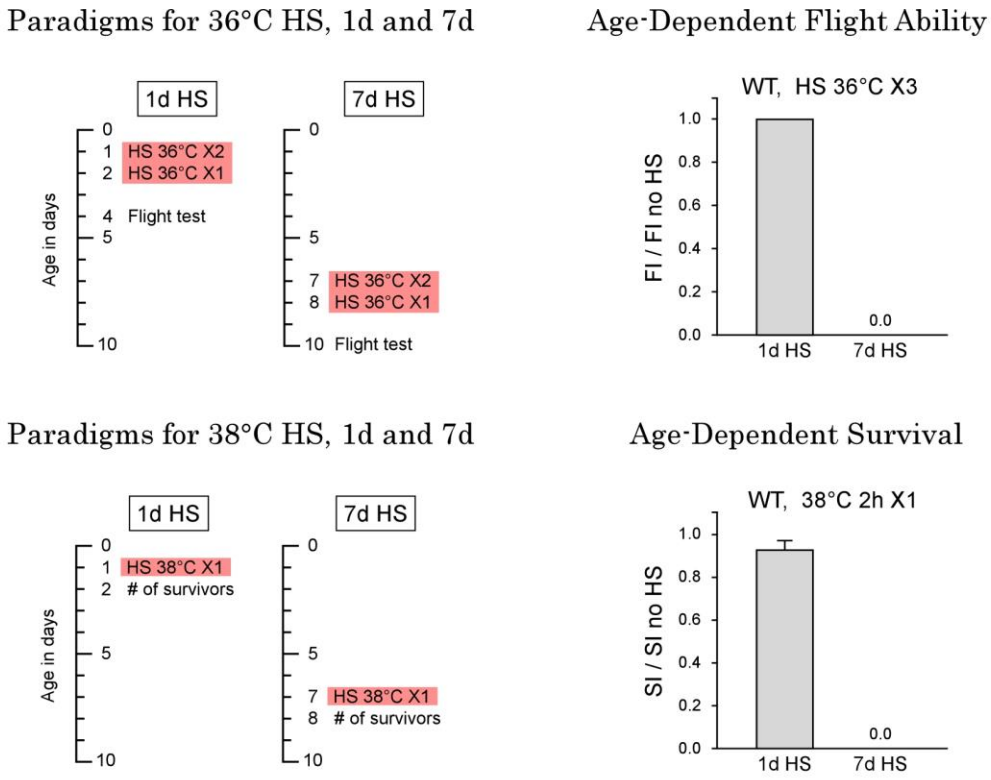

Figure 6. Age dependent loss of flight. Top: Heat shock paradigm used to compare flight ability of flies heat shocked at one day old and seven days old and flight index comparison of respective age groups. Bottom: heat shock paradigm used to compare survival of flies heat shocked at one day old and seven days old. Average percentage of flies which survived the heat shock paradigm.

