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Exploring the Role of Dopamine in Stress Response and Aging in Drosophila Melanogaster - Implications for Neurodegenerative Diseases

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Exploring the role of dopamine in stress response and aging in *Drosophila melanogaster*

– implications for neurodegenerative diseases

By

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Dopamine (DA) is a catecholamine that is involved in several neural functions such as modulation of locomotor behaviors, arousal states to appetitive aversive learning and memory. The relationships between DA, stress response and aging are unclear. This thesis examines numerous physiological, behavioral and biochemical parameters following perturbations in DA synthesis and transport in the *Drosophila melanogaster* model system. Intriguingly, elevated DA pools appear to confer protection, while depleted DA levels or transport increase susceptibility to oxidative insult. Resistance to oxidative stress in mutants with elevated DA levels was attributed to a significant up-regulation of glutathione S-transferase Omega-1. A sexually dimorphic response in aging and senescence characteristics was also recorded among the mutants tested, but no discernable role of DA in these characteristics was observed. Taken together, these results point to a key role played by DA in stress response, which might have implications to age-related neurodegenerative diseases.

DEDICATION

I would like to dedicate this thesis to the countless fallen fruit flies that gave their life for my research.

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I would like to thank my thesis mentor, Dr. Natraj Krishnan for creating a gratifying work environment that has allowed me to grow into a passionate scientist. Your guidance throughout my project, your advice and support for my future endeavors, and your patience dealing with my lab "mishaps" will forever be appreciated. I would also like to thank Dr. Andrea Bednářova for being the greatest teacher, mentor and most importantly, friend over the past few years. My graduate school experience at Mississippi State University would not have been the same without you. Thank you both for believing in me, and pushing me to become a better person and scientist.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND BACKGROUND

1.1 Introduction and thesis overview

An animal's physiologic response to stress involves the activation of multiple biochemical and neuroendocrine cascades. In this, the neuroendocrine environment of the brain is of paramount importance. Stress response and aging are causally linked. Animals that are rendered stress resistant by genetic or pharmacological means generally exhibit an extended lifespan (Lithgow, 2006). Despite the obvious connections between neuroendocrine regulation of stress response and its impact on aging, we have little understanding of the biochemical and physiological events that underpin these relationships. It is hypothesized that neuroendocrine factors responsible for key stress responses may also be involved in the process of aging in a sex – specific manner. This hypothesis is tested using the fruit fly *Drosophila melanogaster*, a valuable model organism historically and currently used for scientific as well as medical research (Jennings, 2011). This well studied, highly tractable genetic model organism would allow us to have a better understanding of the connections between the neuroendocrine regulation of stress response and its effects on aging with implications for neurodegenerative diseases. Transgenic flies with altered levels of dopamine (DA - a key neuro-active catecholamine implicated in diverse physiologic functions including response to stress) synthesis and transport were used in this investigation. The response

of these transgenic fly lines was first tested against oxidative stress (OS) a well-established stressor, and then subsequently the sexually dimorphic role of DA (using the same transgenic flies) in the process of aging was also explored. It is believed that an understanding of the cross-talk between stress response, DA and aging would be potentially interesting and useful since cellular stress responses are linked to the origins of many age-related diseases (particularly neurodegenerative diseases) and such a study could prompt new therapeutic strategies.

In this Chapter a brief overview of stress has been provided followed by: (a) a description of the phenomenon of OS, (b) the process of generation of free radicals, (c) the methods by which OS can be generated in a laboratory setting, (d) the damage caused by such OS, biomarkers of OS with particular reference to protein oxidation and its relevance to aging, (e) the physiology of stress response involving biogenic amines such as DA, its biosynthesis and role in stress response, and (f) the principal antioxidant systems and their coordination by the neuroendocrine system. Following this, an overview of the crosstalk between DA, stress and aging with implications to neurodegeneration has been provided. Additionally, there is a section dealing with sex differences in stress response and aging and the motivation behind sexual dimorphism studies. This Chapter culminates with a description of the over-arching hypotheses and the specific aims and objectives of this work.

1.2 Background

The ensuing sections provide a brief yet comprehensive review of various aspects that are pertinent to the studies embodied in this thesis. While it was not possible to deal with every aspect in its entirety, it is felt that the information provided would be sufficient in providing a setting for the hypotheses that was developed and the subsequent Chapters where these were comprehensively investigated and presented.

1.3 The concept of stress

The term "stress" was defined by Hans Selye (Selye, 1973) as "the non-specific response of the body to any demands made upon it". This was arguably a vague and immeasurable phenomenon that was criticized by physiologists. Selye subsequently clarified this concept by defining the stress response elements, which were involved in the hypothalamo-pituitary-adrenal (HPA) axis system in vertebrates. Currently, the term "stress" has been redefined as "a physiological response that serves as a mechanism of mediation linking any given stressor to its target-organ effect". By viewing the phenomenon of stress within the context of a "linking" mechanism, one can ask questions such as; through what mechanism can stressor stimuli lead to disease and dysfunction? As a response to the stressor stimuli, the organism must conduct an appropriate physiologic response that can restore homeostasis. In other words, the stressful experiences induce a powerful set of behavioral, hormonal, cellular and molecular responses that assist organisms in adapting to its environment. Therefore, stress is now recognized as a valid physiological concept that allows an organism to respond to adverse environmental pressures (McEwen, 2009). Most physiological studies use the word

"stress" to describe negative treatments applied to organisms in an experimental setting, such as nutritional stress, heat stress, oxidative stress etc. The triggering stimulus is generally referred to as the "stressor" while 'stress' is considered as a response syndrome. However, the definition of stress should also take into account the duration and intensity of the stressor involved, thereby classifying it as acute or chronic stress. Thus, "Stress" can also be defined as any factor that reduces an animal's fitness as measured by traits such as reproduction or survival. In general, organisms have a myriad of adaptive responses that minimize the impact of stress on fitness. These range from behavioral adaptations (stress avoidance strategies), major physiological and developmental changes driven by hormonal status, and cellular and molecular responses orchestrated by certain signaling entities such as hormones.

1.4 Oxidative stress – ubiquitous in all aerobic life forms

Molecular oxygen is key to aerobic life but is also converted into cytotoxic by-products referred to as reactive oxygen species (ROS), which can be inherently dangerous to the very survival of aerobic organisms (Davies, 1995). Overproduction of these free radicals coupled with a deficiency of adequate protective mechanisms results in a situation which is termed as oxidative stress (OS). OS causes oxidative damage to biomolecules such as proteins, lipids and DNA. OS thus results from the metabolic reactions that use oxygen, but can be intensified by environmental factors such as pesticides, chemicals, food sources, UV light, irradiation etc. In order to understand the concept of OS, one must first understand the phenomenon of generation of free radicals, the process by which such a stressor can be generated in a laboratory setting, as well as the types of damage caused by OS. The ensuing sections deal with each of these aspects.

1.4.1 Generation of reactive oxygen species (ROS)

Reactive oxygen species (ROS) generation has two opposing facets – redox biology and oxidative stress (OS). They are recognized for having a dual role of beneficial and deleterious species. OS is generated from an increase in intracellular ROS levels that causes damage to lipids, proteins and DNA. These ROS are by-products of aerobic metabolism and have inherent chemical properties that confer reactivity to varying biological targets. On the other hand, redox biology involves a small increase in ROS levels that initiates signaling pathways to activate biological processes. Both of which underlies physiological and pathological conditions (Schieber and Chandel, 2014). The mitochondrial electron transport chain (ETC) is known to be a major contributing factor in cellular generation of ROS such as superoxide (O^{-}_{2}), hydrogen peroxide (H_2O_2) and the hydroxyl free radical (OH) (Loschen et al., 1971; Boveris et al., 1972; Chance et al., 1979; Liu et al., 2002). This occurs by electrons passing through the mitochondrial ETC leaking out to molecular oxygen (O_2) forming superoxide. Subsequently, a number of other species such as hydrogen peroxide and the hydroxyl radical are generated.

1.4.2 Induction of ROS production in the model organism

There are a number of chemicals which can be used to evoke OS in the model organism to study the responses to these stressors *in vivo*. Here, a couple of such stressors have been described since these were used to generate OS in this work (Chapter II, Hanna et al., 2015):

Paraquat (N', N'-dimethyl-4,4'-bipyridinium dichloride) is a typical chemical (often used as a herbicide) having structural moieties that undergo redox cycling in living cells to induce ROS production. This cyclic oxidation/ reduction leads to two potentially

important consequences relevant to the toxicity: (1) Generation of ROS including superoxide anion, hydrogen peroxide, and hydroxyl radical, and (2) Oxidation and depletion of reducing equivalents (e.g.: NADPH, GSH). Both contribute to the induction of OS and damage to the tissues, and to the whole organism in general.

Hydrogen peroxide (H_2O_2) is, in biological systems, generated as a result of dismutation of superoxide radicals. There are also some enzymes that can produce H_2O_2 directly or indirectly. Although H_2O_2 molecules are considered reactive oxygen metabolites, they are not radical by definition, however, they are capable of causing damage to the cell at a relatively low concentration (10 μM). Hence, this compound is very often used to evoke OS in biological systems. Another reason is that the molecules of H_2O_2 are freely dissolved in aqueous solution and can easily penetrate biological membranes. Their deleterious chemical effects can be divided into the categories of direct activity, originating from their oxidizing properties, and indirect activity in which they serve as a source for more deleterious species. Direct activities of H_2O_2 include inactivation of enzymes; and oxidation of DNA, lipids, -SH groups, and keto acids (Kohen and Nyska 2002).

1.4.3 Oxidative damage – protein oxidation a link between aging and neurodegeneration

ROS, as mentioned before, have the ability, either directly or indirectly, to damage all biomolecules, including proteins, lipids, DNA and carbohydrates. Under normal circumstances, there is a well-managed balance between formation and neutralization of ROS so that there is minimal modification of biomolecules. Protein oxidation, in particular, serves as a useful marker for assessing OS *in vivo*. Such protein

oxidation can be formed either by the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary by-products of OS. Agents that lead to protein oxidation include reagents such as H_2O_2 and HOCl and xenobiotics such as paraquat. So, what could be the relationship between protein oxidation, OS, aging and neurodegeneration? It appears that in situations of OS, aging and neurodegeneration the common denominator is protein oxidation. Oxidation of proteins is mediated mainly, but not only, by the hydroxyl radical (OH) that can be produced by the decomposition of hydrogen peroxide in the presence of redox metals (Cu^{+} and Fe^{2+}) (Butterfield and Stadtman, 1997). There are two possible oxidative pathways that can occur: (a) backbone oxidation and (b) side-chain oxidation. It is important to mention that protein oxidation also can lead to protein cross-linking and /or peptide bond cleavage via diamide or α-amidation pathways. Cleavage of the peptide bond will result in formation of carbonyl groups that are often used as a marker of protein oxidation. In this study, protein carbonyls were used as a marker for protein oxidation during aging (in Chapter III).

1.4.3.1 Protein oxidation and its link to aging

Denham Harman, proposed the free radical theory of aging more than fifty years ago stating that aging results from the accumulation of deleterious effects caused by free radicals, and the ability of an organism to cope with cellular damage induced by ROS and the important role it plays in determining the lifespan of the organism (Harman, 1956). Many studies have consistently reported an increase of cellular oxidative damage as a result of aging (Oliver et al., 1987; Fraga et al., 1990) in support of this theory. Further studies (Fucci et al., 1983; Stark-Reed and Oliver, 1989; Stadtman and Oliver, 1991; Stadtman, 1992) have demonstrated that cellular proteins undergo extensive oxidative

modifications, manifested as carbonyl derivatives, as a consequence of aging in several model systems. A direct relationship between protein oxidative damage (caused by generation of ROS) and life expectancy in the housefly was demonstrated by Sohal et al., (1993). However, there are studies disputing the free radical theory despite the vast amount of evidence in support of the linking aspect between ROS and aging (Lapointe and Hekimi, 2010) giving a valuable reason for more detailed studies to be conducted on this topic. Despite intensive debates on the topic, in principle, OS could be related to the process of aging through variations in ROS generation, ROS elimination or both (Barja, 2004), however antioxidants, although possibly involved in protection against various age-related diseases, do not seem to control the rate of aging (Pérez et al., 2009).

1.4.3.2 Protein oxidation and its link to neurodegeneration

Protein oxidation, one of a number of biomarkers of OS, is increased in several age-related neurodegenerative disorders including Alzheimer's disease, Huntington's disease, prion disorders such as Creutzfeldt-Jakob disease, and α-synuclein disorders such as Parkinson's disease. These different disorders caused by different genetic or environmental insults and affecting different brain regions, may have a common underlying molecular basis, namely OS. As aging progresses, the intrinsic OS increases as a consequence of diminished antioxidant defense capabilities (Butterfield and Stadtman, 1997). The free radical damage to neurons caused by the particular molecular alterations (protein oxidation) in the above-mentioned age-related neurodegenerative disorders is compounded by the effects of brain aging. Hence, accumulation of oxidized proteins in general in the organism could be a reflection of an increased susceptibility of the brain to such damage. This is because the brain consumes a large percentage of

inspired oxygen, is rich in polyunsaturated fatty acids, accumulates redox metal ions, is relatively low in antioxidants and is composed of largely non-mitotic cells, hence making this organ especially vulnerable to OS. Gaps do exist in the understanding of relationships between OS, protein oxidation, aggregation and eventual neurotoxicity, thus warranting additional enquiry. However, it is indisputable that protein oxidation is clearly associated with neurodegenerative diseases (Butterfield and Kanski, 2001).

1.5 Understanding the physiology of stress response

The neuroendocrine responses to stressors are considered important survival mechanisms during exposure to life-threatening stimuli. There is a general agreement regarding the role of the hypothalamic-pituitary-adrenal axis and adrenal catecholamines in maintaining homeostasis in higher animals. The role of catecholamines as hormones and neuromodulators in acute stress response is extremely well conserved and documented in vertebrates (Kvetnansky et al., 2009). In higher vertebrates, during physiological stress, the brain orchestrates a series of psychological and behavioral responses to promote survival (Reviewed in Ulrich-Lai and Herman, 2009). These physiological responses include a rapid activation of the sympathetic branch of the autonomic nervous system that results in numerous catecholamine-mediated effects throughout the body, such as increased heart rate and blood pressure. These initial physiological responses to stress are coordinated by a highly-interconnected network of numerous brain structures which are critical for immediate survival in the face of stress. However, excessive or prolonged activation is associated with a number of deleterious side effects including increased neuronal vulnerability to insults both in vertebrates and invertebrates. Indeed in invertebrates catecholamine concentrations are one of a number

of potential indices of sub-lethal stress (Lansing et al., 1993), suggesting that these neuro-active amines are released as a signal to counter stress.

1.5.1 Neurohormones in stress response

Neurohormones are the master regulators of all life processes especially in insects and employ a strategy of triggering anti-stress events. Various stressors of different intensity cause specific changes which influence the neurosecretory neurons activity and synthesis of neurohormones (biogenic amines, ecdysiotropins, ecdysiostatins, alloregulatory neurohormones, adipokinetic neurohormones etc.) (Perić-Mataruga et al., 2006). While, there are numerous neurohormones involved in the concerted response to stress, the following paragraphs will focus only on biogenic amines with particular emphasis on dopamine since that is more germane to the work presented in this thesis.

1.5.2 Biogenic amines in stress response

In the central nervous system of both vertebrates and invertebrates, biogenic amines are important neuroactive molecules. Catecholamines were first detected in insects by Oestlund (1954). Changes in the level of biogenic amines caused by unfavorable conditions are important for stress adaptation particularly in insects (Grutenko et al., 2004). The biogenic amines octopamine (OA) and dopamine (DA) are involved in responses to stressors (Bicker and Menzel, 1989; Ivanovic, 1991; Roeder, 1999; 2005). The fruit fly *Drosophila* makes an ideal model to study the neural role of biogenic amines. Molecules such as DA, serotonin, tyramine, and octopamine are known to mediate diverse physiological and behavioral processes in the fruit fly, and genes encoding relevant synthetic enzymes and receptors have been identified for each of these

molecules allowing for an easier genetic approach to help bring clarity to the complicated biological phenomena (Hardie and Hirsh, 2006). DA is an important catecholamine that exerts widespread effects on neuronal and non-neuronal tissues (Sakar et al., 2010). In the central nervous system, DA binds to specific membrane receptors presented by neurons, and it plays a vital role in the control of locomotion, learning, working memory, cognition, and emotion (Nieullon and Coquerel, 2003; Benturquia et al., 2008). Signaling pathways of DA are affected by stress (Neckameyer and Weinstein, 2005). Stress has been shown to alter normal dopaminergic neurotransmission, and this stress exposure greatly increases dopaminergic activity (Pani et al., 2000). DA is known to be released from cells within several brain regions in response to various stressors. (D'Angio et al., 1988; Abercrombie et al., 1989). A single strongly aversive stress can result in long-term neurochemical and behavioral changes regulated by mesolimbic dopamine systems (Neckameyer and Weinstein, 2005).

1.5.2.1 Dopamine synthesis

The biosynthesis of DA begins with the amino acid, tyrosine (Figure 1.1). A low-affinity amino acid transport system imports blood-borne tyrosine into the brain, then from the brain extracellular fluid into dopaminergic neurons by high and low affinity amino acid transporters. Once tyrosine is inside the nucleus a cytosolic enzyme, tyrosine hydroxylase (TH), then converts tyrosine into dihydroxyphenylalanine (L-DOPA). This is known as the rate-limiting step in the synthesis of DA. The rate of tyrosine hydroxylation is uninfluenced by the amount of tyrosine available. However, when this enzyme is activated, tyrosine levels can affect the rate at which tyrosine is converted to L-DOPA. Dihydropteridin reductase is an enzyme that is indirectly linked to the

synthesis of dopamine. It catalyzes the re-using of the quinonoid dihydrobiopterin to tetrahydrobiopterin, an important cofactor of tyrosine hydroxylase. Tetrahydrobiopterin is dependent on the activity of GTP-cyclohydrolase-1, another enzyme. The cytosolic conversion of L-DOPA to dopamine is catalyzed by the aromatic amino acid decarboxylase (AADC, dopa decarboxylase). This enzyme is extremely efficient that under normal conditions, low levels will persist within the brain (Elsworth and Roth, 1997). DA is the only neuroactive catecholamine in the pathway initiated by tyrosine hydroxylase in *D. melanogaster*. The biosynthesis pathways of DA are highly conserved between *Drosophila* and higher vertebrates (Barron et al., 2009; Vidal-Gadea and Pierce-Shimomura, 2012).

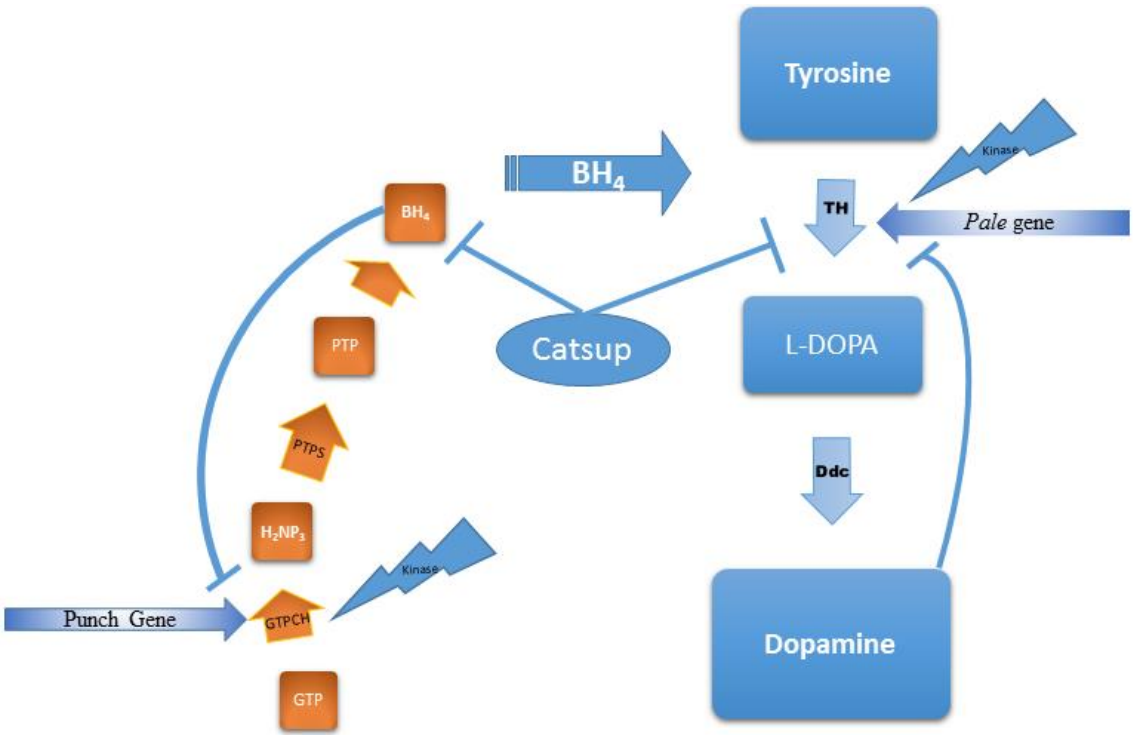

Figure 1.1 Dopamine synthesis pathway.

TH and GTPCH are rate-limiting for their respective pathways. Both are activated by phosphorylation, feedback inhibited by their end products and negatively regulated by Catsup. The *pale* gene encodes for TH, while the *Punch* gene encodes for GTPCH. Abbreviations: **TH**-tyrosine hydroxylase; **Ddc**-dopa decarboxylase; **DA**-dopamine; **GTPCH**-GTP cyclohydrolase; **PTPS**-6-puruvoyl tetrahydropterin synthase; **SR**-sepiaterin reductase; **H_2NP_3**- 7,8-dihydroneopterin triphosphate; **PTP**-6-pyruvoyl tetrahydropterin; **BH_4**- 5,6,7,8-tetrahydrobiopterin; **Catsup** - Catecholamines up.

Dopaminergic signaling pathways are affected by stress. DA is released upon response to varying stressors throughout several brain regions (Abercrombie et al., 1989). However, the discrete steps by which DA potentiates the anti-stress response are largely unknown. Thus, perturbations in catecholamine levels via genetically manipulating various key steps in the synthesis of DA would enable to assess the importance of catecholamines in physiological responses to stress (Chapter II, Hanna et al., 2015).

1.5.2.2 Dopaminergic neurons

Fifteen clusters of DA neurons have been identified distributed throughout the *Drosophila* brain using histofluorescence and immunohistochemistry (Budnik and White, 1988; Nässel and Elekes, 1992). Using a tyrosine hydroxylase *GAL4*-transgene (*TH-GAL4*; Friggi-Grelin et al., 2003) and a UAS-GFP reporter, the dopaminergic neurons were visualized in the brain of *Drosophila* (Figure 1.2). It has been reported that stressors such as paraquat are capable of inducing loss of subsets of dopaminergic neurons (Chaudhuri et al., 2007).

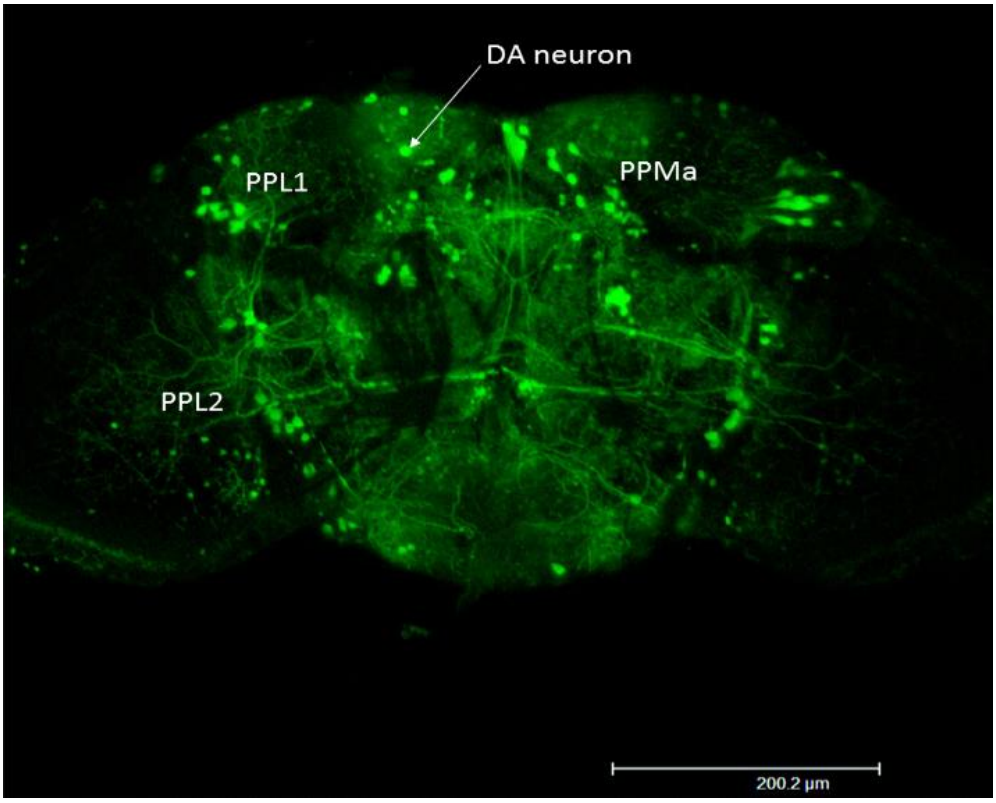

Figure 1.2 Dopaminergic neurons in *Drosophila* brain

(w;TH-Gal4; UAS-GFP).
Posterior view of brain showing Green fluorescent protein (GFP) labeled DA neurons.
PPL: Paired posterior lateral; PPM: Paired posterior medial.

1.5.3 Triggering and regulation of antioxidant systems in response to stress

Antioxidant enzymes are synthesized to intercept and inactivate reactive ROS. These antioxidants are of extreme importance, however they are not completely effective against preventing oxidative damage. Since oxidative damage is inevitable, removal/repair enzymes for proteins, lipids and DNA are generated. Moreover, OS levels are known to vary allowing organisms to adapt to the fluctuation in stress by inducing the synthesis of these enzymes (Davies, 1995).

This intricate defense system of damage removal, replacement and repair, adaptation, growth modulation, and apoptosis make it all possible for aerobic life to live in an oxygen-rich environment (Davies, 2000). Defense mechanisms against free radical-induced oxidative stress involve: (i) preventive mechanisms, (ii) repair mechanisms, (iii) physical defenses and (iv) antioxidant defenses. The antioxidant defense system has a functional connection between its components and thus has been considered as a physiological system (Blagojević and Grubor-Lajšić, 2000). In this section, only the antioxidant defenses elaborated by insects have been dealt with since it is relevant to this thesis.

Insects possess the classical antioxidant system which includes enzymatic scavengers, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (Felton and Summers, 1995). Glutathione S-transferase exhibiting a peroxidase-like activity (GPOx) has also been reported in insects (Ahmad et al., 1989). In addition to these enzymes, a number of non-enzymatic and small molecules are important in scavenging ROS. The non-enzymatic small molecules include glutathione, vitamins C and E, pyruvate, flavonoids, carotenoids, urate and many plant-derived antioxidants.

