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Effects of Altered Superoxide Dismutase Expression on Age-related Functional Declines and Survival in Drosophila

Ian Martin
Virginia Commonwealth University

THE EFFECTS OF ALTERED SUPEROXIDE DISMUTASE EXPRESSION ON AGE-RELATED FUNCTIONAL DECLINES AND SURVIVAL IN *DROSOPHILA*

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

By

Ian Martin

B.Sc. Biochemistry, King's College, London (2003)

Director: Dr. Mike Grotewiel

Associate Professor, Department of Human Genetics

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List of Abbreviations

Ac-DEVD-CHO …….Acetyl-asp-glu-val-asp-7-amido-4-methylcoumarin

AMC …….7-amido-4-methylcoumarin

ANOVA ……...…….....Analysis of variance

aROD ……...…….…..Absolute rate of decline

ATP ……..……..Adenosine 5’-triphosphate

cDNA ……...……...…Complementary DNA

CO_2 ……...Carbon dioxide

DT_{75} …….....Decline time to 75% of initial function

DT_{50} …….....Decline time to 50% of initial function

DT_{25} …….....Decline time to 25% of initial function

DTT …….……Dithiothreitol

EDTA …….....….Ethylenediaminetetraacetic acid

Fe-SOD …….....Fe-superoxide dismutase

H_2O_2 ……...Hydrogen peroxide

Hsp …….…….Heat shock protein

Igf1 receptor ……Insulin-like growth factor 1 receptor

4-MCH ……..…4-methyl cyclohexanol

$MgCl_2$ ……..…….Magnesium chloride

$MnCl_2$ ……...…….Manganese chloride

MSRA …….Methionine sulfoxide reductase A

MSRB…….…….Methionine sulfoxide reductase B

mtDNA ……...…..Mitochondrial DNA

$NADP^+$ …….Nicotinamide adenine dinucleotide phosphate

$O_2•^-$ …….....Superoxide

PAGE ……...…….Polyacrylamide gel electrophoresis

PER ……..……Proboscis extension reflex

pROD ……Proportional rate of decline

RNAi ……....RNA interference

ROS ……...……Reactive oxygen species

SAM-P1 …….....Senescence-accelerated mouse–P1

SOD1 or CuZn-SOD …….……..Superoxide dismutase 1

SOD2 or Mn-SOD ……. Superoxide dismutase 2

SOD3 or EC-SOD ……...Superoxide dismutase 3

Sod2IR ……...……Superoxide dismutase 2 inverted repeat

TEMED ……. N,N,N’,N’-tetramethylethylenediamine

Tris-HCl ……..Tris-hydrochloride

TUNEL ……....Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling

Abstract

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Most organisms experience progressive declines in physiological function as they age. A number of studies in a variety of species support a strong link between oxidative damage, age-related functional declines and life span determination. Here, manipulating the expression levels of superoxide dismutase (SOD) isoenzymes SOD1 and SOD2, resulted in altered functional senescence and survival characteristics in *Drosophila*.

Overexpression of cytosolic *Sod1* using the yeast GAL4/UAS system conferred a 30-34% increase in mean life span and resulted in an attenuated senescence of odor avoidance behavior in aging flies. Tissue-specific *Sod1* overexpression selectively in the nervous system or muscle failed to reproduce these delayed aging phenotypes suggesting that *Sod1* overexpression in these tissues alone was not primarily responsible for the aging effects observed. Graded reduction of mitochondrially localized *Sod2* expression in a series of *Sod2* mutants led to progressive reductions in life span, accelerated age-related functional declines, mitochondrial oxidative damage and neuronal cell death. Tissue-specific *Sod2* knock-down using RNA interference revealed that muscle is a key tissue underlying the accelerated age-related functional decline and mortality observed upon loss of SOD2. *Sod2* knock-down in the musculature caused a degenerative phenotype consisting of a dramatic reduction in muscle mitochondrial content and ATP levels, elevated cell death and progressive locomotor dysfunction which culminated in early-onset mortality. Collectively, these studies highlight the important role of SOD enzymes in protecting against the impact of oxidative damage on senescence and survival. These findings also lend further support to the oxidative damage hypothesis of aging.

Introduction

1. Use of the *Drosophila* model to study aging

Aging is a progressive decline in biological systems that impairs the ability of an organism to maintain homeostasis and consequently increases the organism's susceptibility to disease and death (Harman, 2001). The fruit fly *Drosophila melanogaster* is one of the principal model organisms used for studying the biology of aging. Several features make flies suitable for aging research including their short development period and relatively short life span, both of which facilitate experimental efficiency in aging studies. Flies are also inexpensive to house and to maintain for the extended periods necessary for aging studies. Additionally, flies are appropriate for investigating the molecular-genetic basis of aging because there are powerful tools available to manipulate the fly genome, most of which has been sequenced. Moreover, most fly genes have direct homologues in mammals. To date, most aging studies in model organisms including *Drosophila* have focused on mortality as the main consequence of aging. These longevity studies have produced a vast number of discoveries that have enabled the identification of conserved genetic pathways and common environmental factors that influence survival in nematodes, fruit flies and mice (Barger et al., 2003; Boulianne, 2001; Guarente and Kenyon, 2000; Helfand and Rogina, 2003; Kenyon, 2001; Tatar et al., 2003; Wood et al., 2004).

Functional senescence, defined as the intrinsic age-related decline in functional status, has received very little experimental attention in model organisms. Nearly all organisms

manifest functional declines as a result of aging, although the nature and progression of these declines varies between species and also between individuals of the same species (Arking, 1998). While most aging research using flies has focused on regulation of life span, the fly is emerging as a powerful model system for investigating the biology underlying age-related functional declines. Central to the ability to use flies in this way is the large number of parallels between functional senescence in *Drosophila* and humans.

2. Functional senescence in aging research

Age-related declines in function can have severe consequences on the quality of life in elderly individuals. When questioned, older adults are much more concerned about preserving functionality late in life than simply extending their life span without regard to their functional status (Phelan et al., 2004). A prominent strategy in aging basic research has been attempting to identify manipulations that confer life span extensions in model organisms based on the premise that these may ultimately provide anti-aging benefits to humans. Although a number of genetic and other manipulations extend life span in various species, in most cases it is unclear whether these have global impacts on aging or instead selectively influence processes that support survival. Additionally, when manipulations that extend life span have been found and subsequently examined for their effects on age-related impairments in sensory, locomotor and reproductive function, these are often reported to remain unaltered (Fridell et al., 2005; Holzenberger et al., 2003; Cook-Wiens and Grotewiel, 2002). Hence, although any extension in life span presumably results from the preservation of one or more organ systems vital for life, it

does not necessarily engender a global benefit on age-related functional declines. Functional senescence studies have substantial merit because they can be used to identify key organ systems that fail with age under normal aging conditions and also to discover manipulations that positively impact functional status in aging. While some of these manipulations may attenuate functional senescence and prolong survival, others may delay or attenuate functional senescence without having any impact on survival. Such manipulations would still hold significant promise in aging research since they hold potential to improve the quality of life in aging individuals.

3. Age-related functional declines in flies

The main animal model systems currently used to investigate the molecular-genetic basis of aging are the nematode *C. elegans*, the fruit fly *Drosophila melanogaster*, and the mouse. Each of these has its own strengths and limitations for studies on aging, although the fly is particularly well suited for studying functional senescence. A number of metabolic and organ functions in flies can be assessed in the laboratory and many of these decline with age (summarized in Tables 1 and 2 and reviewed in Grotewiel et al., 2005). Although the fly's maximum life span (50-80 days) is significantly longer than that of the worm (~20 days), it is much shorter than in the mouse (2-3 years). Consistent with its relatively short life span, the fly exhibits many age-related functional deficits fairly quickly and many of the functions that senesce in flies also senesce in humans. Additionally, while the genomes of the worm and fly are similar in size and comparably powerful genetic tools are available to manipulate them, more genes in the fly have

obvious mammalian homologues (Adams et al., 2000). Together these features make the fly an attractive model system for investigating genetic and other factors that influence functional senescence and for gaining insight into aging in humans.

4. The fly as a model for human functional senescence

To evaluate the suitability of *Drosophila* as a model of age-related functional impairment in humans, it is useful to compare functional declines seen in flies to those seen in humans. Nearly all of the age-related functional declines seen in *Drosophila* (Table 1) are also observed in humans, consistent with the commonalities in basic biological systems in both species. Many aspects of locomotor function decline as humans age (Kozakai et al., 2000; McGibbon and Krebs, 2001; Rittweger et al., 2004; Shkuratova et al., 2004; Winter et al., 1990) as does memory function (Grady and Craik, 2000; Perlmutter et al., 1981; Uttl and Graf, 1993), olfactory abilities (Kovacs, 2004; Landis et al., 2004; Larsson et al., 2000), cardiac function (Lakatta 1993; Fleg et al. 1995; AHA 2005), immune system function (Burns, 2004; Plackett et al., 2004), circadian rhythmicity (Copinschi and Van Cauter, 1995; Yoon et al., 2003), quality of sleep (Hood et al., 2004) and reproductive function (Araujo et al., 2004; Arking, 1998; Enzlin et al., 2004; Jung and Schill, 2004; Ng et al., 2004). The similarities between age-related functional decline in humans and *Drosophila* indicate that many aspects of functional senescence are conserved across phyla. Undoubtedly, some functions found in humans are not present in flies and are therefore not amenable to aging studies in this model, yet the commonalities

between these two species provide an opportunity to investigate the molecular-genetic basis for age-related functional decline through studies in *Drosophila*.

Table 1. Organismal and organ system senescence in *Drosophila* (from Grotewiel et al., 2005).

Function	Main Effects of Age	Approximate Age of Onset	Principal References
Negative geotaxis	increased time required to complete task	14-21 days	(Miquel et al., 1976)
	decreased climbing ability during timed test		(Gargano et al., 2005)
Exploratory activity	fewer flies move from release point	14-28 days	(Le Bourg, 1983)
	flies move less far		(Le Bourg and Minois, 1999)
Fast phototaxis	decreased percentage achieving performance criterion	14-21 days	(Leffelaar and Grigliatti, 1984)
Flight	shorter duration free flight	≤11 days @ 28°C	(Leffelaar and Grigliatti, 1984)
Habituation of PER	reduced learning	42 days (maximum effect)	(Fois et al., 1991)
Suppression of PER	reduced learning	28 days	(Brigui et al., 1990)
Olfactory memory	impaired learning	10 days	(Tamura et al., 2003)
	impaired memory	20 days	
Olfaction	decreased innate odor aversion and attraction	14-21 days	(Cook-Wiens and Grotewiel, 2002)
	decreased odor responses in olfactory receptor neurons	5-10 days	
Circadian rhythmicity	shift to later peak in spontaneous activity	42 days	(Driver, 2000)
Noncircadian Rest	decreased rest during night	16 days	(Shaw et al., 2000)
Male reproduction	decreased copulation success	100 days (maximum effect)	(Miquel et al., 1976)
	decreased fertility	90 days (maximum effect)	(Economos et al., 1979)
Sperm competition	decreased sperm defense	≤42 days	(Service and Fales, 1993)
	decreased sperm offense	≤42 days	
Female reproduction	decreased egg-laying	50 days (maximum effect)	(David et al., 1975)
	decreased fertility	50 days (maximum effect)	
Cardiac function	reduced resting heart rate	≤25 days	(Paternostro et al., 2001)
	reduced maximum heart rate		(Wessells and Bodmer, 2004)
	increased pacing-induced fibrillation		
Innate Immunity	exaggerated induction of diptericin (live bacteria)	28 days	(Zerofsky et al., 2005)
	dampened diptericin expression (heat-killed bacteria)	21-28 days	
Stress resistance	decreased resistance to thermal stress	50-60 days (maximum effect)	(Fleming et al., 1992)
	decreased resistance to oxidative stress	10 days	(Bonilla et al., 2002)
	decreased resistance to starvation stress	≤28 days	(Minois and Le Bourg, 1999)
	deceased resistance to desiccation stress	14-21 days	(Nghiem et al., 2000)

Age of onset indicates the earliest age at which defects are observed. PER, proboscis extension reflex

Table 2. Senescence of metabolic functions in *Drosophila* (from Grotewiel et al., 2005).

Metabolic function	Effect of Age	Approximate Age of Onset or Maximum Age Tested	Principal References
Protein synthesis	decreased total protein synthesis	Immediately after emergence	(Webster and Webster, 1979)
	decreased synthesis of mitochondrial proteins	38 days	(Fleming et al., 1986)
	Decreased EF-1α activity	21 days	(Webster and Webster, 1982)
Basal metabolism	decreased heat production	35 days	(Ross, 2000)
	decreased oxygen consumption	42 days	(Lints and Lints, 1968)
	no change in CO_2 production	across entire life span	(Van Voorhies et al., 2004)
ATP synthesis	increased synthesis followed by decreased synthesis	peaks at day 40, declines thereafter	(Vann and Webster, 1977)
Cytochrome C oxidase	decreased activity	14 days	(Schwarze et al., 1998)
Aconitase	decreased activity	25 days	(Das et al., 2001)
α-glycerophosphate dehydrogenase	increased activity followed by decreased activity	peaks at 13 days, declines thereafter	(Baker, 1978)
Ca^{2+}-activated actomyosin ATPase	increased activity followed by decreased activity	peaks at 5 days, declines thereafter	(Rockstein et al., 1981)
Arginine phosphokinase	increased activity followed by decreased activity	peaks at 12 days, declines thereafter	(Rockstein et al., 1981)
Total SOD	no change in activity	measured across life span	(Massie et al., 1980)
	increased activity	28-49 days	(Sohal et al., 1990)
Cu-Zn SOD	no change in activity	measured across life span	(Sohal et al., 1990)
	decreased activity	50 days	(Niedzwiecki et al., 1992)
Mn SOD	decreased activity	10 days	(Massie et al., 1980)
	increased activity	28 days	(Sohal et al., 1990)
Catalase	increased then decreased activity	peaks at 21 days then declines	(Sohal et al., 1990)
	no change in activity	measured across life span	(Niedzwiecki et al., 1992)
Glutathione reductase	increased then decreased activity	peaks at day 20 then declines	(Sohal et al., 1990)

Age of onset indicates the earliest age at which defects are observed or the maximum age tested in cases where no effect of age was found

5. Aims of this investigation

One of the leading mechanistic theories of aging is the oxidative damage hypothesis which postulates that aging occurs as a direct result of the age-dependent accumulation of molecular oxidative damage, causing a progressive functional deterioration of cells, tissues and organ systems (Harman 1956). A number of studies in a variety of species support an important role for oxidative damage in functional senescence and life span determination (reviewed later). A key prediction from these studies is that altering the levels of cellular antioxidant defenses will directly impact oxidative damage in aging organisms and that this in turn will determine the rate of functional senescence and longevity. In *Drosophila*, there is emerging evidence indicating that manipulating the expression levels of the key antioxidant superoxide dismutase (SOD) alters oxidative stress resistance and life span. Increases in SOD activity often result in prolonged survival whereas reducing SOD activity leads to shortening of the fly life span (Sun and Tower, 1999; Orr and Sohal, 1994; Sun et al., 2002; Duttaroy et al., 2003; Phillips et al., 1989). However, the consequences of these manipulations on age-related functional declines are unknown.

The central aim of this investigation was to examine the effects of manipulating antioxidant capacity on age-related functional decline in *Drosophila*. Powerful genetic tools were used to increase or decrease expression levels of SOD, in order to elucidate the consequences of altered SOD activity on functional senescence. A second aim of this study was to investigate whether any of the observed effects on functional aging and

survival were mediated by altered *Sod* expression in individual tissues. This was achieved by comparing the effects of manipulating SOD antioxidant levels in key tissue domains to those obtained by whole-body SOD manipulations. Finally, when specific tissues were found to be instrumental in the effects of altering SOD activity levels on functional senescence, the sub-cellular mechanisms underlying this association were investigated. This overall approach was designed to provide insight into the consequences of manipulating SOD expression levels on the senescence of key tissues and the impact of this on age-related functional declines and survival.

The following introductory sections provide a review of the literature relevant to this study. The first review describes the role of oxidative damage in aging and the second summarizes evidence indicating a role for SOD in aging and age-related pathology.

The Role of Oxidative Damage in Aging

1. Introduction

In recent decades, several theories have been proposed that attempt to explain the underlying biological mechanisms of aging (Weinert and Timiras 2003). The oxidative damage theory of aging postulates that the age-dependent accumulation of oxidative damage to macromolecules causes a progressive functional deterioration of cells, tissues and organ systems that manifests as functional senescence and culminates in death (Harman 1956).

Oxidative damage to lipids, proteins and nucleic acids occurs primarily via the action of reactive oxygen species (ROS). ROS can be generated by several mechanisms but the principal source in aerobic cells is mitochondria (Fridovich 2004). Certain components of the electron transport chain leak electrons to oxygen, promoting the univalent reduction of oxygen to superoxide ($O_2\bullet^-$) (Balaban et al. 2005). Under normal physiological conditions, approximately 0.1% of oxygen (Fridovich 2004) or more (Chance et al. 1979) entering the electron transport chain is reduced to $O_2\bullet^-$. Superoxide itself does not appear to damage all macromolecules at physiologically relevant concentrations; redox reactions involving $O_2\bullet^-$, however, generate other reactive species that oxidatively damage nucleic acids, proteins and lipids (Halliwell and Gutteridge 1999). Although cells are equipped with a variety of antioxidants and multiple enzymatic systems to repair oxidative damage,

such damage is detectable under normal physiological conditions even in young animals (Agarwal and Sohal 1994). This suggests that the endogenous protective mechanisms cannot suppress all oxidative damage even during basal levels of ROS generation (Halliwell and Gutteridge 1999).