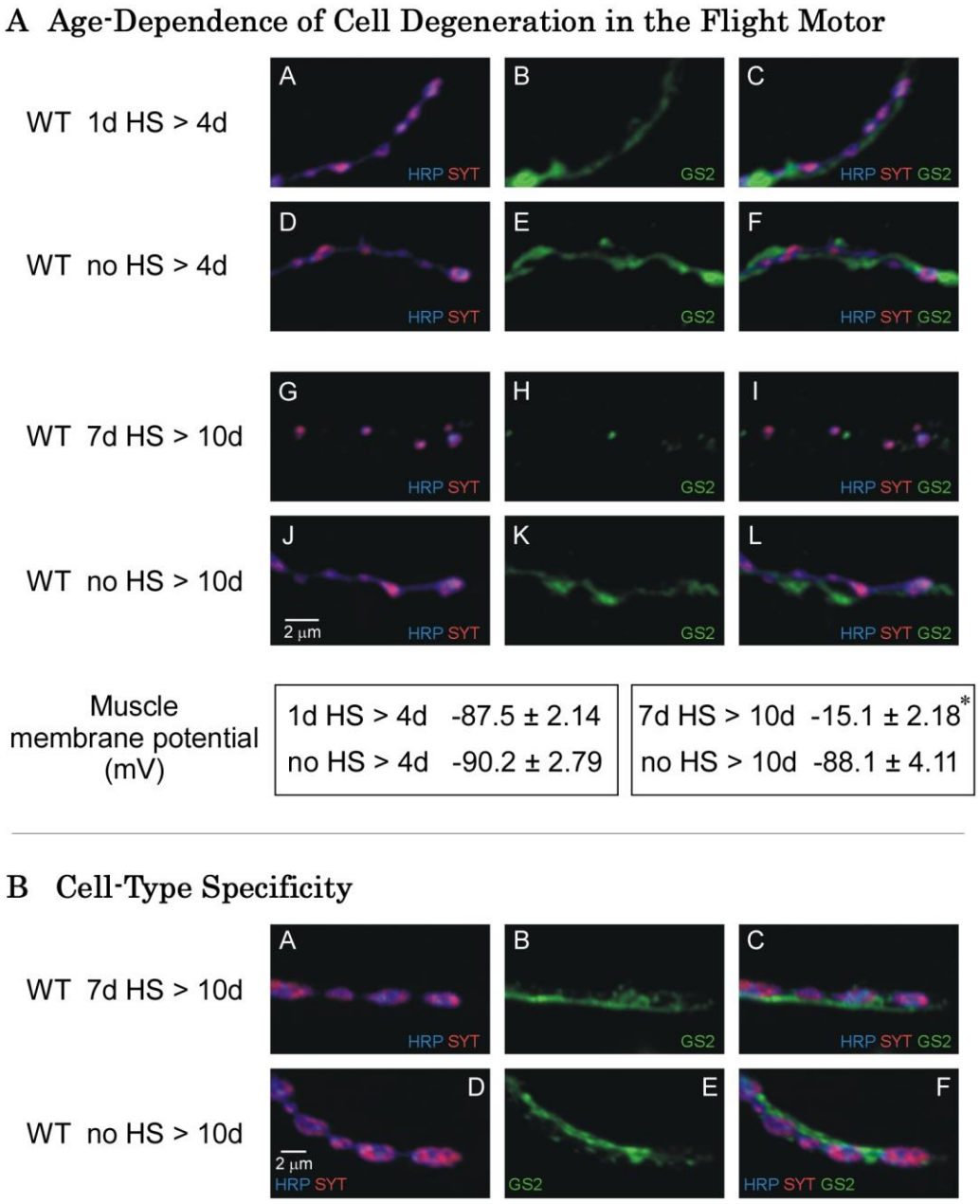

Figure 7. Confocal immunofluorescence images of DLM neuromuscular synapses. (a) DLM neuromuscular synapses in flies of different age groups, with and without heat shock. Muscle membrane potential recorded using single microelectrode current clamp. (b) An adult neuromuscular synapse of the leg (coxal) muscle.

Examination of cell degeneration by immunofluorescence microscopy further supported this conclusion by indicating that, as expected from the flight assays, cell degeneration did not

occur in the younger group of flies (Figure 7a). In non-HS flies, the flight motor cells of younger and older groups of flies are comparable and all fully intact. Flies that were heat shocked at one day old and observed at four days old were significantly more resistant to the degenerative effects of stress compared to flies heat shocked at seven days old and observed at ten days old (Figure 7a). The fluorescent microscopy images show degeneration of neuronal and glial processes. In addition to neurons and glia, muscle cells were also observed for degeneration due to heat shock. When cells degenerate, their membrane potentials become less negative. Compared to non-heat shocked flies, the average membrane potentials of flight motor muscle cells in HS flies was markedly less negative, indicating loss of membrane potential and cellular degeneration (Figure 7a). Furthermore, the muscle degeneration observed in flies exposed to the HS paradigm at 7 days of age was not observed in those exposed when 1 day old (Figure 7a). The dramatically more severe impairment after HS of older flies demonstrates that HS stress-induced cell degeneration is age-dependent.