To have a better understanding of the regulation of stress responses, one must first consider the level of biological organizations in which the responses to adverse environmental changes originate. By doing this, two levels of responses are presented: cell-autonomous and systemic. Cell-autonomous responses consist of molecules operating within cells that enhance the individual cell's chance of survival. In contrast, systemic responses increase the organism's chance of survival. However, in single cell organisms, these two levels of stress response will be one in the same (Johnson and White, 2009). Significant progress has been made in understanding the molecular contributions of cell-autonomous responses. The list of cell-autonomous elements includes well-known and described molecules including the heat-shock proteins (Hsp's), glutathione S-transferase (GST), uncoupling protein, AMP-activated kinase (AMPK), superoxide dismutase (SOD), and many others. Each of these molecules have been highly conserved throughout history (Craig, 1985; Mayer and Bukau, 2005). The prevailing thought concerning the action of these molecules is that they actually serve as sensors and executioners. These molecules are thought to actually sense the stress and its nature, and facilitate the cellular responses to cope with the homoeostatic challenges. For instance, Hsp70 activity is dependent upon structural modifications of the molecular complex, which is influenced by temperature and other stressors (Moro and Muga, 2006; Johnson and White, 2009). The idea behind systemic stress responses is to divert energy away from organs and tissues that are nonessential for survival during homeostatic challenges (Waaij, 2004). Certain cellular stress responders may be impacted differently and coordinated by certain neuro-active amines dependent upon the specific stress situation. There are instances that suggest cell-autonomous activities are differentially connected to

different outputs dependent upon anatomical location. The precise mechanisms linking these two stress response pathways remain somewhat hazy and should still be investigated further (Johnson and White, 2009). The results of such an investigation particularly pertaining to DA and stress response has been presented in Chapter II (Hanna et al., 2015).

1.6 The tip of the ice-berg: Reciprocal links between aging, OS, stress response and neurodegeneration

Aging in *Drosophila* is characterized by a small but across-the-board down-regulation of mitochondrial metabolism and electron transport chain (ETC) genes. This pattern is observed in aging mammalian tissue indicating that aging is a conserved process across species (Landis et al., 2004; Pletcher et al., 2002; Zahn et al., 2006). Normal somatic cells enter an irreversible growth halt after a finite number of cellular divisions and this process is thought to lead to organismal aging (Hayflick and Moorhead, 1961). These senescent cells are associated with high levels of intracellular ROS and accumulated oxidative damage to macromolecules (Chen et al., 1995; Sitte et al., 2000). Oxidatively damaged macromolecules accumulate in every aging organism studied, and oxidative damage is implicated in the etiology of nearly every human aging-related disease (Landis et al., 2004). Correlating evidence suggest stress resistance might be related with both life span and functional senescence. Long lived fly lines selected for late-life reproduction shows enhanced resistance to OS (Arking et al., 1991; Force et al., 1995). Many of these flies have decreased levels of protein and lipid oxidative damage in correlation with an increase in expression or activity of the antioxidant defense genes such as *Sod1* (Cu-Zn SOD), *Sod2* (MnSOD) and *catalase* (Arking et al., 2000). Increased

resistance to paraquat, thermal stress, starvation and desiccation stress was observed in other long lived fly lines as well (Mockett et al., 2001). The idea that OS is involved in age-related functional decline has been directly investigated. Peptide methionine sulfoxide reductase A (MRSA), an enzyme that repairs oxidative damage to proteins was pan-neuronally overexpressed and portrayed extended life span, enhanced locomotor-type behavior in older flies, increased fecundity throughout lifespan and extended fertility periods in females (Ruan et al., 2002). Protein carboxyl methyltransferase (PCMT), and enzyme that also repairs damaged proteins, was ubiquitously overexpressed and reported to have enhanced general locomotor abilities in older flies and extend life span (Chavous et al., 2001). Implications from these studies show links of oxidative damage on proteins and possibly other molecules to locomotor senescence, age-related reproductive declines, and longevity in *Drosophila*. In the subsequent sections an overview of the relationships between stress response, dopaminergic system and aging are presented while briefly touching upon age-related functional senescence since these have been explored more thoroughly and presented in Chapter III of this thesis.

1.6.1 Stress response, dopaminergic system and aging

The relationship among stress response, dopaminergic system and aging is complex and involves the interplay between three major physiological levels: molecular, cellular and neuroendocrine (Roth, 1979). In this context, the molecular level refers to those intracellular biochemical processes directly related to the genome, such as transcription, and how gene expression is modified in response to stress and aging, or a combination of the two. The cellular level refers to the heterogeneous cell populations within complex tissues that proliferate and/or die off during the life-span. A loss or gain

of a particular cell type, or change in the ratio of responsive to non-responsive cells, would likely alter the organism's response to a stressor, as a direct result of the aging process. Thus, changes in the stress response system can either protect against or facilitate the process of aging. The neuroendocrine level refers to the various neurotransmitter and hormonal systems that modulate a host of biological functions, including those systems pre-eminently involved in the organism's response to stress and thus also in the process of aging. Thus, many genetic factors are responsible for the response to stress as well as the aging process, but the interplay between these factors and thus between the organism and its external environment is clearly of paramount importance (Roth, 1979). While catecholamines are critical to surviving a stressor, if persistently elevated or depleted they can cause end-organ damage. Hence, the dysregulation of the neuroendocrine stress-response system (the dopaminergic system) can affect numerous other systems impacting health and longevity during the process of aging. This has been dealt in Chapter III of this thesis.

1.6.2 Stress, the dopaminergic system and its link to neurodegeneration

A continuing matter of investigation in the field of stress research concerns the underlying mechanisms and pathophysiological pathways by which stress possibly influences the onset of diseases. The concrete participation of stress in underlying processes that eventually facilitate these specific disease processes is still found to be controversial. While stress plays an important role in immunological and cardiovascular diseases, the question of stress in connection with its significance in onset, development, and progression of neurological disorders in not as easily understood. This is partly due to the complexity and polymorphism of neurological disorders. A great number of

movement disorders, many of them originating neurologically, are affected by stress and trauma (Grimm, 1996). Neurodegeneration and stress appear to be connected with each other. For example, evidence suggests a linkage between the intensity of the stress response, the rate of age-dependent neurodegeneration, and the individual's life expectancy (Gilad and Gilad, 1995). One of the main factors of neuronal death in neurodegenerative diseases is OS. In particular, the dopaminergic signaling pathways are known to be affected by stress. A single aversive stress can result in a long-term neurochemical and behavioral changes mediated by mesolimbic dopamine systems (Cabid and Puglisi-Allera, 1996). OS is believed to result in neuronal degeneration, and the nigrosriatal dopamine system appears to be particularly vulnerable (Ueda et al., 2000). DA is released from cells in various brain regions in response to diverse stressors (D'Angio et al., 1988; Abercrombie et al., 1989; Neckameyer and Weinstein, 2005). Several studies on Parkinson's and Alzheimer's disease have revealed that the level of natural antioxidants, GSH (glutathione) are lower, while the levels of the oxidative damage markers, lipid peroxidation (LPO), keto-protein formation and DNA oxidation are higher in these patients (Haddadi, 2014; Vaya, 2013), creating an example of the link between anti-oxidative stress responses and neurodegeneration.

1.6.3 Age related functional senescence

Aging is the progressive intrinsic deterioration in physiological status that culminates in death (Arking, 1998). A progressive loss of mitochondrial energetic capacity is also a common hallmark of aging (Wallace, 2005). This may result from a decline in the expression of genes important for mitochondrial electron transport chain function due to aging. This function is conserved throughout diverse organisms including

humans (Rera et al., 2011). A wide range of behaviors in adult *Drosophila* can be quantitatively assessed in the laboratory (Conolly and Tully, 1998). As adult flies age they develop deficits in many of these behaviors including locomotion, olfaction learning and circadian rhythmicity. Studies showing genetic or environmental disruption of the circadian system which leads to premature aging and age-related pathologies reveal the importance circadian regulation has on organismal health. For example, mice lacking the clock protein BMAL1 (homolog of fly CYC protein) show several symptoms of aging, and loss of BMAL1 in the brain may lead to neurodegeneration (Kondrotov et al., 2006; Kondrotova and Kondratov, 2012; Musiek et al., 2013). In flies, a null mutation in the clock gene *per* leads to an increase accumulation of ROS, protein carbonyls, and peroxidated lipids during aging eluding to the idea that antioxidant defenses are compromised by the loss of clock function. (Krishnan et al., 2009; Krishnan et al., 2012). An emerging concern pertaining to circadian clocks involves the regulation of antioxidant defenses and cellular redox (Patel et al., 2014). Studies in *Drosophila* have shown that the levels of ROS and oxidatively damaged (carbonylated) proteins fluctuate in a daily rhythm in the heads of wild type flies and that susceptibility to oxidative challenge is gated by the circadian clock (Krishnan et al., 2009). In this study emphasis has been laid on the deterioration of circadian rhythmicity with age, which has been taken as a read-out of functional senescence (Chapter III).

1.6.4 Links between the dopaminergic and circadian systems

Circadian clocks are endogenous molecular mechanisms that coordinate daily rhythms in gene expression, cellular activities and physiological functions with external day/night cycles. These rhythms enable organisms to adapt to predictable daily

alterations in the environment that are caused by the rotation of the earth are keep a period of approximately 24 hours. Animals are known to adapt to sleep wake cycles by selecting preferred times of sleep and wake. The central clock mechanism, located in the suprachiasmatic nucleus (SCN) is the same in both nocturnal and diurnal mammals. This endogenous time keeping mechanism is controlled at the molecular level by transcription factors, which provide a key advantage to organisms allowing them to prepare in advance for daily environmental changes. The molecular circadian clock also depends on species-specific clock genes and proteins that interact in complex feedback loops to rhythmically control gene transcription (Hardin, 2005; Schibler, 2006). Studies show there are definite links between the dopaminergic system and the circadian sleep wake cycles. Dopamine plays a vital role in initiating arousal in *D. melanogaster* (Kume et al., 2005). Light driving arousal involves a specific group of circadian neurons, known as the large ventral lateral neurons. However, the exact neural circuits that regulate sleep and arousal as well as their integration with circadian circuits remain unclear (Shang et al., 2008). Moreover, recent evidence suggests bidirectional relationships between circadian rhythms and aging. One of the hallmarks of aging is the disruption of sleep/ activity patterns and dampened melatonin oscillations in aged rodents and humans (Turek et al., 1995; Huang et al., 2002; Oster et al., 2003; Hoffman and Swaab, 2006). Fragmentation of sleep/activity was also reported in aged flies (Koh et al., 2006), suggesting that the effects of aging on circadian system are evolutionarily conserved.

Given the role of dopamine in arousal response, we felt it imperative to examine the impact of elevations or depletion of DA or its transport on the molecular clock mechanism and we used circadian locomotor activity pattern as a read-out of the robust

function of the clock (see Chapter II). Subsequently, in Chapter III locomotor activity rhythm decline with age was used as a marker of senescence in DA synthesis and transport mutants.

1.6.5 Aging and its link to neurodegeneration

Aging is marked by a high degree of variability, with some individuals exhibiting little-to-no loss of function ('successful' aging), while others suffer significant loss of function ('unsuccessful' aging). Among the genetic and environmental factors linked with unsuccessful aging, exposure to stress (i.e. a real or perceived threat to homeostasis or well-being), and dysregulated physiological responses to stress have received considerable attention over the past several decades. Aging is a major risk factor for neurodegenerative diseases. Many age-related neurodegenerative diseases are characterized by accumulation of disease-specific misfolded proteins in the cells and tissues of the central nervous system (van Ham et al., 2009). When a cell is facing intrinsic and environmental stressors, the task of maintaining protein homeostasis, or proteostasis is critical. Proteostasis is described as the global regulation of transcription, translation, folding, trafficking, processing, assembly/disassembly, localization and degradation. Thus influencing specific cellular functions enables differentiated cells to change their physiology for successful organismal development and aging when faced with various stress challenges to prevent disease onset (Balch et al., 2008; Douglas and Dillin, 2010). Numerous neurodegenerative diseases are characterized by the misfolding and accumulation of select proteins in insoluble inclusions or aggregates. Clinical onset will not typically occur until middle age or later dependent upon the disease arising from a hereditary or sporadic standpoint. Therefore, neurons can sustain conformationally

challenged, disease proteins in benign states for decades until age-associated alterations in cellular homeostasis predispose different neuronal populations to neurotoxicity (Douglas and Dillin, 2010). However, the molecular mechanisms that link aging and neurodegeneration together are poorly understood. A way to further enrich our understanding of this connection would be to identify the molecular events and pathways that integrate the two. Evidence suggests that a decline in the ability to prevent and eliminate protein misfolding and impaired mitochondrial function are important factors in the increased risk of disease and death associated with aging (Rana et al., 2013). Based on the results of the study presented in Chapter III, the links between aging and its implications to neurodegenerative phenotypes have been discussed in Chapter IV.

1.7 Common pathways and transcription factors involved in oxidative stress response and aging

Since one of the proximal causes of aging may be oxidative damage, oxidative damaged macromolecules accumulate in every aging organism examined, and oxidative damage is implicated in the etiology of practically every human aging-related disease (Landis et al., 2004). It was also demonstrated gene expression changes associated with aging was more similar to the stresses associated most with OS (hyperoxia, hydrogen peroxide, ionizing radiation) (Landis et al., 2012). Thus, it is hypothesized that the pathways triggered in OS response ultimately control the transcriptional processes that maintain cellular homeostasis and these can also deteriorate with age. Cumulatively looking at the gene expression levels of these specific transcription factors, as well as various other parameters among the multiple dopamine phenotypes used within this study

will help to piece together the molecular links between stress response, its relation to aging and implications for neurodegeneration.

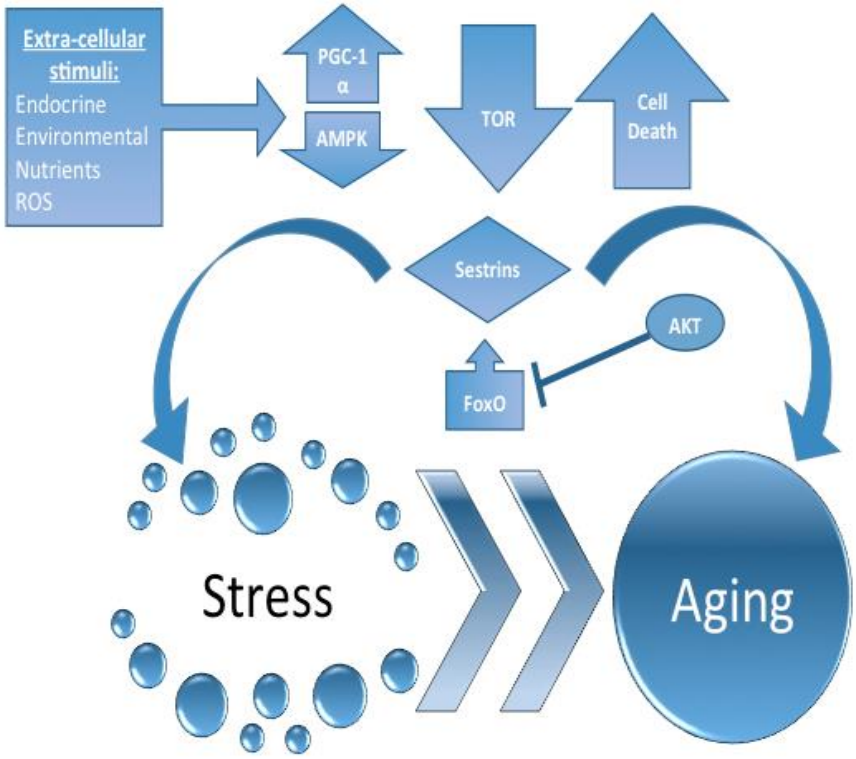

Figure 1.3 A schematic of the relationship between various transcription factors involved in stress response and aging

(For details see Section 1.7).

Numerous signaling pathways and transcription factors have been discovered and insinuate a certain correlation to these processes. A schematic relationship between the various transcription factors in stress and aging has been provided (Figure 1.3). This section will describe the various transcription factors targeted in the study presented in Chapter III.

1.7.1 PGC-1

In recent years, the peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) family of transcriptional co-activators have emerged as key regulators of energy metabolism, including mitochondrial biogenesis and respiration that has been implicated in numerous pathogenic conditions such as neurodegeneration (Rera et al., 2011). It has been reported that an overexpression of *Drosophila* PGC-1 homolog in the digestive tract, can extend adult lifespan (Rera et al., 2011). This up-regulation annuls the early activation of intestinal stem cell proliferation and delays the accumulation of mis-differentiated cells in the intestinal epithelium, which are two signs of aging in this specific tissue. Moreover, up-regulating this gene provides beneficial intestinal functionality against aging in the fruit fly, thus proving the importance of *d*PGC-1 gene activity during aging at the tissue and organismal level (Rera et al., 2011). It has also been found that impairments of this gene play a role in neurodegenerative diseases such as Huntington's and Parkinson's (McGill and Beal, 2006; Shin et al., 2011; Robinson et al., 2014).

1.7.2 FOXO

The forkhead transcription factors (FOXO) are proteins which share an importance throughout pathways regulating numerous cellular functions involved in differentiation, metabolism, proliferation and survival. The function of these molecules is securely controlled by an array of protein-protein interactions and posttranslational modifications including phosphorylation, acetylation and ubiquitination (Obsil and Obsilova, 2008). Numerous mechanisms connect the activation of FOXO transcription factors by OS signals (Brunet et al., 2004; Furukawa-Hibi et al., 2005; Cartert and

Brunet, 2007). OS causes c-Jun N-terminal kinases (JNK) to phosphorylate FOXO proteins leading to its nuclear translocation and activation. The exact opposite reaction occurs when protein kinase A/ phosphoinositide kinase 3 (Akt/PI3) mediates the phosphorylation of FOXO, which then results in the removal of FOXO factors from the nucleus and into the cytoplasm resulting in inhibition of the transcription activity of FOXO. This nuclear translocation of FOXO has led to assumptions involving cellular protection against OS using the transcriptional mechanisms of manganese superoxide dismutase (*MnSOD*) and catalase (*Cat*) gene expression (Glauser and Schelegel, 2007).

1.7.3 Akt

Akt is a serine/threonine kinase that plays an important role in merging cellular responses to growth factors and other extracellular signals (Kandel and Hay, 1999). Akt is activated through the phosphoinositide 3-kinase pathway. It is also an anti-apoptic protein that is activated in response to oxidative injury as well as stresses known to induce OS and toxicity (Ma, 2010).

1.7.4 AMPK

5'-AMP-activated protein kinase (AMPK) is another kinase involved in OS, which is thought to be a sensor and regulator of energy balance at the cellular level responsive to hormonal and nutrient signals. Cellular stress will typically activate this kinase resulting in ATP depletion and an increased AMP:ATP ratio (Kahn et al., 2005; Fuentes et al., 2013). Moreover, efficient control of energy metabolic homeostasis, enhanced stress resistance, and qualified cellular housekeeping are the hallmarks of improved healthspan and extended life span. It has been demonstrated that the AMP-

activated protein kinase controls the aging process via an integrated signaling network (Salminen and Kaarniranta, 2012). This protein transcriptionally inhibits a homolog of the target of rampamycin (Lee et al., 2010).

1.7.5 Sestrin

Sestrins are a group of proteins that were originally discovered in mammals that function antioxidants (Peeters et al., 2003; Budanov et al., 2004). These conserved proteins are thought to be transcriptionally activated by FOXO and in return, increase the levels of AMPK (Budanov and Karin, 2008). However, the precise mechanism is not completely understood. Sestrin also functions as a feedback inhibitor of TOR that prevents age-related pathologies (Lee et al., 2010).

1.7.6 TOR

Target of rapamycin proteins (TOR) are members of the phosphatidylinositol kinase-related kinase (PIKK) family and are extremely conserved throughout organisms, from yeast to mammals. TOR proteins merge signals from growth factors, nutrients, stress and cellular energy levels to control the growth of cells (Inoki et al., 2005) and also in the ageing process (McCormick et al., 2011). This protein is also a player in the FOXO-Sestrin-AMPK-TOR pathway found in *Drosophila*. FOXO is known to induce the transcriptional activation of sestrin, which leads to an increased amount of AMPK, the energy sensor that results in an inhibition of TOR (Lee et al., 2010). Thus, offering protection for the organism against OS (Bednarova et al., 2015).

1.8 A case for sexual dimorphism in stress response and aging

Animals from a broad variety of taxa show sex differences in both stress response as well as lifespan, yet a general biological explanation for this phenomenon is lacking (Williams et al., 1957; Bonduriansky et al., 2008). These examples of sex differences in stress response and neuronal pathways lead to differences in susceptibility to a broad range of neurodegenerative diseases (Kelly et al., 1999). For example, gene expression is sexually dimorphic during brain development, adult life and aging (Bangasser and Valentino, 2012). These differences are orchestrated by the interplay between genetic, hormonal and environmental influences. However, the molecular mechanisms that underpin these differences has not been fully elucidated. Many studies have suggested that gender specific susceptibility for the development of a psychiatric illness as a result of stressful experiences (Figueira and Ouakinin, 2010; Oldehinkel and Bouma, 2011). In addition, a large degree of sexual dimorphism is also observed in several of the health consequences resulting from environmental stressors (Dedovic et al., 2009). In the *D. melanogaster* model, the stress response differs based on gender and the level of sexual maturity and the stress paradigm (Neckameyer and Matsuo, 2008; Neckameyer and Nieto-Romero, 2015). Sex differences in the stress response have also been shown in mammals; for example, male and female rats exposed to chronic mild stress or a forced swim test display differences in dopaminergic activity in discrete brain regions (Dalla et al., 2008). Inappropriate perinatal exposure to glucocorticoids has also been shown to perturb midbrain DA circuitry in a sex-specific manner (McArthur et al., 2007). It has also been reported that mitochondrial maintenance failure during aging exhibits sexual dimorphism (Tower, 2014). These studies and other observations across species suggest

that there is an increasing need to understanding the mechanisms for sexually-dimorphic stress responses which may eventually lead to improved interventions in human age-related diseases based on sex. Thus, gender was an important consideration when the role of DA in aging and senescence was studied (Chapter III).

1.9 Hypothesis and Objectives

This study explores the emerging ill-understood links between the dopaminergic regulatory pathway and its relation to neurodegeneration when faced with stressors or during aging. The overarching hypothesis is that there may be some type of cross-talk between the dopaminergic system, stress-response and aging. The degree of such interaction would determine the development of an efficient response to counter stress and the development of neurodegenerative symptoms during aging. The genetically tractable model organism, Drosophila melanogaster is the model of choice to investigate various aspects involved in the dopaminergic system since this organism is highly studied throughout the past century. The fruit fly has served as an excellent model system for understanding molecular mechanisms and pathogenesis involved in neurodegenerative diseases. Given the conserved nature of the dopaminergic pathway between Drosophila and humans, there is a likelihood that the results produced from this study may have translational significance for humans in the sense of having a greater understanding of the process of aging and the molecular cascade involved in neurodegenerative pathologies.

1.9.1 Objective 1: Exploring perturbations in dopamine synthesis and how they lead to discrete physiological effects and impact oxidative stress response.

Longevity and healthspan are closely correlated with the ability to resist/tolerate stress. Thus, by exposing DA mutant flies (gain-of-function or loss-of-function) to a

stressor such as oxidative stress, and monitoring survival response to stressors at the organismal level and as well as at the biochemical and molecular level will allow us to uncover the connections between the DA regulatory system and stress response pathway.

1.9.2 Objective 2: Determine the sexually dimorphic role of dopamine in aging and its implications to neurodegenerative diseases.

The sexually dimorphic role of the DA system was explored in the context of aging and senescence, to understand the pathologies leading to neurodegenerative phenotypes. An age-related biomarker of oxidative damage (Protein carbonyls) as well as expression of specific genes related to stress response pathways and aging was measured in DA mutants of both sexes at select time points during aging. This will bring clarity to the specific molecular pathways affected in a sexually dimorphic manner and also help unveil the links between aging, sex and neurodegenerative symptoms.

1.10 References

Abercrombie, E.D., Keefe, K.A., DiFrischia, D.S., Zigmond, M.J., 1989. Differential effect of stress on *in vivo* dopamine release in striatum, nucleus accumbens, and medial frontal cortex. *J. Neurochem*. 52: 1655-1658.

Ahmad, S., Beilstein, M.A., Pardini R.S., 1989. Glutathione peroxidase activity in insects: A reassessment. *Arch. Insect Biochem. Physiol*. 12: 31-49.

Arking, R., Buck, S., Berrios, A., Dwyer, S., Baker, G.T.D., 1991. Elevated paraquat resistance can be used as a bioassay for longevity in a genetically based long-lived stain of *Drosophila. Dev. Genet*. 12: 362-370.

Arking, R., Burde, V., Graves, K., Hari, R., Feldman, E., Zeevi, A., Soliman, S., Saraiya, A., Buck, S., Vettraino, J., Sathrasala, K., Wehr, N., Levine, R.L., 2000. Forward and reverse selection for longevity in *Drosophila* is characterized by alteration of antioxidant gene expression and oxidative damage patterns. *Exp. Gerontol.* 35: 167-185.

Balch, W.E., Morimoto, R.I., Dillin, A., Kelly, J.W., 2008. Adapting proteostasis for disease intervention. *Science* 319: 916-919.

Bangasser, D.A., Valentino, R.J., 2012. Sex differences in molecular and cellular substrates of stress. *Cell Mol. Neurobiol.* 32: 709-723.

Barja, G., 2004. Free radicals and aging. *Trends Neurosci.* 27: 595-600.

Barron, A.B., Sovik, E., Cornish, J.L., 2010. The roles of dopamine and related compounds in reward-seeking behavior across animal phyla. *Front Behav. Neurosci.* 4: 1-9.

Bednarova, A., Kodrik, D., Krishnan, N., 2015. Knockdown of adipokinetic hormone synthesis increases susceptibility to oxidative stress in *Drosophila* o a role for dFoxO? *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* 171: 8-14.

Benturquia, N., Courtin, C., Noble, F., Marie-Claire, C., 2008. Involvement of D1 dopamine receptor in MDMA-induced locomotor activity and striatal gene expression in mice. *Brain Res.* 1211: 1-5.

Bicker, G., Menzel, R., 1989. Chemical codes for the control of behavior in arthropods. *Nature.* 337: 33-39.

Blagojević, D.P., Grubor-Lajšić, G., 2000. Multifunctionality of antioxidant system in insects. *Arch. Biol. Sci.* 52: 185-194.

Bonduriansky, R., Maklakov, A., Zajitschek, F., Brooks, R., 2008. Sexual selection, sexual conflict and the evolution of ageing and lifespan. *Funct. Ecol.* 22: 443-453.

Boveris, A., Oshinom N., Chance, B., 1972. Cellular production of hydrogen peroxide. *Biochem. J.* 128: 617-630.