2. Accumulation of oxidative damage in aging

A fundamental prediction from the oxidative damage hypothesis is that oxidative damage should increase with age. Consistent with this, there are many reports of age-related increases in oxidatively damaged nucleic acids, proteins and lipids in various tissues of many species (Sohal et al. 2002). In brain tissue from humans and several other mammalian species, aging is associated with increased oxidative damage to both mitochondrial and nuclear DNA, with damage to mitochondrial DNA being substantially higher than that of nuclear DNA (Mecocci et al. 1993; Barja and Herrero 2000). Additionally, oxidative damage to DNA causes mutations that can impair protein synthesis and lead to cell dysfunction (Wei and Lee 2002), suggesting that oxidative damage to DNA might impact aging. Oxidative damage to proteins also increases with age in a variety of experimental settings including *Drosophila* whole body (Agarwal and Sohal 1994), rat hepatocytes (Starke-Reed and Oliver 1989), canine brain tissue (Head et al. 2002) and human eye lens (Garland et al. 1988) and brain tissue (Smith et al. 1991). The measurement of protein carbonyls in hepatocytes from rats and eye lens and brain from humans suggests that as much as 30% of total cellular protein might be oxidatively damaged in aged organisms at least in some tissues (Starke-Reed and Oliver 1989). Since

oxidized proteins are often non-functional, protein oxidative damage might be central to senescence (Sohal et al. 2002). Oxidation of lipids produces lipid peroxides that can reduce membrane fluidity, inactivate membrane-bound proteins and decompose into cytotoxic aldehydes such as malondialdehyde or hydroxynonenal (Richter 1987). Accumulation of hydroxynonenal increases with age in several *Drosophila* tissues (Zheng et al. 2005) and the level of malondialdehyde and hydroxynonenal-conjugated collagen protein increases with age in rat tissue (Odetti et al. 1994). Hence, lipid oxidation might also impact aging.

In principle, the accumulation of oxidative damage with age could occur via increased generation of oxidizing species, reduced antioxidant capacity, reduced repair of oxidative damage, decreased degradation of oxidized macromolecules, or some combination of these mechanisms (Sohal and Weindruch 1996; Mary et al. 2004). ROS content rises with age in mouse (Sohal et al. 1994), rat (Sohal et al. 1990), gerbil (Sohal et al. 1995) and housefly (Sohal and Sohal 1991), consistent with this mechanism being important for the accumulation of oxidative damage during aging. Expression and enzymatic activity of Methionine Sulfoxide Reductase A and B (MSRA and MSRB, respectively), enzymes that repair oxidative damage to methionine residues, decline with age in rat brain, liver and kidney (Petropoulos et al. 2001) as well as during replicative senescence in human fibroblasts (Picot et al. 2004). Similarly, the activities of three mitochondrial DNA glycosylases involved in base-excision repair of oxidatively damaged DNA decline with age in brain tissue from mice (Imam et al. 2005) and rats (Chen et al. 2002).

Furthermore, the activity of the proteosome, the principal avenue for removal of oxidatively damaged proteins, declines with age in human muscle (Husom et al. 2004), eye lens (Viteri et al. 2004), lymphocytes (Petropoulos et al. 2000) and epidermis (Bulteau et al. 2000; Chondrogianni et al. 2000; Petropoulos et al. 2000) as well as in rat liver (Shibatani et al. 1996), spinal cord (Keller et al. 2000), eye lens (Shang et al. 1997), heart (Bulteau et al. 2002) and retina (Louie et al. 2002). Thus, the ability of cells to control oxidative damage to macromolecules via repair and degradative systems are compromised during aging, suggesting that these changes also underlie the age-related accumulation of oxidatively damaged macromolecules. In contrast, a broad change in antioxidant capacity with age is not well supported. The activities of some antioxidants decline with age whereas the activities of others remain unchanged or even increase (Massie et al. 1980; Sohal et al. 1990; Sohal et al. 1990; Niedzwiecki et al. 1992), indicating that aging is not associated with a global reduction in antioxidant capacity (Sohal and Weindruch 1996). Thus, the principal mechanisms currently thought to drive the increase in oxidative damage with age are increased ROS generation by mitochondria, reduced repair of oxidatively damaged proteins and DNA, and decreased degradation of oxidatively damaged proteins by the proteosome (Sohal and Weindruch 1996; Sohal et al. 2002; Mary et al. 2004).

3. Oxidative damage and life span determination

Many studies using a variety of species implicate oxidative damage in the determination of life span. The rates of mitochondrial $O_2\bullet^-$ and H_2O_2 generation correlate inversely with

maximum life span in non-primate mammals (Ku et al. 1993; Barja 1998). Additionally, age-related oxidative damage to mitochondrial DNA in a number of mammalian species and to proteins in different species of flies inversely correlates with maximum life span (Sohal et al. 1995; Barja and Herrero 2000). Manipulations such as caloric restriction, lowering ambient temperature or eliminating flight activity in flies extends life span, reduces the rates of mitochondrial $O_2\bullet^-$ and H_2O_2 generation, and decreases the rate of oxidative damage accumulation (Buchan and Sohal 1981; Yan and Sohal 2000; Merry 2004). Additionally, flies selected for late-life reproduction are frequently long-lived (Rose and Charlesworth 1981; Clare and Luckinbill 1985; Luckinbill and Clare 1985; Partridge et al. 1999) and resistant to oxidative stress (Harshman et al. 1999; Arking et al. 2000; Arking et al. 2000). These correlations are consistent with oxidative damage being causal in life span determination.

Several pharmacological studies further support a role for oxidative damage in life span determination. Chronic administration of SOD/catalase mimetics nearly doubles the life span of wild-type *C. elegans* and completely rescues the life span of worms with a *mev-1* mutation that elevates age-related oxidative damage (Melov et al. 2000). Similarly, flies fed 4-phenylbutyrate have life span extension coupled with increased oxidative stress resistance (Kang et al. 2002). A number of other studies in rodents and fruit flies show that dietary supplementation with antioxidants can increase life span (Comfort et al. 1971; Oaknin-Bendahan et al. 1995; Bezlepkin et al. 1996; Bains et al. 1997), although extensions of life span are not always found (Lonnrot et al. 1995; Meydani et al. 1998; Le

Bourg 2001). Overall, though, these studies suggest that oxidative damage has a role in determining life span.

Arguably the strongest evidence that connects oxidative damage to life span comes from a number of genetic studies. Mutations in genes including *daf-2* in *C. elegans* (Kimura et al. 1997; Honda and Honda 1999), *methuselah* in *Drosophila* (Lin et al. 1998) and *shc* (Migliaccio et al. 1999) or *Igf1 receptor* (Holzenberger et al. 2003) in mice confer life span extension in conjunction with increased resistance to oxidative stress. Targeted expression of the antioxidant enzyme catalase (normally located in peroxisomes) to mitochondria in mice extends life span and decreases the age-related increase in oxidative damage to DNA (Schriner et al. 2005). In *Drosophila*, life span and oxidative stress resistance are increased by overexpression of MSRA (Ruan et al. 2002), CuZn-SOD (Parkes et al. 1998; Sun and Tower 1999), the DNA methyltransferase gene *dDnmt2* (Lin et al. 2005), and the human mitochondrial uncoupling protein hUCP2 (Fridell et al. 2005). Overexpression of three different heat shock proteins (Hsp22 (Morrow et al. 2004), Hsp26 or Hsp27 (Wang et al. 2004)) reportedly causes similar phenotypes in flies, although the results with Hsp22 are somewhat controversial (Bhole et al. 2004). In yeast, MSRA overexpression significantly increases life span and overexpression of MSRB coupled with caloric restriction yields life span extensions of 119% (Koc et al. 2004). Conversely, mice that lack the *msra* gene have both a reduced life span and enhanced sensitivity to oxidative stress (Moskowitz et al. 2001) and CuZn-SOD or Mn-SOD deficiency in fruit flies reduces life span under normal rearing conditions as well as in the

presence of an oxidative stressor (Phillips et al. 1989; Kirby et al. 2002; Duttaroy et al. 2003). Although the role of oxidative damage in longevity remains at least somewhat controversial (Sohal et al. 2002), the genetic studies reviewed here strongly implicate oxidative damage in life span determination.

4. Contribution of oxidative damage to functional senescence

A variety of functions senesce in animals including memory, locomotor, reproductive, sensory and immune functions (Arking 1998; Grotewiel et al. 2005). In many cases, there is evidence that oxidative damage contributes to these age-related functional declines (Tables 3 and 4).

Table 3. Genetic manipulations that impact oxidative damage and functional senescence (from Martin and Grotewiel, 2006).

Manipulation	Effect on oxidative stress resistance	Effect on functional senescence	References
EC-SOD overexpression	Unknown	Attenuated decline in spatial learning and memory in mice	Levin et al. 2002; Levin et al. 2005
CuZn-Sod knock-out	Unknown	Accelerated loss of hearing sensitivity in mice	McFadden et al. 1999a, 1999b
MSRA overexpression	↑ stress resistance	Attenuated decline in locomotion in *Drosophila* Delayed and attenuated decline in reproduction in *Drosophila*	Ruan et al. 2002 Ruan et al. 2002
Hsp22 overexpression	↑ stress resistance	Delayed decline in locomotion in *Drosophila*	Morrow et al. 2004

See main text for detailed descriptions and additional citations.

Table 4. Pharmacological and dietary manipulations that impact oxidative stress and functional senescence

Pharmacological or dietary manipulation	Effect on oxidative damage	Effect on functional senescence	Principal references
SOD/catalase mimetics	↓ oxidative damage	Attenuated decline in contextual fear learning in mice	Liu et al. 2003
Vitamin E	↓ ROS production	Attenuated decline in spatial learning and memory in rats	Joseph et al. 1999
	Unknown	Attenuated decline in testosterone production in rats	Chen et al. 2005
	Unknown	Attenuated decline in immune response to viral challenge in mice	Hayek et al. 1997
	Unknown	Attenuated decline in cell-mediated immunity in humans	Meydani et al. 1997
Vitamin E and Coenzyme Q	↓ ROS production	Attenuated decline in active avoidance learning and memory in mice	McDonald et al. 2005
Vitamin E and vitamin C	↓ oxidative damage	Reversed decline in LTP in rat dentate gyrus	Murray & Lynch 1998
	Unknown	Attenuated decline in oocyte meiosis and release in mice	Tarin et al. 1998
Vitamin E, vitamin C and phenyl-α-*tert*-butylnitrone	Unknown	Attenuated decline in spatial learning and memory in rats	Socci et al. 1995
Vitamin E, vitamin C, melatonin or lazaroid	↓ mtDNA "common" deletion	Attenuated loss of hearing sensitivity in rats	Seidman et al. 2000
Spinach or strawberry extract	↓ ROS production (strawberry only)	Attenuated decline in spatial learning and memory in rats	Joseph et al. 1999
Blueberry extract	↓ ROS production	Attenuated decline in locomotion in rats	Joseph et al. 1999
	↓ ROS production	Attenuated decline in spatial learning and memory in rats	Joseph et al. 1999
Caloric restriction	↓ oxidative damage	Attenuated decline in locomotion in mice	Dubey et al. 1996
	↓ oxidative damage	Attenuated decline in lymphocyte proliferation in rats	Tian et al. 1995
Thioproline and N-acetylcysteine	Unknown	Attenuated decline in cell-mediated immune response in mice	De la Fuente et al. 2002
4-phenylbutyrate	↑ oxidative stress resistance	Attenuated decline in locomotion in *Drosophila*	Kang et al. 2002
ROS-generating compounds	Unknown	Attenuated loss of hearing sensitivity in guinea pigs	Clerici & Yang 1996

Complete description and additional citations are provided in main text. mtDNA, mitochondrial DNA; LTP, long-term potentiation

4.1 Senescence of learning and memory

Aging in humans and other animals is associated with declines in a number of cognitive functions including short-term memory, problem-solving abilities and information processing speed (Christensen 2001). As noted above, oxidative damage accumulates in brain from many species as they age (Sohal et al. 1995; Dubey et al. 1996; Aksenova et al. 1998; Head et al. 2002). A growing amount of evidence suggests that this oxidative damage contributes to the age-related impairment of learning and memory.

The age of onset and extent of cognitive declines in individuals within the same population can vary substantially in humans and laboratory animals (Arking 1998). An important prediction from the oxidative damage theory is that the onset a magnitude of decline in a particular cognitive function should correlate with the amount of oxidative damage to the brain regions mediating that function (Sohal et al. 2002). This prediction has been tested by evaluating young (4-month-old) and aged (22-month-old) mice in a behavioral battery and then measuring protein oxidative damage in multiple brain regions from each mouse individually (Forster et al. 1996). In these studies, age-associated impairment of learning in the Morris water maze correlated with the amount of protein oxidative damage in the cortex; i.e., animals with more oxidative damage in the cortex displayed a greater impairment in memory function (Forster et al. 1996). These data support the hypothesis that the nature and severity of age-related memory deficits in an individual depend on the extent of oxidative damage to specific brain regions.

If senescence of memory is caused by oxidative damage, reducing this damage by enhancing antioxidant capacity in aging animals should preserve memory function. Superoxide dismutase (SOD) is an integral enzymatic antioxidant that reduces $O_2\bullet^-$ to H_2O_2, which is in turn converted to water by catalase or glutathione peroxidase (Balaban et al. 2005). Consistent with the oxidative damage hypothesis, overexpression of extracellular SOD (EC-SOD) throughout the lifetime of transgenic mice protects them from age-dependent declines in spatial learning and memory assessed in an 8-arm radial maze (Levin et al. 2002; Levin et al. 2005). Pharmacological intervention with antioxidants has also been used to assess the role of oxidative damage in senescence of memory. Continuous systemic administration of two SOD/catalase mimetics from 8 to 11 months of age in mice reduces the age-related increase in oxidative damage to protein, lipid and DNA during this period (Liu et al. 2003). Additionally, mimetic-treated mice have better memory performance in a fear-conditioning paradigm than vehicle-treated controls (Liu et al. 2003). This is consistent with oxidative damage playing a role in the senescence of spatial memory in mice and suggests that age-related decline in memory function can be reversed by antioxidant treatment. Further studies will be required, however, to determine whether the SOD/catalase mimetics are retarding the age-related decline in memory or, instead, are acting as cognitive enhancers that are elevating behavioral performance at all ages.

Other studies indicate that dietary antioxidants can also attenuate age-related declines in learning and memory in rodents. For example, young (6-months-old) rats fed a diet

supplemented with α-tocopherol (vitamin E) for 8 months have reduced age-related deficits in a spatial learning and memory task, the Morris water maze (Joseph et al. 1998). Interestingly, late-life supplementation of rodent diets with vitamin E combined with other antioxidants can protect against age-related memory impairments and age-associated defects in neural plasticity. Aged (24-month-old) mice fed vitamin E plus coenzyme Q for 14 weeks have improved learning in an active avoidance assay, although learning is not significantly improved in mice receiving either antioxidant alone (McDonald et al. 2005). Similarly, supplementing the diet of aged (22-months-old) rats with vitamin E and ascorbate (vitamin C) for 12 weeks reverses age-associated deficits in long-term potentiation (Murray and Lynch 1998), a leading model of synaptic plasticity thought to underlie learning and memory (Murase and Schuman 1999). Finally, aged (24-month-old) rats receiving daily injections of the spin-trapping compound phenyl-α-*tert*-butylnitrone and vitamin E plus vitamin C in their diet for 2 months have better memory performance in the Morris water maze (Socci et al. 1995) as do 19-month-old rats fed strawberry, spinach, or blueberry extracts for 8 weeks (Joseph et al. 1999). Collectively, these studies indicate that consuming a diet rich in antioxidants or antioxidant supplements might prevent or even reverse age-related memory defects. High doses of vitamin E, however, are associated with an increase in mortality in patients with chronic disease (Miller et al. 2005), highlighting the need to carefully investigate antioxidant treatment regimens for positive as well as negative health effects. Other therapeutic approaches aimed at delaying aging could also be explored. For example, since oxidative damage in the brain is associated with up-regulation of genes mediating inflammatory

responses, inflammation might be tied to senescence of memory via oxidative stress (Blalock et al. 2003). If so, anti-inflammatory drugs also might be of therapeutic value in retarding cognitive senescence.

4.2 Senescence of sensory function

Age-related functional declines in the auditory, visual and olfactory systems are among the most prominent age-related changes observed in humans (Doty 1991; Keller et al. 1999). Age-related hearing loss in humans manifests as a progressive impairment of auditory sensitivity, mainly affecting the detection of high frequency sounds (Seidman et al. 2002). Similar age-related auditory defects are seen in mice, which experience a loss of sensory hair cells progressing from the base to the apex of the cochlea (high to low sound frequency) (McFadden et al. 1999). Applying ROS-generating compounds to the guinea pig cochlea results in an increase in the sound intensity threshold required to elicit cochlear action potentials (Clerici and Yang 1996). This suggests that inducing oxidative stress in the cochlea leads to an impairment of auditory sensitivity that mimics normal aging (Clerici and Yang 1996). The antioxidant enzyme CuZn-SOD, found mainly in the cytosol (Landis and Tower 2005), normally protects against age-related hearing loss. Mice with knockout of *Sod1*, the gene that encodes CuZn-SOD, experience an accelerated loss of hair cells along the length of the cochlea between 2 and 19 months of age (McFadden et al. 1999). Decreased expression of *Sod1* also reduces auditory sensitivity in 13 month-old mice and exacerbates the normal age-related loss of auditory nerve fibers and spiral ganglion cells (sensory cells located in the spiral ganglion of the

cochlea) found at this age (McFadden et al. 1999). Although reduced expression of CuZn-SOD appears to accelerate senescence of the auditory system, CuZn-SOD overexpression in mice provides no detectable protection against age-related hearing loss up to 7 months of age (Coling et al. 2003). This suggests that normal CuZn-SOD levels are sufficient to prevent defects in auditory function caused by oxidative damage during the initial stages of aging. It would be informative to determine whether overexpression of CuZn-SOD confers protection from age-related auditory dysfunction in mice older than 7 months of age. Additionally, it would be interesting to further assess the ability of supplementation with dietary antioxidants or overexpression of antioxidant enzymes to protect or possibly reverse auditory system senescence in mice. For example, one study suggests that dietary supplementation with vitamin E, vitamin C, melatonin or a synthetic lazaroid antioxidant confers partial protection against age-related loss of auditory sensitivity in rats (Seidman 2000).