In addition to age dependency, the extent of degenerative effects was also found to be dependent on cell type. Although HS stress induced degeneration in the flight motor, microscopic observation of neurons and glia associated with leg muscles did not show degeneration after heat shock (Figure 7b). This corresponded with findings that climbing ability was preserved after heat shock. Thus, it was determined that even cells comprising different types of neuromuscular synapses had different susceptibilities to stress induced cell degeneration.

Chapter 4

Investigation of the Heat Shock Response

One major focus in our investigation of HS stress-induced degeneration was to examine a possible role for the universal heat shock response that uses molecular chaperones to mitigate stress-induced proteotoxicity. As introduced in Chapter 1, the heat shock factor (HSF) is a transcription factor that exists in cytoplasmic complex with HSP90. Activation of HSP90 as a molecular chaperone releases HSF, which enters the nucleus as a multimeric transcription factor. HSF eventually promotes the transcription of other heat shock proteins such as HSP70, HSP40, and small HSPs (Figure 1). These induced HSPs work to mitigate protein misfolding, aggregation and toxicity. Since HSF plays such a key role in the heat shock response pathway, it was hypothesized that enhancing the heat shock response through HSF overexpression would reduce protein misfolding and aggregation and possibly protect against HS stress-induced degeneration.

In vivo overexpression of HSF with temporal and spatial control was achieved using the GAL4-UAS transgenic expression system (Figure 8). For studies of the HSR pathway involving overexpression of HSF, overexpression was performed in muscle on the basis of other studies indicating that HS induced an increase in misfolded proteins selectively in muscle. A UAS transgene expressing HSF was crossed with a muscle-specific Gal4 driver, MHC-GAL4, and thus flies overexpressing HSF in muscle cells were created.

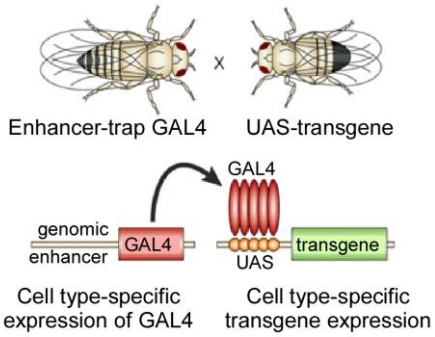

Figure 8. The Gal-4 expression system. Adapted from Muqit and Feany (2002) Nat. Rev. Neurosci.

Transgenic flies in which HSF was overexpressed in muscle were tested for resistance to HS stress-induced flight loss and cell degeneration after exposure of flies to the HS paradigm at 4 or 7 days of age. While wild type flies exhibit little to no flight ability after heat shock, HSF transgenic flies exhibit strong protection (Figure 9). Protection against cell degeneration was observed by immunofluorescence microscopy. Images of wild type and HSF overexpression flies exposed to the HS paradigm at 7 days of age showed severe degeneration in wild type but no degeneration in HSF overexpressing flies (Figure 9). These results show that HSF overexpression in muscle provided strong protection against HS stress-induced degeneration. Importantly, although it was restricted to muscle, HSF overexpression also provided protection of neurons and glia. Protection of these other cell types represents a cell non-autonomous effect and indicates that intercellular communication influences the degeneration process. For example, degeneration in one cell type may produce a toxic signal which promotes degeneration in other cell types. Such mechanisms may be involved in spreading of degenerative mechanisms in neurodegenerative disease (Douglas 2010).