Brunet, A., Sweeny, L., Sturgill, J.F., Chua, K.F., Greer, P., Lin, Y., et al 2004. Stress dependent regulation of FOXO transcription factors by SIRT1 deacetylase. *Science* 303: 2011-2015.

Budanov, A.V., Sablina, A.A., Feinstein, E., Koonin, E.V., Chumakov, P.M., 2004. Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. *Science* 304: 596-600.

Budanov, A.V., Karin, M., 2008. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. *Cell* 134: 451-460.

Budnik, V., White, K., 1988. Catecholamine-containing neurons in *Drosophila melanogaster*: distribution and development. *J. Comp. Neurol.* 268: 400-413.

Butterfield, D.A., Kanski, J., 2001. Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. *Mech. Age. Dev.* 122: 945-962.

Butterfield, D.A., Stadtman, E.R., 1997. Protein oxidation process in aging brain. *Adv. Cell Aging Gerontol*. 2: 161-191.

Cabib, S., Puglisi-Allegra, S., 1996. Stress, depression and the mesolimbic DA system. *Psychopharmacol* (Berl) 128: 331-342.

Carter, M.E., Brunet, A., 2007. FOXO transcription factors. *Curr. Biol.* 17: R113-R114.

Chance, B., Sies, H., Boveris, A., 1979. Hydroperoxide metabolism in mammalian organs. *Pysiol. Rev.* 59: 527-605.

Chaudhuri, A., Bowling, K., Funderburk, C., Lawal, H., Inamdar, A., Wang, Z., O'Donnell, J.M., 2007. Interaction of genetic and environmental factors in a *Drosophila* parkinsonism model. *J. Neurosci.* 27: 2457-2467.

Chavous, D.A., Jackson, F.R., O'Connor, C.M., 2001. Extension of the *Drosophila* lifespan by overexpression of a protein repair methltransferase. *Proc. Natl Acad. Sci.* USA 98: 14814-14818.

Chen, Q., Fischer, A., Reagan, J.D., Yan, L.J., Ames, B.N., 1995. Oxidative DNA damage and senescence of human diploid fibroblasts cells. *Proc. Natl. Acad. Sci.* USA 92: 4337-4341.

Connolly, J.B., Tully, T., 1998. Behavior, learning and memory. In Roberts, D.B., (Ed.), *Drosophila*, A practical approach. IRL Press at Oxford University Press, Oxford, 1998, pp. 265-317.

Craig, E.A., 1985. The heat shock response. *Critical Rev. Biochem. Mol. Biol.* 18: 239-280.

Dalla, C., Antoniou, K., Kokras, N., Drossopoulou, G., Papathanasiou, G., Bekris, S., Daskas, S., Papadopoulou-Daifoti, Z., 2008. Sex differences in the effects of two stress paradigms on dopaminergic neurotransmission. *Physiol. Behav.* 93: 595-605.

D'Angio, M., Serrano, A., Driscoll, P., Scatton, B., 1988. Stressful environmental stimuli increase extracellular DOPAC levels in the prefrontal cortex of hypoemotional (Roman high-avoidance) but not hyperemotional (Roman low-avoidance) rats. An *in vivo* voltametric study. *Brain Res.* 451: 237-247.

Davies, K.J., 1995. Oxidative stress: the paradox of aerobic life. *Biochem. Soc. Symp.* 61: 1-31.

Davies, K.J.A., 2000. An overview of oxidative stress. *IUBMB* 50: 241-244.

Dedovic, K., Wadiwalla, M., Engert, V., Pruessner, J.C., 2009. The role of sex and gender socialization in stress reactivity. *Dev. Psychol.* 45: 45-55.

Douglas, P.M., Dillin, A., 2010. Protein homeostasis and aging in neurodegeneration. *J. Cell. Bio.* 190: 719-729.

Elsworth, J.D., Roth, R.H., 1997. Dopamine synthesis, uptake, metabolism, and receptors: relevance to gene therapy of Parkinson's disease. *Exp. Neruol.* 144: 4-9.

Felton, G. W., Summers, C.B., 1995. Antioxidant systems in insects. *Arch. Insect Biochem. Physiol*. 29: 187-197.

Figueira, M.L., Ouakinin, S., 2010. Gender-related endocrinological dysfunction and mental disorders. *Curr. Opin. Psychiatry* 23: 369-372.

Force, A.G., Staples, T., Soliman, S., Arking, R., 1995. Comparative biochemical and stress analysis of genetically selected *Drosophila* strains with different longevities. *Dev. Genet.* 17: 340-351.

Fraga, C.G., Shigenega, M.K., Parks, J.W., Degan, P., Ames, B.N., 1990. Oxidative damage to DNA during aging: 8-Hydroxy-2'-deoxyguanosine in rat organ DNA and urine. *Proc. Natl. Acad. Sci.* USA 87: 4533-4537.

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., Birman, S., 2003. Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol*. 54: 618-627.

Fucci, L., Oliver, C.N., Coon, M.J., Stadtman, E.R., 1983. Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implications in protein turnover and aging. *Proc. Natl. Acad. Sci.* USA 80: 1521-1525.

Fuentes, E.N., Safian, D., Einarsdottir, I.E., Valdés, J.A., Elorza, A.A., Molina, A., Björnsson, B.T., 2013. Nutritional status modulates plasma leptin, AMPK and TOR activation, and mitochondrial biogenesis: Implications for cell metabolism and growth in skeletal muscle of the fine flounder. *Gen. Comp. Endocrinol.* 186: 172-80.

Gilad, G.M., Gilad, V.H., 1995. Strain, stress, neurodegeneration and longevity. *Mech. Ageing Dev.* 78: 75-83.

Glauser, D.A., Schlegel, W., 2007. The emerging role of FOXO transcription factors in pancreatic beta cells. *J. Endocrinol.* 193: 195-207.

Grimm, R.J., 1996. A discussion of movement disorders. *Nurse Pract. Forum* 7: 154-159.

Grutenko, N.E., Chentsova, N.A., Andreenkova, E.V., 2004. The effect of mutations altering biogenic amine metabolism in *Drosophila* viability and the response to heat stress. *Arch. Insect Biochem. Physiol.* 55: 55-67.

Haddadi, M., Jahromi, S.R., Sagar, B.K.C., Patil, R.K., Shivanandappa, T., Ramesh, S.R., 2014. Brain agining, memory impariment and oxidative stress: A study in *Drosophila melanogaster*. *Behav. Brain Res.*259: 60-69.

Hanna, M.E., Bednářová, A., Rakshit, K., Chaudhuri, A., O'Donnell, J.M., Krishnan, N., 2015. Perturbations in dopamine synthesis lead to discrete physiological effects and impact oxidative stress response in *Drosophila*. *J. Insect Physiol.* 73: 11-19.

Hardie, S.L., Hirsh, J., 2006. An improved method for the separation and detection of biogenic amines in adult *Drosophila* brain extracts by high performance liquid chromotography. *J. Neruosci. Methods* 153: 243-49.

Hardin, P.E., 2005. The circadian timekeeping system of *Drosophila*. *Curr. Biol.* 15: R714-722.

Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. *J Gerontol.* 11: 298–300.

Hayflick, L., Moorhead, P.S., 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 25: 585-621.

Hofman, M.A., Swaab, D.F., 2006. Living by the clock: the circadian pacemaker in older people. *Ageing Res. Rev.* 5: 33-51.

Huang, Y.L., Liu, R.Y., Wang, Q.S., Van Someren, E.J., Xu, H., Zhou, J.N., 2002. Age-associated difference in circadian sleep-wake and rest-activity rhythms. *Physiol. Behav.* 76: 597-603.

Inoki, K., Ouyang, H., Li, Y., Guan, K-L., 2005. Signaling by Target of Rapamycin Proteins in Cell Growth Control. *Microbiol. Mol. Biol. Rev.* 69: 79-100.

Ivanovic, J., 1991. Metabolic response to stressor. In: Ivanovic J, Jankovic-Hladni M, editors. *Hormones and Metabolism in Insect stress*. Boca Raton, FL, USA: CRC Press. p p.27.

Jennings, B.H., 2011. *Drosophila* – a versatile model in biology & medicine. *Materials today* 14: 190-195.

Johnson, E.C., White, M.P., 2009. Stressed-out insects: Behavioural modifications and hormonal Actions. In: *Hormones, Brain and Behavior*. (2nd Edition) Academic Press; 2: 1069-1096.

Kahn B.B., Alquier, T., Carling, D., Hardie, D.G,. 2005. AMP-activated protein kinase:ancient energy gauge provides clues to modern understanding of metabolism. *Cell. Metab.* 1: 15-25.

Kelly, S.J., Ostowski, N.L., Wilson, M.A., 1999. Gender differences in brain and behavior: Hormonal and neural bases. *Pharmacol. Biochem. Behav.* 64: 655-664.

Kandel, E.S., Hay, N., 1999. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp. Cell. Res.* 253: 210-229.

Kenyon, D.H., 2010. The genetics of ageing. *Nature* 464: 504-512.

Kohen, R., Nyska, A., 2012. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol. Pathol.* 30: 620-650.

Koh, K., Evans, J.M., Hendricks, J.C., Sehgal, A., 2006. A *Drosophila* model for age-associated changes in sleep:wake cycles. *Proc. Natl. Acad. Sci.* USA 103:13843-13847.

Kondratov, R.V., Kondratova, A.A., Gorbacheva, V.Y., Vykhovanets, O.V., Antoch, M.P., 2006. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev.* 20: 1868–1873.

Kondratova, A.A., Kondratov, R.V., 2012. The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci.* 13: 325–335.

Krishnan, N., Kretzschmar, D., Rakshit, K., Chow, E., Giebultowicz, J.M., 2009. The circadian clock gene period extends healthspan in aging *Drosophila melanogaster. Aging* 1: 937-948.

Krishnan, N., Rakshit, K., Chow, E.S., Wentzell, J.S., Kretzschmar, D., Giebultowicz, J.M., 2012. Loss of circadian clock accelerates aging in neurodegeneration-prone mutants. *Neurobiol. Dis.* 45: 1129-1135.

Kume, K., Kume, S., Park, S.K., Hirsh, J., Jackson, F.R., 2005. Dopamine is a regulator of arousal in the fruit fly. *J. Neurosci.* 25: 7377-7384.

Kvetnansky, R., Sabban, E.L., Palkovits, M., 2009. Catecholaminergic systems in stress: structural and molecular genetic approaches. *Physiol. Rev.* 89: 535-606.

Landis, G.N., Abdueva, D., Skvortov, D., Yang, J., Rabin, B.E., Carrick, J., Tavare, S., Tower, J., 2004. Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* USA 101: 7663-7668.

Landis, G., Shen, J., Tower, J. 2012. Gene expression changes in response to aging compared to heat stress, oxidative stress and ionizing radiation in *Drosophila melanogaster*. *Aging* 4: 768-789.

Lansing, M.B., Gardner, W.S., Eadie, B.J., 1993. Catechomalines as potential sub-lethal stress indicators in great lakes macrobenthic invertebrates. *J. Great Lakes Res.* 19: 569-581.

Lapointe, J., Hekimi, S., 2010. When a theory of aging ages badly. *Cell. Mol. Life Sci.* 67: 1-8.

Lee, J.H., Budanov, A.V., Park, E.J., Birse, R., Kim, T.E., et al 2010. Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. *Science* 327: 1223-1228.

Lithgow, G.J., 2006. Stress response and aging. *Drug Discov. Today Dis. Mech.* 3: 27-31.

Liu,Y., Fiskum, G., Schubert, D., 2002. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J. Neurochem.* 80: 780-787.

Loschen, G., Flohe, L., Chance, B., 1971. Respiratory chain linked production in pigeon heart mitochondria. *FEBS Lett.* 18: 261-264.

Ma, Q., 2010. Transcriptional responses to oxidative stress: Pathological and toxicological implications. *Pharmacol. Therap.* 125: 376-393.

Mayer, M., Bakau, B. 2005. Hsp70 Chaperones: Cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* 62: 670-684.

McArthur, S., McHale, E., Gillies, G., 2007. The size and distribution of mid-brain dopaminergic populations are permanently altered by perinatal glucocorticoid exposure in a sex-, region-, and time-specific manner. *Neuropsychopharm.* 32: 1462-1476.

McCormick, M.A., Tsai, S-Y., Kennedy, B.K., 2011. TOR and ageing: a complex pathway for a complex process. *Phil. Trans. R. Soc. B* 366: 17-27.

McEwen, B.S., 2009. The brain is the central organ of stress and adaptation. *NeuroImage* 47: 911-913.

McGill, J.K., Beal, M.F., 2006. PGC-1alpha, a new therapeutic target in Huntington's disease? *Cell* 127: 465-468.

Mockett, R.J., Orr, W.C., Rahmandar, J.J., Sohal, B.H., Sohal, R.S., 2001. Antioxidant status and stress resistance in long- and short-lived lines of *Drosophila melanogaster*. *Exp. Gerontol.* 36: 441-463.

Moro, F., Muga, A., 2006. Thermal adaptation of the yeast mitochondrial Hsp70 system is regulated by the reversible unfolding of its nucleotide excchange factor. *J. Mol. Biol.* 358: 1367-1377.

Musiek, E.S., Lim, M.M., Yang, G., Bauer, A.Q., Qi, L., et al. 2013. Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. *J. Clin. Invest.* 123: 5389–5400.

Nässel, D.R., Elekes, K., 1992. Aminergic neurons in the brain of blowflies and *Drosophila*: dopamine- and tyrosine hydroxylase - immunoreactive neurons and their relationship with putative histaminergic neurons. *Cell Tissue Res*. 267: 147-167.

Neckameyer, W.S., Matsuo, H., 2008. Distinct neural circuits reflect sex, sexual maturity, and reproductive status in response to stress in *Drosophila melanogaster*. *Neuroscience* 156: 841-856.

Neckameyer, W.S., Nieto-Romero, R.A., 2015. Response to stress in *Drosophila* is mediated by gender, age and stress paradigm. *Stress* 1-13.

Neckameyer, W.S., Weinstein, J.S., 2005. Stress affects dopaminergic signaling pathways in *Drosophila melanogaster*. *Stress* 8(2): 1-15.

Nieoullon, A., Coquerel, A., 2003. Dopamine: a key regulator to adapt action, emotion, motivation and cognition. *Curr. Opin. Neurol.* 16: S3-S9.

Obsil, T., Obsilova, V., 2008. Structure/function relationships underlying regulation of FOXO transcription factors. *Oncogene* 27: 2263-2275.

Oestlund, E., 1954. The distribution of catecholamine in lower animals and their effects on the heart. *Acta. Physiol. Scand.* 112: 1-67.

Oldehinkel, A.J., Bouma, E.M.C., 2011. Sensitivity to the depressogenic effect of stress and HPA-axis reactivity in adolescence: A review of gender differences. *Neurosci. Biobehav. Rev.* 35: 1757-1770.

Oliver, C.N., Ahn, B.W., Moerman, E.J., 1987. Age-related changes in oxidized proteins. *J. Biol. Chem.* 262: 5488-5491.

Oster, H., Baeriswyl, S., Van Der Horst, G.T., Albrecht, U., 2003. Loss of circadian rhythmicity in aging mPer1-/-mCry2-/- mutant mice. *Genes Dev.* 17: 1366-1379.

Pani, L., Porcella, A., Gessa, G.L., 2000. The role of stress in the pathophysiology of the dopaminergic system. *Mol. Psych.* 5: 14-21.

Patel, S.A., Velingkaar, N.S., Kondratov, R.V., 2014. Transcriptional control of antioxidant defense by the circadian clock. *Antioxid. Redox Signal* 20: 2997-3006.

Peeters, H., Debeer, P., Bairoch, A., Wilquet, V., Huysmans, C., et al 2003. PA26 is a candidate gene for heterotaxia in humans: identification of a novel PA26-related gene family in human and mouse. *Hum. Genet.* 112: 573-580.

Pérez, V.L., Bokov, A., Van Rammen, H., Mele, J., Ran, Q., Ikeno, Y., Richardson, A., 2009. Is the oxidative stress theory of aging dead? *Biochem. Biophys. Acta.* 1790: 1005-1014.

Perić-Mataruga, V., Nenadović, V., Ivanović, J., 2006. Neurohormones in insect stress: a review. *Arch. Biol. Sci.* Belgrade 58: 1-12.

Pletcher, S.D., Macdonald, S.J., Marguerie, R., Certa, U., Stearns, S.C., Goldstein, D.B., and Partridge, L., 2002. Genome-wide transcript profiles in aging and calorically restricted *Drosophila melanogaster*. *Curr. Biol.* 12: 712-723.

Rana, A., Rera, M., Walker, D.W., 2013. Parkin overexpression during aging reduces proteotoxicity, alters mitochondrial dynamics and extends lifespan. *PNAS* 110: 8638-8643.

Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., Ansari, W.S., Lo. T. Jr., Jones, D.L., Walker, D.W., 2011. Modulation of longevity and tissue homeostasis by the *Drosophila* PGC-1 homolog. *Cell. Metab.* 14: 623-634.

Robinson, A. Grösgen, S., Mett, J., Zimmer, V.C., Haupenthal, V.J., Hundsdörfer, B., Stahlmann, C.P., Slobodskoy, Y., Muller, U.C., Hartmann, T., Stein, R., Grimm, M.O.W., 2014. Upregulating of PGC-1α expression by Alzheimer's disease-associated pathway: presenilin 1/amyloid precursor protein (APP)/intracellular domain of APP. *Aging Cell* 13: 263-272.

Roeder, T., 1999. Octopamine in invertebrates. *Prog. Neurobiol.* 59: 533-561.

Roeder, T., 2005. Tyramine and octopamine: ruling behavior and metabolism. *Annu. Rev. Entomol.* 50: 447-77.

Roth, G.S., 1979. Hormone action during aging: alterations and mechanisms. *Mech. Aging Dev.* 9: 497-514.

Ruan, H., Tang, X.D., Chen, M.L., Joiner, M.L., Sun, G., Brot, N., Weissbach, H., Heinemann, S.H., Iverson, L., Wu, C.F., Hoshi, T., Joiner, M.A., 2002. High-quality life extension by the enzyme peptide methionine sulfoxide reductase. *Proc. Natl. Acad. Sci. USA* 99: 2748-2753.

Salminen, A., Kaarniranta, K., 2012. AMP-activated protein kinase (AMPK) controls the aging process via an integrated signaling network. *Ageing Res. Rev.* 11: 230-241.

Sarkar, C., Basu, B., Chakroborty, D., Dasgupta, P.S., Basu, S., 2010. The immunoregulatory role of dopamine: an update. *Brain, Behav. Immun.* 24: 525-528.

Selye, H., 1973. The evolution of the stress concept. *Am. Scientist* 61: 692-699.

Shang, Y., Griffith, L.C., Rosbash, M., 2008. Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. *Proc. Natl. Acad. Sci.* USA 105: 19587-19594.

Schibler, U., 2006. Circadian time keeping: the daily ups and downs of genes, cells and organisms. *Prog. Brain Res.* 153: 271-282.

Schieber, M., Chandel, N.S., 2014. ROS function in redox signaling and oxidative stress. *Curr. Biol.* 24: R453-R462.

Shin, J.H., Ko, H.S., Kang, H., Lee, Y., Lee, Y.I., Pletinkova, O., Troconso, J.C., Dawson, V.L., Dawson, T.M., 2011. PARIS (ZNF746) repression of PGC-1α contributes to neurodegeneration in Parkinson's disease. *Cell* 144: 689-702.

Sitte, N., Merker, K., Von Zglinicki, T., Grune, T., 2000. Protein oxidation and degradation during proliferative senescence of human MRC-5 fibroblasts. *Free Radic. Biol. Med.* 28: 701-708.

Sohal, R.S., Mockett, R.J., Orr, W.C., 2002. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. *Free Radic. Biol. Med.* 33: 575-586.

Stadtman, E.R., 1992. Protein oxidation and aging. *Science* 257: 1220-1224.

Stadtman, E.R., Oliver, C.N., 1991. Metal-catalyzed oxidation of proteins. *J. Biol. Chem.* 266: 2005-2008.

Stark-Reed, P.E., Oliver, C.N. 1989. Protein oxidation and proteolysis during aging and oxidative stress. *Arch. Biochem. Biophys.* 275: 559-567.

Tower, J., 2014. Mitochondrial maintenance failure in aging and role of sexual dimorphism. *Arch. Biochem. Biophys.* (In Press doi:10.1016/jab.2014.10.008.)

Turek, F.W., Penev, P., Zhang, Y., van Reeth, O., Zee. P., 1995. Effects of age on the cirdadian system. *Neurosci. Biobehav. Rev.* 19: 53-58.

Ueda, S., Aikawa, M., Ishizuya-Oka, A., Yamaoka, S., Koibuchi, N., Yoshimoto, K., 2000. Age-related dopamine deficiency in the mesostriatal dopamine system of zitter mutant rats: Regional fiber vulnerability in the striatum and olfactory tubule. *Neurosci.* 85: 389-398.

Ulrich-Lai, Y.M., Herman, J.P., 2009. Neural regulation of endocrine and autonomic stress responses. *Nat. Rev. Neurosci.* 10: 397-409.

Van Ham, T.J., Breitling, R., Swertz, M.A., Nollen, E.A., 2009. Neurodegenerative diseases: Lessons from genome-wide screens in small model organisms. *EMBO Mol. Med.* 1: 360-370.

Vaya, J., 2013. Exogenous markers for the characterization of human diseases associated with oxidative stress. *Biochimie* 95: 578-584.

Vidal-Gadea, A.G., Pierce-Shimomura, J.T., 2012. Conserved role of dopamine in the modulation of behavior. *Common Integr. Biol.* 5: 440-447.

Waaij, E.H.van der., 2014. A resource allocation model describing consequences of artificial selection under metabolic stress. *J. Animal Sci.* 82: 973-81.

Wallace, D.D., 2005. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* 39: 359-407.

Williams, G.C., 1957. Pleiotropy, natural-selection, and the evolution of senescence. *Evolution* 11: 398-411.

Zahn, J.M., Sonu, R., Vogel, H., Crane, E., Mazan-Mamczarz, K., Rabkin, R., Davis, R.W., Becker, K.G., Owen, A.B., Kim, S.K., 2006. Transcriptional profiling of aging in human muscle reveals a common aging signature. *PLoS Genet.* 2: e115.

CHAPTER II

PERTURBATIONS IN DOPAMINE SYNTHESIS LEAD TO DISCRETE PHYSIOLOGICAL EFFECTS AND IMPACT OXIDATIVE STRESS RESPONSE IN *DROSOPHILA*

2.1 Abstract

The impact of mutations in four essential genes involved in dopamine (DA) synthesis and transport on longevity, motor behavior, and resistance to oxidative stress was monitored in Drosophila melanogaster. The fly lines used for this study were: (i) a loss of function mutation in Catecholamines up (Catsup26), which is a negative regulator of the rate limiting enzyme for DA synthesis, (ii) a mutant for the gene pale (ple2) that encodes for the rate limiting enzyme tyrosine hydroxylase (TH), (iii) a mutant for the gene Punch (PuZ22) that encodes guanosine triphosphate cyclohydrolase, required for TH activity, and (iv) a mutant in the vesicular monoamine transporter (VMATΔ14), which is required for packaging of DA as vesicles inside DA neurons. Median lifespans of ple2, PuZ22 and VMATΔ14 mutants were significantly decreased compared to Catsup26 and wild type controls that did not significantly differ between each other. Catsup26 flies survived longer when exposed to hydrogen peroxide (80 μM) or paraquat (10 mM) compared to ple2, PuZ22 or VMATΔ14 and controls. These flies also exhibited significantly higher negative geotaxis activity compared to ple2, PuZ22, VMATΔ14 and controls. All mutant flies demonstrated rhythmic circadian locomotor activity in general,

albeit Catsup26 and VMATΔ14 flies had slightly weaker rhythms. Expression analysis of some key antioxidant genes revealed that glutathione S-transferase Omega-1 (GSTO1) expression was significantly up-regulated in all DA synthesis pathway mutants and especially in Catsup26 and VMATΔ14 flies at both mRNA and protein levels. Taken together, we hypothesize that DA could directly influence GSTO1 transcription and thus play a significant role in the regulation of response to oxidative stress. Additionally, perturbations in DA synthesis do not appear to have a significant impact on circadian locomotor activity rhythms per se, but do have an influence on general locomotor activity levels.

2.2 Introduction

Dopamine (DA) is a catecholamine that modulates fast neurotransmission in the central nervous systems of both vertebrates and invertebrates. In insects such as the fruit fly, Drosophila melanogaster, DA has several roles in neural functions, from modulation of locomotor behaviors and arousal states, to appetitive and aversive learning and memory (Restifo and White, 1990; Barron et al., 2010; Waddell, 2013). The dopaminergic system in Drosophila is highly rhythmic, as supported by rhythmicity in responsiveness to DA agonists, and by the rhythmic transcription of the rate-limiting enzyme tyrosine hydroxylase (TH) encoded by the gene pale (ple) (Hirsh et al., 2010). pale mutants have been reported to show decreased locomotor activity (Pendleton et al., 2002). DA has been implicated in promoting arousal in Drosophila as well as promoting higher nocturnal activity in the ClkJrk clock mutant (Kumar et al., 2012; Kume et al., 2005). In rats, DA regulates the expression of the clock protein PER2 (Hood et al., 2010). However, precise links between DA synthesis levels and circadian locomotor activity

behavior are unclear. It has been reported that disruptions in the circadian clock network result in increased sensitivity to stress as well as neurodegeneration (Krishnan et al., 2008; Krishnan et al., 2012; Wulff et al., 2010), whereas DA release is triggered from cells in response to diverse stressors (Abercrombie et al., 1989). In particular, oxidative stress is believed to cause neuronal degeneration, and the nigrostriatal DA system appears to be particularly sensitive (Ueda et al., 2000). This begs the question if there are reciprocal links between the two systems in their behavioral and stress responses or if they act independent of each other.

Components of DA biosynthesis are highly conserved across a divergent range of animal phyla and have been well described in mammalian and *Drosophila* systems (Barron et al., 2010). Therefore, investigations on the links between perturbations in DA biosynthesis and their physiological effects on behavioral and stress responsive pathways in *Drosophila* would likely inform its fundamental role in stress physiology in mammalian systems. DA synthesis requires closely regulated cooperation of two enzymatic pathways, and is highly sensitive to external cues. In *D. melanogaster*, tyrosine hydroxylase (TH), encoded by the gene *pale,* converts tyrosine to DA during catecholamine synthesis (Neckameyer and White, 1993). TH catalytic activity requires and is regulated by the cofactor, tetrahydrobiopterin (BH_4). The enzyme GTP cyclohydrolase I (GTPCH) is the initiating and limiting component of BH_4 biosynthesis and therefore also in DA production (Hsouna et al., 2007). Once catecholamines such as DA are produced, they can be transported by vesicular monoamine transporters (VMAT) from the cytoplasm into synaptic vesicles (Greer et al., 2005). Interestingly, DA is also a self-oxidizing catecholamine known to generate reactive oxygen species including H_2O_2,

making catecholaminergic neurons extremely susceptible to higher oxidative stress and more free radical damage than other types of neurons (Graham, 1978; Hald and Lotharius, 2005). *Catecholamines up* (*Catsup*), works as a negative regulator of DA production that acts on TH and GTPCH, both of which are rate-limiting enzymes (Stathakis et al., 1999). Moreover, loss-of-function mutations in *Catsup* hyperactivate TH by a post-translational mechanism that also corresponds to increased catecholamine pool levels. Paradoxically, *Catsup* mutants are resistant to oxidative stress induced by paraquat (Chaudhuri et al., 2007) and have also been reported to cause dominant hyperactivation of both TH as well as GTPCH (Wang et al., 2011). The latter study also established that VMAT was also hyperactivated by Catsup, and the excess DA is both transported into synaptic vesicles and released into the synapse at higher rates in *Catsup* mutants. However, reasons behind the resistance of *Catsup* mutants to oxidative stress remains unclear.