There is emerging evidence that oxidative damage plays a role in the pathogenesis of age-related macular degeneration, a common cause of blindness in humans over 60 years of age (Liang and Godley 2003). Although the visual loss associated with macular degeneration results from photoreceptor cell damage in the central retina (i.e. the macula), initial development of pathology involves degeneration of the retinal pigment epithelium (Green et al. 1985). Retinal pigment epithelial cells exist in a highly oxidative environment due to a high oxygen partial pressure from underlying capillaries and because of they are exposed to light that generates ROS via photochemical reactions

(Liang and Godley 2003). Consequently, the retinal pigment epithelium is rich in a variety of antioxidant defenses to cope with this environment (Beatty et al. 2000). The levels of some antioxidants are reduced in eyes or serum from individuals with macular degeneration (Liles et al. 1991; Cohen et al. 1994; Bernstein et al. 2002; Simonelli et al. 2002) and lipid peroxidation products are elevated in plasma from these individuals (Totan et al. 2001; Gu et al. 2003; Yildirim et al. 2004). These data suggest a role for reduced antioxidant capacity and elevated oxidative damage in the pathogenesis of macular degeneration. Consistent with this mechanism, individuals with high dietary intake of antioxidant carotenoids (Seddon et al. 1994) or vitamin E, vitamin C and beta-carotene (AREDSRG 2001) have a reduced risk of developing advanced stages of macular degeneration. Thus, oxidative damage might have a central role in the etiology of macular degeneration. The development of animal models for macular degeneration would greatly facilitate our understanding of oxidative damage in this important cause of blindness

Another prominent sensory system that senesces in humans is the olfactory system. As much as two-thirds of the elderly population has at least some form of olfactory impairment (Murphy et al. 2002). Although age-related olfactory impairments also occur in model organisms such as rodents and *Drosophila*, relatively few studies address the role of oxidative damage in olfactory system senescence. Age-related defects in olfactory system function are exacerbated in the senescence-accelerated mouse (SAM-P1) (Getchell et al. 2003). Electrophysiological response of the olfactory epithelium to the

odorant vanillin decreases substantially with age between 10 and 50 weeks in these mice and is associated with a loss of olfactory receptor cells (Nakayasu et al. 2000). SAMP-P1 mice also display an age-related atrophy of the anterior olfactory nucleus (which receives direct projections from the olfactory bulb), a change not found in controls (Shimada et al. 1994). Importantly, oxidative damage to protein and lipid are increased in aged SAM-P1 mice (Yagi et al. 1995; Butterfield et al. 1997), suggesting that oxidative damage could be responsible for the rapid decline in olfactory system function in these animals. It would be interesting to determine whether genetic manipulations that enhance antioxidant defenses or the repair of oxidative damage can ameliorate olfactory system senescence in SAM-P1 and normal mice.

Fruit flies also develop defects in their behavioral response to odorants as they age (Cook-Wiens and Grotewiel 2002; Tamura et al. 2003). Curiously, the *Drosophila* mutant *methuselah* has enhanced resistance to oxidative stress (Lin et al. 1998), but does not appear to have a detectable change in the senescence of olfactory behavior (Cook-Wiens and Grotewiel 2002). Assuming that increased resistance to oxidative stress leads to a reduced accumulation of oxidative damage in aging *methuselah* flies, this finding suggests that senescence of the olfactory system in *Drosophila* might be independent of oxidative damage. Other interpretations, however, are also possible. One alternative possibility is that mutation of *methuselah* protects organ systems important for survival in the presence of a strong oxidizing agent but not those that mediate olfactory behavior. Another possibility is that mutation of *methuselah* protects the fly from oxidative damage

only during defined time periods and that the critical periods for oxidative damage impacting the olfactory system and survival are distinct. Additional independent strategies that mitigate oxidative damage will be key to further exploring the possible connection between oxidative damage and senescence of the olfactory system in flies and other animals.

4.3 Senescence of locomotor function

In humans, deficits in the ability to perform locomotor tasks such as lifting, walking, turning, standing and sitting occur with age (Ridgel and Ritzmann 2005). Analogous changes also occur in mice (Ingram et al. 1981) and fruit flies (Grotewiel et al. 2005). In principle, locomotor senescence could arise from functional deficits in regions of the nervous system or the musculature that have specific motor roles. Both nervous and muscle tissue are thought to be especially prone to oxidative damage due to their high metabolic rate and high rate of ROS generation without commensurate enhancement of antioxidant defenses (Halliwell and Gutteridge 1999).

Locomotor senescence in humans and other animals is associated with an age-related loss of muscle mass and function (Ridgel and Ritzmann 2005). Consistent with the oxidative damage model of aging, skeletal muscle exhibits age-dependent increases in oxidative damage to DNA, lipids and proteins (Mecocci et al. 1999). Deletions in mitochondrial DNA, which can be caused by oxidative damage (Short et al. 2005), accumulate focally in the skeletal muscle of humans (Melov et al. 1995). Deletions in mitochondrial DNA

co-localize with sites of muscle fiber atrophy in rats (Wanagat et al. 2001), suggesting that oxidative damage to mitochondrial DNA could contribute to the age-associated loss of muscle fibers (Melov et al. 1995; Short et al. 2005).

A stronger link connecting mitochondrial DNA deletions and muscle atrophy is derived from additional studies. Cytochrome *c* oxidase is a key enzyme in the respiratory chain of mitochondria (Wilson 1982) and consists of subunits encoded by both nuclear and mitochondrial genes (Coenen et al. 2001). In rhesus monkeys, rats and probably humans, the number of muscle fibers with defects in cytochrome *c* oxidase activity increases with age (Muller-Hocker 1990; Boffoli et al. 1996; Aspnes et al. 1997; Lee et al. 1998). Importantly, the activity of cytochrome *c* oxidase is substantially lower or absent at sites of mitochondrial DNA deletions and muscle fiber atrophy in senescent rhesus monkeys (Lee et al. 1998; Wanagat et al. 2001). Moreover, muscle fibers with larger regions of impaired cytochrome *c* oxidase activity are more likely to exhibit atrophy (Wanagat et al. 2001). Decreases in cytochrome *c* oxidase sub-unit expression in aging human muscle are associated with decreased mitochondrial DNA content, decreased ATP production and reduced aerobic capacity (Short et al. 2005). Similarly, reduced cytochrome *c* oxidase activity in aged fruit flies is associated with decreased mitochrondial respiration (Ferguson et al. 2005), reduced ATP levels and increased lipid peroxidation (Schwarze et al. 1998). Decreased ATP synthesis in muscle fibers would presumably impair their ability to contract, thereby reducing their functionality (Volpi et al. 2004). Collectively, these studies support a model (Fig. 1) in which oxidative damage to mitochondrial DNA

accumulates focally in aging muscle and leads to reduced expression and function of cytochrome *c* oxidase. This, in turn, compromises ATP production, which impairs muscle contractility and ultimately manifests as muscle atrophy (Aiken et al. 2002).

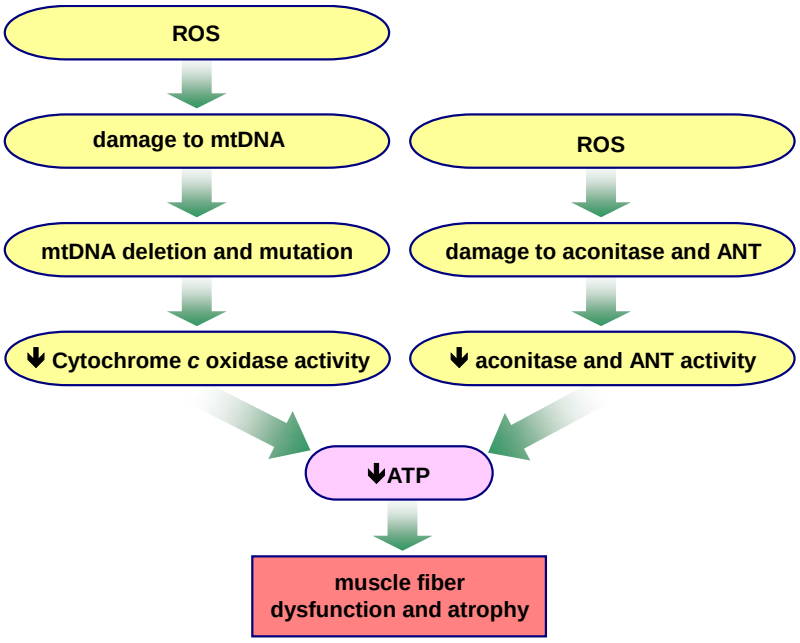


Figure 1. Schematic representation of a model linking oxidative damage to muscle fiber atrophy. See text for details. Reductions are indicated by narrow downward arrows. ROS, reactive oxygen species; mtDNA, mitochondrial DNA; ANT, adenine nucleotide translocase.

A second route leading to impaired ATP generation, muscle atrophy and locomotor senescence might proceed through direct oxidative damage to mitochondrial proteins. The activity of aconitase declines with age in *Drosophila* and housefly flight muscle (Das et al. 2001). The activity of adenine nucleotide translocase also declines with age in the housefly (Yan and Sohal 1998). The decrease in activity of these proteins coincides with their accumulation of protein carbonyls (Yan and Sohal 1998; Das et al. 2001),

suggesting that protein oxidative damage underlies the observed decreases in enzymatic activity. Since both of these enzymes are important for mitochondrial ATP production (Yarian and Sohal 2005), it seems likely that loss of activity would result in ATP deficits that over time could lead to muscle atrophy (Figure).

Locomotor senescence has also been investigated at the whole-organism level. Laboratory selection for late-life reproduction in *Drosophila* females produced a number of long-lived strains (Rose and Charlesworth 1981; Clare and Luckinbill 1985; Luckinbill and Clare 1985; Partridge et al. 1999). Many of the long-lived strains have enhanced resistance to oxidative stress (Harshman et al. 1999; Arking et al. 2000; Arking et al. 2000) and at least one of them exhibits delayed senescence of negative geotaxis (Arking and Wells 1990), a locomotor behavior. These studies are consistent with oxidative damage driving locomotor senescence. Several other studies suggest that locomotor senescence might be caused by oxidative damage specifically in the nervous system. In mice, age-related decline in bridge-walking, a motor coordination skill, is associated with increased protein oxidation in the cerebellum, a structure important for balance and fine movement (Forster et al. 1996). Caloric restriction, a reduction in caloric intake that extends life span in a number of species (Bordone and Guarente 2005; Masoro 2005; Partridge and Brand 2005), reduces oxidative damage to proteins in the mouse cerebellum with a concomitant reduction in senescence of locomotor skills (Dubey et al. 1996). This suggests that protein oxidative damage underlies age-dependent defects in locomotion. Supplementing the diet of aged rats with an antioxidant-rich blueberry

extract improves performance in two locomotor tasks involving rod walking (Joseph et al. 1999). The enhanced locomotor performance of the extract-fed animals is associated with improved biochemical measures of neuronal function, consistent with the improved aging of locomotor behavior being due to reduced oxidative damage in the brain (Joseph et al. 1999). In *Drosophila*, overexpression of either Methionine Sulfoxide Reductase A (MSRA, an enzyme that repairs oxidative damage to methionine residues) (Ruan et al. 2002) or the mitochondrial heat shock protein Hsp22 (Morrow et al. 2004) in the nervous system enhances resistance to oxidative stress and delays senescence of locomotion. Together, these studies in rodents and *Drosophila* strongly implicate oxidative damage to the nervous system in age-related declines in locomotor skills.

Other studies suggest that mechanisms other than oxidative damage might also be involved in locomotor senescence. Flies with a mutation in the *methuselah* gene have enhanced resistance to oxidative stress (Lin et al. 1998), but no obvious change in senescence of two locomotor behaviors (Cook-Wiens and Grotewiel 2002). Conversely, reduced expression of the *myospheroid* and *chico* genes, which encode a β integrin (MacKrell et al. 1988) and an insulin signaling molecule (Bohni et al. 1999), respectively, retard locomotor senescence in *Drosophila* without significantly altering resistance to oxidative stress (Clancy et al. 2001; Goddeeris et al. 2003; Gargano et al. 2005). Although it remains to be determined whether oxidative damage is decreased in *methuselah* and unchanged in *myospheroid* and *chico* mutants (as predicted from studies on stress resistance), the simplest interpretation of these data is that mechanisms in

addition to oxidative damage might influence locomotor senescence in *Drosophila*. It will be important to identify these mechanisms.

4.4 Senescence of reproductive function

Multiple aspects of reproductive function decline with age in males and females of many species (Arking 1998). Although the nature of reproductive senescence varies considerably across different species, certain commonalities are found throughout much of the animal kingdom. For example, males typically experience decreases in sperm production and motility along with a variety of changes in their sexual behavior as they age (Kidd et al. 2001; DeLamater and Sill 2005). Similarly, females experience an accelerated loss of oocyte reserves, decreased oocyte quality, as well as changes in reproductive behavior during aging (te Velde and Pearson 2002; DeLamater and Sill 2005; Lobo 2005). Oxidative damage to reproductive tissues in both sexes is implicated in several of these reproductive declines (Abidi et al. 2004).

Reproductive function is dependent on the synthesis and secretion of steroid hormones in male and female reproductive tissues (Nussey and Whitehead 2001). During aging, steroid hormone synthesis and secretion decline in humans and other animals (Danilovich et al. 2002; Cao et al. 2004). In males, there is evidence that oxidative damage plays a role in the senescence of steroid hormone biosynthesis in the testes (Diemer et al. 2003; Cao et al. 2004). Steroidogenic Leydig cells isolated from the testes of aged rats display higher levels of mitochondrial superoxide than those from young rats (Chen et al. 2001).

Additionally, Leydig cell membrane preparations from aged (24 months-old) male rats have 2- to 3-fold more lipid peroxides than do young (5-month-old) male rats (Cao et al. 2004). This increase in lipid peroxidation is associated with reduced antioxidant capacity, including declines in glutathione content and activities of CuZn-SOD, Mn-SOD and glutathione peroxidase-1 (Cao et al. 2004). Furthermore, aged rats fed a vitamin E-deficient diet between 6 and 24 months of age display higher levels of hydroxynonenal in the epididymis than controls (Jervis and Robaire 2004) and supplementing the diet of aging rats with vitamin E between 6 and 25 months of age attenuates the senescence of Leydig cell testosterone production (Chen et al. 2005). An increase in oxidant content and a decrease in antioxidant capacity, therefore, may underlie the age-associated decline in steroid production in male reproductive tissues. In support of this possibility, treatment of mouse Leydig tumor cells with H_2O_2 inhibits progesterone production in a dose-dependent manner (Stocco et al. 1993) and exposure of cells to ROS impairs the transport of cholesterol to the inner mitochondrial membrane, the rate-limiting step in the biosynthesis of steroid hormones (Diemer et al. 2003). Collectively, these studies indicate that oxidative damage to the testes contributes to the age-related decline in steroid hormone production that, in turn, leads to reproductive senescence in males.

In females, oocyte quality declines with age in many mammalian species (te Velde and Pearson 2002). This decline is thought to be caused by an increase in the frequency of oocyte aneuploidy in aged animals resulting from non-disjunction during meiosis (te Velde and Pearson 2002). Consequently, embryos from women aged 40 or older often

exhibit chromosomal abnormalities and fail to develop more frequently than do those from younger women (teVelde and Pearson 2002). Supporting a role for oxidative damage in senescence of oocyte quality, aging female mice receiving dietary supplementation with vitamins C and E continuously from birth produce fewer aneuploid oocytes (Tarin et al. 1998). The age-related decline in gonadotropin-stimulated oocyte release is also ameliorated in vitamin C- and E-supplemented mice (Tarin et al. 1998). Lastly, experimentally-induced oxidative stress in dividing mouse oocytes results in an increased frequency of aneuploidy and spindle disorganization (Tarin et al. 1996). It is possible that oxidative damage to chromosomes and microtubule spindle proteins could account for the meiotic dysfunction observed in aged females (Tarin et al. 1998); this possibility remains to be directly tested.

A number of studies indicate a role for oxidative damage in the senescence of female reproductive function in fruit flies. Several strains of *Drosophila* selected for late-life female fecundity have enhanced resistance to oxidative stress (Harshman et al. 1999; Arking et al. 2000; Arking et al. 2000), suggesting that oxidative damage might contribute to senescence of reproduction in *Drosophila* females. An additional study supports this possibility by suggesting that oxidative damage to proteins might be involved in reproductive senescence (Ruan et al. 2002). Although proteins can be oxidized at all amino acid residues, methionine residues are the most susceptible to oxidation and, together with cysteine, are the only amino acids that when oxidized can be repaired (Stadtman et al. 2005). Methionine sulfoxide reductases A and B (MSRA and

MSRB) catalyze the reduction of methionine sulfoxide back to methionine (Stadtman et al. 2005). MSRA content in various rat tissues declines with age (Petropoulos et al. 2001), raising the possibility that reduced repair of oxidative damage to proteins might influence various aspects of senescence. This possibility was explored by overexpressing MSRA throughout the *Drosophila* nervous system and then assessing female reproductive senescence. Flies that overexpress MSRA exhibit enhanced resistance to oxidative stress as expected (Ruan et al. 2002). MSRA-overexpressing females also have significant delays in the onset of reproductive senescence and an extension in the total reproductive period (Ruan et al. 2002). These data suggest that oxidative damage to protein methionine residues might drive reproductive senescence in *Drosophila*. It would be interesting to determine whether additional enzymes that repair oxidative damage are involved in reproductive senescence in flies and other animals.

4.5 Senescence of immune system function

Age-related changes in immune function occur in humans and a number of other species. These changes include abnormalities in the function of many immune cells which results in impaired cell-mediated immune responses in aged organisms (Linton and Dorshkind 2004). Consequently, immune system senescence is associated with an increase in the incidence of infections and cancer in the elderly (De la Fuente 2002). The ability of caloric restriction or dietary supplementation with antioxidants to suppress senescence of the immune system has been investigated in numerous studies (Meydani et al. 2004). Aged (17 month-old) mice receiving a diet supplemented with the antioxidants

thioproline and N-acetylcysteine for 4 weeks exhibit enhanced phagocytic activity of macrophages and neutrophils, increased natural killer cell activity and enhanced proliferation of lymphoid cells in response to mitogen (De La Fuente et al. 2002). In another study, aged mice received a vitamin E-supplemented diet for 6 weeks and were subsequently infected with influenza virus (Hayek et al. 1997). In contrast to control animals, vitamin E-fed mice do not experience weight loss in the week following infection (indicating that they maintain consumption of food), have significantly lower pulmonary viral titers (consistent with greater pathogen clearance), and exhibit an increase in cell-mediated immune function. The effects of vitamin E supplementation on immune function in young mice in this study were much smaller than those observed in aged mice (Hayek et al. 1997), indicating that vitamin E supplementation protects mice from age-related declines in immune function. Similarly, supplementing the diet of healthy humans older than 65 years of age with vitamin E for 4.5 months results in a dose-dependent increase in cell-mediated immune function and an increase in antibody titers to hepatitis B and tetanus vaccines (Meydani et al. 1997). The observations that dietary supplementation with certain antioxidants can ameliorate specific age-related defects in immune system function suggest that pharmacological intervention might be beneficial for maintaining a healthy immune system throughout adulthood. Several issues related to the role of oxidative damage in immune system senescence, however, remain to be further addressed. For example, it will be important to determine whether the positive effects of dietary supplements on the immune system are due to the antioxidant properties of these supplements or some other biochemical property.