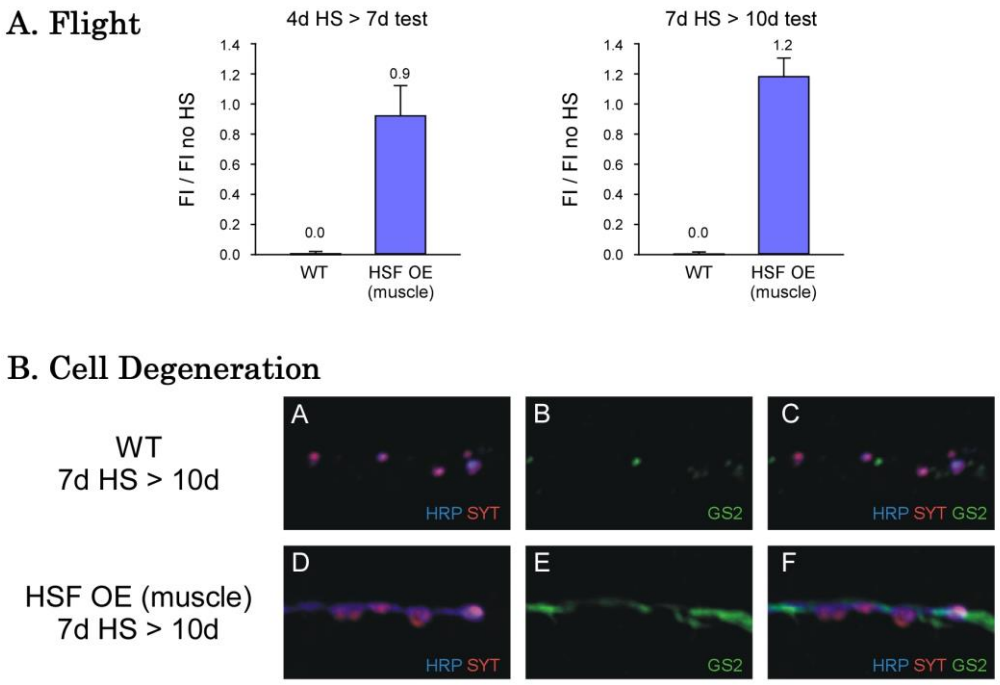

Figure 9. Heat shock factor overexpression (HSF OE) in muscle cells. (a) Flight abilityof four day old and seven day old wild type and HSF OE flies after heat shock (b) confocal immunofluorescence images of DLM neuromuscular synapses in seven day old wild type and HSF OE flies after heat shock.

Because HSF is a transcription factor that promotes the expression of heat shock response genes, Western blot analysis was used to investigate the downstream effects of HSF overexpression in the transgenic flies. HSF overexpression resulted in increased expression of molecular chaperones, including HSP70 and HSP23 (Figure 10). Note that the increase in HSP70 expression occurs only upon HS, whereas HSP23 expression in increased markedly in the absence of HS. These results raised the possibility that increased expression of HSP23 in the absence of HS preconditioned the fly to withstand an acute HS stress. This was investigated by examining whether direct overexpression of HSP23 in muscle would be sufficient to confer the same protective effect observed with HSF overexpression.

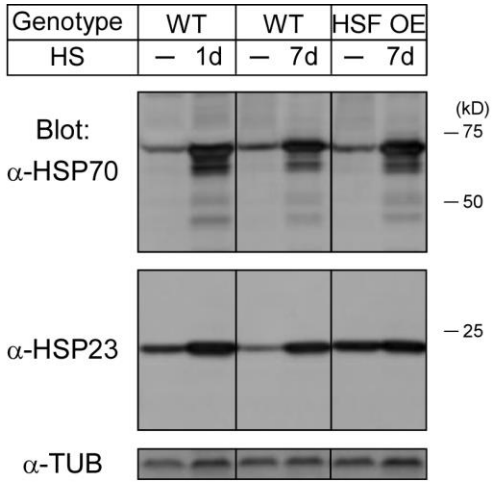

Figure 10. Western analysis of HSP70 and HSP23 in HSF OE flies. HSF OE in muscle cells was achieved using a GAL4 driver. Western blotting compared expression levels of HSP70 and HSP23 in HSF OE flies and wild type flies

Overexpression of HSP23 in muscle cells was achieved using a UAS-HSP23 transgene. As for HSF, HSP23 overexpression in muscle provided cell-autonomous protection of the muscle and cell-nonautonomous protection of neurons and glia (Figure 11). Thus, muscle-specific overexpression of HSP23 is sufficient to protect all three cell types. In addition, the UAS-HSP23 transgene was crossed to other Gal4 drivers in order to achieve HSP23 overexpression in neurons and glia and these studies further defined the mechanism of HSP23 protection. HSP23 overexpression in neurons or glia did not provide protection of any cell types from HS stress-induced degeneration (Figure 11). Thus HSP23 overexpression can protect neurons (and glia) when it occurs in muscle but not when it occurs in neurons. These results indicate that cell stress responses and the mechanisms of protection by molecular chaperones differ in various cell types and also highlight the importance of intercellular signaling mechanisms in mediating protection from environmental stress. These results are further

indication that the heat shock response is a complex system that works differently in various cell types.