Our objectives in this study were to investigate if perturbations in DA synthesis (by elevating DA pools or reducing DA synthesis) as well as DA transport could impact longevity, behavioral characteristics (circadian locomotor activity and negative geotactic response), and response to oxidative stress in *D. melanogaster*. Insights obtained from this investigation will contribute to a deeper understanding of DA involvement in response to oxidative stress and its link to the circadian clock network.

2.3 Materials and Methods

2.3.1 Drosophila stocks and husbandry

D. melanogaster were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25 °C in 12 h light:12 h dark (LD 12:12) cycles (with an average

light intensity of ~2000 lux). Two different fly lines w^{1118} and Canton-S, which are wild type for the catecholaminergic pathway mutations employed in this study, were used as control strains. No significant differences in longevity, circadian rhythmicity, or response to oxidative stress was observed between these two control fly lines. Mutant fly lines used in this study were as follows: *Catsup*^{26}*/CyO* (Stathakis et al., 1999), a deletion extending 600 bp into the gene from immediately upstream of the start codon produces no detectable protein and was derived from the mobilization of a 5´P-element insertion in *Catsup*^{KO5042}. As *Catsup* mutant alleles are homozygous lethal, all experiments in this study were conducted using heterozygous strains. The *Pu* mutant allele employed in this study was generated in an ethylmethane sulfonate (EMS) mutagenesis screen and the genotype is *dp cn Pu*^{Z22} *a px sp/*SM1. Genetic characteristics of this strain are reported elsewhere (Mackay et al., 1985; Reynolds and O'Donnell, 1988). The homozygous lethal *ple*^{2} is a loss-of-function allele recovered in an EMS screen and the heterozygous mutant *w; ple*^{2}*/TM3 Sb e* was used (Neckameyer and White, 1993). For mutations in the transporter of DA, we used the *VMAT* loss of function mutant *w; VMAT*$^{\Delta 14}$*/CyO*, (Romero-Calderon et al., 2008). All behavioral studies were conducted on mutant heterozygotes crossed into the appropriate wild type background to eliminate balancers. Other stocks used in this study were the UAS-*Catsup* RNAi, w; TH-GAL4 (III) and w; GAL4-*elav* (II). Only male flies were used in this study, since female flies have altered physiological status because of the reproductive development.

2.3.2 Lifespan measurements

For lifespan measurement, 3 cohorts of 80 mated male flies of each genotype (n=240) were housed in 8 oz round bottom polypropylene bottles (Genesee Scientific,

San Diego, CA, USA) inverted over 60 mm Falcon Primaria tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) containing 15 mL of diet. Diet dishes were replaced daily without CO_2, after tapping flies to the bottom of the bottle. Mortality was recorded daily. Median survival of flies was calculated using the Kaplan-Meier survival curve.

2.3.3 Survival in response to oxidative stress

To test the resistance of the fly lines with increased or decreased DA pools, or with impaired transporter function (along with their wild type controls), adult male flies (6-8 days old) were exposed to 80 µM hydrogen peroxide (Krishnan et al., 2008) or 10 mM paraquat in 5% sucrose (Lawal et al., 2010). Untreated controls were exposed to 5% sucrose. In all instances, flies were starved for 6 h before being transferred to vials containing a 22 mm filter paper disks soaked with 80 µM H_2O_2 (HP) or with 10 mM paraquat (PQ, methyl viologen, Sigma Chemical Co, St. Louis, MO) in a 5% sucrose solution. The number of dead flies was scored every 18 h, 24 h, and the experiment was terminated 72 h post exposure. The choice of scoring time was determined by the time of initiation of experiment as well as convenience of access to the incubators housed in the laboratory. HP or PQ was replenished once daily till the end of the experiment. Each vial contained 25 flies per genotype in 3 independent replicates. The percentage of flies that survived at the time of scoring were plotted for each genotype over the course of the experiment. For subsequent experiments related to oxidative stress exposure and gene expression and protein blot analysis, only HP treatment of 4 h was used, since it has been demonstrated earlier to be a potent elicitor of oxidative stress and the exposure time is optimal without leading to mortality (Krishnan et al., 2008).

2.3.4 Rapid iterative negative geotaxis assay (RING)

Genotype-specific negative geotaxis was tested using the RING assay at room temperature (25 ± 1°C) (Gargano et al., 2005). Three groups of 25 male flies (6-8 days old) of each genotype were transferred into empty narrow vials that were loaded onto the RING apparatus. Following a 3 min acclimatization, the apparatus was rapped sharply 3x to initiate a negative geotaxis response. The flies' negative geotaxis climbing movement in tubes was recorded as a movie and digital images captured 4 s after initiating the behavior were used for data analyses. Five consecutive trials were interspersed with at least a 30 s rest. Thus, genotypes involved in the first trial had time to recover before the next trial which involved other genotypes and so on. The average height climbed by all flies in each vial during the 4 s interval was calculated, and the climbing performance was averaged for three vials of a given genotype. To preclude any difference between the groups in exhaustion in the behavioral response during consecutive trials, care was taken to randomize each trial as well as analysis among the replicate vials of each genotype.

2.3.5 Locomotor activity analysis

Flies were entrained in LD 12:12 at 25 °C to acclimatize them to the activity monitoring tubes in the *Drosophila* activity monitor. Locomotor activity of 6-8 days old males was recorded for 3 d in LD 12:12, followed by 10 d in constant darkness (DD) using the Trikinetics locomotor activity monitor (Waltham, MA, USA) as described by Pfeiffenberger et al., (2010). Locomotor activity, as counted by number of infrared beam crossings by the individual flies was collected in 15 min bins and represented as actograms. For daily activity profiles, the number of beam crossings in LD cycles was averaged for the 3 days in LD for all flies of a particular genotype. For a quantitative

measure of circadian rhythmicity in DD, Fast Fourier Transform (FFT) analysis was conducted using CLOCKLAB software (Actimetrics; Coulbourn Instruments, Whitehall, PA, USA). Flies with FFT values <0.04 were classified as arrhythmic, ones with values of 0.04–0.08 were classified as weakly rhythmic, whereas flies with FFT values >0.08 were considered strongly rhythmic. Flies with both weak and strong rhythms that showed a single peak in the periodogram were included in the calculation of the free-running period using the CLOCKLAB software.

2.3.6 Quantitative real-time polymerase chain reaction

Three independent bio-replicates of male flies (6-8 days old) were collected following 4 h exposure to HP stress from each genotype. In parallel, flies from untreated groups were also collected in a similar manner. Total RNA was extracted from whole body homogenates of flies (25) using Tri Reagent (Sigma, St. Louis, MO, USA). The samples were purified and treated with Takara Recombinant DNase I (Clontech Laboratories Inc., Mountain View, CA, USA). Synthesis of cDNA was achieved with the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed on the Eppendorf realplex^2 Mastercycler (Eppendorf, USA) under default thermal cycling conditions, with a dissociation curve step. Every reaction contained Power SYBR Green (Applied Biosystems), 10 ng cDNA, and 400 nM primers. Primer sequences are given in Table A.1. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized to the gene *rp49*. Relative mRNA amplitude was calculated with respect to untreated control wild type w^{1118}, or wild type Canton-S flies whose expression for a particular gene was set as 1.

2.3.7 Western blotting

Three independent bio-replicates of 6-8 days old males of different genotypes were collected following 4 h exposure to HP stress. In parallel, flies from untreated groups were also collected in a similar manner. About 20 flies were homogenized on ice in 50 mM phosphate buffer, sonicated, and centrifuged at 10,000 g for 10 min at 4 °C. The protein content was equalized using the bicinchoninic acid method (Smith et al., 1985) to ensure equal protein loading. Samples were then separated by polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% resolving gel (Laemmli, 1970) followed by transfer onto PVDF Immobilon membranes (Millipore Billerica, MA, USA) and incubated in 1x TBST (10 mM Tris, 0.15 M NaCl, 0.1% Tween-20, pH 7.5) + 5% milk for 2 h. Then the membranes were incubated overnight at 4 °C with primary antibody 1:10,000 for MnSOD (procured from Acris Antibodies, Inc.); and 1:1000 for GST Omega 1 (kind gift from Dr. K. Kim, Seoul National University, Korea), in blocking buffer. Membranes were treated for 2 h with 1:20,000 goat anti-rabbit IRDye680 (LI-COR Biosciences, Lincoln, NE, USA). Blots were scanned using the LI-COR Odyssey Infrared Imaging System (CLx) and imaging software Image Studio v. 3.0 (LI-COR Biosciences, Lincoln, NE, USA). Blots were quantified using ImageJ, v. 1.47 (National Institutes of Health, USA, http://imagej.nih.gov/ij).

2.3.8 Statistical analyses

Lifespan and survival curves were plotted using Kaplan-Meier survival curves and statistical significance of curves assessed using the Log-Rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests (GraphPad Prism v 5.01; GraphPad software Inc. San Diego, CA). For statistical analysis of locomotor activity, mortality to oxidative stress

(the percentage data of surviving flies at each time point were transformed prior to statistical analysis), RING assay, gene expression and western blot analyses post-quantification, one-way ANOVA with Tukey's post-hoc tests were conducted (GraphPad Instat v 3.0).

2.4 Results

2.4.1 Lifespan analysis

The median lifespan (MLS) of *ple*^{2}, *Pu*^{Z22} and *VMAT*$^{\Delta 14}$ mutants was significantly reduced to 58, 61 and 59 d respectively, when compared to wild type controls *w*^{1118} (68 d) and Canton-S flies (70 d) (Figure 1). Increased DA pools in *Catsup*^{26} mutants did not result in enhanced lifespan (MLS ~ 68 d) and the MLS was not significantly different from the control strains. However, this longevity was significantly greater ($p<0.001$) than the *ple*^{2}, *Pu*^{Z22} and *VMAT*$^{\Delta 14}$ mutants (Figure 2.1).

2.4.2 Survival response to oxidative stress

A differential survival response to oxidative stress induced by hydrogen peroxide (HP, 80 µM) or paraquat (PQ, 10 mM) was observed in the fly lines tested (Figure 2.2 A, B). Median survival of wild-type control flies (*w*^{1118} and Canton-S) was only 24 h when exposed to HP, which was similar to *ple*^{2} mutants (Figure 2.2 A). On the other hand, *Catsup*^{26} flies survived longer ~ 45.5 h upon exposure to HP which was not significantly different from *Pu*^{Z22} or *VMAT*$^{\Delta 14}$ flies (43 h) (Figure 2.2 A).

In case of PQ exposure, median survival of control flies (*w*^{1118} and Canton-S) was 43 h which was significantly ($p<0.05$) more than the survival of *ple*^{2} flies (33.5 h) but not significantly different from *Catsup*^{26} (45.5 h), *Pu*^{Z22}, or *VMAT*$^{\Delta 14}$ flies (43 h) (Figure 2.2

B). Since HP was a more potent stressor in terms of average survival duration of control flies, all subsequent experiments were conducted following exposure to HP (see also Section 2.3 for choice of HP).

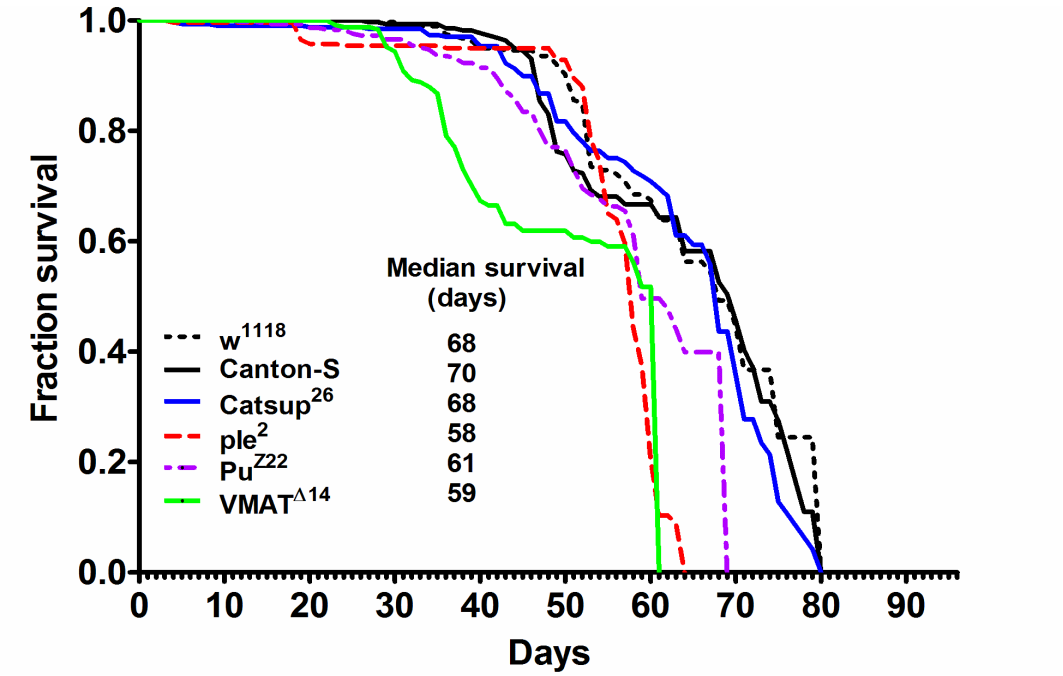

Figure 2.1 Kaplan-Meier survival curves of male flies of w^{1118}, Canton-S, $Catsup^{26}$, ple^{2}, Pu^{Z22} and $VMAT^{\Delta 14}$ strains under 12h light: dark (LD) cycles and *ad libitum* feeding conditions.

Data was obtained from three independent replicates of 80 flies each for each genotype (n=240).

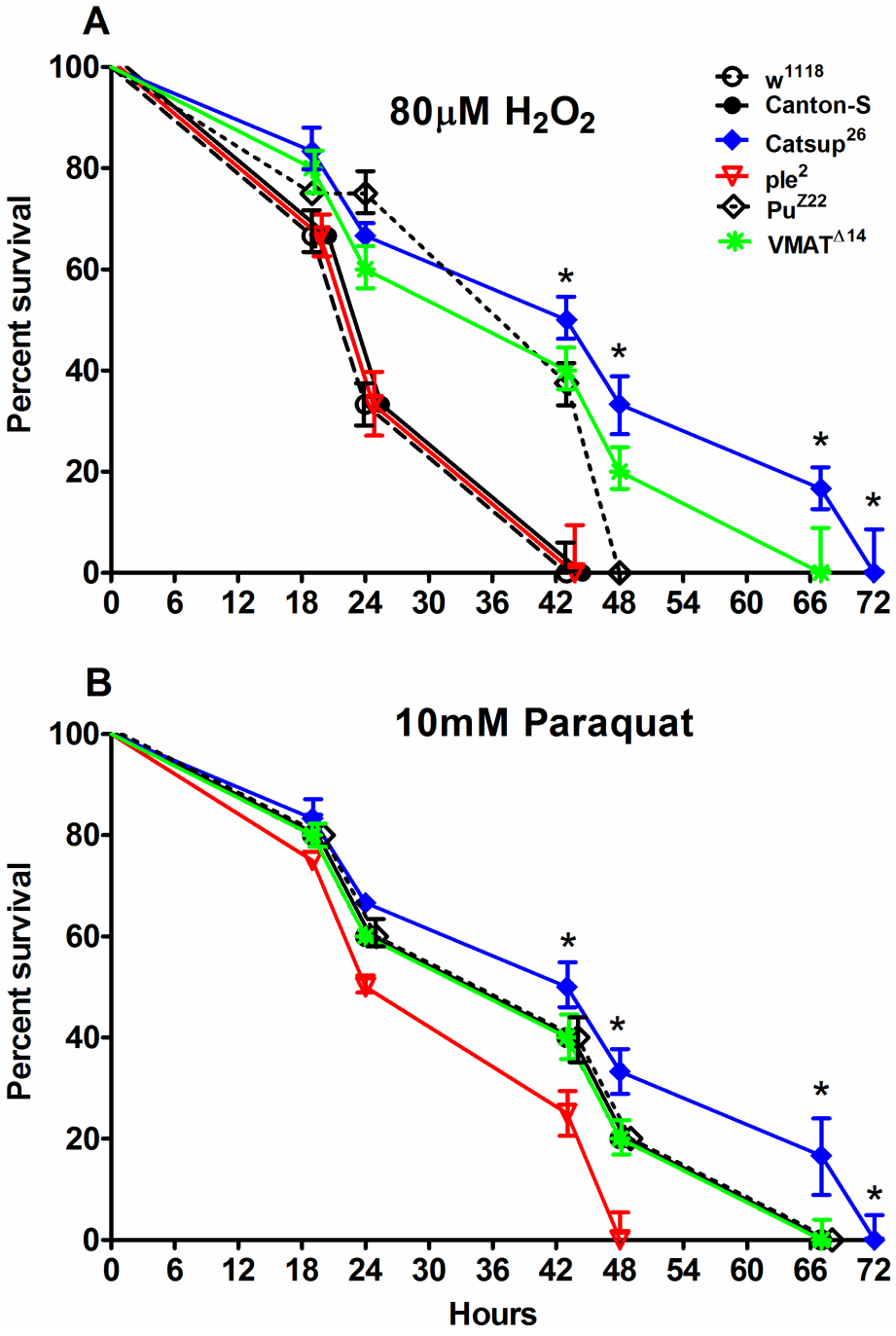

Figure 2.2 Mortality test of the experimental 6-8 days male flies exposed for 72 hours

(**A**) 80μM H_2O_2 (HP) or to (**B**) 10 mM paraquat (PQ).
Mortality curves were plotted and values represent mean survival (%) ± SD at various hours post-exposure. Bars with * are significantly different at $p<0.05$ among genotypes.

2.4.3 Negative geotaxis response

RING assay indicated that *Catsup*^{26} mutants exhibited enhanced climbing ability ($p<0.001$) compared to all other fly lines tested (Figure 2.3). No significant differences in the negative geotaxis response was recorded among control flies, which was however significantly more ($p<0.001$) than the climbing ability displayed by *ple*^{2}, *Pu*^{Z22}, or *VMAT*$^{\Delta 14}$ mutants. *ple*^{2} and *Pu*^{Z22} flies with impaired DA synthesis did not exhibit any difference in the negative geotactic response between each other, but recorded significantly less climbing ability than *Catsup*^{26} or the wild-type controls. The *VMAT*$^{\Delta 14}$ mutants recorded the least climbing ability (Figure 2.3).

2.4.4 Circadian locomotor activity rhythms

Average daily activity of the fly lines tested revealed that *w*^{1118}, *Catsup*^{26} and *VMAT*$^{\Delta 14}$ flies showed the highest daily activity averaging ~830 horizontal beam crossings per day (Table 2.1, Figure 2.4 A, C, F). In contrast, *Pu*^{Z22} flies recorded the least daily activity (415 ± 30) among all fly lines studied (Figure 2.4 E). *ple*^{2} mutants on the other hand showed higher average daily activity (629 ± 27) than *Pu*^{Z22} flies but this was significantly less ($p<0.05$) than any of the other mutants or their controls. Interestingly, average daily activity of Canton-S flies was recorded to be significantly less (731 ± 43, $p<0.05$) than the *w*^{1118}, *Catsup*^{26} or *VMAT*$^{\Delta 14}$ fly lines (Table 2.1, Figure 2.4 A, C, F).

While control flies exhibited 100% rhythmicity in their circadian locomotor activity (Table 2.1), this was marginally reduced in *Pu*^{Z22} mutants (94% rhythmic).

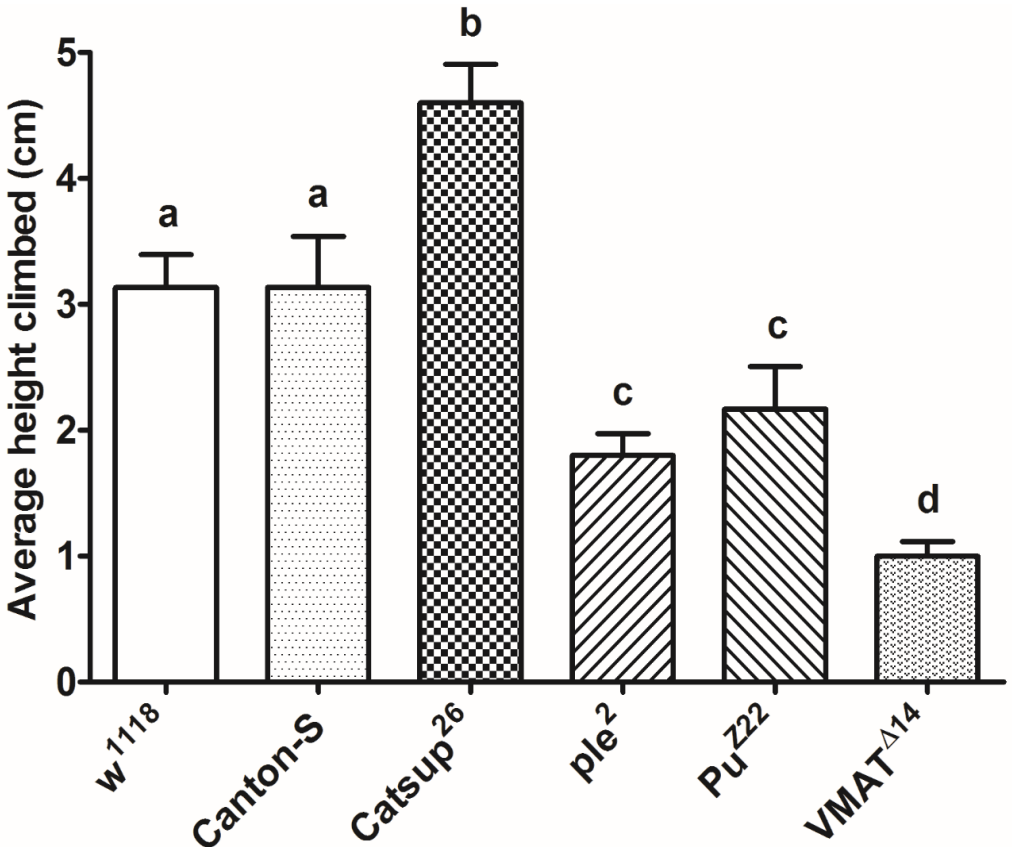

Figure 2.3 Rapid iterative negative geotaxis (RING) assay of experimental 6-8 days old male flies.

Bar graphs represent the average height climbed by each genotype. Average values for each mutant and respective wild-type (w^{1118} and Canton-S) control were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Bars with different superscripts are significantly different at $p<0.001$.

Table 2.1 Data representing the average daily activity per fly, the percentage of rhythmic flies, the strength of the rhythm exhibited by rhythmic flies, the period of rhythm (in constant darkness –DD) and the number of individuals of a genotype tested.

Genotype	Average daily activity/fly	% Rhythmic	FFT (strength of rhythm)	Period of rhythm in DD (Hrs)	Sample size (n)
w^{1118}	836 ± 39^{a}	100	0.18 ± 0.02^{a}	23.7 ± 0.2	32
Canton-S	731 ± 43^{b}	100	0.13 ± 0.08^{b}	23.9 ± 0.2	32
$Catsup^{26}$	813 ± 29^{a}	70*	0.055 ± 0.03^{c}	23.4 ± 0.2	48
ple^{2}	629 ± 27^{c}	67	0.12 ± 0.05^{b}	23.5 ± 0.2	48
Pu^{Z22}	415 ± 30^{d}	94	0.16 ± 0.09^{a}	23.0 ± 0.1	48
$VMAT^{\Delta 14}$	841 ± 43^{a}	75*	0.052 ± 0.7^{c}	24.0 ± 0.7	48

Average daily activity was computed as beam crossings in 15 min bins over 3 days of LD cycles (see Materials and Methods Section 2.5 for more details). Values with different superscripts are significantly different at $p<0.05$ in a column (one-way ANOVA with Tukey's multiple comparison test). Data are represented as mean ± SD. * weakly rhythmic

Elevation of DA pools in $Catsup^{26}$ flies or impaired transporter activity in $VMAT^{\Delta 14}$ flies resulted in decreased strength of rhythm (FFT~0.05) as well as a decrease in number of individuals displaying rhythmic locomotor behavior (Table 2.1). Interestingly Pu^{Z22} mutants displayed strong rhythmic locomotor behavior comparable to w^{1118} wild type flies. There was no marked difference in the period length of rhythms in every fly line investigated.

2.4.5 Expression of antioxidant genes and levels of some key translated products

As mentioned in Section 3.2, for studying gene expression and the translated protein products of antioxidant enzymes, only HP exposure was employed, since it was a more potent stressor than PQ. A general up-regulation in *Catalase* (*Cat*) gene expression was recorded in all the fly lines upon exposure to HP (Figure 2.5 A). In general, $Catsup^{26}$, ple^{2} and Pu^{Z22} mutants showed markedly lower basal levels of *Cat* transcript under

control unstimulated conditions compared to wild type controls as well as to the *VMAT*$^{\Delta 14}$ mutant. A similar pattern was observed in copper/zinc superoxide dismutase (*Cu/Zn SOD*) mRNA levels (Figure 2.5 B). mRNA levels of manganese superoxide dismutase (*MnSOD*) were either unchanged or decreased upon exposure to stress in all the fly lines. Interestingly, the *MnSOD* transcript markedly declined in *Pu*^{Z22} mutants compared to all other fly lines tested upon exposure to HP (Figure 2.5 C). Glutathione S-transferase Omega 1 (*GSTO1*) transcript level was up-regulated in all DA mutant flies as well as *w*^{1118} control upon HP exposure with the exception of Canton-S where we observed a marked down-regulation of mRNA levels upon exposure to stressor (Figure 2.5 D). Interestingly, basal levels of *GSTO1* were markedly higher in *Catsup*^{26} and *VMAT*$^{\Delta 14}$ compared to all other fly lines (Figure 2.5 D).