Additionally, it will be necessary to determine whether all antioxidant-induced improvements in immune system function are due to a bona fide reduction in immune system senescence as opposed to an acute stimulation of the immune system. This issue is particularly important given that treating macrophages isolated from mice with a number of antioxidants (vitamin E, vitamin C, glutathione, N-acetylcysteine, thioproline, or thiazolidine-4-carboxylic acid) *in vitro* results in an acute improvement in the function of these cells (Del Rio et al. 1998).

Caloric restriction decreases the accumulation of oxidative damage in rodents (Chipalkatti et al. 1983; Koizumi et al. 1987; Youngman 1993; Sohal et al. 1994), monkeys (Zainal et al. 2000), and *Drosophila* (Zheng et al. 2005). The mechanisms underlying this effect, which might include either a reduction in ROS generation or enhancement of antioxidant defenses, are currently under debate (Masoro 2005). Interestingly, splenic lymphocytes of male rats fed a calorie-restricted diet display an attenuated loss of proliferative response to phytohemagglutinin and concanavalin A between 5 and 31 months of age (Tian et al. 1995). The age-related accumulation of lipid peroxides and protein carbonyls in splenic lymphocytes correlates with their decline in proliferative capacity and is reduced in caloric restricted rats (Tian et al. 1995). This suggests that a reduction in caloric intake might delay immune system senescence via a reduction in oxidative damage to immune system cells.

5. Functional senescence: a link between oxidative damage and life span determination?

The studies reviewed above indicate that the accumulation of oxidative damage with age is a significant cause of functional senescence and an important determinant of life span in a number of species. Are oxidative damage, functional senescence and longevity connected? This question has begun to be addressed in several studies that determined whether life span and age-related functional decline were impacted by manipulations that reduce oxidative damage or enhance resistance to oxidative stress. In *Drosophila*, senescence of locomotor activity and reproductive function is delayed while life span is extended and oxidative stress resistance is enhanced in strains selected for late life reproduction (Arking and Wells 1990) and by overexpression of MSRA in the nervous system (Ruan et al. 2002). The administration of SOD/catalase mimetics can almost double the life span of *C. elegans* and these same compounds can prevent the appearance of age-related cognitive defects in mice (Melov et al. 2000; Liu et al. 2003). Feeding a diet supplemented with 4-phenylbutyrate elevates resistance to oxidative stress, increases life span and delays loss of locomotor function in *Drosophila* (Kang et al. 2002). Overexpression of the heat shock protein Hsp22 in motor neurons reportedly has similar effects in flies (Morrow et al. 2004), although the positive changes in stress resistance and life span via Hsp22 expression is controversial (Bhole et al. 2004). Nevertheless, these data suggest that oxidative damage, life span extension and age-related functional declines are linked. These connections are consistent with a model in which the functions being assessed in these studies are directly involved in life span determination.

Other studies, however, have not found such connections between oxidative damage, life span and age-related declines in specific functions. In *Drosophila*, targeted expression of hUCP2 to neurons extends life span and increases resistance to oxidative stress, but spontaneous locomotion in aged flies and senescence of fertility were unchanged by this manipulation (Fridell et al. 2005). Similarly, mutation of the *Ifg1 receptor* gene in mice extends life span and enhances resistance to oxidative stress, but does not delay senescence of female fertility (Holzenberger et al. 2003). Additionally, the *Drosophila* mutant *methuselah*, despite being long-lived and resistant to oxidative stress (Lin et al. 1998), has normal age-related declines in exploratory activity, locomotor activity and olfactory behavior (Cook-Wiens and Grotewiel 2002). Furthermore, extension of life span and delayed age-related decline in locomotor activity in *Drosophila* with mutations in *chico* (Clancy et al. 2001; Tu et al. 2002; Gargano et al. 2005) or *myospheroid* (Goddeeris et al. 2003) occur in the absence of changes in oxidative stress resistance. Collectively, these data demonstrate that life span, oxidative damage and at least some aspects of functional senescence can be experimentally uncoupled. Any extension in life span, however, presumably results from preservation in the function of one or more vital organ systems that allows the organism to live longer. Hence, in studies where attenuating oxidative damage extends life span, but does not protect specific functions from senescence, it is likely that at least one untested function is positively impacted by manipulating oxidative damage. It will be important to identify the organ systems and functions that directly mediate the effects of reduced oxidative damage on life span and

to determine whether these key organ systems and functions differ between various species.

6. Summary

Oxidative damage accumulates with age in tissues that govern many senescent functions. Functional senescence can be accelerated by manipulations that increase oxidative damage and also retarded by manipulations that reduce it (Tables 3 and 4). These data support a role for oxidative damage in functional senescence. The molecular mechanisms through which oxidative damage contributes to age-related impairment of physiological function, however, remain to be systematically investigated. Continuing pharmacological and genetic studies aimed at attenuating oxidative damage in conjunction with assessing functional senescence should further illuminate the role of oxidative damage in age-related physiological decline. Additional studies that incorporate life span analyses should identify age-related functional losses critical to survival and determine whether oxidative damage plays a role in the demise of key organ systems that impact life span. Such studies will allow us to better understand the biology of aging through delineation of the connections between oxidative damage, functional senescence and longevity.

The Role of Superoxide Dismutases in Aging

1. The family of superoxide dismutase isoenzymes

The mitochondrial electron transport chain is the primary source of reactive oxygen species in eukaryotes, with 0.1% or more of oxygen entering the chain being univalently reduced to superoxide (Fridovich, 2004; Chance et al., 1979). The escape of superoxide presents a direct threat to mitochondria and the whole cell due to the potential damage this reactive intermediate and downstream reactive species can inflict (Balaban et al., 2005). The superoxide dismutases are a family of antioxidant enzymes present across all phyla that catalyze the dismutation of superoxide to oxygen and hydrogen peroxide (Halliwell and Gutteridge, 1999) as in equation 1.

$$O_2\bullet^- + O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(equation 1)}$$

Hydrogen peroxide is subsequently converted to water in a reaction catalyzed by catalase or peroxidase. Hence, superoxide dismutases act in concert with other cellular antioxidant enzymes to eliminate superoxide in order to prevent its toxicity. The accumulation of oxidative damage with age in the tissues of many species indicates that this protection is not complete.

CuZnSOD (SOD1) is present in nearly all eukaryotic and some prokaryotic cells where it is principally located in the cytosol but also found in lyzosomes, nuclei and inter-

mitochondrial membrane space (Halliwell and Gutteridge, 1999). SOD1 has a molecular weight of about 32 kDa and is dimeric, with each subunit containing an active site. MnSOD (SOD2), also found in all kingdoms, is tetrameric in higher organisms and contains manganese at the active site instead of copper and zinc. SOD2 is almost exclusively localized to the mitochondrial matrix, in close proximity to the primary source of superoxide. An extracellular type of CuZnSOD, named SOD3, is also found in many species which exists as tetrameric glycoproteins mostly bound to cell surfaces. Finally, an Fe-SOD was discovered in E.coli and later found in several other bacteria, algae and higher plants.

2. The role of superoxide dismutases in aging

The oxidative damage hypothesis of aging is supported by many reports of age-related increases in oxidatively damaged macromolecules in numerous tissues of many species (Sohal et al., 2002). Another causal link between oxidative damage and aging has been provided by genetic and pharmacological studies in which antioxidant levels have been manipulated in model organisms resulting in altered survival and functional senescence (reviewed in Martin and Grotewiel, 2006). Changes in SOD expression levels have been shown to impact model organisms in a manner consistent with these antioxidants having a direct role in aging and survival. Studies that illustrate this connection are briefly reviewed below.

The importance of SOD antioxidant function is underscored by the consequences observed upon eliminating it from living organisms. Genetic knock-out of *Sod1* in mice was shown to cause elevated endogenous oxidative stress, female infertility (Ho *et al.* 1998), motor impairments (Shefner *et al.* 1999), accelerated age-related hearing loss (McFadden *et al.* 1999) and an ~30% reduction in mean life span (Elchuri et al. 2005). Conversely, overexpression of extracellular SOD3 improved learning and memory in aged mice (Levin *et al.* 2002) while combined administration of SOD/catalase mimetics to middle aged mice prevented macromolecular oxidative damage and almost completely prevented cognitive deficits that appeared in controls during the 3-month test period (Liu *et al.* 2003). Treating *C. elegans* with SOD/catalase mimetics resulted in a substantial extension in life span in normal worms and completely rescues the life span of worms with a *mev-1* mutation that elevates age-related oxidative damage (Melov et al., 2000). In *Drosophila*, the *Sod1*-null phenotype was shown to include increased spontaneous genomic damage (Woodruff et al., 2004) adult sensitivity to hyperoxia and paraquat, male sterility, female semisterility and early-onset mortality (Reveillaud et al., 1994, Parkes *et al.* 1998). Conversely, whole-body or motor neuron-specific overexpression of *Sod1* resulted in significant life span extension and attenuation of oxidative damage accrual (Sun *et al.* 2004; Sun and Tower 1999; Parkes *et al.* 1998, Orr and Sohal 1994). Furthermore, *Sod1* overexpression in *Drosophila* motorneurons was reported to partially restore the reduced life span of flies carrying a mutation in SOD1 commonly found in individuals with familial Amyotrophic Lateral Sclerosis (Parkes *et al.* 1998). Although

the life span-extending effects of *Sod1* overexpression have been demonstrated in *Drosophila*, little is known about its effects on functional senescence.

Complete loss of SOD2 activity in mice results in mitochondrial disease (Melov et al., 1999), neurodegeneration, motor impairments consistent with underlying motor cortex degeneration, cardiomyopathy and perinatal death (Li et al., 1995; Lebovitz et al., 1996). Transgenic mice overexpressing *Sod2*, on the other hand, exhibit slight extensions in life span, decreased mitochondrial superoxide in the hippocampus but no rescue of impaired synaptic plasticity or memory function in old mice (Hu et al., 2007). The consequences of *Sod2* knock-out in *Drosophila* are similarly devastating on survival, with all flies being dead by 36 hrs of age (Duttaroy et al., 2003). Increasing *Sod2* expression throughout adulthood, on the other hand, prolongs survival in proportion to the degree of increase in SOD2 activity, up to a maximum mean life span extension of 33% (Sun et al., 2002).

These studies in model organisms establish an important role of superoxide dismutases in modulating the impact of ROS-mediated oxidative damage on senescence and survival. While some of the phenotypes resulting from *Sod* mutations resemble those commonly seen in aged organisms, others e.g. cardiomyopathy in *Sod2* null mice are more suggestive of an underlying pathology. In practice, however, it is difficult to definitively determine whether any of these phenotypes resulting from a loss of SOD function truly represent an accelerated form of normal aging or alternatively, are pathological in nature. This is because phenotypes that resemble accelerated aging could be caused by a

global pathological state unrelated to aging that still manifests as accelerated senescence. Conversely, changes in certain organ systems that result in phenotypes not typically seen in normal aging may only appear pathological in nature because they are not expressed within the normal boundaries of aging, i.e. before mortality intervenes. Nonetheless, age-related phenotypes are altered by both decreasing and increasing *Sod* expression levels in a manner consistent with the oxidative damage hypothesis of aging. This evidence reinforces the role of SOD antioxidant function in the aging process. While the effects of SOD manipulations on *Drosophila* life span are established, it remains to be determined how changes in SOD activity affect other important aspects of aging such as age-related functional senescence. From a human perspective of aging, an extension of life span via increased antioxidant function without commensurate enhancement in healthy function span (the portion of life span that an animal remains functional) would be considered fruitless. Since many of the functional declines in humans are also seen in model organisms, work seeking manipulations to suppress functional senescence such as increasing SOD activity in *Drosophila* will offer important insights into their potential benefit in humans. A central aim to the studies reported in this dissertation was to discover the effects of such manipulations in SOD activity to age-related functional declines in *Drosophila*.

Research Chapter 1.

Effects of Whole-Body and Tissue-Specific *Sod1* Overexpression on Life Span and Age-Related Functional Declines in *Drosophila*

1. Introduction

An important prediction from the oxidative damage hypothesis of aging is that enhancing the antioxidant capacity of cells by elevating levels of endogenous antioxidants or by administering exogenous antioxidants might attenuate organismal aging (Orr and Sohal, 1994). Indeed, augmenting the levels of numerous antioxidants individually or in combination can increase life span and/or ameliorate age-related functional declines in several model organisms (reviewed in Martin and Grotewiel, 2006). Interestingly, some manipulations positively impact both functional and survival parameters of aging whereas others confer benefit to functional status or survival alone, indicating the complex nature of the association between oxidative damage, functional senescence and life span regulation. In *Drosophila*, *Sod1* overexpression either alone or in conjunction with catalase has been shown to result in a substantial increase in life span although it remains to be determined how *Sod1* overexpression affects other important aspects of aging. Here, we sought to investigate the consequences of *Sod1* overexpression on age-related functional declines and to determine whether the effects observed were mediated by elevated SOD1 activity in certain tissues essential to those functions.

2. Materials and Methods

2.1 Fly stocks and husbandry

Flies were reared to adulthood at 25°C and 55% relative humidity under a 12 hour light–dark cycle on a sugar : yeast : cornmeal : agar medium (10% : 2% : 3.3%: 1% w/v) supplemented with 0.2% Tegosept (Sigma Chemical Co., St. Louis, MO, USA) and active yeast. Flies carrying the 2nd chromosome *UAS-human Sod1* (*hSod1*) transgene were provided by Gabrielle Boulianne and were back-crossed into our standard laboratory stock, *w[CS]*, which harbors the w^{1118} allele backcrossed to Canton-S (Cook-Wiens & Grotewiel, 2002; Gargano *et al*., 2005) for six generations. The *actin 5C-Gal4* strain (*a5CGal4*, supplied by David Arnosti) carries a 2nd chromosome GAL4 enhancer-trap that expresses the GAL4 transcriptional activator ubiquitously and was also backcrossed into a *w[CS]* background. To generate flies with *a5CGal4*-driven expression of the *hSod1* transgene and appropriate non-activated controls in an isogenic background, *a5CGal4* and *hSod1* flies were mated to each other or to *w[CS]* recipient flies, respectively. A second independent ubiquitous *hSod1*-expressing line plus control groups were generated by mating the ubiquitous *Gal4* driver strain, *Daughterless-Gal4* (*DaGal4,* from John Phillips) to *hSod1* and *w[CS]* flies, respectively. Flies expressing *hSod1* targeted to muscle plus controls were generated by mating two independent muscle-specific *Gal4* drivers, *24B-Gal4* (Bloomington stock center) and *Mef2-Gal4* (gift from Sunita Gupta Kramer) to *hSod*1 or *w[CS]* flies, respectively. Flies expressing *hSod1* pan-neuronally plus controls were generated by mating two independent pan-neuronal GAL4

drivers, *Appl-Gal4* (from Lawrence Goldstein) and *elaV-Gal4* (Bloomington stock center) to *hSod1* or *w[CS]* flies, respectively. In the *Gal4* screen of odor avoidance behavior, the *Gal4* lines indicated were mated to *hSod1* and *w[CS]* flies to generate progeny overexpressing *Sod1* in various nervous system components and controls, respectively.

2.2 Odor avoidance

All flies for behavioral tests were reared and aged at 25°C, 60% relative humidity under a 12 hour light/dark cycle. Avoidance of flies to 4-methylcyclohexanol (MCH, Sigma Chemical Co. St. Louis, MO, USA, dilution factor 1:100) was assessed. One- to four-day-old adults were briefly anesthetized with CO_2, separated by gender, and males were transferred in groups of 25 to fresh food vials. Male flies at various ages were transferred to a T-maze. After one minute of rest, flies were allowed two minutes to choose between a maze arm containing an air stream with MCH and an opposing arm containing an air stream without an explicit odorant. After each two-minute choice test, flies were briefly anesthetized with CO_2. Flies that moved into the two arms of the T-maze were counted and (for longitudinal studies) transferred together into a fresh food vial for aging until the next assessment. Odor avoidance scores were calculated as the percentage of flies that moved into the arm without odorant minus the percentage of flies that moved into the arm with odorant. A score of 100 results if all flies avoid the arm that contains odorant. A score of zero results if flies do not respond to the odorant. Six to ten vials of flies were tested for each genotype to derive N =6-10.

2.3 Negative Geotaxis

Groups of 25 male flies were collected under brief CO_2 anesthesia and allowed to recover at least 18 hours at 25°C and 60% relative humidity prior to assay. Flies were transferred to the RING apparatus (described in Gargano et al., 2005) After a 1 minute rest, the apparatus was rapped sharply on a table three times in rapid succession to initiate negative geotaxis responses. The flies' positions in the tubes were captured in digital images taken 4 sec after initiating the behavior. This constituted one trial. Five trials separated by 30 sec inter-trial rest periods were performed in all experiments. After testing, flies were transferred to food vials and housed until the next test. Digital images of the flies were transferred to a PC and analyzed to determine the positions for each fly in each tube as an X-Y coordinates. The performance of flies in a single vial was calculated as the average of 5 consecutive trials to generate a single datum. Five vials of flies were tested per genotype to derive N=5.

2.4 Survival

200 adult flies of each genotype 0-3-days old were collected under brief CO_2 anesthesia and transferred to food vials at a density of 25 flies per vial. Surviving flies were counted at each transfer to fresh food, every 3-4 days. All genotypes were aged in parallel at 25°C, 60% relative humidity under a 12 hour light/dark cycle.