The mechanisms by which cell non-autonomous protection of neurons and glia is mediated by overexpression of HSP23 in muscle remain to be determined, however several different types of mechanism are under consideration. It was found that damaging muscle cells by mechanical means does not cause subsequent degeneration of neurons and glia. This does not support the hypothesis that the muscle is emitting a positive factor maintaining the health of neurons and glia. One viable possibility is that degeneration of the muscle could cause secretion of a negative factor that damages neighboring neurons and glia.

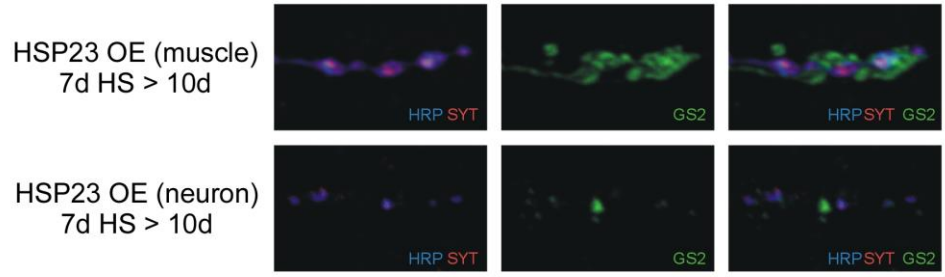

Figure 11. Degeneration in HSP23 OE in muscle and neuronal cells. Confocal immunofluorescence images of DLM neuromuscular synapses. Muscle-specific expression of a UAS-HSP23 transgene provided protection against HS stress-induced degeneration of neurons, glia, and muscle. Neuronal expression of thesame UAS-HSP23 transgene failed to provide protection.

Deeper investigation of the protective mechanism mediated by HSP23 overexpression will be facilitated by generation of UAS-HSP23-EGFP transgenic fly lines. This tool will facilitate both live imaging and biochemical studies of HSP23 function. Generation of the transgene construct has been completed and transgenic fly lines were tested through Western blot for fusion protein expression levels. Since the transgene expresses a larger fusion protein, α-GFP antibody could be used in Western blotting to detect expression of the transgene product, while the smaller endogenous HSP23 expression could be detected separately by α-HSP23. Lines

103C, 25, and 93 were chosen to keep as permanent stocks due to strong overexpression of HSP23 (Figure 12).

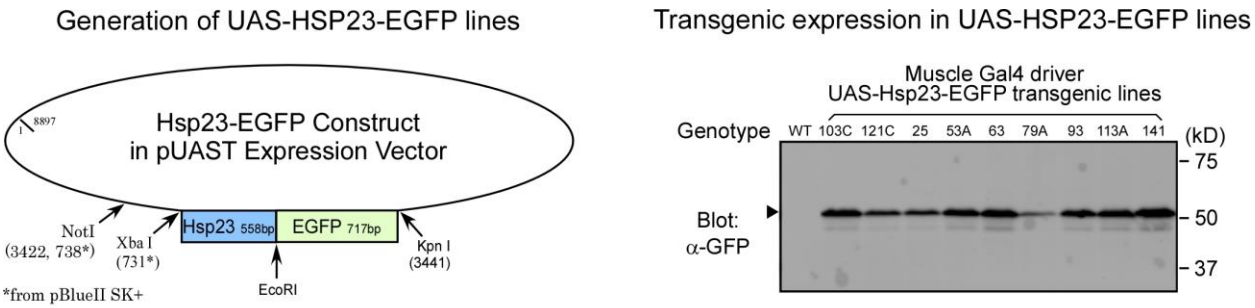

Figure 12. Transgenic expression of HSP23-EGFP. (a) Transgenic construct of HSP23-EGFP (b) Western blot analysis of GFP expression levels in transgenic HSP23-EGFP fly lines (indicative of fusion protein expression).