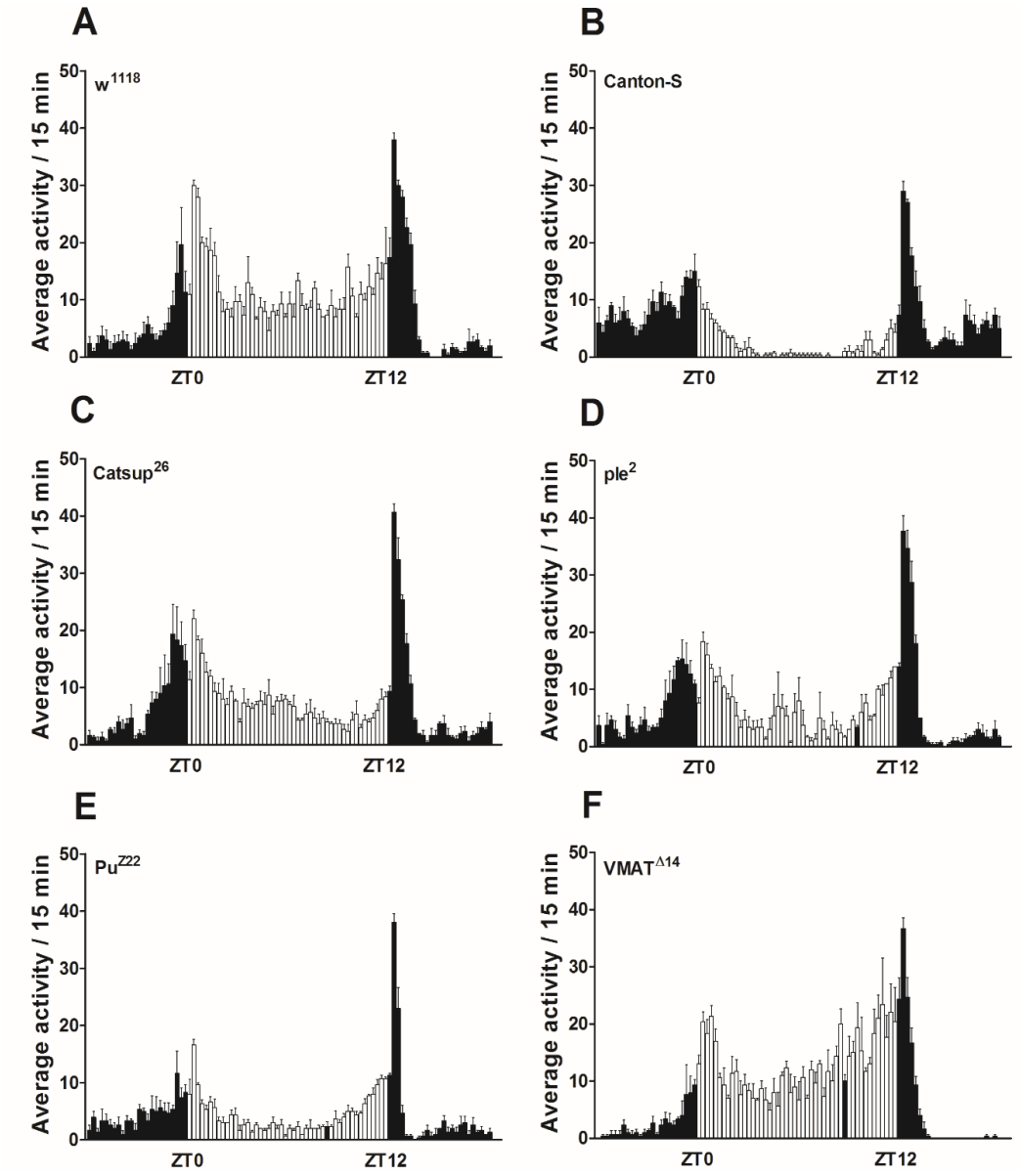

Figure 2.4 Daily activity profiles (indicating a circadian rhythmic pattern) per 15 minutes of the experimental flies 6-8 days post-eclosion averaged over a period of 3 days.

The open bars represent lights-on between 9:00 am (ZT0) and 9:00 pm (ZT12) while the filled bars represent averages of activity profiles under lights-off between 9:00 pm (ZT12) and 9:00 am (ZT0).

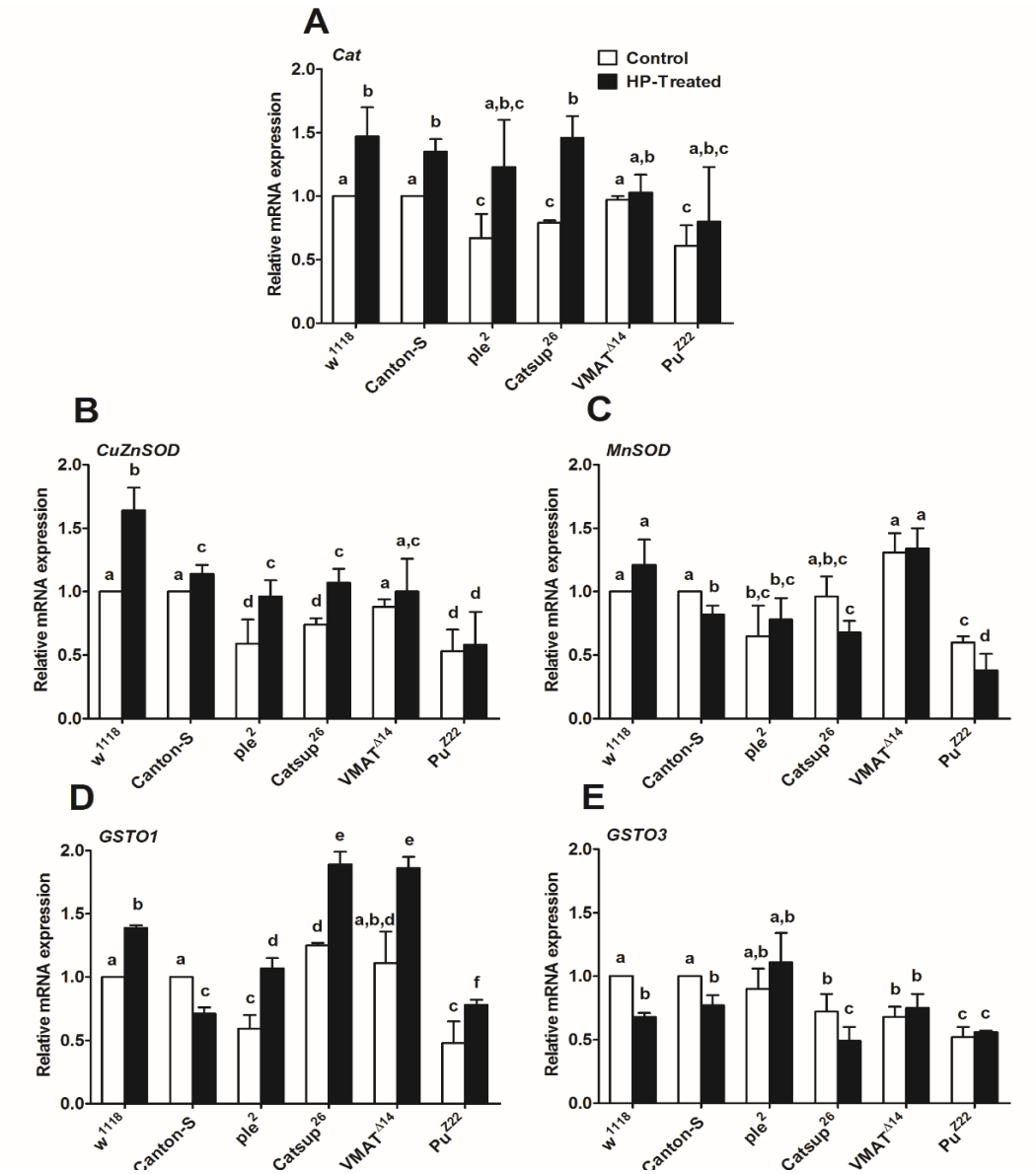

Figure 2.5 Quantitative-Reverse Transcription PCR of several genes encoding key antioxidant enzymes

(**A**) *Catalase*, (**B**) *CuZnSOD*, (**C**) *MnSOD,* (**D**) *GSTO1* and (**E**) *GSTO3* after 4 hours exposure to 80 µM H_2O_2 (HP-treated).
Relative mRNA expression of different fly lines was quantified using w^{1118} or Canton-S control lines (without H_2O_2 exposure-control) which was set as reference (= 1). Values represent mean ± SD of three independent biological replicates. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

Also, there is a significant up-regulation of *GSTO1* mRNA levels in these two fly lines upon exposure to HP, which was the highest among all fly lines tested. Pu^{Z22} showed the least amount of up-regulation of *GSTO1* mRNA, which was only slightly higher than the expression observed in Canton-S. Glutathione S-transferase Omega 3 (*GSTO3*) transcript levels were either down-regulated or remained unchanged following exposure to oxidative stress by HP in all mutants and wild-type controls used in this study (Figure 2.5 E).

An investigation of the translated protein for MnSOD and GSTO1 (Figure 2.6) revealed a similar pattern as exhibited by gene expression (see Figure 2.5C and 2.5D). In case of MnSOD, only w^{1118} flies revealed a marked increase in protein levels upon exposure to HP. In all other fly lines tested, there was no significant difference in MnSOD between unexposed and exposed flies. However, we did detect differences in levels of MnSOD among the genotypes tested (Figure 2.6 A). In case of GSTO1, ple^{2} and Pu^{Z22} flies showed significantly lower basal levels of GSTO1 compared to all other fly lines. Only $Catsup^{26}$ showed higher basal levels of GSTO1 which was significantly up-regulated upon exposure to HP, followed by $VMAT^{\Delta 14}$ flies (Figure 2.6). While in general all genotypes revealed an increase of GSTO1 upon HP exposure, Canton-S flies were an exception and showed a marked decrease in GSTO1 levels following exposure to HP (Figure 2.6 B). This was exactly the pattern that was revealed in gene expression (Figure 2.5D).

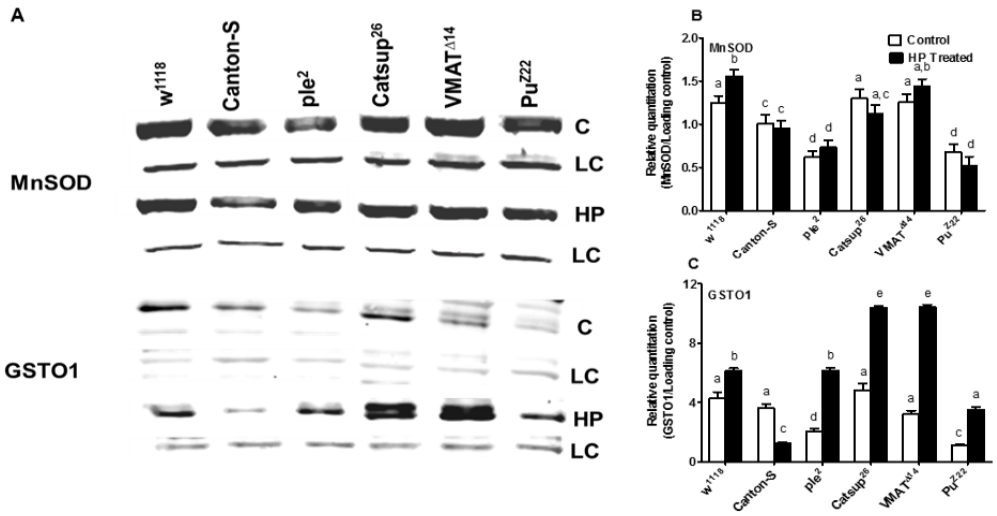

Figure 2.6 Western blots (A) and their quantifications (B, C) for two key proteins likely to be involved in response to OS in the experimental fly lines.

Blots were performed for MnSOD, and for GSTO1, both with (HP) and without (C) exposure to hydrogen peroxide. LC represents the loading control, a non-specific protein band indicative of equal protein loading in both cases. Figures are representative of one of three independent blots. Blots were quantified by mean grey scale value relative to LC and bars are represented as mean ± SD of three independent blots. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

2.5 Discussion

This study demonstrates that perturbations in DA synthesis or transport have a negative impact on the lifespan of fruit flies. However, elevation of DA pools as in the *Catsup*^{26} mutant does not appear to impact longevity, which is similar to the lifespan of wild type flies. DA has been shown to be a marker of neuronal senescence in *Drosophila*, since its levels decrease with increasing age, accompanied by deficits in dopaminergic modulated behaviors in aging flies (Neckameyer et al., 2000). It has been shown earlier that variation in the biosynthesis of biogenic amines such as DA could be a factor contributing to natural variation in lifespan of *Drosophila* (De Luca et al., 2003). The concept that lifespan is a function of the capacity to withstand extrinsic stress is well

known. In agreement with this concept, one would expect that long-lived individuals are often empowered with an increased resistance against a variety of stresses throughout life. Therefore, genes that underlie stress response may have the ability to affect lifespan. The finding that *Catsup*^{26} mutants are more resistant to stress in general (Chaudhuri et al., 2007) would lead to the inference that these mutants should have significantly extended lifespan compared to control flies. However, this was not the case as observed in this study. It is plausible that stress resistance genes triggered by higher DA pools only get activated in response to certain stress situations and determine survival responses. Thus, the efficiency of stress responses will determine lifespan only under adverse circumstances. This is precisely what we observed in flies with DA synthesis or trafficking perturbed, in their response to oxidative stress. *Catsup*^{26} mutants were more resistant to both HP stress as well as PQ stress in general. Interestingly, it was pointed out in a previous study that *Catsup*^{26} mutants are resistant only to PQ stress and not to HP (Chaudhuri et al., 2007). In fact, *Punch* mutants were reported as more resistant to HP stress and less sensitive to PQ. We believe that this discrepancy in results could be due to differences in HP and PQ dose used in the present study. In fact, *Pu*^{Z22} mutants showed lesser mortality at 24 h post exposure to HP compared to *Catsup*^{26} flies but eventually *Catsup*^{26} mutants survived the longest upon exposure to this stressor. The levels of DA are high in the heads of *Catsup*^{26} mutants whereas they are significantly depleted in *ple*^{2} and *Pu*^{Z22} mutants, on the other hand BH_4 levels are also elevated in *Catsup* mutants and depleted in *Pu*^{Z22} flies but not in *ple*^{2} flies (Chaudhuri et al., 2007). In the case of *VMAT*$^{\Delta 14}$ flies ~65% reduction in DA levels is reported compared to controls (Simon et al., 2009). It is possible that the variation in the levels of resistance to oxidative stress that

we observe in these flies is in part contributed by the variation in levels of DA pools. It was later demonstrated that *Catsup* mutants have elevated TH (3-fold) and GTPCH (7-fold) activity along with enhanced synaptic activity such that VMAT activity is enhanced with *Catsup* loss-of-function mutations (Wang et al., 2011).

The enhanced negative geotactic response in $Catsup^{26}$ flies as demonstrated by the RING assay indicates that DA pools generated in excess are synaptically active. Other studies on polymorphisms in *Catsup* gene locus have also shown an association with locomotion (Carbone et al., 2006) as well as modulation of sleep (Harbison et al., 2009). Interestingly, in bees it was shown that exogenous injection of DA decreased walking behavior while increasing other behaviors (Mustard et al., 2010). This relationship between DA and mobility led us to investigate whether it is such elevated or depleted DA pools, or impaired synaptic trafficking, that leads to overt changes in locomotory rhythms. While we observe that average daily activity of flies with depleted DA levels was significantly decreased compared to $Catsup^{26}$ mutants or wild type controls, yet, impairing DA's trafficking (in $VMAT^{\Delta 14}$) did not result in any change in average daily activity compared to w^{1118} or $Catsup^{26}$ flies. This implies that there are obviously other transporters of DA at work, such as the *Drosophila* dopamine transporter, encoded by the *dDAT* gene, which takes up DA from the extracellular space. It has been demonstrated that *fumin* (*fmn*) mutants with a mutation in *dDAT* gene, have abnormally high activity levels coupled with reduced rest (sleep) (Kume et al., 2005). dDAT transporters are expressed almost exclusively in the dopaminergic neurons (Porzgen et al., 2001) and function in the presynaptic membrane. They re-uptake the released DA, thereby diminishing DA signaling (Makos et al., 2009). Thus, a mutation in this transporter

results in augmentation of DA signaling and causes an increased ratio in the duration of the active state, which is the enhanced negative geotaxis phenotype. Interestingly, the power-law property of the distribution of rest bouts is unchanged in these mutants though DA modulates the rest period length (Ueno et al., 2012). VMAT regulates cytosolic DA levels and impaired trafficking has a negative effect on the negative geotactic response but intriguingly has an opposite effect in the horizontal mobility response. It has been demonstrated previously that the $dVMAT^{P1}$ homozygous mutant have an impaired negative geotactic response but this response is potentiated in the heterozygote such that they demonstrate an enhanced escape response compared to their controls (Simon et al., 2009). The observed deviation from this result in our studies could be the effect of the specific mutation in this transporter (Romero-Calderon et al., 2008).

Flies with either elevated DA pools ($Catsup^{26}$) or depleted DA levels (ple^{2} or Pu^{Z22}) or with impaired DA trafficking ($VMAT^{\Delta 14}$) were all rhythmic in their locomotor activity behavior (Figure A.1). However, strength of the rhythms was affected in case of $Catsup^{26}$ flies as well as in the flies with mutated *VMAT*, such that these flies were only "weakly" rhythmic. This is consistent with the idea that elevated DA pools would lead to enhanced arousal response (less sleep) (Kumar et al., 2012; Kume et al., 2005) and have an impact on the locomotor activity rhythms. On the other hand, ple^{2} and Pu^{Z22} flies showed substantially increased rhythm strength comparable to wild type controls, despite depleted levels of DA. This could be due to the fact that these mutations in the heterozygous state would be still capable of producing sufficient critical levels of DA required to sustain robust circadian locomotor activity behavior. Interestingly, transgenic flies with *Catsup* gene impaired in all nervous tissues (*elav*-Gal4/UAS-*Catsup*-RNAi)

resulted only in 7% rhythmic (weak, FFT 0.019 ± 0.01) flies (Figure A.2). On the other hand, their average daily activity was 874 ± 57, marginally higher than the wide type controls. Using a Gal4 driver specific to TH (*ple*), we observed that RNAi of *Catsup* resulted in 13% rhythmic (weak, FFT 0.023 ± 0.01) flies (Figure A.2) but the average daily activity plummeted to 474 ± 33. These results are in apparent agreement with the findings of Mustard et al., (2010) on honey bees. Thus, *Catsup* has pronounced impact on locomotor activity as well as its rhythms, when impaired throughout the nervous system. However, the neuronal and non-neuronal effects of DA is far from clear at this point and demand a more intensive investigation using the GAL4-UAS approach more extensively.

Despite being present in substantial levels in the heads, DA is equally rather more abundant in the body of *Drosophila* (Ream et al., 2003). This is probably due to the fact that DA is also functional in the cuticle, and immune response, in addition to its central role as a neurotransmitter. Thus, it would be reasonable to assume that mutations in *Catsup*, *pale* or *Punch* genes would reflect in stress tolerance signatures of the whole body. Therefore, we asked whether the primary antioxidant responsive systems such as catalase, superoxide dismutase, glutathione S-transferases would be affected by mutations that would result in enhanced or depleted DA levels or its impaired trafficking. Catalase gene expression was uniformly up-regulated in all mutant and control flies upon exposure to HP, indicating that all flies were responding to the stressor (catalase being involved in the decomposition of HP to water and oxygen). A differential response to oxidative stress was observed in the expression of *Cu/Zn SOD*, *MnSOD,* and *GSTO3*. Over-expression of *Cu/Zn SOD* has been demonstrated to protect dopaminergic neurons in *Drosophila* (Botella et al.,2008), however, in the present study, we did not observe any

significant up-regulation of *Cu/Zn SOD* associated with increased tolerance to oxidative stress. While we quantified the gene expression patterns of five major antioxidant enzymes, we were able to target protein quantitation of only two antioxidative enzymes viz. MnSOD and GSTO1 since reliable antibodies were available only for them. Among the two, only glutathione S-transferase Omega 1 (*GSTO1*) gene expression and protein levels indicated that elevated DA pools could be triggering an up-regulation in the expression of this gene along with enhanced activity. In general, GSTs are evolutionarily conserved enzymes that are important in the detoxification of many xenobiotic compounds. These enzymes catalyze the conjugation of GSH to electrophile substrates, producing compounds that are generally less reactive and more soluble. This facilitates their removal from the cell via membrane based GSH conjugation pumps. The broad substrate specificity of GSTs allows them to protect cells against a range of toxic products (Salinas and Wong, 1999). *DmGSTO1* has been demonstrated to be a novel genetic suppressor of the *parkin* dysfunction and has a protective role in a model of Parkinson's disease (PD) (Kim et al., 2012). Active sites of GST Omega have a unique cysteine residue that can form a disulfide bond with glutathione (GSH) and facilitate the conjugation of lipid-derived carbonyls species. Cells respond to oxidative stress by inducing the expression of genes whose products protect the cell, and one such cellular defense mechanism is the antioxidant response element (ARE), a *cis*-acting enhancer element that is upstream of many phase II detoxification and antioxidant systems such as GSTs (Rushmore et al., 1991; Rushmore and Pickett, 1990). 6-hydroxydopamine (6-OHDA) a hydroxylated analog of DA has been shown to activate antioxidant response elements (Jakel et al., 2005) and we hypothesize that elevated DA levels in *Catsup*^{26}

mutants could be acting in a similar manner potentiating an antioxidant response through GSTO1 and conferring the observed protective effect against oxidative stress. Taken together, these results add to an evolving picture of DA regulated defense against oxidative stress. Our blots confirm the validity of our real-time qRT-PCR results indicating a novel functional role of GSTO1 in this response. In this study we have not explored the role of DA receptors such as D1-like and D2-like and its likely interaction with the insulin signaling pathway as well as the circadian clock network (Gruntenko et al., 2012; Rauschenbach et al., 2014; Yujnovsky et al., 2006). There may be many such key players in the neuroendocrine regulation of stress response. Further analysis on the precise signaling pathways triggered by DA, as well as the balance between critical and toxic levels of DA would likely yield new insights on DA homeostasis and its relevance to stress physiology.

2.6 References

Abercrombie, E.D., Keefe, K.A., DiFrischia, D.S., Zigmond, M.J., 1989. Differential effect of stress on in vivo dopamine release in striatum, nucleus accumbens, and medial frontal cortex. *J. Neurochem.* 52: 1655-1658.

Barron, A.B., Søvik, E., Cornish, J.L., 2010. The roles of dopamine and related compounds in reward-seeking behavior across animal phyla. *Front. Behav. Neurosci.* 4: 163 eCollection.

Botella, J.A., Bayersdorfer, F., Schneuwly, S., 2008. *Superoxide dismutase* overexpression protects dopaminergic neurons in a *Drosophila* model of Parkinson's disease. *Neurobiol. Dis.* 30: 65-73.

Carbone, M.A., Jordan, K.W., Lyman, R.F., Harbison, S.T., Leips, J., Morgan, T.J., De Luca, M., Awadalla, P., Mackay, T.F., 2006. Phenotypic variation and natural selection at *Catsup*, a pleiotropic quantitative trait gene in *Drosophila*. *Curr. Biol.* 16: 912-919.

Chaudhuri, A., Bowling, K., Funderburk, C., Lawal, H., Inamdar, A., Wang, Z., O'Donnell, J.M., 2007. Interaction of genetic and environmental factors in a *Drosophila* parkinsonism model. *J. Neurosci.* 27: 2457-2467.

Civelli, O., Bunzow, J.R., Grandy, D.K., 1993. Molecular diversity of the dopamine receptors. *Annu. Rev. Pharmacol. Toxicol.* 33: 281-307.

De Luca, M., Roshina, N.V., Geiger-Thornsberry, G.L., Lyman, R.F., Pasyukova, E.G., Mackay, T.F., 2003. Dopa decarboxylase (*Ddc*) affects variation in *Drosophila* longevity. *Nat. Genet.* 34: 429-433.

Graham. D.G., 1978. Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol. Pharmacol.* 14: 633-643.

Greer, C.L., Grygoruk, A., Patton, D.E., Ley, B., Romero-Calderon, R., Chang, H.Y., Houshyar, R., Bainton, R.J., Diantonio, A., Krantz, D.E., 2005. A splice variant of the *Drosophila* vesicular monoamine transporter contains a conserved trafficking domain and functions in the storage of dopamine, serotonin, and octopamine. *J. Neurobiol.* 64: 239-258.

Gruntenko, N.E., Laukhina, O.V., Bogomolova, E.V., Karpova, E.K.,Menshanov, P.N., Romanova, I.V., I.Yu.Rauschenbach., 2012. Downregulation of the dopamine D2-like receptor in corpus allatum affects juvenile hormone synthesis in *Drosophila melanogaster* females. *J. Insect Physiol.* 58: 348-355.

Hald, A., Lotharius, J., 2005. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? *Exp. Neurol.* 193: 279-290.

Harbison, S.T., Carbone, M.A., Ayroles, J.F., Stone, E.A., Lyman, R.F., Mackay, T.F., 2009. Co-regulated transcriptional networks contribute to natural genetic variation in *Drosophila* sleep. *Nat. Genet.* 41: 371-375.

Hirsh, J., Riemensperger, T., Coulom, H., Iche, M., Coupar, J., Birman, S., 2010. Roles of dopamine in circadian rhythmicity and extreme light sensitivity of circadian entrainment. *Curr. Biol.* 20: 209-214.

Hood, S., Cassidy, P., Cossette, M.P., Weigl, Y., Verwey, M., Robinson, B., Stewart, J., Amir, S., 2010. Endogenous dopamine regulates the rhythm of expression of the clock protein PER2 in the rat dorsal striatum via daily activation of D2 dopamine receptors. *J. Neurosci.* 30: 14046-14058.

Hsouna, A., Lawal, H.O., Izevbaye, I., Hsu, T., O'Donnell, J.M., 2007. *Drosophila* dopamine synthesis pathway genes regulate tracheal morphogenesis. *Dev. Biol.* 308: 30-43.

Jakel, R.J., Kern, J.T., Johnson, D.A., Johnson, J.A., 2005. Induction of the protective antioxidant response element pathway by 6-hydroxydopamine *in vivo* and *in vitro*. *Toxicol. Sci.* 87: 176-186.

Kim, K., Kim, S-H., Kim, J., Kim, H., Yim, J., 2012. Glutathione S-transferase omega 1 activity is sufficient to suppress neurodegeneration in a *Drosophila* model of Parkinson disease. *J. Biol. Chem.* 287: 6628-6641.

Krishnan, N., Davis, A.J., Giebultowicz, J.M., 2008. Circadian regulation of response to oxidative stress in *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 374: 299-303.

Krishnan, N., Rakshit, K., Chow, E.S., Wentzell, J.S., Kretzschmar. D., Giebultowicz. J.M., 2012. Loss of circadian clock accelerates aging in neurodegeneration-prone mutants. *Neurobiol. Dis.* 45: 1129-1135.

Kumar, S., Chen, D., Sehgal, A., 2012. Dopamine acts through cryptochrome to promote acute arousal in *Drosophila*. *Genes. Dev.* 26: 1224-1234.