2.5 SOD activity

Groups of 25 adult males (0-4 days old) per genotype were collected under brief CO_2 anesthesia and homogenized in extraction buffer (50 mM potassium phosphate/0.1 mM EDTA/2% Triton-X-100, pH 7.8) on ice. Samples were then probe sonicated for 20 sec and incubated at 4^{o}C for 45 mins. Next, samples were centrifuged at 14,000 rpm for 15 mins at 4^{o}C and the resulting supernatant was harvested and stored at 4^{o}C until use. Protein concentration measurements were performed using the Lowry method (Bio-rad DC Protein Assay). Samples containing equal amounts of protein were electrophoresed using discontinuous Native PAGE (4% stacking gel pH 6.8, 20% resolving gel pH 8.8) in sample buffer (0.5 M Tris-HCl/50% glycerol/0.01% bromophenol blue) at 80-100 V. SOD activity was measured colorimetrically using a modified version of an "in-gel" SOD assay previously described (Kirby et al., PNAS 2002). Briefly, gels were first soaked in a solution containing 2.5 mM nitroblue tetrazolium in 50 mM potassium phosphate buffer for 20 mins in the dark under gently agitation. Gels were next washed briefly in 50 mM phosphate buffer then transferred to a second solution (28 mM TEMED/28 μM riboflavin/50 mM potassium phosphate) for 15 mins in the dark under gentle agitation. After a second brief wash in 50 mM pottasium phosphate buffer, gels were placed on a light box and exposed to white light to allow color development for approximately 15 mins. Gels were imaged and an index of SOD activity was quantified by densitometry using Alpha Imager software (Alpha Innotech Corp., San Leandro, CA).

2.6 Statistical analyses

Survival statistical analyses were done using JMP (SAS, Cary, NC, USA) to derive mean and median life span. All other statistical analyses were done using either JMP or GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Specific statistical analyses performed are indicated in each Figure legend.

3. Results

3.1 Sod1 overexpression increases SOD1 activity, extends life span and improves odor avoidance behavior in 4-week old flies

Sod1 overexpression was achieved in *Drosophila* using the GAL4/UAS system as described in materials and methods. Expression of a human *Sod1* transgene (*hSod1*) using either of two ubiquitous *Gal4* drivers resulted in a two- to three-fold increase in whole-body SOD1 activity relative to flies containing *Gal4* or *hSod1* transgenes alone, confirming that functional human SOD1 protein was expressed (Fig. 2). A life span-extending effect of ubiquitous *Sod1* overexpression has previously been reported (Sun and Tower, 1999; Orr and Sohal, 1994) and was confirmed here (Fig 3). Mean life span of *Sod1* overexpressor flies was increased by 30-34% relative to controls while median life span (age at which 50% of initial cohort are still alive) was increased by 17-29%.

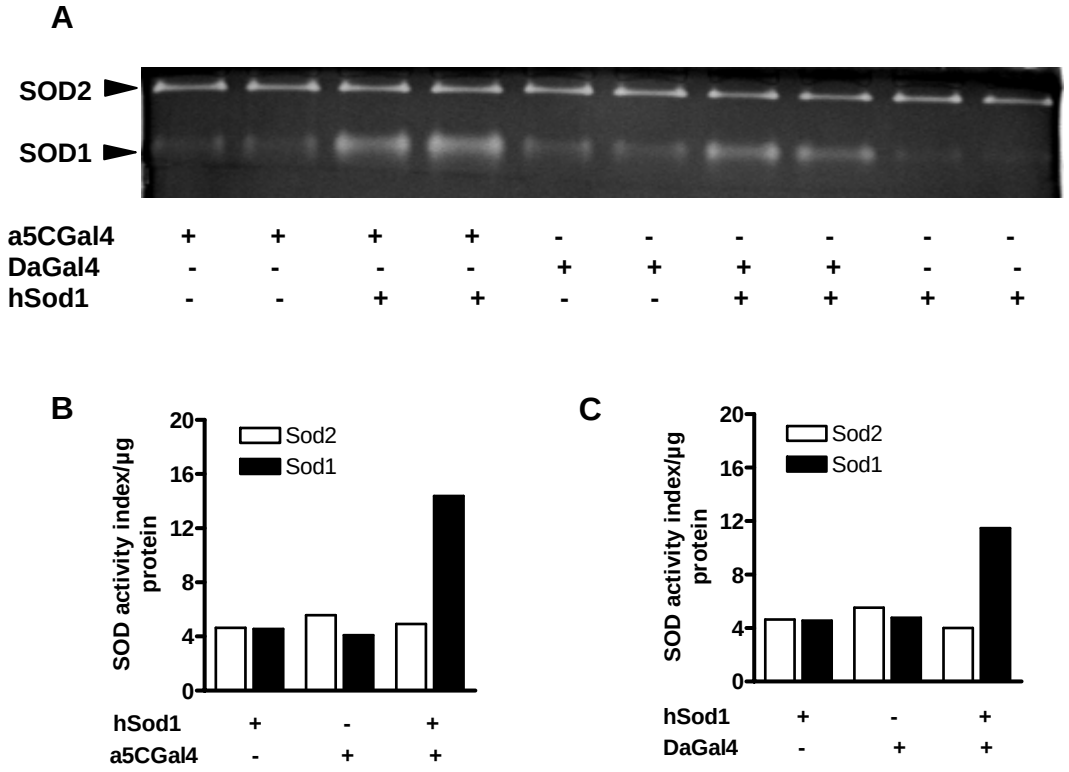


Figure 2. SOD activity in *Sod1* overexpressing flies. (A) In-gel SOD activity assay of whole-body extracts (containing 30µg protein) from flies expressing the *UAS-hSod1* transgene via two independent ubiquitous GAL4 drivers (*a5CGal4* or *DaGal4*). *Sod1* overexpressors and controls were generated as described in materials and methods. Densitometric analysis revealed a significant increase in SOD1 activity via *a5CGal4* (ANOVA, $p<0.05$) (B) but not via *DaGal4* (ANOVA) (C). Tukey's honestly significant different (HSD) post-test revealed that in (B), SOD1 activity was significantly increased compared to both control groups ($p<0.05$). There was no effect of *hSod1* overexpression on SOD2 activity via either GAL4 driver (individual ANOVAs). Densitometry data are mean of duplicate measurements.

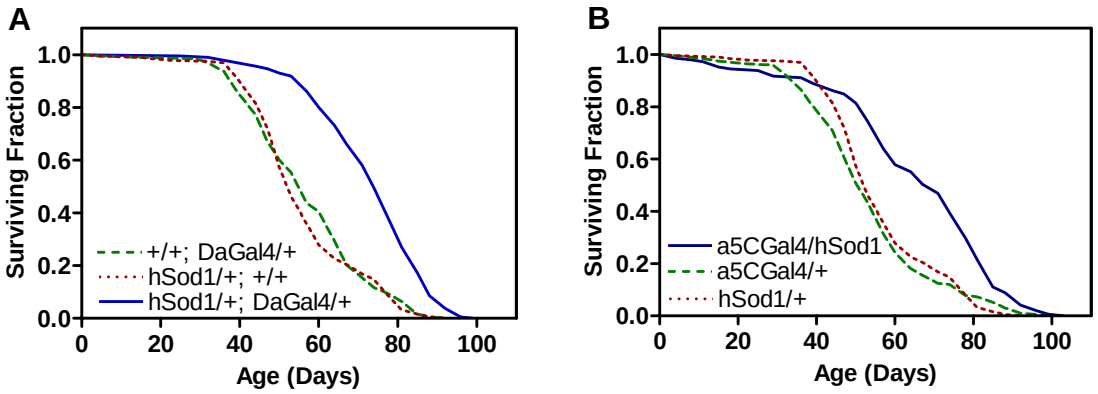


Figure 3. Survival in *Sod1* overexpressing flies. Adult males (200 per genotype) overexpressing *Sod1* via *DaGal4* (A) or *a5CGal4* (B)-mediated *hSod1* expression plus relevant controls (see materials and methods) were maintained at 25°C and 55% relative humidity in shell vials (25 per vial). Flies were transferred to fresh medium and scored for survivorship twice weekly. The mean and median life spans (days) were as follows: *hSod1/+; +/+* (mean =57, median =53); *DaGal4/+; +/+* (mean =57, median =57); *hSod1/+; DaG4/+* (mean =74, median =74); *a5CGal4/+* (mean =54, median =53); *a5CGal4/hSod1* (mean =66, median =71). Data are representative of two independent experiments.

The substantial increase in life span observed suggested that other manifestations of aging sensitive to oxidative damage accumulation, such as age-related functional declines, might be altered in flies overexpressing *Sod1*. The avoidance of noxious odors (in a T-maze assay) is a behavior seen robustly in young flies that declines progressively with age due to an impairment in the ability to detect and/or move against an aversive stimulus (Cook-Wiens and Grotewiel, 2002). Movement toward light or away from electric shock in the same T-maze apparatus is not affected by age over the time period in which declines in odor avoidance behavior manifest suggesting that this decline is not due to age-related locomotor impairment (Cook-Wiens and Grotewiel, 2002). Additionally, olfactory receptor neurons exhibit an age-associated loss of odor-evoked electrophysiological responses (Ayer and Carlson 1992) consistent with a loss of sensory function at least partly underlying senescence of odor avoidance behavior. *Sod1* overexpressing flies tested at 4 weeks of age (the age at which large deficits in odor avoidance behavior typically occur in standard laboratory strains) for avoidance to 4-methylcyclohexanol (4-MCH) performed significantly better than controls (Fig. 4). Avoidance indices in control flies were at the low levels expected for 4-week old flies whereas the performance of *Sod1* overexpressors was at the level typically seen in young flies (Cook-Wiens and Grotewiel, 2002) suggesting that the period of youthful function may have been prolonged in these animals. To further explore this possibility, it was necessary to measure odor avoidance behavior in young flies overexpressing *Sod1* and then at intervals throughout the life span.

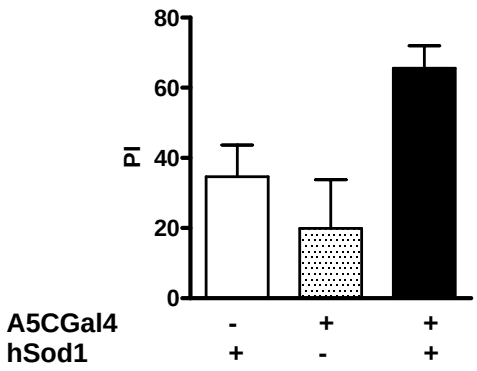


Figure 4. Odor avoidance in *Sod1* overexpressing flies. There was a significant effect of genotype on avoidance behavior (ANOVA, p =0.0215, n =8). Tukey's HSD post-test revealed that *Sod1* overexpressors performed significantly better than *a5CG4/+* controls but not *hSod1/+* controls. Data are mean ± S.E.M.

Measurement of odor avoidance behavior across age had previously been carried out in this lab using cross-sectional studies (i.e. by measuring performance of multiple fly cohorts with each cohort assessed at a separate age point and then discarded), which involves generating and maintaining very large numbers of flies. An alternative, more resource-conserving approach is the longitudinal assessment of a single cohort of flies across age. Before applying this type of longitudinal assay to *Sod1* overexpressors, it was necessary to validate the use of longitudinal assays to measure olfactory behavior declines in general.

3.2 Longitudinal odor avoidance assay validation

There are several differences between cross-sectional and longitudinal behavioral assays that could impact behavior and therefore needed to be addressed to validate the use of longitudinal assays. First, in cross-sectional studies, cohort size (number of flies tested in each trial) was kept constant across age whereas in longitudinal assessments, cohort size would inevitably decline with age due to mortality within sample groups. Hence, we asked whether variability in the number of flies tested in each trial (i.e. fly density) would have an effect on odor avoidance performance within that trial. The results show that in young or aged males, a difference in fly density does not lead to altered performance in the T-maze assay (Fig. 5). Second, we asked whether repeated exposure to the odorant (4-MCH), T-maze apparatus or brief anesthesia following testing would affect behavior in flies tested at multiple ages. To address these possibilities, longitudinal and cross-sectional studies were carried out in parallel on the same *w[CS]* standard laboratory strain

of flies. The results showed that the performance of the cohort assessed longitudinally was indistinguishable from that of the cohorts assessed cross-sectionally throughout the assessment period. This demonstrates that when tested at weekly intervals, repeated exposure to 4-MCH, the T-maze apparatus or anesthesia does not alter their behavior or confound our ability to accurately measure the effects of aging on performance.

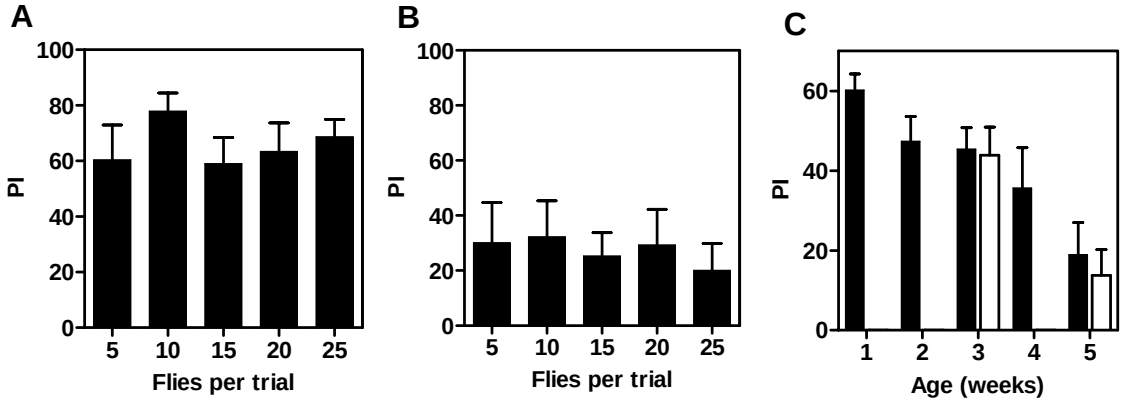


Figure 5. Longitudinal odor avoidance assay validation. There was no significant effect of varying the number of young 1-week old (A) or aged 5-week old (B) flies tested per trial on avoidance of w[CS] males to 4-MCH (individual ANOVAs, n =8). (C) There was no significant effect of longitudinal (closed bars) vs. cross-sectional (open bars) assessment of odor avoidance to 4-MCH in w[CS] males between 1 and 5 weeks of age (two-tailed unpaired t-tests performed on 3- and 5-week old flies separately). Data are mean ± S.E.M.

3.3 SOD1 overexpression ameliorates senescence of odor avoidance

Flies overexpressing *Sod1* were tested for odor avoidance behavior across age in longitudinal studies (Fig. 6). *Sod1* Overexpression driven by both *a5CGal4* and *DaGal4* resulted in attenuated decline of odor avoidance behavior across age. As mentioned before, this behavior contains both sensory and motor output components and hence it's possbile that the effects of *Sod1* overexpression could occur due to an attenuated decline of sensory detection or processing, stimulus-evoked locomotor response or some combination of these functions. Using the GAL4/UAS system, it was possible to directly address these possibilities by targeting *Sod1* overexpression to key tissues underlying olfactory sense and locomotor functions.

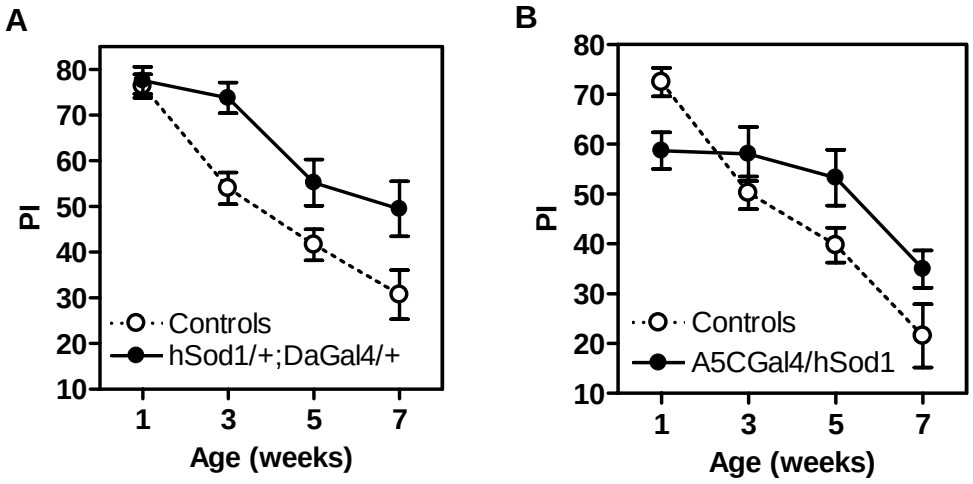


Figure 6. Odor avoidance across age in *Sod1* overexpressing flies. Odor avoidance performance index (PI) was assessed at the indicated ages. (A) There was a significant effect of age and genotype on odor avoidance behavior (two-way ANOVA, $p<0.0001$, n =8). Tukey's HSD post-test revealed that *Sod1*-overexpressing *hSod1/+; DaGal4/+* flies performed significantly better than both control groups (*hSod1/+; +/+* or *+/+; DaGal4/+*) across age and that control groups were not statistically different (hence presented here in combined form). (B) *a5CGal4/+* and *hSod1/+* control groups performed indistinguishably across age (two-way ANOVA, n =8) and are presented here in combined form. Comparison of *Sod1*-overexpressing *a5CGal4/hSod1* flies to control groups combined revealed an effect of age but not genotype on behavior and a significant interaction between age and genotype (two-way ANOVA, $p = 0.0149$, n =8-16). Data (mean ± S.E.M.) are compiled from two independent experiments.

3.4 Tissue-targeted SOD1 overexpression studies

It has long been held that muscle and nervous system tissues are inherently more susceptible to oxidative damage due to their extreme metabolic demands (resulting in high rates of ROS generation) without commensurate levels of antioxidant defenses (Halliwell and Gutteridge, 1999). To determine whether aging of olfactory behavior and survival were altered in *Sod1*-overexpressing flies due to an effect of increased SOD1 on locomotor capacity, *Sod1* overexpression was driven specifically in the musculature or motor neurons and flies were assessed for odor avoidance across age and survival (Fig. 7). There was no significant effect of muscle- or motor neuron-specific *Sod1* overexpression on odor avoidance declines although it did appear that there might be a subtle effect of muscle-targeted overexpression (Fig 7C and 7E), conferred by a large increase in SOD1 activity (Fig. 7G and 7H). Likewise, targeted expression in these groups did not prolong survival, suggesting that the life span extension effect of ubiquitous *Sod1* overexpression was not principally due to expression specifically in these tissues. Additionally, assessment of negative geotaxis across age in flies with ubiquitously elevated SOD1 indicated that locomotor function was either unchanged or only modestly affected by ubiquitous *Sod1* overexpression (Fig. 8). Collectively, these findings suggest that the effects of ubiquitous *Sod1* overexpression on odor avoidance senescence and survival are not primarily due to effects on age-related decline in locomotor function.