Finally, our analysis has extended to the HSP90 class of molecular chaperones, for which no UAS-transgenic lines were previously available. A UAS transgene was generated for the fly HSP90 type protein, HSP83. UAS-HSP83 transgenic fly lines were crossed to various Gal4 drivers and analyzed through heat shock experiments and Western blotting. Overexpression of HSP83 did not show any significant protection against stress-induced flight loss or degeneration. Western blots were used to compare HSP83 expression levels in various transgenic lines in order to decide which to keep as permanent stocks (Figure 13).

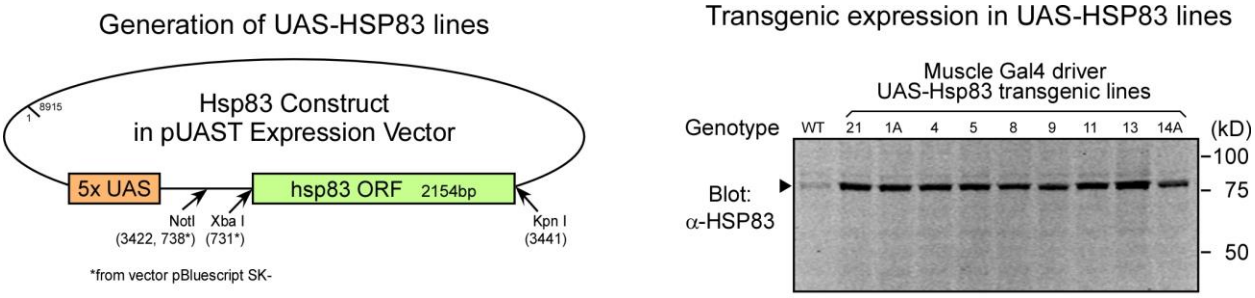

Figure 13. Transgenic expression of HSP90. (a) Transgenic construct of HSP90, also known as HSP83 in *Drosophila melanogaster*. (g) Western blot analysis of HSP90 expression levels in transgenic HSP90 fly lines.

Chapter 5

Conclusion and Future Directions

The research explained in this thesis all focuses toward a mechanistic understanding of the heat shock response to stress-induced cellular degeneration. Heat shock was used as a model of environmental stress, causing protein misfolding and aggregation, cellular degeneration, and loss of flight in *Drosophila melanogaster*. Increasing age is correlated with higher susceptibility to degeneration, strongly indicating that the decline in proteostasis via molecular chaperone efficacy plays a role in the age-dependence of degenerative disease. It was also found that unique characteristics of different cells create varied levels of vulnerability to stress-induced degeneration. Impairment of the flight motor specifically suggests that high metabolic demands in these cell types are one factor that may increase susceptibility to HS stress. This opens up an interesting avenue of research into the etiology of degenerative disorders, and possible mechanisms of disease initiation and progression.

Using the heat shock experimental model, it was found that muscle-specific overexpression of the HSF transcription factor or one of its downstream chaperones was found to protect flies from flight loss and cellular degeneration. These findings suggest that HSF generally elevates the entire heat shock response, and supports a role for HSPs in protecting organisms from the toxicity of protein misfolding and aggregation.

Overexpression of small HSP23 in muscle cells showed cell non-autonomous protection of neurons and glia, but overexpression of HSP23 in neurons did not protect from stress-induced degeneration. These observations indicate that intercellular signaling mechanisms operating

among the three cell types play important roles in degeneration and raise interesting questions about the mechanisms of protection and cell non-autonomous spreading of degeneration into adjacent cells and tissues. Although it has been found that misfolded proteins are able to spread among cells through extracellular space and axon processes (Aguzzi 2009), it is still uncertain whether molecular chaperones and the protection they offer behave similarly. It is important to note that our results do not support a protection mechanism involving spread of HSP23 from muscle to other cell types. This is evident from our finding that overexpression of HSp23 in either neurons or glia does not protect any of the three cell types. Furthermore, these results show that the protective functions of HSPs differ depending upon the specific type of cell in which they are expressed. This research could contribute to a more thorough understanding of degenerative disease progression and therapeutic applications of protection against spreading. The role of each individual heat shock protein in different cell types is a rich area that remains undiscovered.