Kume, K., Kume, S., Park, S.K., Hirsh, J., Jackson, F.R., 2005. Dopamine is a regulator of arousal in the fruit fly. *J. Neurosci.* 25: 7377-7384.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Lawal, H.O.,Chang, H-Y., Terrell, A.N., Brooks, E.S., Pulido, D., Simon, A.F., Krantz, D.E., 2010. The *Drosophila* vesicular monoamine transporter reduces pesticide-induced loss of dopaminergic neurons. *Neurobiol. Dis.* 40: 102-112.

Mackay, W., Reynolds, E.R., O'Donnell, J.M., 1985. Tissue-specific and complex complementation patterns in the *Punch* locus of *Drosophila melanogaster*. *Genetics* 111: 885-904.

Makos, M.A., Kim, Y.C., Han, K.A.,Heien, M.L., Ewing, A.G., 2009. In vivo electrochemical measurements of exogenously applied dopamine in *Drosophila melanogaster*. *Anal. Chem.* 81: 1848-1854.

Mustard, J.A., Pham, P.M., Smith, B.H., 2010. Modulation of motor behavior by dopamine and the D1-like dopamine receptor AmDOP2 in the honey bee. *J. Insect Physiol.* 56: 422-430.

Neckameyer, W.S., White, K., 1993. *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. *J. Neurogenet.* 8: 189-199.

Neckameyer, W.S., Woodrome, S., Holt, B., Mayer, A., 2000. Dopamine and senescence in *Drosophila melanogaster*. *Neurobiol. Aging.* 21: 145-152.

Pendleton, R.G., Rasheed, A., Sardina, T., Tully, T., Hillman, R., 2002. Effects of tyrosine hydroxylase mutants on locomotor activity in *Drosophila*: a study in functional genomics. *Behav. Genet.* 32: 89-94.

Pfeiffenberger, C., Lear, B.C., Keegan, K.P., Allada, R., 2010. Locomotor activity level monitoring using the Drosophila Activity Monitoring (DAM) System. *Cold Spring Harb. Protoc.* 11, pdb.prot5518.

Porzgen, P., Park, S.K., Hirsh, J., Sonders, M.S., Amara, S.G., 2001. The antidepressant-sensitive dopamine transporter in *Drosophila melanogaster*: a primordial carrier for catecholamines. *Mol. Pharmacol.* 59: 83-95.

Rauschenbach, I.Yu., Karpova, E.K., Adonyeva, N.V., Andreenkova, O.V., Faddeeva, N.V., Burdina, E.V., Alekseev, A.A., Menshanov, P.N., Gruntenko, N.E. 2014. Disruption of insulin signaling affects the neuroendocrine stress reaction in *Drosophila* females. *J.Exp. Biol.* (In Press: doi: 10.1242/jeb.106815)

Ream, P.J., Suljak, S.W., Ewing, A.G., Han, K-A., 2003. Micellar electro-kinetic capillary chromatography-electrochemical detection for analysis of biogenic amines in *Drosophila melanogaster*. *Anal. Chem.* 75: 3972-3978.

Restifo, L., White, K., 1990. Molecular and genetic approaches to neurotransmitter and neuromodulator systems in *Drosophila*. *Adv. Insect Physiol.* 22: 116-219.

Reynolds, E.R., O'Donnell, J.M., 1988. Characterization of new *Punch* mutations: identification of two additional mutant classes. *Genetics* 119: 609-617.

Romero-Calderon, R., Uhlenbrock, G., Borycz, J., Simon, A.F., Grygoruk, A., Yee, S.K., Shyer, A., Ackerson, L.C., Maidment, N.T., Meinertzhagen, I.A., Hovemann. B.T., Krantz, D.E., 2008. A glial variant of the vesicular monoamine transporter is required to store histamine in the *Drosophila* visual system. *PLoS Genet.* 4: e1000245.

Rushmore, T.H., Morton, M.R., Pickett, C.B., 1991. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem*. 266: 11632-11639.

Rushmore, T.H., Pickett, C.B., 1990. Transcriptional regulation of the rat glutathione S-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. *J. Biol. Chem.* 265: 14648-14653.

Salinas, A.E., Wong, M.G., 1999. Glutathione S-Transferases - A Review. *Curr. Med. Chem.* 6: 279-309.

Simon, A.F., Daniels, R., Romero-Calderon, R., Grygoruk, A., Chang, H-Y., Najibi, R., Shamouelian, D., Salazar, E., Solomon, M., Ackerson, L.C., Maidment, N.T., DiAntonio, A., Krantz, D.E., 2009. *Drosophila* vesicular monoamine transporter mutants can adapt to reduced or eliminated vesicular stores of dopamine and serotonin. *Genetics* 181: 525-541.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson B.J., Klenk D.C., 1985. Measurement of protein using bicinchonininc acid. *Anal. Biochem.* 150: 76-85.

Stathakis, D.G., Burton, Y., McIvor, W.E., Krishnakumar, S., Wright, T.R.F., O'Donnell, J.M., 1999. The catecholamines up (Catsup) protein of *Drosophila melanogaster* functions as a negative regulator of tyrosine hydroxylase activity. *Genetics* 153: 361-382.

Ueda, S., Aikawa, M., Ishizuya-Oka, A., Yamaoka, S., Koibuchi, N., Yoshimoto, K., 2000. Age-related dopamine deficiency in the mesostriatal dopamine system of zitter mutant rats: regional fiber vulnerability in the striatum and the olfactory tubercle. *Neuroscience* 95: 389-398.

Ueno, T., Masuda, N., Kume, S., Kume, K., 2012. Dopamine modulates the rest period length without perturbation of its power law distribution in *Drosophila melanogaster*. *PloS One.* 7: e32007.

Waddell, S., 2013. Reinforcement signaling in *Drosophila*: dopamine does it all after all. *Curr. Opin. Neurobiol.* 23: 324- 329.

Wang, Z., Ferdousy, F., Lawal, H., Huang, Z., Daigle, J.G., Izevbaye, I., Doherty, O., Thomas, J., Stathakis, D.G., O'Donnell, J.M., 2011. *Catecholamines up* integrates dopamine synthesis and synaptic trafficking. *J. Neurochem.* 119: 1294-1305.

Wulff, K., Gatti, S., Wettstein, J.G., Foster, R.G., 2010. Sleep and circadian rhythm disruption in psychiatric and neurodegenerative disease. *Nat. Rev. Neurosci.* 11: 589-599.

Yujnovsky, I., Hirayama., Dol, M., Borrelli, E., Sassone-Corsi, P. 2006. Signaling mediated by the dopamine D2 receptor potentiates circadian regulation by CLOCK:BMAL1. *Proc. Natl. Acad. Sci. USA.* 103: 6386-6391.

CHAPTER III

DOES DOPAMINE PLAY A SEXUALLY DIMORPHIC ROLE IN AGING AND SENESCENCE IN *DROSOPHILA*?

3.1 Abstract

The neurotransmitter dopamine (DA) is known to be involved in stress response, and certain age-related disease pathologies are known to stem from dysfunction within the DA system in mammals. This led to the presumption that there is a correlation between aging/senescence and DA. Using the fruit fly *Drosophila melanogaster*, the sexually dimorphic effects of perturbations of DA synthesis (*Catsup* – with elevated DA levels and *pale* and *Punch* mutants with depleted DA levels) and transport (a mutant in the vesicular monoamine transporter, *VMAT*) and its relationship on aging and senescence was investigated. In general, wild type (w^{1118} and Canton-S), $Catsup^{26}$ and Pu^{Z22} mutant females were longer lived than their male counterparts, unlike the ple^{2} and $VMAT^{\Delta 14}$ whose males had a longer average lifespan. A marked decline in circadian locomotor rhythmicity was recorded in old female flies compared to age-matched males, however, walking activity increased in old females compared to males. A significant increase of protein damage was recorded in both male and female fly lines with age across all genotypes but the increase was more so in the males. A sexually dimorphic response was also observed in the expression of key stress and age-related transcription factors. Taken together, our results point to a sexually dimorphic pattern in male and

female aging and senescence, however, DA *per se* may not be a strong contributing factor to the observed differences.

3.2 Introduction

Aging studies on diverse species ranging from yeast to man have resulted in the delineation of several signaling pathways that influence the process of aging and the consequent senescent decline. The fruit fly, *Drosophila* is one of the principal model organisms used for studying the biology of aging, and such studies have led to a remarkable series of discoveries regarding conserved genetic pathways and common environmental factors that influence survival across a range of species (Takahashi et al., 2000; Boulianne, 2001; Kenyon, 2001; Helfand and Rogina, 2003a;b; Barger et al., 2003; Tatar et al., 2003; Wood et al., 2004; Antosh et al., 2011; Iliadi et al., 2012). The consequences of aging of the neuroendocrine system have been incriminated in the development of various age-dependent pathologies (Rehman and Masson, 2001). Moreover, it is known that aging affects the endocrine system by altering the endocrine cells, the hormones produced by these cells and the hormone receptors or post-receptor processes in the target cells (Chahal and Drake, 2007; Noth and Mazzaferri, 1985). The neuroendocrine system has a bidirectional communication with the immune/ stress response system (Besedovsky and del Ray, 1996). Thus, with aging, not only does a functional decline occur in the nervous system with consequent changes to the immune or stress response system, but also an impaired relationship between these two regulatory systems becomes evident, with the resulting loss of homeostasis and higher risk of death (Fabris, 1991; De La Fuente, 2002). However, dysregulation of the neuroendocrine system and its influence on aging and senescence has been little explored. The

implications of such a dysregulation would be acceleration of the process of aging through changes in neural and endocrine functions that would be crucial for: (a) coordination and responsiveness of different systems to the external environment; (b) programming physiological responses to environmental stimuli; and (c) the maintenance of an optimal functional status for reproduction and survival. Such changes, not only selectively affects the regulation of evolutionarily significant functions such as reproduction, growth and development, but also influences the regulation of survival through adaptation to stress.

In mammals, the neurotransmitter dopamine (DA) modulates movement, motivation, and cognition (Aarts et al., 2011). Abnormal functional states of the DA system in humans is thought to underlie the behavioral abnormalities in Parkinson's disease, Attention Deficit Hyperactivity disorder (ADHD), and schizophrenia (Winterer and Weinberger, 2004; Villar-Cheda et al., 2014). Catecholamines such as DA are also known to play key roles in orchestrating the response to stress (Sabban, 2007). DA release from cells in several brain regions is stimulated in response to diverse stressors (D'Angio et al., 1988; Abercrombie et al., 1989). Studies in mammals have demonstrated altered regulation of catecholaminergic systems in aged animals. The mammalian brain DA levels decrease significantly as a function of age in the striatum, ventral tegmental area, ventral pallidum and substantia nigra (Demarest et al., 1980; Gozlan et al., 1990; Woods and Druse, 1996; Goicoechea et al., 1997). Parkinson's disease which is correlated with a drastic decline in dopaminergic neurons, may result from a combination of age-related loss of dopaminergic neurons and environmentally-induced damage to the substantia nigra (Kish et al., 1992). In insects, like *Drosophila*, DA is involved in

locomotion (Pendleton et al., 2002), sleep and arousal (Andretic et al., 2005; Foltenyi et al., 2007; Kume et al., 2005), courtship behavior (Liu et al., 2008; Neckameyer, 1998), inhibition of startle induced hyper-excitability (Friggi-Grelin et al., 2003) and in mediating response to stress (Hanna et al., 2015). The role of biogenic amines has also been implicated in determining longevity (De Luca et al., 2003). It has been demonstrated earlier that DA levels are reduced in aging flies and that dopaminergic modulated behaviors are perturbed with increasing age (Neckameyer et al., 2000). However, it is unclear if perturbations in DA synthesis and transport can impact aging or senescence characteristics. Although *Drosophila* and mammalian neuroanatomy differ considerably, many molecular elements of aminergic neurotransmission are conserved (Corey et al., 1994; Blenau and Baumann, 2001; Porzgen et al., 2001). Thus, *Drosophila* provides a powerful system to explore the mechanisms by which perturbations in aminergic neurotransmission may impact aging and senescence.

An aspect that is little understood is the impact of sexual dimorphism on aging and senescence characteristics. Several previous studies in mammalian systems have shown sexually dimorphic behaviors, neuroendocrine changes, and alterations in neurotransmitter release in response to stress (see Sullivan, 2004 for review). In *Drosophila*, DA levels are also sexually dimorphic (Neckameyer et al., 2000). Moreover, the stress response circuitry (involving DA) also differs based on sex (Argue, 2012). In our previous study, we demonstrated that perturbations in DA levels and transport has a distinct impact on response to oxidative stress as well as some other physiological characteristics (Hanna et al., 2015). Importantly, it has also been demonstrated that similar gene expression patterns characterize oxidative stress response and aging (Landis

et al., 2004; 2012). Thus, in the present study, our objective was to determine if perturbations in DA synthesis and transport could impact aging and senescence in *Drosophila* in a sexually dimorphic manner. We have examined the longevity characteristics, oxidative protein damage which accumulates during aging, dampening of the circadian locomotor activity rhythm associated with aging as a read-out of senescence, as well as the pattern of expression of some key transcription factors involved in both stress response and aging. This is important, since many age-related pathologies exhibit a sexually dimorphic trend. Insights of the links between perturbations in the dopaminergic system and its impact on aging and senescence in a sexually dimorphic manner could eventually lead to novel gender based therapeutic strategies.

3.3 Material and Methods

3.3.1 Drosophila stocks and husbandry

D. melanogaster were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25 °C in 12 h light:12 h dark (LD 12:12) cycles (with an average light intensity of ~2000 lux). Fly lines w^{1118} and Canton-S were used as the control strains for the wild types of the catecholaminergic pathway mutations employed in this study. In situations where no significant differences were observed in the experiments conducted using these two control fly lines, the data was pooled. The following mutant fly lines were used in this study: $Catsup^{26}/CyO$, a 600 bp deletion extending immediately upstream of the start codon produced no detectable protein and was derived from the mobilization of a 5'P-element insertion in $Catsup^{KO5042}$(Stathakis et al., 1999). Since *Castsup* mutant alleles are homozygous lethal, all experiments in the study were

performed using heterozygous strains. The *Pu* mutant allele (*dp cn* Pu^{Z22} *a px sp*/SM1) utilized in this study was derived in an ethylmethane sulfonate (EMS) mutagenesis screen (Mackay et al., 1985; Reynolds and O'Donnell, 1988). The homozygous lethal ple^2 is a loss-of-function allele recovered in an EMS screen and the heterozygous mutant *w;* ple^2*/TM3 Sb e* was used (Neckameyer and White, 1993). For mutations in the transporter of DA, we used the *VMAT* loss of function mutant *w;* $VMAT^{\Delta 14}$*/CyO,* (Romero-Calderon et al., 2008). All behavioral studies were conducted on mutant heterozygotes crossed into the appropriate wild type background to eliminate balancers. Both male and female flies were examined separately within this study to observe what characteristics were affected by sexual dimorphism.

3.3.2 Lifespan measurements

For measuring lifespan, 3 cohorts of approximately 80 mated male and 80 mated female flies of each genotype (n=240) were housed separately in 8 oz round bottom polypropylene bottles (Genesee Scientific, San Diego, CA, USA) inverted over 60 mm Falcon Primaria tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) containing 15 mL of diet. Diet dishes were replaced on alternate days without anesthesia (CO_2), after tapping flies to the bottom of the bottle. Mortality was recorded daily and mortality calculations and Gompertz-Makeham maximum likelihood estimates were done using WinModest (v.1.0.2) (Pletcher, 1999; Krishnan et al., 2009).

3.3.3 Circadian locomotor activity analysis

Flies of each gender, genotype and age were entrained in LD 12:12 at 25 °C for 3 days to acclimatize them to the activity monitoring tubes in the Drosophila activity

monitor. Locomotor activity of young (3-5 days) and old (45-50 days) male and female flies of each genotype were recorded for 3 d in LD 12:12, followed by 10 d in constant darkness (DD) using the Trikinetics locomotor activity monitor (Waltham, MA, USA) as described by Pfeiffenberger et al., (2010). Locomotor activity, counted by number of infrared beam crossings of the individual flies collected in 15 min bins is represented as actograms. For daily activity profiles, the number of beam crossings in LD cycles was averaged for the 3 days in LD for all flies of a particular gender and genotype. For a quantitative measure of circadian rhythmicity in DD, Fast Fourier Transform (FFT) analysis was conducted using CLOCKLAB software (Actimetrics; Coulbourn Instruments, Whitehall, PA, USA). Flies with FFT values <0.04 were classified as arrhythmic, ones with values of 0.04-0.08 were classified as weakly rhythmic, whereas flies with FFT values > 0.08 were considered strongly rhythmic. Flies with both weak and strong rhythms that showed a single peak in the periodogram were included in the calculations of the free-running period using the CLOCKLAB software.

3.3.4 Total protein carbonyl content assay

The amount of protein carbonyls was quantified in whole body homogenates (25 flies in each replicate in three bioreplicates) of young (3-5 days) and old (45-50 days) flies of each gender and genotype. Samples were derivatized after reaction with 2,4 dinitrophenylhydrazine (DNPH) as described before (Krishnan et al., 2007). Results were expressed as nmol mg-1 protein using an extinction coefficient of 22,000 M-1 cm-1 at absorbance maxima of 370 nm in BioTek H1M Synergy plate reader. Bovine serum albumin (BSA) standard curve was used for protein concentrations in guanidine solutions

(Abs 280 nm). Protein carbonyl values were corrected for interfering substances by subtracting the Abs 370 nm mg-1 protein measured in control samples.

3.3.5 Quantitative real-time polymerase chain reaction

Three independent bio-replicates of young (5-8 days) and old (45-50 days) male and female flies of each genotype was collected separately. Total RNA was extracted from whole body homogenates of flies (~25 flies) using Tri Reagent (Sigma, St. Louis, MO, USA). The samples were treated with Takara Recombinant DNase I (Clontech Laboratories Inc., Mountain View, CA, USA). Synthesis of cDNA was achieved with the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed on the Eppendorf realplex^2 Mastercycler (Eppendorf, USA) under default thermal cycling conditions, with a dissociation curve step. Every reaction contained Power SYBR Green (Applied Biosystems), 10 ng cDNA, and 400 nM primers. Primer sequences are given in Table B.1. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized to the gene *rp49*. Relative mRNA amplitude was calculated with respect to pooled data of wild type w^{1118}, and wild type Canton-S flies whose expression for a particular gene was set as 1.

3.3.6 Statistical analyses

Analysis of longevity and mortality characteristics was conducted in WinModest (v. 1.0.2) followed by two-way ANOVA with Tukey's post hoc tests (GraphPad Instat v 3.0). Gender and genotype were the two factors whereas average survival and other Gompertz-Makeham estimates were the parameters evaluated. Locomotor activity data, protein carbonyl content and gene expression analysis was subjected to one or two-way

ANOVA with Tukey's post-hoc tests as appropriate. Graphs were developed in GraphPad Prism v 5.01 (GraphPad software Inc. San Diego, CA).

3.4 Results

3.4.1 Lifespan characteristics

There was a significant difference in lifespan among genotypes as well as between males and females of each genotype (Table 3.1). Among males of different genotypes studied with increased DA (*Catsup*^{26}) or decreased DA (*ple*^{2} and *Pu*^{Z22}) or with impaired DA trafficking (*VMAT*$^{\Delta 14}$) and the wildtype controls (w^{1118} and Canton-S), it was observed that there was no significant difference between wild-type controls (mean longevity 53.9 ± 1.3 days), or *Catsup*^{26} mutants (54.6 ± 1.6). However, the mean longevity was significantly reduced in *ple*^{2} (51.4 ± 1.4 days), *Pu*^{Z22} (37.8 ± 1.2 days) and *VMAT*$^{\Delta 14}$ (47.1 ± 0.8 days) mutants respectively compared to wildtype controls or *Catsup*^{26} flies (Table 3.1). Among females, the wildtype controls (w^{1118} and Canton-S) exhibited significantly enhanced mean longevity (61.5 ± 0.9 days) compared to other genotypes studied. This was followed by *Catsup*^{26} mutants (57.9 ± 1.1 days). All other genotypes with decreased DA levels or impaired DA trafficking showed reduced longevity compared to either wildtype controls or *Catsup*^{26} mutants (Table 3.1). This male or female specific mortality characteristics were also reflected in the mortality parameters derived from the Gompertz-Makeham model and maximum likelihood estimates (MLE) (Table B.2).

When males and females of a particular genotype was compared, we found sex specific differences in the mean longevity (Table 3.1). In general female wildtype, *Catsup*^{26} and *Pu*^{Z22} mutants were significantly longer lived than the males for those

genotypes. On the other hand, male *ple*^{2} and *VMAT*$^{\Delta 14}$ mutants lived longer than their female counterparts. These sexually dimorphic differences among genotypes were also noted in the mortality parameters from the Gompertz-Makeham model fitting and MLE estimates (Table B.2).

Table 3.1 Mean longevity (in days) of male and female flies with mutations in DA synthesis or transport.

Genotypes	Males	Females
Wild type (*w*^{1118} or Canton-S)	53.9$^{a\psi}$ ± 1.3 (n=237)	61.5$^{b\psi}$ ± 0.9 (n=230)
***Catsup*^{26}**	54.6$^{a\psi}$ ± 1.6 (n=291)	57.9^{b‡} ± 1.1 (n=253)
***ple*^{2}**	51.4^{a‡} ± 1.4 (n=291)	48.7$^{b\&}$ ± 1.0 (n=179)
***Pu*^{Z22}**	37.8$^{a\&}$ ± 1.2 (n=285)	50.5$^{b\&}$ ± 1.0 (n=140)
***VMAT*$^{\Delta 14}$**	47.1$^{a@}$ ± 0.8 (n=312)	42.1$^{b@}$ ± 0.6 (n=231)

Values are mean ± SD. Values in parenthesis are total number of individuals studied. In each row (Male vs Female, for a particular genotype) mean values followed by different alphabetic superscripts are significantly different at $p<0.0001$. In each column, mean values followed by different symbols in superscripts are significantly different at $p<0.001$.

3.4.2 Age-related senescence of circadian locomotor activity

Significant changes in gender and age related decline in circadian locomotor activity rhythms were recorded across all genotypes tested (Table 3.2). In male flies the wild type flies exhibited a decline in rhythmicity from 100% in young flies to 63% in old flies. In females such a decline was drastic with only 6% of old flies remaining rhythmic. In *Catsup*^{26} mutants with elevated DA in males there was only a marginal decline in rhythmicity with age (70% in young to 63 % in old), however, in old females, there were

no rhythmic individuals (0%). Only Pu^{Z22} mutants of females showed a marginal decline in rhythmicity with age (63%), whereas all other mutant fly lines exhibited a significant decline in rhythmicity with age (Table 3.2).

Interestingly, while in males the average daily activity tended to decline across genotypes in general with age, in females, this was quite the opposite with older females either exhibiting comparable activity levels to the young for a genotype, or even elevated activity with age (Figure 3.1A). In females, only $Catsup^{26}$ mutants with elevated DA levels exhibited a marked decline in average daily activity with age. The strength of the rhythm revealed a tendency to decline with age among both males and females in general, however, there was no significant differences between the genders within a specific genotype (Figure 3.2B).

Table 3.2 Data representing the percentage of rhythmic flies, the period of rhythm (in constant darkness –DD) and the number of individuals of a genotype tested.

Sex/Age	Genotype	% Rhythmic	Period of rhythm in DD (Hrs)	Sample size (n)
Male (young) 3-5 days	wild type	100	23.7± 0.2	16
	Catsup^{26}	70	23.4 ± 0.2	16
	ple^{2}	67	23.5 ± 0.2	16
	Pu^{Z22}	94	23.0 ± 0.1	16
	VMAT$^{\Delta 14}$	59	24.0 ± 0.7	16
Male (old) ~ 45 days	wild type	63	24.3 ± 7.7	16
	Catsup^{26}	63	26.0 ± 7.0	16
	ple^{2}	69	26.6 ± 3.9	16
	Pu^{Z22}	25	20.3 ± 10.3	16
	VMAT$^{\Delta 14}$	31	27.2 ± 6.1	16
Female (young) 3-5 days	wild type	100	23.9± 0.2	16
	Catsup^{26}	94	23.7 ± 0.1	16
	ple^{2}	75	23.6 ± 0.1	16
	Pu^{Z22}	100	23.8 ± 0.3	16
	VMAT$^{\Delta 14}$	75	23.7 ± 0.4	16
Female (old) ~ 45 days	wild type	6	24.7 ± 11.1	16
	Catsup^{26}	0	17.5 ± 12.0	16
	ple^{2}	56	26.3 ± 5.3	16
	Pu^{Z22}	63	23.1 ± 7.1	16
	VMAT$^{\Delta 14}$	13	19.6 ± 10.3	16

Flies of each sex (male or female) and age (young or old) from each genotype was tested separately. Data for wild type flies is pooled data of w^{1118} and Canton-S flies which showed similar circadian locomotor behavior patterns. Period of rhythm is represented as mean (hours) ± SD.

3.4.3 Protein carbonyl accumulation with age

In general there was a significant accumulation of protein carbonyls with age (young vs old) in both males and females of each genotype (Figure 3.2 A, B). Among genotypes in young males, *ple*^{2} mutants showed significantly higher levels of protein carbonyls and *Pu*^{Z22} mutants the least (Figure 3.2A), no significant differences were observed among young male flies of wildtype (w^{1118} or Canton-S), *Catsup*^{26} and *VMAT*$^{\Delta 14}$

flies. Among old male flies, only $VMAT^{\Delta 14}$ flies showed lesser accumulation of protein carbonyls compared to all other genotypes studied (which were not significantly different from each other). In young female flies only Pu^{Z22} flies displayed the least amount of carbonyls ($p<0.05$) while there was no significant difference in carbonyl content in all other genotypes. In old females however, $Catsup^{26}$ flies showed the highest accumulation of carbonyls and $VMAT^{\Delta 14}$ flies the least (Figure 3.2B).

On comparing young males and females of the various mutant flies it was observed that only Pu^{Z22} flies showed least amount of carbonyls in both sexes (Figure 3.2C). On comparing old flies of both sexes there was a tendency of lesser accumulation of protein carbonyls in females compared to males across genotypes (Figure 3.2D). In general, it could be stated that males accumulated more carbonyls across genotypes compared to females during aging.