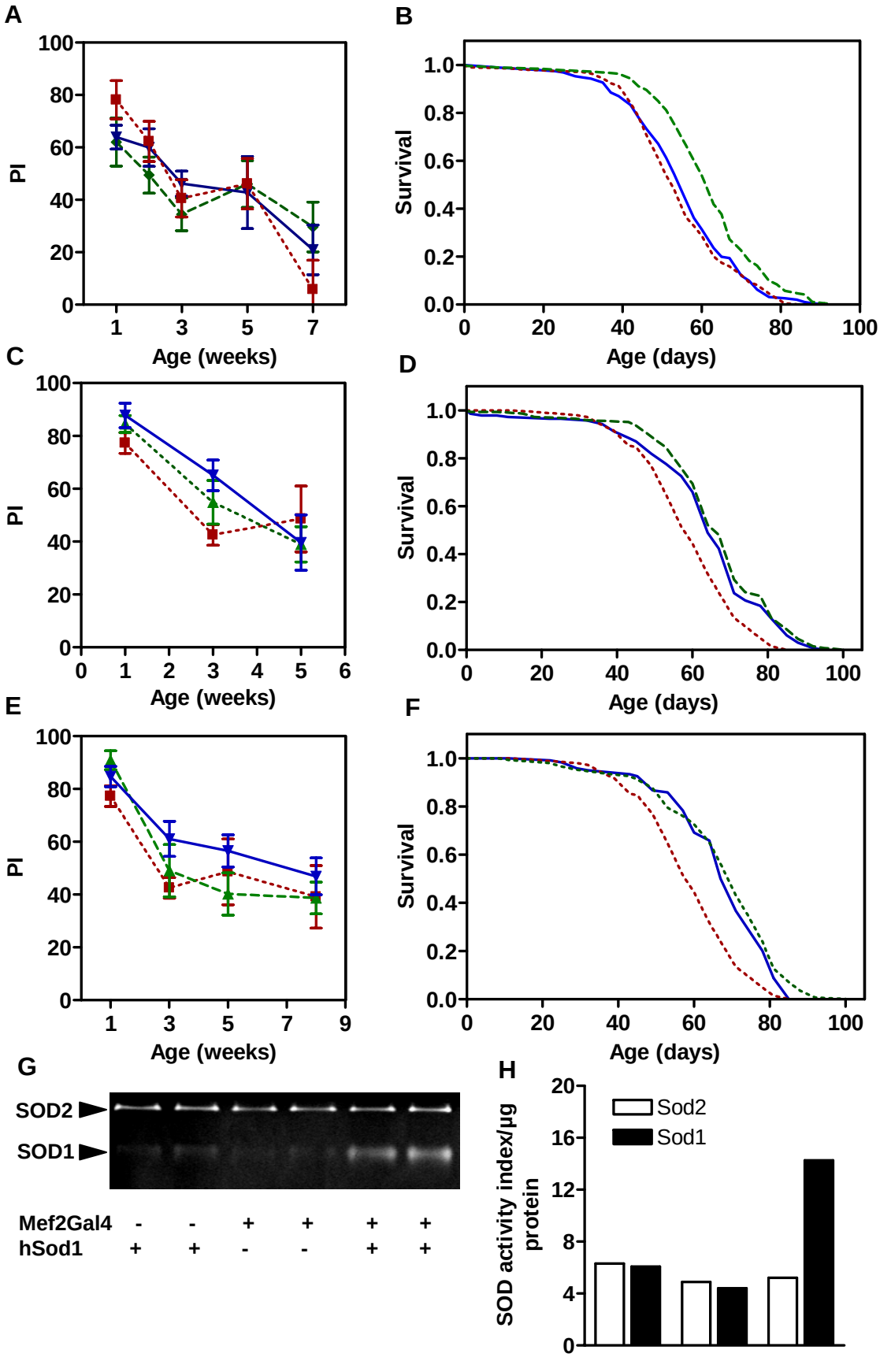

A
PI
Age (weeks)
B
Survival
Age (days)
C
PI
Age (weeks)
D
Survival
Age (days)
E
PI
Age (weeks)
F
Survival
Age (days)
G
SOD2
SOD1
Mef2Gal4 - - + + + +
hSod1 + + - - + +
H
SOD activity index/µg protein
Sod2
Sod1

Figure 7 (previous page). Odor avoidance and life span following targeted *Sod1* overexpression in muscle and motor neurons. Odor avoidance performance index (PI) (A,C,E) and survival studies (B,D,F) were performed on flies expressing *hSod1* driven in motor neurons (A and B) using *D42Gal4*, or muscle using *24BGal4* (C and D) or *Mef2Gal4* (E and F). In odor avoidance assays, comparison of *Sod1* overexpressors (*Gal4/hSod1*, ▼), *Gal4/+* (▲) and *hSod1/+* (■) controls in each data set revealed a significant effect of age but not genotype on 4-MCH odor avoidance (individual two-way ANOVAs, n =8). In survival studies, *Sod1* overexpressors (blue solid line) were not longer lived than *Gal4/+* (green dashed line) and *hSod1/+* (red dotted line) controls. (G) In-gel SOD activity assay of whole-body extracts (containing 30µg protein) from flies expressing the *UAS-hSod1* transgene via the muscle-specific *Mef2Gal4* driver and controls generated as described in materials and methods. Densitometric analysis (H) revealed a significant increase in SOD1 activity (ANOVA, $p<0.001$, n =2). Tukey's HSD post-test revealed that SOD1 activity in *Sod1* overexpressors was significantly higher than both controls, $p<0.01$). There was no effect of *Sod1* overexpression on SOD2 activity (ANOVA, n =2). Odor avoidance data are mean ± S.E.M. Densitometry data are mean of duplicate measurements.

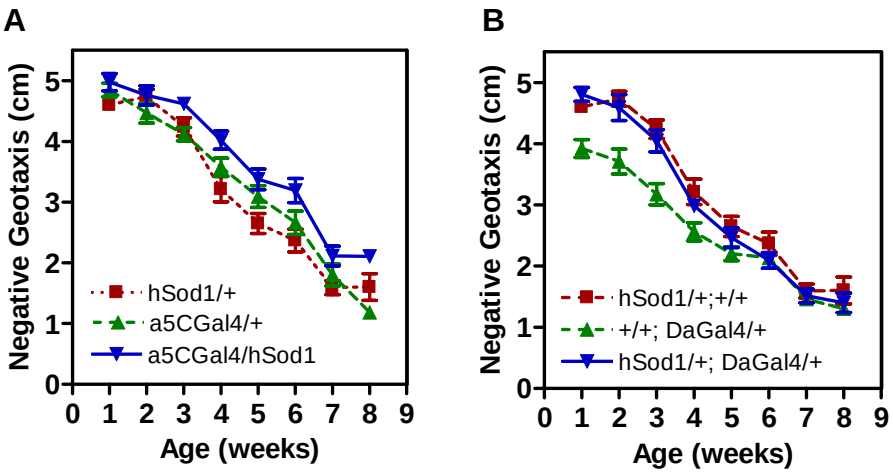


Figure 8. Negative geotaxis behavior in flies ubiquitously overexpressing *Sod1*. There were significant effects of age and genotype on negative geotaxis (individual two-way ANOVAs, $p<0.0001$, n =5-10) in flies expressing *hSod1* via *a5CGal4* (A) or *DaGal4* (B) drivers compared to controls harboring *Gal4* or *hSod1* transgenes alone. Tukey's HSD post-test revealed that in (A) *Sod1* overexpressors performed significantly better than both control groups across age ($p<0.05$) and in (B) that *Sod1* overexpressors performed better than *DaGal4/+; +/+* controls ($p<0.05$) but not *hSod1/+; +/+* controls across age. Data (mean ± S.E.M.) are compiled from two independent experiments.

To investigate whether *Sod1* overexpression in tissues required for olfactory chemoreception or central processing of stimuli accounted for the effects on odor avoidance senescence, a multitude of fly lines were generated that overexpressed *Sod1* in olfactory sense organs and/or individual regions of the brain thought to be important for olfactory response behavior (Table 5). These lines were screened for odor avoidance behavior at 4 weeks of age. From this screen, only one line that overexpressed *Sod1* in the central complex, a region implicated in controlling locomotor responses exhibited elevated performance. Subsequent assessment of this line across age, however, produced contradicting results indicating that odor avoidance senescence was not actually attenuated by overexpressing *Sod1* in this brain region (Fig. 9A). Also, survival analyses indicated that life span was not altered through *Sod1* overexpression in the central complex (Fig. 9B).

Table 5. Screen for odor avoidance behavior in *Sod1* overexpressors

Region of expression	*Gal4* enhancer trap line	Effect on odor avoidance
Antennal lobe *(receives olfactory sensory input)*	59Y[CS]Gal4	N
Antennal lobe	C133[CS]Gal4	N
Antennal lobe	H24[CS]Gal4	N
Antennal lobe	MT14[CS]Gal4	N
Antennal lobe	KL116[CS]Gal4	N
Mushroom body *(implicated in olfactory response)*	201Y[CS]Gal4	N
Mushroom body	238Y[CS]Gal4	N
Mushroom body	C309[CS]Gal4	N
Mushroom body	C747[CS]Gal4	N
Mushroom body	72Y[CS]Gal4	N
Mushroom body	30Y[CS]Gal4	N
Mushroom body & eye	107-E[CS]Gal4	N
Antennal lobe & mushroom body	C739[CS]Gal4	N
Antennal lobe & mushroom body	C492[CS]Gal4	N
Central Complex *(implicated in movement control)*	78Y[CS]Gal4	Y ($p = 0.0283$)
Central Complex	C232[CS]Gal4	N
Central Complex	C42[CS]Gal4	N
Central Complex	C507[CS]Gal4	N
Central Complex	OK348[CS]Gal4	N

Odor avoidance was measured in all lines at 4 weeks of age, n =8. *Sod1* overexpressor lines in each data set were compared to their relevant *Gal4/+* control and the *hSod1/+* control tested in parallel for effects on odor avoidance by ANOVA. N indicates no significant effect; Y indicates a significant effect.

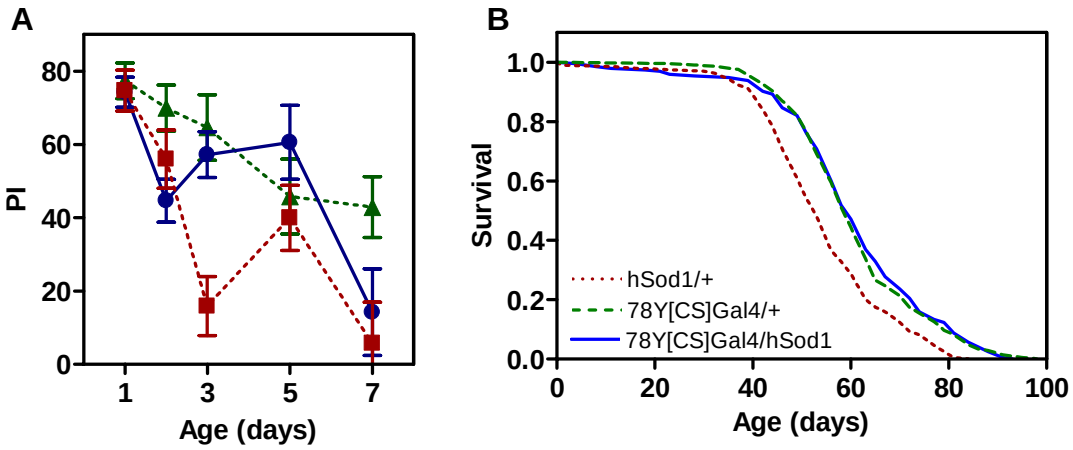


Figure 9. Odor avoidance senescence and survival following *Sod1* overexpression in central complex. (A) Odor avoidance performance index (PI) analyses revealed significant effects of age and genotype and a significant interaction between these two parameters (two-way ANOVA, $p \leq 0.0075$, $n = 8$) on the performance of *Sod1* overexpressing flies, *78Y[CS]Gal4/hSod1*(●) and controls *78Y[CS]Gal4/+* (▲), and *hSod1/+* (■). Tukey's HSD post-test revealed, however, that *Sod1* overexpressors did not perform significantly better than either control group. Odor avoidance data are mean ± S.E.M. (B) There was no change in mean or median life span following *Sod1* overexpression in the central complex.

Next, the possibility that attenuated decline in olfactory behavior seen via elevated SOD1 activity requires *Sod1* overexpression throughout the entire nervous system was examined using pan-neuronal or pan-glial expressing *Gal4* drivers of *Sod1* overexpression (no available *Gal4* driver expresses throughout both neuronal and glial cells). Pan-glial *Sod1* overexpression had no effect on behavioral decline (Fig 10C) whereas pan-neuronal expression, which augmented SOD1 activity in fly heads (Fig. 10D) appeared to exert a subtle delay in odor avoidance senescence (Fig 10A and 10B). Furthermore, pan-neuronal *Sod1* overexpression did not confer any positive effect on age-related decline of negative geotaxis behavior or life span suggesting that there were no clear beneficial effects of pan-neuronal *Sod1* overexpression on functional senescence or survival (Fig. 11).

4. Discussion

Whole-body *Sod1* overexpression reproducibly attenuated olfactory behavior aging and extended the life span of *Drosophila*. There has been some debate in the literature concerning the validity of previously reported life span extensions following *Sod1* overexpression. This concern surrounds the fact that increased longevity has been reported in comparison to controls with fairly short life spans and therefore may represent a weak genetic background rescue phenotype and not a *bona fide* attenutation of aging in an otherwise healthy fly. An important finding in the current study was that *Sod1* overexpression increased longevity in long-lived genetic backgrounds (control mean life

spans ranged from 54 to 61 days) reinforcing the tenet that *Sod1* overexpression genuinely impacts aging.

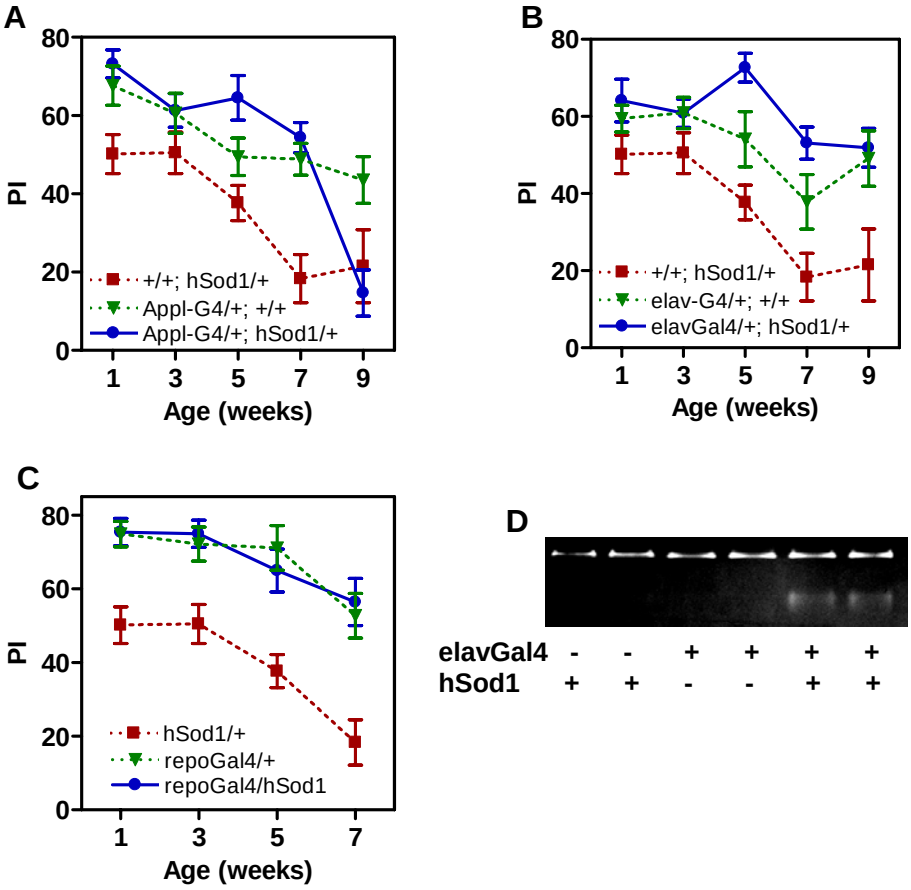


Figure 10. Odor avoidance behavior in flies overexpressing *Sod1* in the nervous system. Pan-neuronal GAL4 drivers *ApplGal4* and *elavGal4* (A and B) and the pan-glial driver *repoGal4* (C) were used to achieve *Sod1* overexpression in the nervous system. In (A), there were significant effects of age and genotype and significant interaction between these factors on odor avoidance behavior (two-way ANOVA, $P \leq 0.0258$, n =16). Tukey's HSD test revealed that *Sod1* overexpressors performed significantly better than *+/+; hSod1/+* controls but not *ApplGal4/+; +/+* controls although there was a significant interaction between age and genotype between these last two groups ($p=0.0041$). In (B), analyses revealed significant effects of age and genotype on behavior (two-way ANOVA, $P<0.0001$ for both factors, n =16). Tukey's HSD post-test indicated that *Sod1* overexpressors performed significantly better than the *+/+; hSod1/+* control ($p<0.05$) but not the *elavGal4/+; +/+* control. In (C), there were significant effects of age and genotype (two-way ANOVA, $p<0.0001$, n =16) although Tukey's HSD test revealed that *Sod1* overexpressors only performed significantly better than *hSod1/+* controls and not repoGal4/+ controls. (D) An in-gel SOD activity assay revealed an increase in SOD1 activity in heads of flies overexpressing *Sod1* pan-neuronally via *elavGal4*. Odor avoidance data (mean ± S.E.M.) are compiled from two independent experiments.