Ongoing studies will utilize newly constructed transgenic lines in order to further examine the protective roles of HSPs in HS stress induced degeneration in the flight motor. Generation of the transgene construct for HSP90 and initial studies with resulting transgenic lines did not show significant protection against HS stress-induced cellular degeneration when overexpressed in the muscle. However, the tools created in this portion of the research can be used in future genetic analysis in order to explore the activity of this particular class of molecular chaperones. Generation of the transgene construct for HSP23-EGFP provides a useful tool for further molecular research into the mechanisms and functions of small HSPs. Because the fusion protein includes a fluorescent EGFP tag, expression of this transgene can be used in a wide array of imaging endeavors. Immunofluorescence can be used to visualize HSP23 in living cells and

analyze its subcellular distribution after varying extents of induced stress. Additionally, the fusion protein can be used in biochemical assays to determine what other proteins or molecules HSP23 interacts with in different stages of the heat shock response. Co-eluted proteins could give insight into the mechanisms of HSp23, which would open doors to a myriad of other research paths or therapeutic applications.

This work with HS stress-induced degeneration, HSF, HSP23, and HSP90 can be extended to many further studies addressing the etiology of neurodegeneration and mechanistic protection against it. Additionally, application of these powerful genetic approaches in *Drosophila* allow further understanding of the underlying mechanisms and common features of degenerative disease in general, which may provide new insights into disease processes and potential therapeutic approaches. Much remains to be learned about the contributions and mechanisms of age-dependence, cell type-susceptibility, and cell non-autonomous signaling mechanisms, and advances in these studies can contribute to our understanding of a broad range of diseases involving tissue degeneration.

BIBLIOGRAPHY

Abravaya K., Phillips B., Morimoto RI. (1991). Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures. *Genes Dev* . 5: 2117–2127.

Aguzzi A., Calella A.M. (2009). Prions: protein aggregation and infectious diseases. *Physiol. Rev*. 89: 1105–1152.

Balch W.E., Morimoto R.I., Dillin A., Kelly J.W. (2008). Adapting proteostasis for disease intervention. *Science*. 319(5865):916-9.

Biskup S., Moore D.J. (2006). Detrimental deletions: mitochondria, aging and Parkinson's disease. *Bioessays*. 28:963–967.

Brand, A.H, Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*. 118: 401-415.

Bucciantini, M. (2002). Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature.* 416.6880: 507-511.

Chan C.S., Guzman J.N., Ilijic E., Mercer J.N., Rick C., Tkatch T., Meredith G.E., Surmeier D.J. (2007). ‘Rejuvenation’ protects neurons in mouse models of Parkinson's disease. *Nature*. 447: 1081–1086.

Cole S.L., Vassar R. (2009). Linking vascular disorders and Alzheimer's disease: potential involvement of BACE1. *Neurobiol. Aging*. 30:1535–1544.

Danjo, R., Kawasaki, F., Ordway, R.W. (2011). A tripartite synapse model in *Drosophila*. *PlosONE*. 6.2e17131.

Decarli C. (2004). Vascular factors in dementia: an overview. *J. Neurol. Sci*., 226:19–23.

Douglas, P.M., Dillin, A. (2010). Protein homeostasis and aging in neurodegeneration. *The Journal of Cell Biology*. 190: 719-729.

Frost B., Diamond M.I. (2010). Prion-like mechanisms in neurodegenerative diseases. N*at. Rev. Neurosci*. 11:155–159.

Gan L., Mucke L. (2008). Paths of convergence: sirtuins in aging and neurodegeneration. *Neuron*. 58:10–14.

Gasch AP, Spellman PT, Kao CM, Carmel-Harel O, Eisen MB, Storz G, Botstein D, Brown PO. (2000). Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol* Cel.l 11: 4241–4257.

Kim, Y.E., Hipp, M.S., Bracher, A., Hayer-Hartl, M. and Hartl, F.U. (2013). Molecular chaperone functions in protein folding and proteostasis. *Annual Review of Biochemistry*. 82: 323-55.