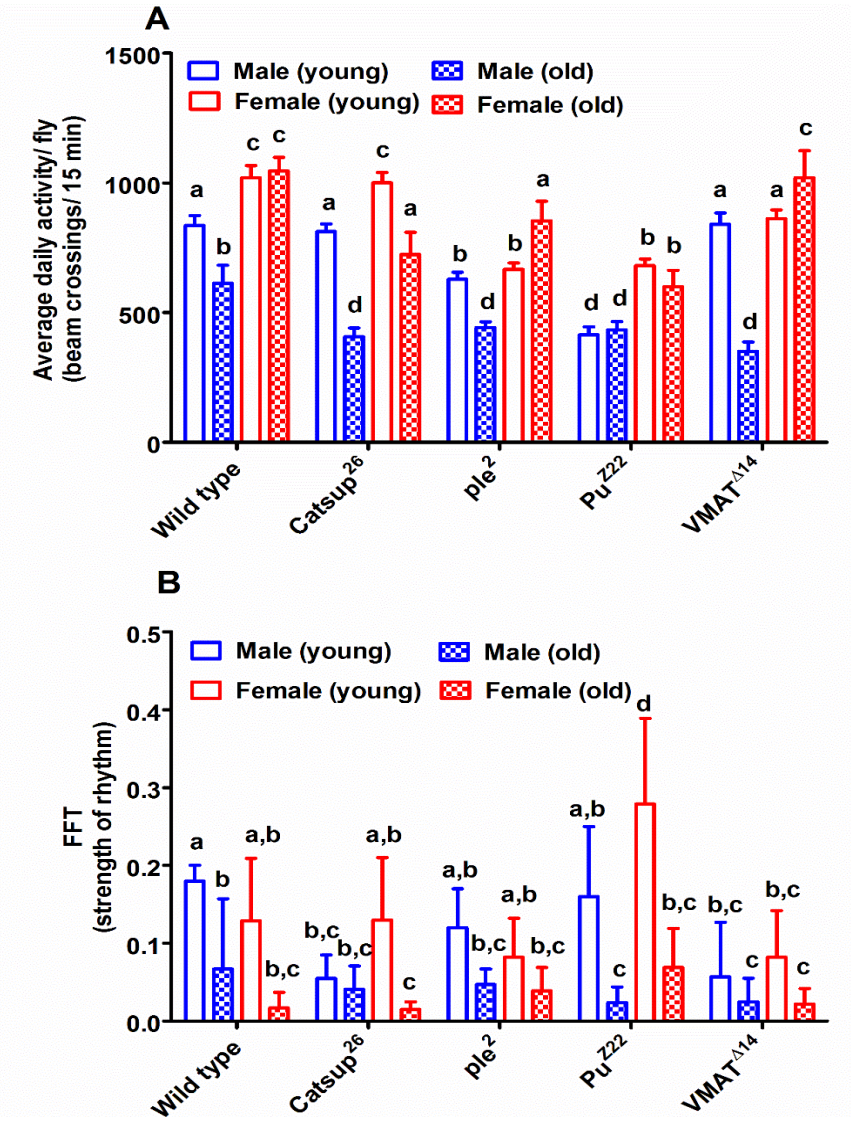

Figure 3.1 Graphical representation of the average daily activity per fly.

(A) The strength of the rhythm exhibited by rhythmic flies **(B)** Data for wild type flies is pooled data of w^{1118} and Canton-S flies which showed similar circadian locomotor behavior patterns. Average daily activity was computed as beam crossings in 15 min bins over 3 days of LD cycles (see Materials and Methods Section 2.3 for more details). Comparisons for various parameters were made between male (young vs old), female (young vs old) and young (male vs female) and old (male vs female) for each genotype as well as among genotypes. Bars with different superscripts are significantly different at $p<0.05$ (two-way ANOVA with Bonferroni multiple comparisons test). Data are represented as mean ± SD. (See Table B.3 and B.4 for Statistical analysis)

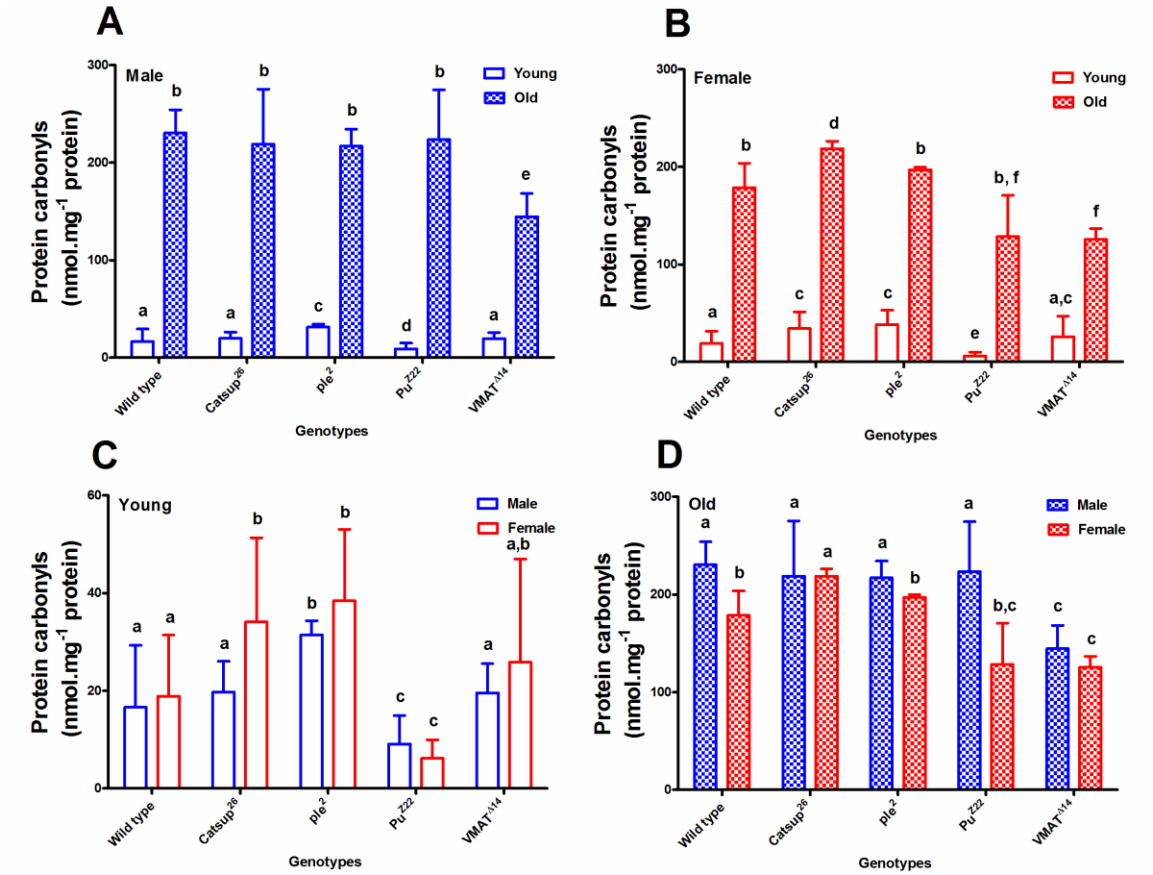

Figure 3.2 Accumulation of total protein carbonyls with age.

(A) Male (young vs old) **(B)** Female (young vs old) **(C)** Young (male vs female) and **(D)** Old (male vs female). Data are mean ± SD of assays from three independent bioreplicates. Bars with different superscripts are significantly different at $p<0.05$ using one-way ANOVA with Tukey's multiple comparison test.

3.4.4 Differential expression of target genes involved in stress response and aging

A sexually dimorphic as well as age dependent response of some key genes involved in general stress response and aging was observed (Figure 3.3). A list of forward and reverse primers used for all genes are shown in (Table B.1). *dPGC-1* expression was dramatically up-regulated in all young female fly lines of various genotypes compared to age-matched males (Figure 3.3A). In case of males, no significant changes in expression of *dPGC-1* was observed among genotypes except in case of the

Pu^{Z22} mutant where in aged males a significant up-regulation was observed when compared to young males. We did not find any expression of *dPGC-1* in old *ple*^{2} mutant males, similarly, in old *VMAT*$^{\Delta 14}$ females no expression of *dPGC-1* was recorded (Figure 3.3A). In general in females of all genotypes a reduction or no change in *dPGC-1* expression was observed when young flies were compared to old ones.

Expression of the gene encoding the energy sensor protein AMPK was variable across the different genotypes tested. In general, where expression was recorded in old individuals, we found a marked reduction in *AMPK* expression levels with age (Figure 3.3B). Interestingly, in male wild-type flies and the *Pu*^{Z22} mutant we were not able to detect *AMPK* expression levels in old flies. Similarly, in old females no expression of AMPK was recorded in *Pu*^{Z22} and *VMAT*$^{\Delta 14}$ mutants (Figure 3.3B).

Interestingly in old male flies of all genotypes tested *dTOR* expression was significantly elevated when compared with younger male flies, except in case of *Catsup*^{26} mutant (where there was no difference) and *ple*^{2} mutant where no expression was recorded in old flies (Figure 3.3C). This was the opposite of what was observed in female flies where the expression of *dTOR* always decreased in old flies compared to young individuals with the exception of *ple*^{2} mutant where *dTOR* expression was significantly up-regulated in old flies (Figure 3.3C). A similar expression trend was also recorded for *Akt* (Figure 3.3D).

In general *dFoxO* expression levels was higher in females compared to age-matched males across all genotypes, except in case of wild-type where expression of *dFoxO* in old females was on par with old males (Figure 3.3E). There was also a

tendency of dFoxO expression with age to either decline or remain at par with young individuals across all genotypes regardless of gender (Figure 3.3E).

The expression of *dSesn* was markedly up-regulated in young females of all genotypes compared to age-matched males (Figure 3.3F). However, in old females there was also a significant down-regulation in expression of *dSesn* among all genotypes studied except in case of ple^{2} mutants where the expression of *dSesn* in old females was on par with young females. In males on the other hand, *dSesn* expression increased in old males when compared to young males except in $Catsup^{26}$ and $VMAT^{\Delta 14}$ mutants (Figure 3.3F).

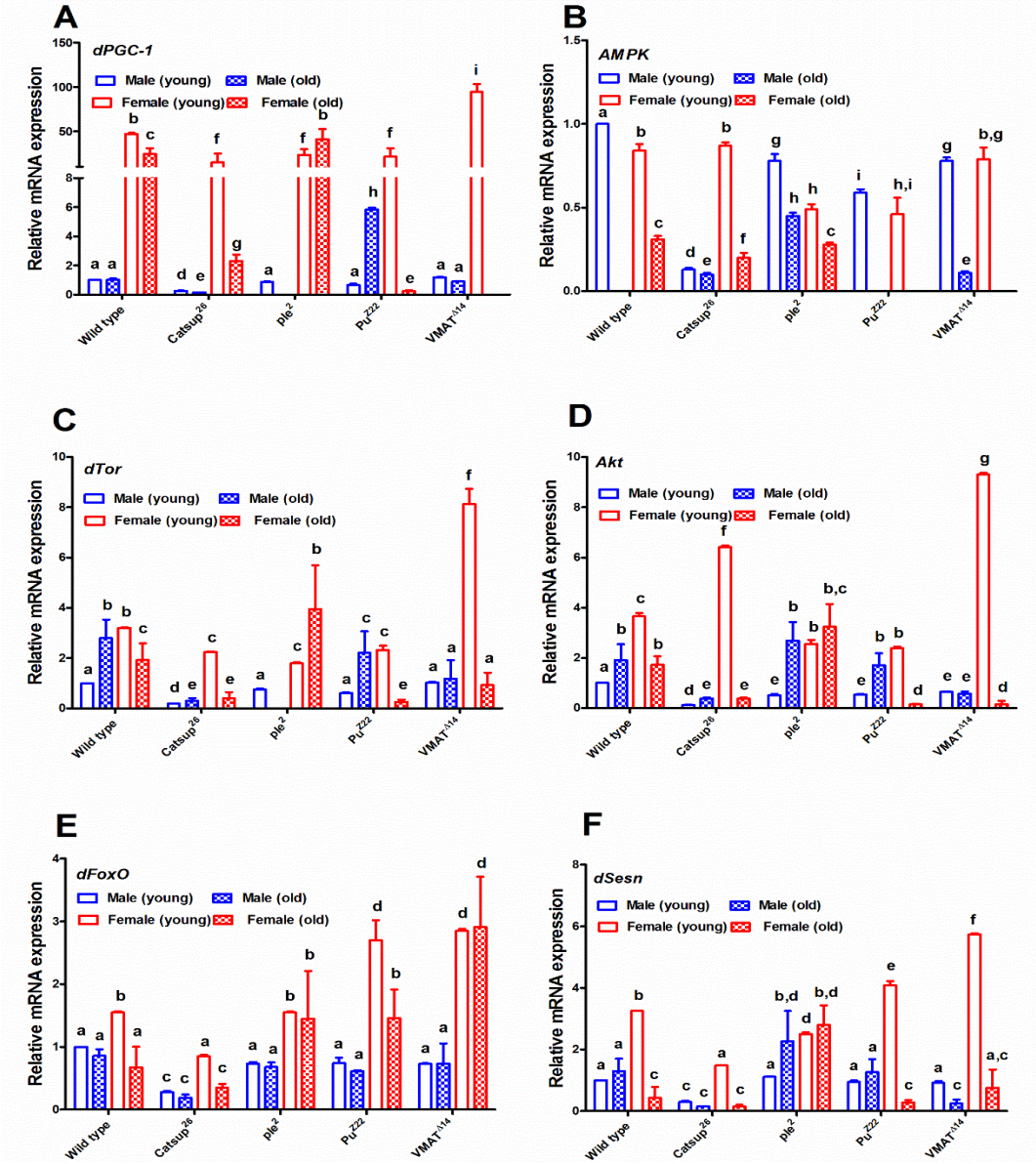

Figure 3.3 Quantitative-Reverse Transcription PCR of key signaling members involved in stress response and aging.

(A) dPGC-1 (B)AMPK (C) dTOR (D)Akt (E) dFoxO and (F) dSesn in male and female flies of two different ages from different fly lines with mutations in synthesis or transport of DA. Relative mRNA expression of different fly lines was quantified using wild type controls (w1118 and Canton-S) which was set as a reference (=1). Values represent mean ± SD of two independent biological replicates. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

3.5 Discussion

The results of this study clearly indicate a sexually dimorphic response in aging and senescence and the possible involvement of DA in the process. In general, elevation of DA levels in *Catsup*^{26} mutants resulted in enhanced longevity in both males and females compared to mutants with impaired DA synthesis (*ple*^{2} and *Pu*^{Z22}) or transport (*VMAT*$^{\Delta 14}$). This enhanced longevity of *Catsup*^{26} was not significantly different from wild type controls in case of males while in case of females, this was markedly lower than wild type controls. Also, in each of the different mutants, a sexually dimorphic difference in longevity profiles was recorded. Thus, increased DA levels were not detrimental to flies of either sex, but depleted DA levels or their transport did have an impact on their longevity. This is in agreement with the results that were reported earlier (Vermeulen et al., 2006; Hanna et al., 2015). However, it has also been reported that there are behavioral consequences to DA deficiency in the adult brain but this does not impact their longevity significantly compared to wild-type flies (Riemensperger et al., 2011). The apparent discrepancy observed between their results and our present study could be genotype specific.

A sexually dimorphic decline in whole body DA levels with age has been reported earlier (Neckameyer et al., 2000), however, apparently brain DA levels do not get impacted significantly with age (White et al., 2010). We report that perturbed DA levels have a significant impact on both longevity and senescence characteristics in a sexually dimorphic manner. Thus, age-related senescence showed a marked sexually dimorphic response with female flies exhibiting higher average daily activity compared to males, except in young *ple*^{2} and *VMAT*$^{\Delta 14}$ mutants. Elevated DA levels in *Catsup*^{26}

mutants resulted in enhanced walking activity in females compared to age-matched males. While in males across all genotypes (except in Pu^{Z22}), there was a decreased walking activity in old individuals compared to young, the walking activity was either enhanced or remained at par when old females were compared to young ones. Enhanced walking activity in aged *Drosophila* has also been demonstrated earlier (White et al., 2010), which is very different from startle-induced locomotion and negative geotaxis. A rapid decline in rhythmicity was observed in both males and females with age across genotypes and this was more so in case of females. Age-related behavioral changes has been reported earlier in *Drosophila* (Iliadi and Boulianne, 2010). The effects of aging on circadian rhythms have been reported in numerous studies in different animal species (Kondratov, 2007). It has been suggested that changes in circadian rhythm can alter metabolic processes that are associated with circadian timing (Aschoff, 1984), eventually resulting in accelerated senescence. Yet, despite being arrhythmic with age $Catsup^{26}$ flies with elevated DA levels displayed enhanced longevity in both males and females compared to mutants with depleted DA levels (ple^{2} or Pu^{Z22}) or its transport ($VMAT^{\Delta 14}$). Obviously, such elevated DA levels in the $Catsup^{26}$ mutants might have a stress-protective function as reported earlier (Hanna et al., 2015) that might contribute to enhanced lifespan compared with mutants with depleted DA or impaired transport.

Protein oxidative damage showed a tendency to accumulate more slowly in females compared to males, despite the fact that young females in general had slightly more protein carbonyls in general compared to age-matched males. The slower accumulated of oxidatively damaged proteins correlates with enhanced longevity observed in females compared to males across genotypes barring certain exceptions. This

strengthens the link between protein oxidative damage and the aging process as reported earlier (Sohal et al., 1993). However, correlation cannot be taken as causation. Thus, a stress paradigm would need to be introduced in order to tease apart the link between stress induced damage and lifespan as reported earlier (Hanna et al., 2015).

According to several aging theories lifespan is causally related to the ability to withstand extrinsic or intrinsic stresses (Vermeulen and Loeschcke, 2007). Hence, longevity should positively correlate with the ability to resist stress (Kirkwood and Austad, 2000). Thus, genes involved in the stress response should be relevant candidates for lifespan determination at the mechanistic level. To explore this aspect, several candidate genes involved in both stress response and aging were targeted in this study. The dramatic up-regulation of *dPGC-1* in age-matched females compared to males across genotypes indicates a strong link between this transcription factor and aging. The peroxisome proliferator activated receptor gamma co-activator (PGC-1) family of transcriptional co-activators play a central role in regulation of mitochondrial biogenesis and function (Scarpulla, 2008). The *Drosophila* homolog of this is Spargel/ dPGC-1, and shares certain functional similarities and divergences with the mammalian PGC-1 protein (Mukherjee et al., 2014). While Spargel/dPGC-1 apparently does not influence antioxidant enzymes, it does play a significant role in growth, longevity and aging because of its critical role in energy homeostasis (Rera et al., 2011; Mukherjee et al., 2014). The AMP-activated protein kinase (AMPK) is involved in the adaptive response to energy deficit. Activation of AMPK induces PGC-1. In this study, a marked up-regulated of AMPK expression was recorded in female *Catsup*^{26} mutants compared to their male counterparts. While in mutants with depleted DA levels *AMPK* expression in

females was markedly lower. With aging, there is a blunted metabolic response to AMPK activation (Reznick et al., 2007). This was also observed in our present study. AMPK is involved in PGC-1 autoregulation, such that direct phosphorylation by AMPK promotes PGC-1 dependent induction at the PGC-1 promoter (Jager et al., 2007). In addition to activating PGC-1, AMPK also inhibits TOR (Gwinn et al., 2008), a nutrient activated factor that regulates PGC-1 gene expression (Cunningham et al., 2007). In this study, *dTOR* expression levels were significantly down-regulated especially in young males compared to age-matched females except in case of the $VMAT^{\Delta 14}$ mutants. Inhibition of TOR has been shown to extend lifespan in yeast, worms and flies (Anderson and Weindruch, 2007). dTOR is also a repressor of dFoxO. The serine threonine kinase AKT is a negative regulator of PGC-1 (Anderson and Prolla, 2009). This was also significantly down-regulated in young males compared to age-matched females. AKT has also been demonstrated to have a repressive effect on *dFoxO* transcription by phosphorylating it at specific sites (Brunet et al., 1999). Both *dFoxO* and *dSesn* expression levels were up-regulated in age-matched females compared to males across genotypes. The FoxO transcription factors are involved in several physiological and pathological processes, including aging, cancer and neurological diseases (Maiese et al., 2008; Greer and Brunet, 2008). However, enhanced dFoxO expression does not necessarily correlate with enhanced longevity since DA depleted mutants or mutants with mutation in DA transport showed higher expression levels in females and these mutants did not have enhanced longevity. Sestrins encoded by *dSesn* are a family of stress inducible proteins that can function as antioxidants and inhibitors of TOR and play key roles in stress response and

aging (Lee et al., 2010). *dSesn* expression in general declined with age in females except in the ple^2 mutants.

Taken together, our data does indicate that there is a sexually dimorphic response between males and females during aging and senescence but the role played by DA in such a process is as yet not very clear-cut. We did observe some genotype specific changes in gene expression but it is difficult to attribute these changes to the process of longevity and senescence.

3.6 References

Aarts, E., van Holstein, M., Cools, R., 2011. Striatal dopamine and the interface between motivation and cognition. *Front. Physiol.* 2: 163.

Abercrombie, E.D., Keefe, K.A., DiFrischia, D.S., Zigmond, M.J., 1989. Differential effect of stress on *in vivo* dopamine release in striatum, nucleus accumbens, and medial frontal cortex. *J. Neurochem*. 52: 1655-1658.

Anderson, R., Prolla, T., 2009. PGC-1α in aging and anti-aging interventions. *Biochim. Biophys. Acta.* 1790: 1059-1066.

Anderson, R.M., Weindruch, R., 2007. Metabolic reprogramming in dietary restriction. *Interdiscipl. Top. Gerontol.* 35: 18-38.

Andretic, R., van Swinderen, B., Greenspan, R.J., 2005. Dopaminergic modulation of arousal in *Drosophila*. *Curr. Biol*. 15: 1165-1175.

Antosh, M., Fox, D., Helfand, S.L., Cooper, L.N., Neretti, N., 2011. New comparative genomics approach reveals a conserved health span signature across species. *Aging* 3: 576-583.

Argue, K.J., 2012. Differential recruitment of dopamine neurons into the stress response in *Drosophila melanogaster* depends on sex and level of sexual maturity. PhD Thesis. Saint Louis University, 217 pages.

Aschoff, J., 1984. Circadian timing. *Ann. NY Acad. Sci.* 423: 442-468.

Barger, J.L., Walford, R.L., Weindruch, R., 2003. The retardation of aging by caloric restriction: its significance in the transgenic era. *Exp. Gerontol*. 38: 1343-1451.

Besedovsky, H., Del Ray, A., 1996. Immune-neuro-endocrine interactions: facts and hypothesis. *Endocrinol. Rev*. 17: 64-102.

Blenau, W., Baumann, A., 2001. Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol*. 48: 13-38.

Boulianne, G.L., 2001. Neuronal regulation of lifespan: clues from flies and worms. *Mech. Ageing Dev*. 122: 883-894.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell* 96: 857-868.

Chahal, H.S., Drake, W.M., 2007. The endocrine system and ageing. *J. Pathol.* 211: 173-180.

Corey, J.L., Quick, M.W., Davidson, N., Lester, H.A., Guastella, A., 1994. A cocaine-sensitive Drosophila serotonin transporter: cloning, expression, and electrophysiological characterization. *Proc. Natl. Acad. Sci. USA*. 91: 1188-1192.

Cunningham, J.T., Rodgers, J.T., Arlow, D.H., Vazquez, F., Mootha, V.K., Puigserver, P., 2007. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 450: 736-740.

D'Angio, M., Serrano, A., Driscoll, P., Scatton, B., 1988. Stressful environmental stimuli increase extracellular DOPAC levels in the prefrontal cortex of hypoemotional (Roman high-avoidance) but not hyperemotional (Roman low-avoidance) rats. An *in vivo* voltametric study. *Brain Res.* 451: 237-247.

De La Fuente, M., 2002. Effects of antioxidants on immune system ageing. *Eur. J. Clin. Nutr.* 56: S5-S8.

De Luca, M., Roshina, N.V., Geiger-Thornsberry, G.L., Lyman, R.F., Pasyukova, E.G., Mackay, T.F., 2003. Dopa decarboxylase (*Ddc*) affects variation in *Drosophila* longevity. *Nat. Genet.* 34: 429-433.

Demarest, K.T., Riegle, G.D., Moore, K.E., 1980. Characteristics of dopaminergic neurons in the aged male rat. *Neuroendocrinol*. 31: 222-227.

Fabris, N., 1991. Neuroendocrine-immune interactions: a theoretical approach to ageing. *Arch. Gerontol. Geriatr.* 12: 219-230.

Foltenyi, K., Andretic, R., Newport, J.W., Greenspan, R.J., 2007. Neurohormonal and neuromodulatory control of sleep in *Drosophila. Cold Spring Harb. Symp. Quant. Biol*. 72: 565-571.

Friggi-Grelin, F., Coulom, H., Meller, M., Gomez, D., Hirsh, J., Birman, S., 2003. Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol*. 54: 618-627.

Goicoechea, C., Ormazabal, M., Alfaro, M., Martin, M., 1997. Age-related changes in nociception, behavior and monoamine levels in rats. *Gen. Pharm.* 28: 331-336.

Gozlan, H., Daval, G., Verge, D., Spampinato, U., Fattacini, C., Gallisor, M., el Mestikawy, S., Harmon, M., 1990. Aging-associated changes in serotonergic and dopaminergic pre- and post-synaptic neurochemical markers in the rat brain. *Neurobiol. Aging* 11: 437-449.

Greer, E.L., Brunet, A., 2008. FOXO transcription factors in ageing and cancer. *Acta. Physiol.* 192: 19-28.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., Mihaylova, M.M., Mery, A., Vasquez, D.S., Turk, B.E., Shaw, R.J., 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. *Mol. Cell* 30: 214-226.

Hanna, M.E., Bednářová, A., Rakshit, K., Chaudhuri, A., O'Donnell, J.M., Krishnan, N., 2015. Perturbations in dopamine synthesis lead to discrete physiological effects and impact oxidative stress response in *Drosophila*. *J. Insect Physiol.* 73: 11-19.

Helfand, S.L., Rogina, B., 2003a. Genetics of aging in the fruit fly, *Drosophila melanogaster*. *Annu. Rev. Genet.* 37: 329-348.

Helfand, S.L., Rogina, B., 2003b. Molecular genetics of aging in the fly: is this the end of the beginning? *Bioessays* 25: 134-141.

Iliadi, K.G., Boulianne, G.L., 2010. Age-related behvaioral changes in *Drosophila*. *Ann. NY Acad. Sci.* 1197: 9-18.

Iliadi, K.G., Knight, D., Boulianne, G.L., 2012. Healthy aging – insights from *Drosophila*. *Front. Physiol.* 3: 106.

Jager, S., Handschin, C., St-Pierre, J., Speigelman, B.M., 2007. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1aplha. *Proc. Natl. Acad. Sci.* USA 104: 12017-12022.

Kenyon, C., 2001. A conserved regulatory system for aging. *Cell* 105: 165-168.

Kirkwood, T.B.L., Austad, S.N., 2000. Why do we age? *Nature* 408: 233-238.

Kish, S.J., Shannak, K., Rajput, A.deck, J.H., Hornykiewicz, O., 1992. Aging produces a specific pattern of striatal loss: implications for the etiology of idiopathic Parkinson's disease. *J. Neurochem*. 58: 642-648.

Kondratov, R.V., 2007. A role of the circadian system and circadian proteins in aging. *Ageing Res. Rev.* 6: 12-27.

Krishnan, N., Večeřa, J., Kodrík, D., Sehnal, F., 2007. 20-Hydroxyecdysone prevents oxidative stress damage in adult *Pyrrhocoris apterus*. *Arch. Insect Biochem. Physiol*. 65: 114-124.