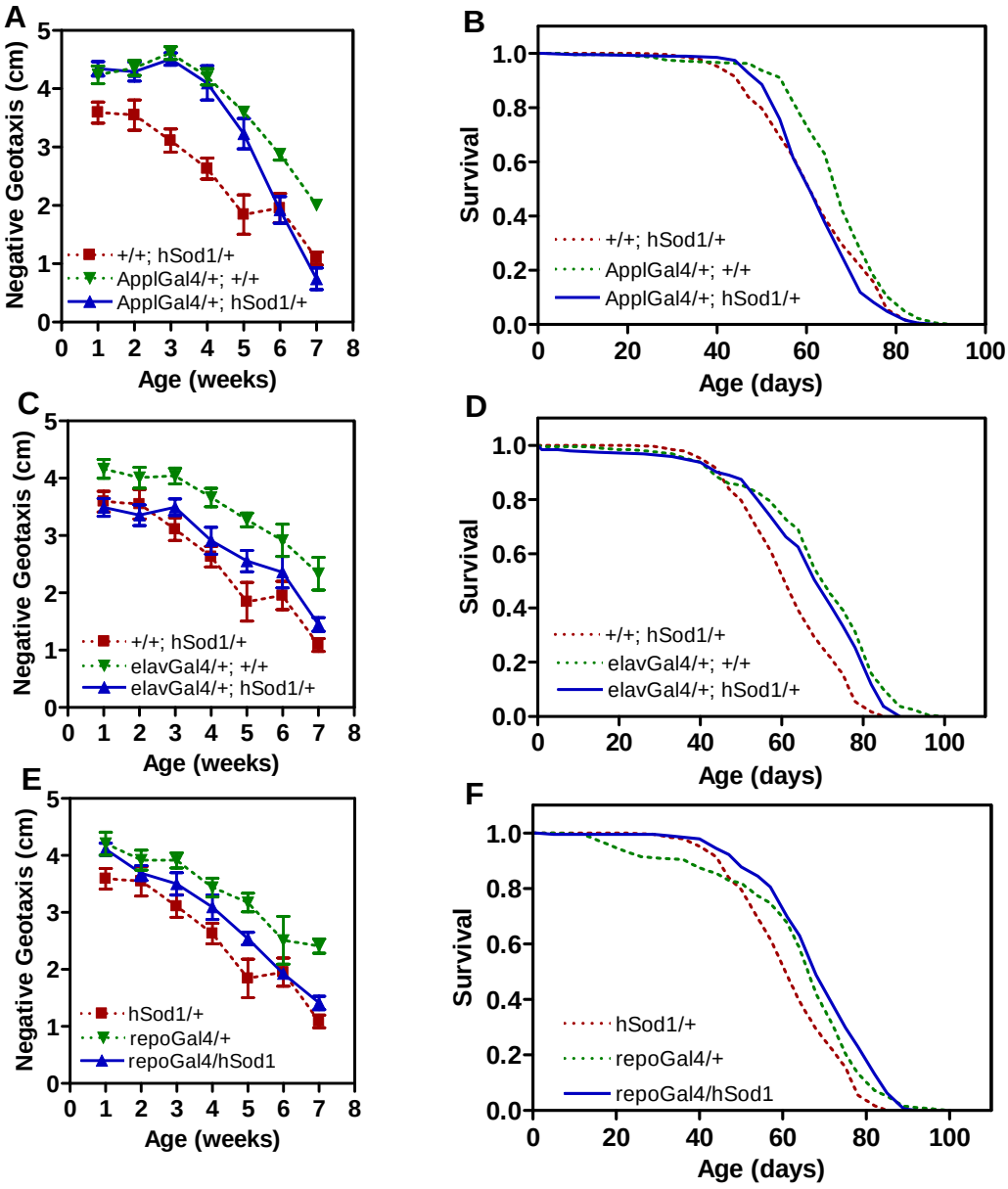


Figure 11. Negative geotaxis senescence and life span in flies overexpressing Sod1 in the nervous system. Survival studies (B,D,F) indicated that there was no substantive increase in mean or median life span conferred by *Sod1* overexpression in all lines tested. Negative geotaxis analyses in (A) revealed significant effects of age and genotype and a significant interaction between these factors on behavior across age (two-way ANOVA, $P<0.0066$, $n=16$). Tukey's HSD test revealed that *Sod1* overexpressors performed better than *+/+; hSod1/+* controls but not *ApplGal4/+; +/+* controls. In both (B) and (C), there were significant effects of age and genotype on performance (individual two-way ANOVA, $p<0.0001$ in both cases for both factors) although Tukey's HSD post-test revealed that *Sod1* overexpressors did not perform statistically better than either control group in either data set. Life span data are representative of two independent experiments. Negative geotaxis data are mean ± S.E.M.

Surprisingly, motor neuron-specific *Sod1* overexpression in our studies did not lead to an increase in mean life span in contrast with a previous report (Parkes et al., 1998). Attempts by other investigators to reproduce these results have similarly failed (personal communication) although the reasons underlying these discrepancies are currently unknown. While much research effort has been focused on discovering genetic manipulations that prolong life span in various model organisms, few attempts have been made to determine how such manipulations affect physiological function during aging. Here, ubiquitous *Sod1* overexpression was shown to significantly attenuate aging of olfactory response behavior suggesting a protective effect in tissues mediating this behavior. Tissue-specific *Sod1* overexpression studies indicate that neither muscle nor the nervous system alone mediates the effects of enhanced SOD1 activity on odor avoidance behavior. Interestingly, odor avoidance senescence was slightly mitigated by pan-neuronal (Fig. 10) or pan-muscle (Fig. 8) *Sod1* overexpression individually, suggesting that combined expression throughout muscle and the nervous system might bestow more substantial effects similar to those seen via ubiquitous increase in SOD1 activity. The survival studies conducted here also indicated that *Sod1* overexpression in neither muscle nor the nervous system recapitulated the life span extension conferred by ubiquitous *Sod1* overexpression. Hence, these effects may also depend on combined overexpression in muscle and nervous system tissues. Several other feasible explanations could also account for the lack of behavioral and survival effects seen in tissue-specific studies: (i) there may be optimal levels of SOD1 activity induction required that were not achieved in these studies; (ii) The temporal dynamics of *Gal4* expression and therefore *Sod1*

overexpression throughout the fly life span for most *Gal4* drivers used in this study are unknown and could be central to the effects observed in ubiquitous and tissue-specific studies (iii) there may be untested tissues that mediate the positive effects of *Sod1* overexpression on odor avoidance senescence and survival.

The lack of clear tissue-specific effects notwithstanding, the protective effect of ubiquitous SOD1 augmentation against senescence of odor avoidance behavior is informative since it provides preliminary evidence that increasing SOD1 activity might preserve functionality in aging organisms. The search for interventions that promote "healthy" aging by protecting against age-related functional declines is becoming increasingly important in an aging society. Manipulations in the levels of key antioxidants that extend life span are good candidates for having such effects on functional senescence and should be explored further.

Research Chapter 2.

Effect of Graded Reduction in *Sod2* expression on Mitochondrial Oxidative Damage, Functional Senescence, Neurodegeneration and Life Span

1. Introduction

SOD2 is a key enzymatic antioxidant located at the primary source ROS generation in cells, the mitochondria (Kirby et al., 2002). Oxidative damage due to the attack of ROS on cellular macromolecules accumulates with age and is thought to be a primary driving force in aging and age-related pathology (Sohal et al., 2002). Complete loss of SOD2 in mice leads to a striking phenotype which includes mitochondrial dysfunction, neurodegeneration, cardiomyopathy and dramatic life span reduction. Genetic knock-out of *Sod2* in *Drosophila* was also shown to severely reduce life span (Duttaroy et al., 2003). To further explore the role of SOD2 in protecting flies from aging and age-related pathology, a series of *Drosophila* mutants were generated with progressively reduced *Sod2* expression. Indices of mitochondrial oxidative damage, nervous system integrity, functional senescence and life span were measured to assess how graded reductions in *Sod2* expression would affect each age-related parameter. Accelerated appearance of mitochondrial oxidative damage, behavioral senescence and neuronal loss was found only in flies with the most severe *Sod2* reductions whereas all mutants experienced life span reductions in proportion to the extent of *Sod2* silencing.

2. Materials and Methods

2. 1 Western blots (Atanu Duttaroy lab)

Total protein was extracted in sample buffer (0.5 M Tris-HCl pH 6.8, 3% glycerol; 0.4% SDS and 10 mM DTT) from age-matched young flies. Samples containing equal amounts of protein were electrophoresed using 9% discontinuous SDS-PAGE and transferred to PVDF membranes. Transferred proteins were probed with rabbit anti-SOD2 primary antibody (Stressgen, Canada) at 1:5000 dilution and subsequently with HRP-conjugated anti-rabbit secondary antiserum (Calbiochem, USA) at 1:5000 dilution and detected with ECL using the manufacturer's protocol. Western blots were quantified by scanning the exposed films. Luminescence units derived from SOD2 bands for each genotype were expressed as percentage values of $Sod2^{KGr/KGr}$ samples. The final values were compiled from three blots.

2.2 Aconitase activity (Atanu Duttaroy lab)

Total aconitase activity (most of which was shown to be mitochondrially located (see Paul et al., 2007) was measured from whole fly extracts in reaction mixtures containing 0.6 mM $MnCl_2$, 2 mM citric acid, 50 mM Tris-HCl, 0.2 mM $NADP^+$ and isocitrate dehydrogenase (~0.5 units/µl), pH 8.0. Aconitase converts citrate to isocitrate which in turn is converted to α-ketoglutarate by isocitrate dehydrogenase with concomitant generation of NADPH from $NADP^+$. Aconitase activity was calculated from the increase in absorbance at 340 nm due to NADPH generation.

2.3 Survival (Atanu Duttaroy lab)

All lifespan studies were done in population cages with approximately 500 flies per cage. 2-3 day old flies were briefly anesthetized under CO_2, separated according to sex and counted. Males and females were allowed to recover for 24 hours and equal number of males and females were added to each population cage. Mortality cages were kept in insect chambers maintained at 24°C. Flies were cultured on regular fly media containing maize, yeast, agar and molasses. The number of dead flies was assessed daily.

2.4 Odor avoidance

All flies for behavioral tests were reared and aged at 25°C, 60% relative humidity under a 12 hour light/dark cycle. Avoidance of 4-methylcyclohexanol (MCH, Sigma Chemical Co. St. Louis, MO, USA, dilution factor 1:100) was measured. One- to four-day-old adults were briefly anesthetized with CO_2, separated by sex, and males were transferred in groups of 25 to fresh food vials. Male flies at various ages were transferred to a T-maze. After one minute of rest, flies were allowed two minutes to choose between a maze arm containing an air stream with MCH and an opposing arm containing an air stream without an explicit odorant. After each two-minute choice test, flies were briefly anesthetized with CO_2. Flies that moved into the two arms of the T-maze were counted and transferred together into a fresh food vial for aging until the next assessment. Avoidance indices were calculated as described in Chapter 1 and then normalized to the performance of 3-5 day old w[CS] control flies tested in parallel during each assessment.

2.5 Negative Geotaxis

Groups of 25 male flies were collected under brief CO_2 anesthesia and allowed to recover at least 18 hours at 25°C and 55% relative humidity prior to assay. Flies were transferred to the RING apparatus (described in Gargano et al., Exp Gerontol. 2005). After a 1 minute rest, the apparatus was rapped sharply on a table three times in rapid succession to initiate negative geotaxis responses. The flies' positions in the tubes were captured in digital images taken 4 sec after initiating the behavior. This constituted one trial. Five trials separated by 30 sec inter-trial rest periods were performed in all experiments. After testing, flies were transferred to food vials and housed until the next test. Digital images of the flies were transferred to a PC and analyzed to determine the positions for each fly in each tube as an X-Y coordinates. The performance of flies in a single vial was calculated as the average of 5 consecutive trials to generate a single datum. Five vials of flies were tested per genotype to derive N=5.

2.6 TUNEL assays (Atanu Duttaroy lab)

TUNEL assays were performed using the *In-Situ* Cell Death Detection Kit, AP (Roche Diagnostics, USA), according to the manufacturer's instructions. Age-matched specimen heads were dissected, fixed in FAAG (4% formaldehyde, 5% acetic-acid, 1% glutaraldehyde dissolved in 80% ethanol), embedded in paraplast, and sectioned at 7μm.

TUNEL detection was performed using the optional alkaline phosphatase detection procedure (Roche Diagnostics, USA) and digitally imaged in bright field at 400X on a Zeiss Axioskop 2 plus microscope.

2.7 Statistical analyses

All statistical analyses were done using either JMP (SAS, Cary, NC, USA) or GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Specific statistical analyses performed are indicated in each Figure legend.

3. Results

3.1 Generation of flies with progressive reduction in Sod2 expression

A series of *Sod2* mutants were generated in the laboratory of Dr. Atanu Duttaroy and assessed for *Sod2* expression levels by Western blot in his lab. P-element insertion KG06854 is located in the 5'-untranslated region of the first exon in *Sod2* (Fig. 12). Flies homozygous for this insert ($Sod2^{KG/KG}$) produced about 46% of SOD2 protein compared to flies homozygous for a revertant allele, $Sod2^{KGr/KGr}$ generated via precise excision of the P-element (Fig. 12). Imprecise excision of KG06854 resulted in a strong loss of function allele, $Sod2^{n283}$ that has undetectable SOD2 protein or activity (Duttaroy et al., 2003). Further molecular analyses revealed that the $Sod2^{n283}$ chromosome carries a 167 bp deletion that removes portions of the first exon and intron of *Sod2*. Flies heterozygous for this allele ($Sod2^{n283/+}$) expressed about 50% of SOD2 protein relative to revertant controls. A $Sod2^{KG/n283}$ transheterozygote expresses only 22% of SOD2 protein relative to $Sod2^{KGr/KGr}$. These experiments show that $Sod2^{KG}$ is a partial loss-of-function allele whereas $Sod2^{n283}$ is a null allele. Additionally, our studies show that by using these alleles alone or *in trans*, flies with progressively reduced *Sod2* expression can be generated.

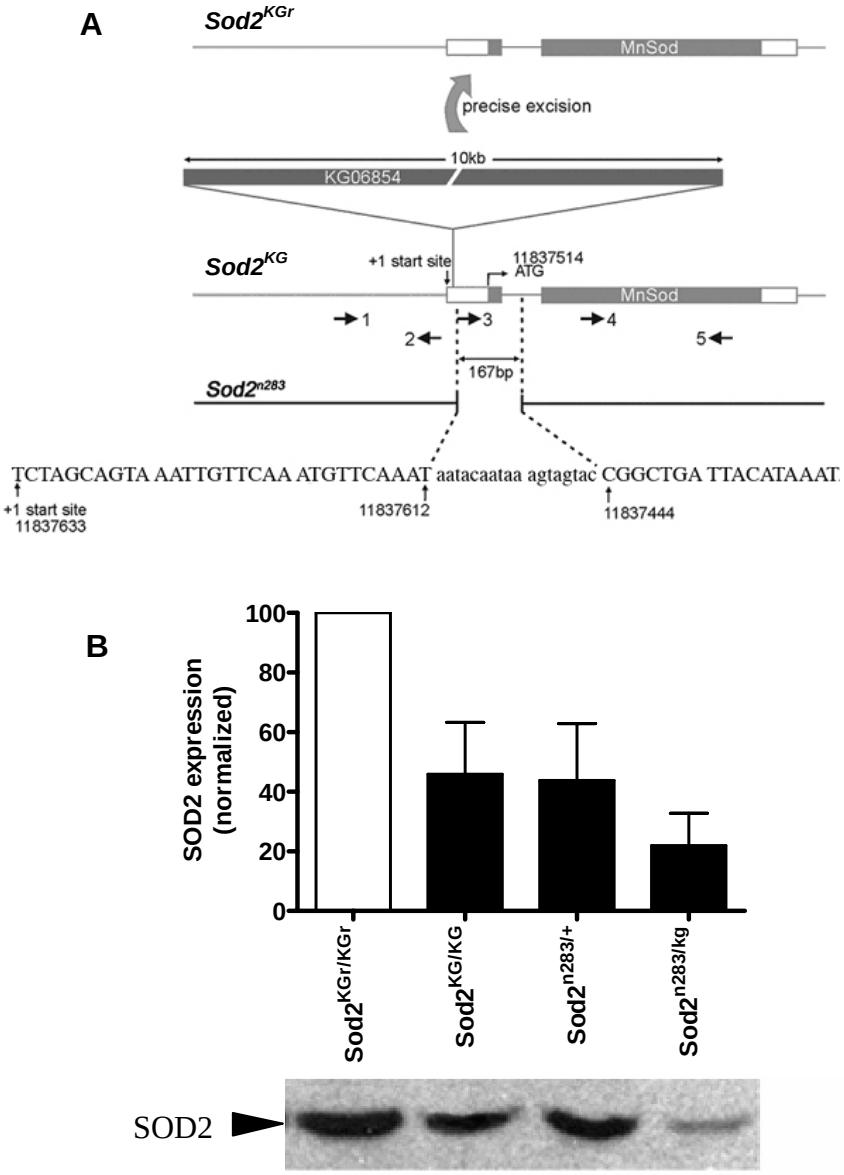


Figure 12. *Sod2* mutant generation and SOD2 protein levels. (A) *Sod2*^{KG} is the original P-insertion located in the 5' untranslated region of *Sod2*. *Sod2*^{n283} has a 167 bp deletion within exon 1 and part of intron 1. The span of the deletion was confirmed by PCR sequencing using a combination of primers 1 and 5. Sod2^{KGr} is a precise excision derivative of *Sod2*^{KG} as revealed by DNA sequencing. Molecular coordinates are according to FlyBase release 3.0. (B) Densitometric analysis of SOD2 protein normalized to levels in Sod2$^{KGr/KGr}$ revertant controls from Western blots. Data are mean ± SD (n =3). Experiments were performed in Atanu Duttaroy's lab.

3.2 Reduced Sod2 expression causes mitochondrial oxidative damage

Aconitase is a citric acid cycle principally localized to the mitochondrial matrix in *Drosophila* (Das et al., 2001). Aconitase contains an iron-sulfur cluster which renders it susceptible to oxidative inactivation specifically by superoxide (Kirby et al., 2002). Hence, reductions in aconitase activity can be indicative of increased superoxide flux in cells. Since aconitase is integral to the citric acid cycle, reduced activity also indicates impaired mitochondrial function and is a hallmark of normal aging in *Drosophila* (Das et al., 2001). Aconitase activity was reduced by about 75% in $Sod2^{n283/n283}$ homozygotes and $Sod2^{n283/KG}$ transheterozygotes compared to revertant controls (Fig. 13). In contrast, the activity of fumarase, a citric acid cycle enzyme that is not sensitive to oxidative inactivation, was unaffected by reduced *Sod2* expression (data not shown). Interestingly, aconitase activity was found to be normal in young $Sod2^{n283/+}$ heterozygotes, suggesting that reducing SOD2 by approximately 50% did not significantly alter superoxide levels in young flies.