Morimoto, R.I. (2011). The heat shock response: systems biology of proteotoxic stress in aging and disease. *Cold Spring Harb Symp Quant Biol*. 76: 91-9.

Prahlad V., Morimoto R.I. 2009. "Integrating the stress response: lessons for neurodegenerative diseases from *C. elegans*." *Trends Cell Biol*., 19:52–61.

Richter, K., Haslbeck, M. and Buchner, J. (2010). The heat shock response: life on the verge of death. *Molecular Cell*. 40: 253-66.

Saibil H. (2013). Chaperone machines for protein folding, unfolding, and disaggregation. *Nature*. 14: 630-642.

Saxena, S. and Caroni, P. (2011). Selective neuronal vulnerability in neurodegenerative diseases: from stressor thresholds to degeneration. *Neuron*. 71: 35-48.

Sherer T.B., Betarbet R., Testa C.M., B.B. Seo B.B., Richardson J.R., Kim J.H., Miller G.W., Yagi T., Matsuno-Yagi A., Greenamyre J.T. (2003). Mechanism of toxicity in rotenone models of Parkinson's disease. *J. Neurosci*. 23:10756–10764.

Sherman MY, Goldberg AL. (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases. *Neuron*. 29:15-32.

Subhankar, P., Sailendra M. (2013). Association of heat-shock proteins in various neurodegenerative disorders: is it a master key to open the therapeutic door?. *Molecular Cell Biochemistry*. 386:45-61.

ACADEMIC VITA

Permanent Address
504 Ironwood Way
Dresher PA 19025

Annie E. Lin
ael5270@psu.edu

Current Address
111 South Allen Street
State College PA 16801

EDUCATION

The Pennsylvania State University | Schreyer Honors College | Class of 2015
Major: Biology – Genetics and Development Option
Minor: Neuroscience

HONORS

The Pennsylvania State University Summer Discovery Grant | Summer 2013
J. Ben and Helen D. Hill Memorial Fund | 2014-2015
Schreyer Honors College Scholarship | 2011-2015
Dean's List | 2011-2015

PROFESSIONAL EXPERIENCE

The Pennsylvania State University | September 2012 to Present
Undergraduate Researcher in Neuroscience and Molecular Genetics
Principle Investigators: Dr. Richard Ordway and Dr. Fumiko Kawasaki
Assisted in the research of neurodegenerative mechanisms. Genes found to protect *Drosophila* from neurodegeneration are isolated, engineered, and used to create transgenic flies with the desired resistance to neuron decay. Experiments done on these transgenic lines are used to deduce molecular mechanisms of neurodegeneration, which serve as targets for gene therapy for neurodegenerative disorders such as Huntington's, Parkinson's, or Alzheimer's Diseases. Lab work includes molecular transgene design, Western blot for protein analysis, and *Drosophila* care and experimentation.

Volunteer at Mt. Nittany Medical Center | State College, PA; August 2012 to Present
Physical Therapy Department | August 2012 to May 2013
Assisted physical therapists with patient exercises and transportation
Emergency Department | may 2013 to Present

Volunteer at Centre County PAWS | State College, PA; November 2014 to Present

PRESENTATIONS

"A New *Drosophila* Mutant Exhibiting Resistance to Neurodegeneration" | 2013
Yunzhen Zheng, Alexandra L. Strauss, Annie E. Lin, Fumiko Kawasaki and Richard W. Ordway

"Heat Shock Induces Selective and Age-Dependent Cellular Degeneration in *Drosophila*" | 2013
Alexandra L. Strauss, Yunzhen Zheng, Annie E. Lin, Fumiko Kawasaki, and Richard W. Ordway

PUBLICATIONS

Flightless Flies: Heat Shock Induces Selective Cell Degeneration in the Flight Motor | 2014 *Nature Communications*
Alexandra L. Strauss, Yunzhen Zheng, Annie E. Lin, Fumiko Kawasaki and Richard W. Ordway
Submitted manuscript currently under revision for re-submission

STUDENT INVOLVEMENT

Global Medical Brigades | August 2011 to May 2013
Science LionPride | August 2013 to May 2014
Campus Orchestra | August 2012 to Present