Krishnan, N., Kretzschmar, D., Rakshit, K., Chow, E., Giebultowicz, J.M., 2009. The circadian clock gene period extends healthspan in aging *Drosophila melanogaster. Aging* 1: 937-948.

Kume, K., Kume, S., Park, S.K., Hirsh, J., Jackson, F.R., 2005. Dopamine is a regulator of arousal in the fruit fly. *J. Neurosci.* 25: 7377-7384.

Landis, G.N., Abdueva, D., Skvortov, D., Yang, J., Rabin, B.E., Carrick, J., Tavare, S., Tower, J., 2004. Similar gene expression patterns characterize aging and oxidative stress in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* USA 101: 7663-7668.

Landis, G., Shen, J., Tower, J. 2012. Gene expression changes in response to aging compared to heat stress, oxidative stress and ionizing radiation in *Drosophila melanogaster*. *Aging* 4: 768-789.

Lee, J.H., Bodmer, R., Bier, E., Karin, M., 2010. Sestrins at the crossroad between stress and aging. *Aging* 2: 369-374.

Liu, T., Dartevelle, L., Yuan, C., Wei, H., Wang, Y., Ferveur, J.F., Gua, A., 2008. Increased dopamine level enhances male-male courship in *Drosophila*. *J. Neurosci*. 28: 5539-5546.

Mackay, W., Reynolds, E.R., O'Donnell, J.M., 1985. Tissue-specific and complex complementation patterns in the *Punch* locus of *Drosophila melanogaster*. *Genetics* 111: 885-904.

Maiese, K., Chong, Z.Z., Shang, Y.C., 2008. OutFOXOing disease and disability: the therapeutic potential of targeting FoxO proteins. *Trends Mol. Med.* 14: 219-277.

Mukherjee, S., Basar, M.A., Davis, C., Duttaroy, A., 2014. Emerging funcitonal similarities and divergences between *Drosophila* spargel/dPGC-1 and mammalian PGC-1 protien. *Front. Genet.* doi:3389/fgene.2014.00216.

Neckameyer, W., 1998. Dopamine and mushroom bodies in *Drosophila*: experience-dependent and –independent aspects of sexual behavior. *Learn. Mem*. 5: 157-165.

Neckameyer, W.S., White, K., 1993. *Drosophila* tyrosine hydroxylase is encoded by the *pale* locus. *J. Neurogenet.* 8: 189-199.

Neckameyer, W.S., Woodrome, S., Holt, B., Mayer, A., 2000. Dopamine and senescence in *Drosophila melanogaster*. *Neurobiol. Aging.* 21: 145-152.

Noth, R.H., Mazzaferri, E.L., 1985. Age and the endocrine system. *Clin. Geriatr. Med.* 1: 223-250.

Pendleton, R.G., Rasheed, A., Sardina, T., Tully, T., Hillman, R., 2002. Effects of tyrosine hydroxylase mutants on locomotor activity in *Drosophila*: a study in functional genomics. *Behav. Genet.* 32: 89-94.

Pfeiffenberger, C., Lear, B.C., Keegan, K.P., Allada, R., 2010. Locomotor activity level monitoring using the Drosophila Activity Monitoring (DAM) System. *Cold Spring Harb. Protoc.* 11, pdb.prot5518.

Pletcher, S.D., 1999. Model fitting and hypothesis testing for age-specific mortality data. *J. Evol. Biol*. 12: 430-439.

Porzgen, P., Park, S.K., Hirsh, J., Sonders, M.S., Amara, S.G., 2001. The antidepressant-sensitive dopamine transporter in *Drosophila*: a primordial carrier for catecholamines. *Mol. Pharmacol*. 59: 83-95.

Rehman, H.U., Masson, E.A., 2001. Neuroendocrinology of ageing. *Age Ageing* 30: 279-287.

Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., Ansari, W.S., Lo. T. Jr., Jones, D.L., Walker, D.W., 2011. Modulation of longevity and tissue homeostasis by the *Drosophila* PGC-1 homolog. *Cell. Metab.* 14: 623-634.

Reynolds, E.R., O'Donnell, J.M., 1988. Characterization of new *Punch* mutations: identification of two additional mutant classes. *Genetics* 119: 609-617.

Reznick, R.M., Zong, H., Li, J., Morino, K., Moore, I.K., Yu, H.J., Liu, Z-X., Dong, J., Mustard, K.J., Hawley, S.A., Befroy, D., Pypaert, M., Hardie, G.G., Young, L.H., Shulman, G.I., 2007. Aging-associated reductions in AMP-activated protein kinase acitivity and mitochondrial biogenesis. *Cell. Metab.* 5: 151-156.

Riemensperger, T., Isabel, G., Coulom, H., Neuser, K., Seugnet, L., Kume, K., Iché-Torres, M., Cassar, M., Strauss, R., Preat, T., Hirsh, J., Birman, S., 2011. Behavioral consequences of dopamine deficiency in the *Drosophila* central nervous system. *Proc. Natl. Acad. Sci.* USA 108: 834-839.

Romero-Calderon, R., Uhlenbrock, G., Borycz, J., Simon, A.F., Grygoruk, A., Yee, S.K., Shyer, A., Ackerson, L.C., Maidment, N.T., Meinertzhagen, I.A., Hovemann. B.T., Krantz, D.E., 2008. A glial variant of the vesicular monoamine transporter is required to store histamine in the *Drosophila* visual system. *PLoS Genet.* 4: e1000245.

Sabban, E.L., 2007. Catecholamines in stress: molecular mechanisms of gene expression. *Endocr. Regul.* 41: 61-73.

Scarpulla, R.C., 2008. Transcriptional paradigms in mammalian mitochondrial biogenesis and function. *Physiol. Rev.* 88: 611-638.

Sohal, R.S., Agarwal, S., Dubey, A., Orr, W.C., 1993. Protein oxidative damage is associated with life expectancy of houseflies. *Proc. Natl. Acad. Sci.* USA 90: 7255-7259.

Stathakis, D.G., Burton, Y., McIvor, W.E., Krishnakumar, S., Wright, T.R.F., O'Donnell, J.M., 1999. The catecholamines up (Catsup) protein of *Drosophila melanogaster* functions as a negative regulator of tyrosine hydroxylase activity. *Genetics* 153: 361-382.

Sullivan, R., 2004. Hemispheric assymetry in stress processing in rat prefrontal cortex and the role of mesocortical dopamine. *Stress* 7: 131-143.

Takahashi, Y., Kuro, O.M., Ishikawa, F., 2000. Aging mechanisms. *Proc. Natl. Acad. Sci. USA*. 97: 12407-12408.

Tatar, M., Bartke, A., Antebi, A., 2003. The endocrine regulation of aging by insulin-like signals. *Science* 299: 1346-1351.

Vermeulen, C.J., Cremers, T.I., Westerink, B.H., Van De Zande, L., Bijlsma, R. 2006. Changes in dopamine levels and locomotor activity in response to selection on virgin lifespan in Drosophila melanogaster. *Mech. Ageing Dev.* 127: 610-617.

Vermeulen, C.J., Loeschcke, V., 2007. Longevity and the stress response in *Drosophila*. *Exp. Geront.* 42: 153-159.

Villar-Cheda, B., Moninguez-Meijide, A., Valenzuela, R., Granado, N., Moratalla, R., Labandeira-Garcia, J.L., 2014. Aging- related dysregulation of dopamine and angiotensin receptor interaction. *Neurobiol. Aging*. 35: 1726-1738.

White, K.E., Humphrey, D.M., Hirth, F., 2010. The dopaminergic system in the aging brain of *Drosophila*. *Front. Neurosci.* doi: 10.3389/fnins.2010.00205.

Winterer, G., Weinberger, D.R., 2004. Genes, dopamine and cortical signal-to-noise ratio in schizophrenia. *Trends Neurosci.* 27: 683-690.

Wood, J.G., Rogina, B., Lavu, S., Howitz, K., Helfand, S.L., Tatar, M., Sinclair, D., 2004. Sirtuin activation mimic caloric restriction and delay ageing in metazoans. *Nature* 430: 686-689.

Woods, J.M., Druse, M.J., 1996. Effects of chronic ethanol consumption and aging on dopamine, serotonin, and metabolites. *J. Neruochem.* 66: 2168-2178.

CHAPTER IV

CONCLUSIONS AND FUTURE PERSPECTIVES

The major objectives of this thesis was to examine the effects of perturbations in DA synthesis and transport on stress response followed by investigations if DA has a sexually dimorphic role in aging and senescence in *Drosophila*. The major conclusions emanating from this thesis are as follows:

Perturbations in DA synthesis and transport have a significant impact on OS response. Elevated DA levels in $Catsup^{26}$ mutants result in GSTO1 transcriptional up-regulation upon exposure to OS thus contributing to resistance to OS. Depleted DA levels (in ple^{2} and Pu^{Z22} mutants) lead to increased susceptibility to OS by down-regulation of GSTO1 transcription.

A sexually dimorphic effect on aging and senescence characteristics was observed in *Drosophila*, however, DA may not be a strong contributing factor on the observed differences.

DA signaling pathways have been reported to be affected by stress (Neckameyer and Weinstein, 2005). Stress can affect normal dopaminergic neurotransmission, and hence, exposure to stress greatly increases dopaminergic activity (Pani et al., 2000). The work presented in this thesis clearly demonstrates that elevated DA pools can have a stress-protective function especially against OS. While depleted DA levels have a negative impact on OS resistance (Chapter II).

4.1 Linking stress-response, aging and neurodegeneration

While stress response and aging are causally linked, our results do not support a strong role for DA in aging and senescence. There is, however, a sexually dimorphic response in *Drosophila* on aging and senescence characteristics. The ramifications aging has on the neuroendocrine system alludes to the development of various age-dependent pathologies (Rehman and Masson, 2001). Aging culminates a functional decline in the nervous system resulting in alterations of the immune or stress response systems, and an impaired relationship between the two regulatory systems becomes apparent and also leads to homeostatic disruptions and a higher risk of death (Fabris, 1991; De La Fuenta, 2002). Aging represents a major risk factor for a plethora of age-related diseases (Squier, 2001), with a particular pre-disposition for neurodegenerative diseases (Bishop et al., 2010). Many age-related neurodegenerative diseases are characterized by accumulation of disease-specific misfolded proteins in the central nervous system (van Ham et al., 2009). These include β-amyloid peptides and tau/ phosphorylated tau proteins in Alzheimer's disease, α-synuclein in Parkinson's disease, superoxide dismutase in amyotrophic lateral sclerosis (Durham et al., 1997), and mutant huntingtin in Huntinton's disease (Scherzinger et al., 1997). The association between age and protein misfolding is not clear yet. However, accumulation of oxidatively damaged proteins is common during both aging as well as in neurodegenerative diseases (Grune et al., 2001). In our study (Chapter III), a distinct sexually dimorphic response on accumulation of oxidatively damaged proteins was recorded, with females having a slower accumulation rate compared to males. This might be indicative of males having a higher propensity for age-related neurodegenerative diseases particularly Parkinson's disease where the

dopaminergic system is significantly affected (Gillies et al., 2014). However, this might not explain why females have a higher prevalence and severity in Alzhimer's disease (Carter et al., 2012).

One of the clear themes that has emerged from several lines of aging research is that OS is a major factor in aging and cellular senescence (Carrard et al., 2002; Finkel and Holbrook, 2000). Mitochondrial and cytoplasmic production of hydrogen peroxide, superoxide free radicals and hydroxyl free radicals can cause substantial modifications of DNA, lipids and proteins (Stadtman, 1992). The notion that these alterations result in cumulative macromolecular damage, which contributes to senescent decline is the foundation of the free radical theory of aging as originally outlined by Harman in 1957 (Harman, 1988). OS also plays an important role in neurodegenerative disorders; the concept that OS occurs in Parkinson's disease derives from the fact that OS can be initiated by a decline in the anti-oxidative defense system or a decrease of antioxidant concentration caused by other factors all leading to a loss of DA neurons. Glutathione (GSH) is an important intracellular antioxidant; the most robust and significant alteration in antioxidant defense in Parkinson's disease is a decrease of GSH concentration. Another consistent finding in Parkinson's disease patients is a defect in oxidative phosphorylation due to a decrease in the electron transport chain complex I activity in the substantia nigra. It remains controversial whether a decrease in GSH concentrations precedes the defect of oxidative phosphorylation or vice versa (Schulz et al., 2000). There is mounting evidence that OS is also involved in the pathogenesis of Alzheimer's disease. Our finding that elevated DA pools confer protection against OS by up-regulating GSTO1 is unique and can lead to new insights on the role DA plays in stress response

and neurodegenerative pathologies. However, DA by itself may not be a major player in aging and senescence related changes.

4.2 Future perspectives

The key questions with regard to DA in all age-related changes is whether they have a role in predisposing to age-related neurodegenerative diseases or modulating their severity and progression. This enigma remains largely unsolved. Studies in simple invertebrate model organisms such as *Drosophila* should help address this question. Future studies should focus on exploring the role of specific DA receptors such as DopR, D1-like or D2R in stress response and age-related neurodegenerative pathologies. Since knockout of DA synthesis is embryonically lethal, conditional knock-out of receptor targets should provide more information on such aspects. Given newly developed experimental approaches and well defined genetic systems available in the *Drosophila* model, future work should now enable the link between DA, stress response and neurodegeneration to be addressed in greater detail.

4.3 References

Bishop, N.A., Lu, T., Yankner, B.A., 2010. Neural mechanisms of ageing and cognitive decline. *Nature* 464: 529-535.

Carrard, G., Dieu, M., Raes, M., Toussaint, O., Friguet, B., 2002. Impact of ageing on proteasome structure and function in human lymphocytes. *Int. J. Biochem. Cell Biol*. 1447: 1-12.

Carter, C.L., Resnick, E., Mallampalli, M., Kalbarczyk, A., 2012. Sex and gender differences in Alzheimer's disease: recommendations for future research. *J. Women's Health* 21: 1018-1023.

De La Fuente, M., 2002. Effects of antioxidants on immune system ageing. *Eur. J. Clin. Nutr.* 56: S5-S8.

Durham, H.D., Roy, J., Dong, L., Figlewicz, D.A., 1997. Aggregation of mutant Cu/Zn superoxide dismutase proteins in a culture model of ALS. *J. Neuropathol. Exp. Neurol.* 56: 523-530.

Fabris, N., 1991. Neuroendocrine-immune interactions: a theoretical approach to ageing. *Arch. Gerontol. Geriatr.* 12: 219-230.

Finketl, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239-247.

Gillies, G.E., Pienaar, I.S., Vohra, S., Qamhawi, Z., 2014. Sex differences in Parkinson's disease. *Front. Neuroendocrinol.* 35: 370-384.

Grune, T., Shringarpure, R., Sitte, N., Davies, K.J.A., 2001. Age related changes in preotien oxidation and proteolysis in mammalian cells. *J. Gerontol. Biol. Sci.* 56: 459-467.

Harman, D., 1988. Free radicals in aging. *Mol. Cell. Biochem.* 84: 155-161.

Neckameyer, W.S., Weinstein, J.S., 2005. Stress affects dopaminergic signaling pathways in *Drosophila melanogaster*. *Stress* 8(2): 1-15.

Pani, L., Porcella, A., Gessa, G.L., 2000. The role of stress in the pathophysiology of the dopaminergic system. *Mol. Psych.* 5: 14-21.

Rehman, H., Masson, E., 2001. Neuroendocrinology of ageing. *Age Ageing* 30: 279-287.

Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollenbach, B., Hasenbank, R., Bates, G.P., Davies, S.W., Lehrach, H., Wanker, E.E., 1997. Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell* 90: 549-558.

Schulz, J., Dehmer, T., Schöls, L., Mende, H., Hardt, C., Vorgerd, M., Bürk, K., Dichgans, J., Beal, M.F., Bogdanov, M.B., 2000. Oxidative stress in Friedreich ataxia. *Neurology* 55: 1719-1721.

Squier, T.C., 2001. Oxidative stress and protein aggregation during biological aging. *Exp. Gerontol.* 36: 1539-1550.

Stadtman, E.R., 1992. Protein oxidation and aging. *Science* 28: 1220-1224.

Van Ham, T.J., Breitling, R., Swertz, M.A., Nollen, E.A.A., 2009. Neurodegenerative diseases: Lessons from genome-wide screens in small model organisms. *EMBO Mol. Med.* 1: 360-370.

APPENDIX A

SUPPLEMENTAL DATA FOR CHAPTER II

Table A.1 List of primers and their sequences used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
rp49	ACG TTG TGC ACC AGG AAC TT	CCA GTC GGA TCG ATA TGC TAA
Cat	AGA TGC TGC ATG GTC GTC TGT TGT TCT	TCC ATC CCG CTG GAA GTT CTC AAT
MnSOD	ACA TCA CCG ACT CCA AGA TTA C	TTG CCC GTT GAC TTG CT
CuZnSOD	TAA ATT GAT TAA TTC ATT CG	ACA TCG GAA TAG ATT ATC GC
GSTO1	CAT ATG AGC AAT ACT CAG CAC TTA ACT AT	GGA TCC CTA CCC CAA TTT GAC ACG TTT G
GSTO3	CAT ATG AGT TCT GGT AAA CAT TTG GCC AA	GGA TCC CTA AGC CAG CAG ATC GTA GTT T

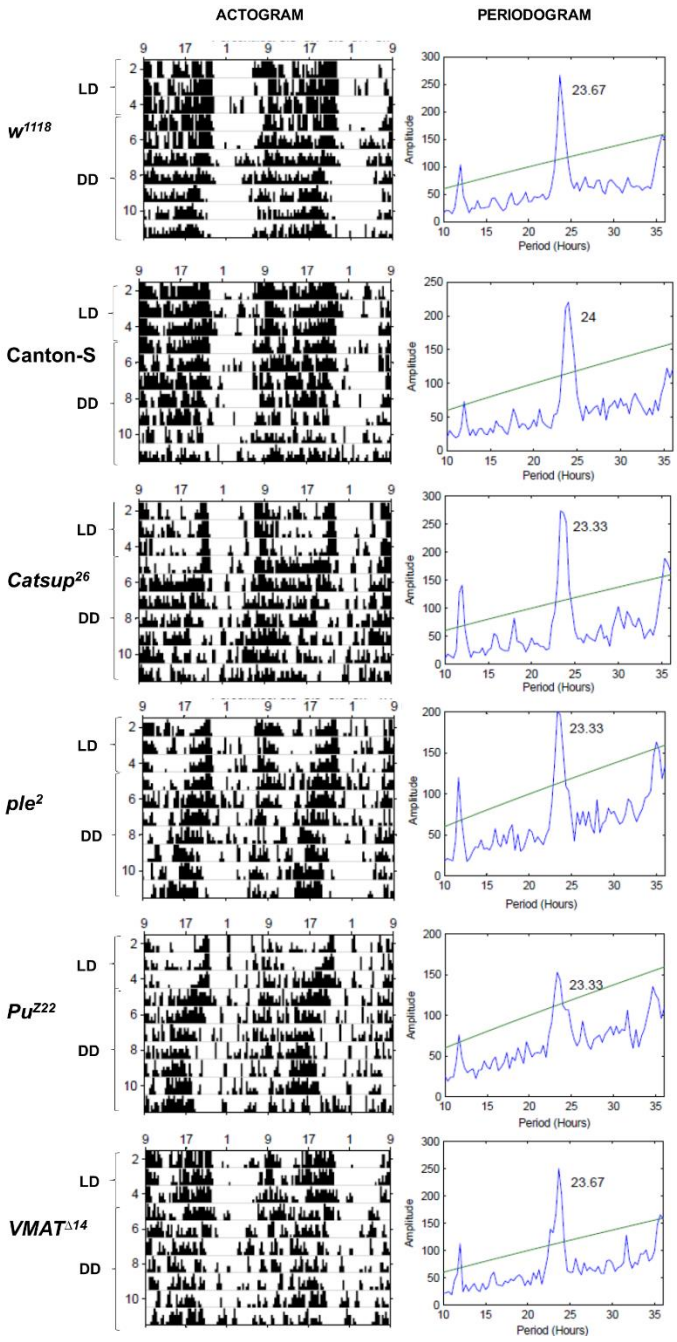

Figure A.1 Representative double-plotted actograms and periodograms of w^{1118}, Canton-S, $Catsup^{26}$, ple^{2}, Pu^{Z22} and $VMAT^{\Delta 14}$ flies ~ 6-8 days after adult eclosion.

Flies were entrained for 3 days in 12h:12h LD (Light/Dark) cycles before being maintained in constant darkness (DD) for 7-10 days. Actograms and periodograms were generated using ClockLab (Actrimetrics). The Y-axis on actograms represents days, whereas the top axis denotes hours in military time (for further details refer to the text).

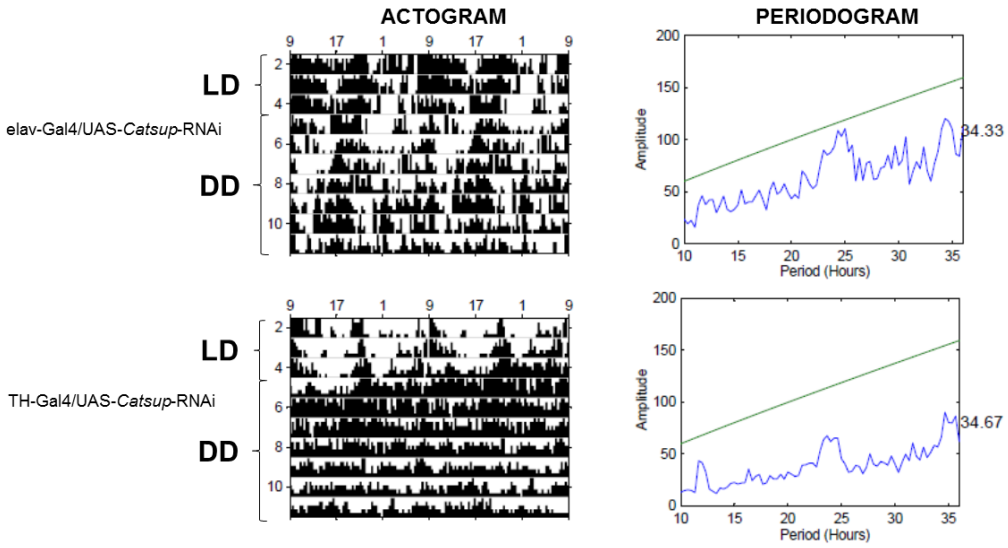

Figure A.2 Representative double-plotted actograms and periodograms of *elav*-Gal4/UAS-*Catsup*-RNAi and TH-Gal4/UAS-*Catsup*-RNAi flies ~ 6-8 days after adult eclosion.

Flies were entrained for 3 days in 12h:12h LD (Light/Dark) cycles before being maintained in constant darkness (DD) for 7-10 days. Actograms and periodograms were generated using ClockLab (Actrimetrics). The Y-axis on actograms represents days, whereas the top axis denotes hours in military time (for further details refer to the text).

APPENDIX B

SUPPLEMENTAL DATA FOR CHAPTER III

Table B.1 Primer sequences of transcription factors used in this study.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
rp49	CAG TCG GAT CGA TAT GCT AAG GTG	TAA CCG ATG TTG GGC ATC AGA TAC T
PGC-1	GGA TTC ACG AAT GCT AAA TGT GTT CC	GAT GGG TAG GAT GCC GCT CAG
AMPK	CAT CCG CAC ATC ATC AAG TT	TTC TCT GGC TTC AGG TCT CG
Tor	CAG GTT ATC CCG CAG CTT ATT	GCG GGT GAT TCT TTC CTA TGT
Akt	GCA GAG AAA TTC AGC TGG CAG CAA	TGA GTC TGT TCC GTA AGC GCA TGA
FOXO	CCG CCA GCT TGG AAG ATA ATA	CAC GGG AAA GTT CTC CAG ATT
Sestrin	CTC GAC TCG ATC CCC TCC G	CAG GTC ATC GAG CTC GTC C

Table B.2 Mortality parameters derived from fitted Gompertz-Makeham model and maximum likelihood estimates (MLE).

Genotype	Gompertz-Makeham parameters			Actual lifespan	Fitted lifespan	% Error in lifespan
	a (intercept) MLE value	*b* (slope) MLE value	*c* (constant) MLE value			
Males						
wild type (w^{1118} or Canton-S)	$4.1(10^{-4})$	0.091	$2.1(10^{-9})$	53.9	53.3	0.01
***Catsup*^{26}**	$6.9(10^{-4})$	0.075	$3.0(10^{-4})$	54.6	54.5	0.0009
***ple*^{2}**	$2.1(10^{-9})$	0.321	$3.3(10^{-3})$	51.4	51.7	0.006
***Pu*^{Z22}**	$5.8\ (10^{-4})$	0.097	$2.1(10^{-9})$	47.1	46.9	0.002
***VMAT*$^{\Delta 14}$**	$1.4(10^{-3})$	0.101	$2.1(10^{-9})$	37.8	36.7	0.03
Females						
wild type (w^{1118} or Canton-S)	$2.3(10^{-3})$	0.072	$2.1(10^{-9})$	61.5	61.0	0.01
***Catsup*^{26}**	$8.0(10^{-5})$	0.133	$2.6(10^{-3})$	57.9	57.8	0.002
***ple*^{2}**	$2.4(10^{-4})$	0.108	$1.6(10^{-3})$	48.7	48.8	0.001
***Pu*^{Z22}**	$2.3(10^{-4})$	0.110	$1.7(10^{-4})$	50.5	50.6	0.002
***VMAT*$^{\Delta 14}$**	$2.8(10^{-4})$	0.133	$2.1(10^{-9})$	42.1	41.9	0.005

Mortality μx is given as $\mu x = ae^{bx} + c$, where a is the baseline mortality rate (intercept), b is the age-dependent increase in mortality (slope), and c is the age-independent mortality.

Table B.3 Two-Way ANOVA of mean daily activity with Bonferroni's post-hoc test.

Source of Variation	**% of total variation**	**P value**		
Interaction	16.19	< 0.0001		
Gender and Age	52.22	< 0.0001		
Genotype	26.87	< 0.0001		
Source of Variation	**P value summary**	**Significant?**		
Interaction	****	Yes		
Gender and Age	****	Yes		
Genotype	****	Yes		
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	12	2.621e+006	218443	85.69
Gender and Age	3	8.453e+006	2.818e+006	1105
Genotype	4	4.349e+006	1.087e+006	426.5
Residual	300	764775	2549	

Table B.4 Two-Way ANOVA of FFT values with Bonferron's post-hoc test.

Source of Variation	**% of total variation**	**P value**		
Interaction	16.58	< 0.0001		
Gender and Age	29.77	< 0.0001		
Genotype	12.98	< 0.0001		
Source of Variation	**P value summary**	**Significant?**		
Interaction	****	Yes		
Gender and Age	****	Yes		
Genotype	****	Yes		
Source of Variation	**Df**	**Sum-of-squares**	**Mean square**	**F**
Interaction	12	0.3840	0.03200	10.19
Gender and Age	3	0.6894	0.2298	73.19
Genotype	4	0.3007	0.07517	23.94
Residual	300	0.9420	0.00314	