3.3 Progressive reduction in Sod2 expression shortens life span

Survival studies were performed to determine the impact of progressive reduction of *Sod2* expression on life span. Revertant control ($Sod2^{KGr/KGr}$) flies had mean and maximum life spans of 50.4 and 86 days, respectively (Table 6). Mean and maximum life spans were reduced by 20-24% in $Sod2^{n283/+}$ heterozygotes and $Sod2^{wk/wk}$ homozygotes, flies with ~50% of normal SOD2 levels.

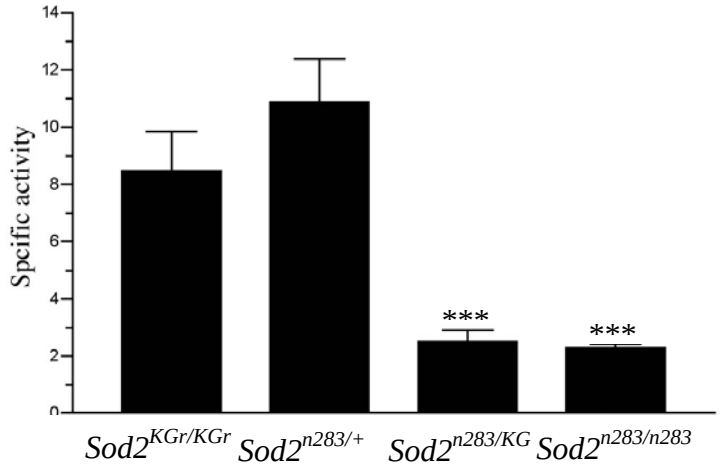


Figure 13. Reduced aconitase activity in *Sod2* mutants. Aconitase activity data are mean ± S.D. of at least three independent determinations. ***, significantly different from revertant control ($Sod2^{KGr/KGr}$), $p<0.001$. Experiments were performed in Atanu Duttaroy's lab

Hence, although young *Sod2*$^{n283/+}$ flies had no measurable reduction in aconitase activity (Fig. 13), their substantial life span reduction suggests that they are under increased oxidative stress. Mitochondrial superoxide generation increases with age in many organisms including flies (Sohal et al., 2002) and loss of *Sod2* expression to the extent found in *Sod2*$^{n283/+}$ flies may lead to an age-dependent increase in oxidative stress. Further reduction of *Sod2* expression by ~75% in *Sod2*$^{n283/KG}$ transheterozygotes shortened mean and maximum life spans by 38% and 43%, respectively (Table 6). Complete loss of SOD2 function was previously shown to results in a mean life span less than 24 hours and maximum life span around 36 hours (Duttaroy et al., 2003). These data demonstrate that SOD2 activity is crucial for normal life span and that graded reduction in *Sod2* expression results in progressively shortened life span in *Drosophila*.

Table 6. *Sod2* mutant life spans

Genotype	Mean Life Span (days)
Sod2^{KGrKGr} (n = 679)	50.4
Sod2$^{n283/+}$ (n = 1038)	41.5
Sod2$^{KG/KG}$ (n = 704)	40.0
Sod2$^{KG/n283}$ (n = 687)	31.1

See main text for detailed description

3.4 Functional senescence is accelerated by reduced Sod2 expression

Olfactory abilities decline with age in a number of species including *Drosophila* (Grotewiel et al., 2005). Odor avoidance was assessed as a function of age in *Sod2* mutants males to investigate whether elevated ROS influences age-related decline in this behavior (Fig. 14). Male flies were used in all behavioral assays since their behavioral

aging is not complicated by egg laying and reproductive history. Odor avoidance decreased across the first seven weeks of age in control flies (*Sod2*$^{KGr/KGr}$) as expected (Fig. 14). Decline in this behavior was indistinguishable between *Sod2*$^{KG/KG}$ flies and controls but was substantially accelerated in *Sod2*$^{n283/KG}$ and even more so in *Sod2*$^{n283/n283}$ flies. The DT_{50} values (age at which performance of a cohort has declined by 50% of initial levels measured in young animals) for controls and *Sod2*$^{KG/KG}$ were approximately 6 weeks whereas DT_{50}s for *Sod2*$^{n283/KG}$ and *Sod2*$^{n283/n283}$ were about 3 weeks and 10 hours of age, respectively. Hence, accelerated declines in odor avoidance performance emerged once SOD2 was reduced more than 50% and were seen to be progressively pronounced thereafter.

To investigate whether SOD2 reductions affected the rate of locomotor function decline, negative geotaxis performance was measured across age in *Sod2* mutants (Fig. 14). Akin to the odor avoidance results ~50% reduction of SOD2 did not significantly affect the rate of negative geotaxis decline although in contrast to the odor avoidance results, *Sod2*$^{n283/KG}$ flies with ~75% reduction in SOD2 also exhibited no change in the *rate* of negative geotaxis decline (Fig. 14E). Complete loss of SOD2 resulted in profound locomotor deficits compared to controls even 2 hours into adult life, and this behavior was absent in *Sod2*$^{n283/n283}$ flies by about 6 hours of age (Fig. 14F). These results indicate that reducing SOD2 levels by more than 75% produced locomotor deficits in flies whereas declines in negative geotaxis behavior were largely unaffected by lesser reductions in SOD2.

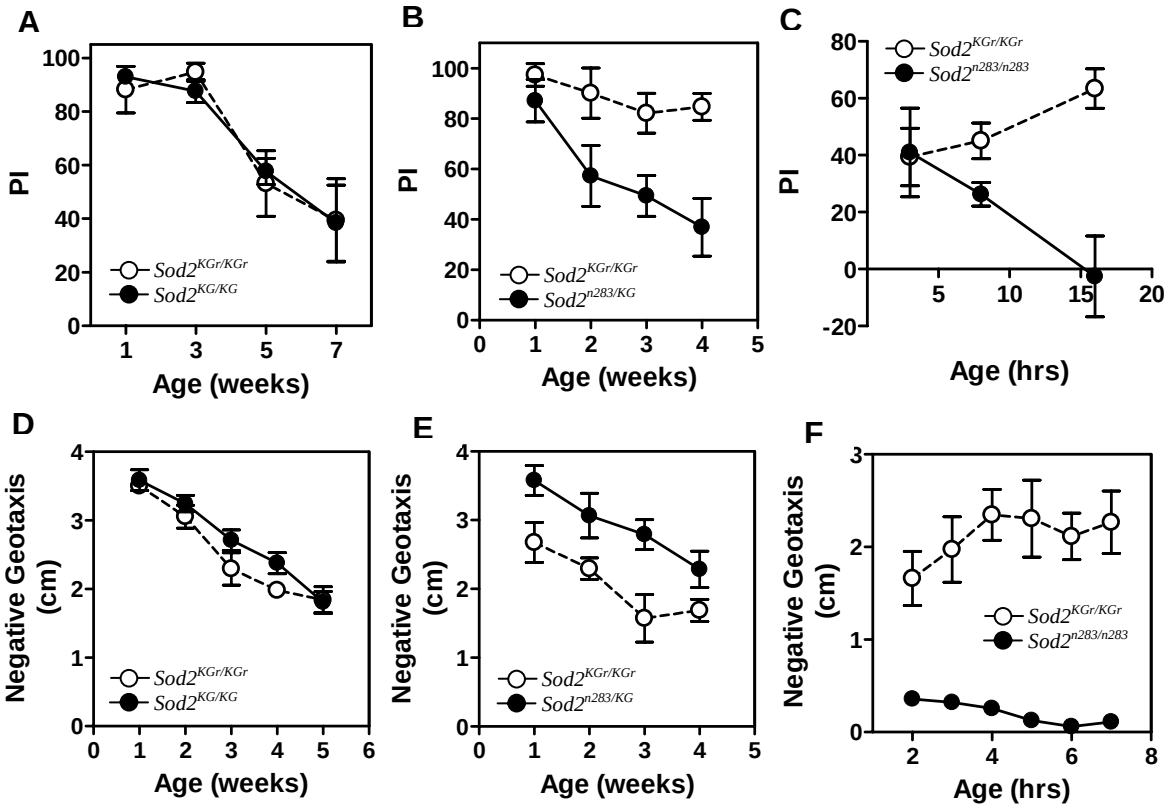


Figure 14. Senescence of odor avoidance and negative geotaxis behavior in *Sod2* mutants. Odor avoidance (A-C) and negative geotaxis (D-F) behaviors were assessed in male revertant control (open circles) and *Sod2* mutant (closed circles) flies at the ages indicated. For $Sod2^{KG/KG}$ (A,D), there was a significant effect of age but not genotype on odor avoidance behavior (two-way ANOVA $p<0.0001$ for effect of age) and effects of age and genotype on negative geotaxis (two-way ANOVA, $p<0.05$) but no effect of genotype on the *rate* of decline of geotaxis (determined from measurements of aROD over the assessment period). For $Sod2^{n283/KG}$ (B,E), there were effects of age and genotype on both odor avoidance and negative geotaxis (individual two-way ANOVAs) but again no effect of genotype on *rate* of geotaxis decline (determined from measurements of aROD over the assessment period). For $Sod2^{n283/n283}$, a two-way ANOVA revealed an effect of genotype on odor avoidance and negative geotaxis behavior ($p <0.002$) and a significant interaction between age and genotype on odor avoidance ($p<0.005$). Data are mean ± S.E.M., n = 8-16 for odor avoidance and n = 5-10 for negative geotaxis.

3.5 Complete loss of SOD2 causes neuronal cell death

Exposure to increased oxidative stress via loss of SOD2 can result in neuronal degeneration in mice (Lynn et al., 2005). To determine whether reduced *Sod2* expression caused neuronal cell death in flies, DNA strand breakage was assessed by TUNEL staining (Fig 15). Brain sections from $Sod2^{n283/n283}$ homozygotes showed widespread TUNEL-positive nuclei within hours after eclosion (Fig. 15B) whereas those from newly-emerged $Sod2^{n283/+}$ heterozygotes and revertant controls (Fig. 15C) did not. Similar levels of neuronal cell death were observed in brains from both of these lines at about 9 weeks of age (Fig. 15D), suggesting that an ~50% reduction in *Sod2* expression did not affect the normal course of age-related neuronal cell death.

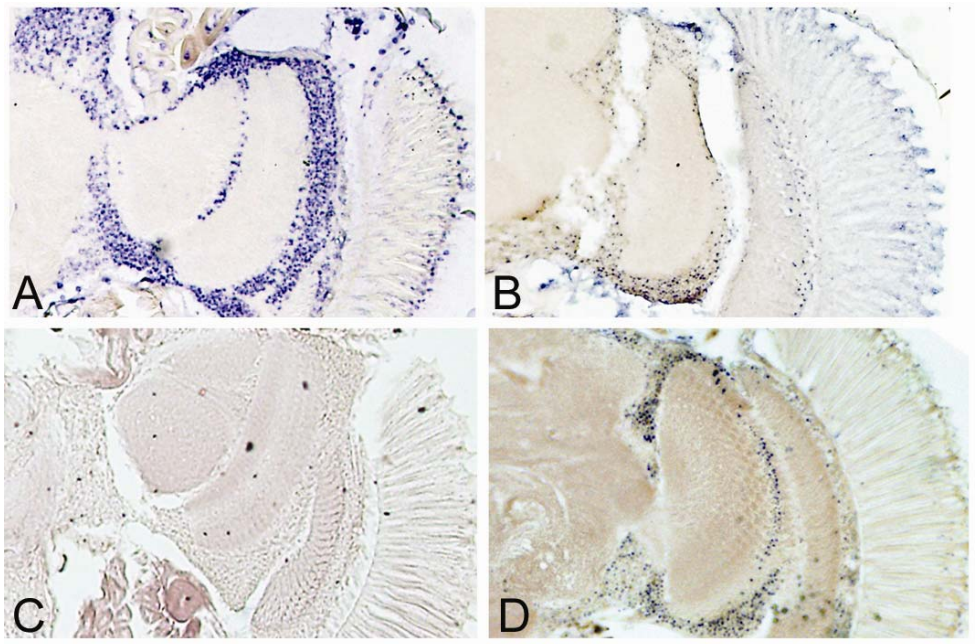


Figure 15. TUNEL staining in *Sod2* mutants. (A) TUNEL positive nuclei (blue) in adult brain sections following induction of DNA fragmentation with DNase I. (B) *Sod2*$^{n283/n283}$ flies (~20 hrs old) exhibit high levels of fragmented DNA indicative of neuronal cell death. (C) Young *Sod2*$^{KGr/KGr}$ flies displayed little DNA fragmentation. By 9 weeks of age, both *Sod2*$^{n283/+}$ (D) and *Sod2*$^{KGr/KGr}$ flies (not shown) began to exhibit DNA fragmentation. Experiments were performed in Atanu Duttaroy's lab.

4. Discussion

Studies examining the effects of SOD2 reduction in animals have been conducted in mice heterozygous or homozygous for a *Sod2* null allele, resulting in partial or full loss of *Sod2* expression, respectively. *Sod2* knock-out mice display a number of severe phenotypes including neonatal lethality, neurodegeneration, motor impairments and cardiomyopathy (Li et al., 1995). Interestingly, despite the fact that $Sod2^{+/-}$ heterozygous mice exhibit elevated mitochondrial oxidative damage and apoptosis, these mice do not display any gross phenotype or change in life span (Kokoszka et al., 2001). Here, we investigated the effects of graded reduction in *Sod2* expression on mitochondrial oxidative damage, age-related functional declines, neuronal cell death and life span in *Drosophila*. Collectively, our studies demonstrate a progressive effect of reducing *Sod2* expression on these aging parameters, supporting a link between oxidative damage, age-related functional declines and life span. Moreover, these studies indicate that life span in flies is closely correlated with the ability to eradicate ROS at their primary source, the mitochondria. Our studies and those in mice can be used to highlight important similarities and differences in SOD2 function between insects and mammals. Complete loss of SOD2 in flies and mice results in severe mitochondrial oxidative damage, neurodegeneration and behavioral phenotypes leading to early-onset mortality (Kirby et al., 2002; Li et al., 1995; Duttaroy et al., 2003). Additionally, both species exhibit substantive behavioral phenotypes only when the majority of *Sod2* expression is lost. An important difference exists, however, in that flies with ~50% loss of *Sod2* expression experience a 20-24% reduction in life span whereas mice with comparable SOD2

reductions have a normal life span relative to controls despite evidence of macromolecular damage. This raises the possibility that oxidative damage and life span in mice may not be as intimately linked as in flies. Supporting this hypothesis, overexpression of *Sod1* has been shown to increase mean life span in flies but not in mice (Huang et al., 1999; Gallagher et al., 2000; Sun et al., 1999; Parkes et al., 1998; Orr and Sohal, 1994). Furthermore, mean life span is reduced severely by 78% in *Sod1* null mutant *Drosophila* (Phillips et al., 1989) but only by about 30% in *Sod1* null mice (Elchuri et al., 2005) indicating that elevated levels of ROS in mice and flies do not have comparable consequences on longevity. An alternative possibility in the case of *Sod1* null mutants is that mice are better able to tolerate reductions in SOD1 levels because they generate less ROS under physiological conditions as a species, and/or because they possess a better armament of antioxidants acting to compensate for loss of SOD1. These possibilities seem unlikely given the extensive oxidative damage to proteins, lipids and DNA observed in *Sod1*-null mice from an early age onwards (Elchuri et al., 2005). Currently, despite a well-established association between oxidative damage and life span regulation, the mechanism underlying this link remains to be discovered. Given the apparent variability in the effect of altering SOD1 or SOD2 in flies and mice, it is tempting to speculate that these mechanisms may possibly manifest differently in separate species.

Research Chapter 3.

Tissue-Specific Studies Reveal a Degenerative Muscle Phenotype Underlying Locomotor Dysfunction and Death Upon *Sod2* RNAi in *Drosophila*

1. Introduction

The studies reported in Chapter 2 describe how reduced *Sod2* expression in flies results in mitochondrial oxidative damage, accelerated age-related functional declines and truncated life span. Although there is a well-established connection between oxidative damage and aging (Sohal et al., 2002), it is unclear whether this association is mediated by global oxidative damage throughout the body or whether oxidative damage to certain key tissues underlies this connection. Accordingly, the accelerated age-related phenotypes in *Sod2* mutants could result from loss of SOD2 activity throughout the body or primarily due to its elimination in specific tissues which are more susceptible to oxidative damage. Identifying key tissues that mediate the effects of oxidative damage on age-related functional declines and life span determination will provide mechanistic insight into the effects of oxidative damage on organisms in the context of aging. Muscle and nervous system tissues are thought to be especially prone to oxidative damage because of their high metabolic rate which gives rise to high levels of mitochondrial ROS generation (Halliwell and Gutteridge, 1999). Here, using the yeast GAL4/UAS system to achieve gene knock-down in specific tissues, the effects of pan-neuronal and muscle-specific *Sod2* silencing on survival and age-related functional declines were investigated.

2. Materials and Methods

2.1 Fly stocks and husbandry

Flies were reared to adulthood at 25°C and 55% relative humidity under a 12 hour light–dark cycle on a sugar : yeast : cornmeal : agar medium (10% : 2% : 3.3%: 1% w/v) supplemented with 0.2% Tegosept (Sigma Chemical Co., St. Louis, MO, USA) and active yeast. Silencing of *Sod2* gene expression by RNA interference in specific tissues was achieved using the yeast GAL4/UAS system as described elsewhere (Bhandari et al., 2006). Ubiquitous or spatially-restricted *Sod2* knock-down was achieved using GAL4 enhancer trap lines with tissue-specific expression patterns as desired. Once expressed, GAL4 binds to UAS sequences flanking a *Sod2* inverted repeat (*Sod2IR*) transgene resulting in expression of sense and anti-sense *Sod2* cDNA, and thereby producing a double-stranded RNA species (via hairpin formation) that elicits RNAi-mediated knockdown of native *Sod2* gene expression. The *UAS-Sod2IR24*, *UAS-Sod2IR15* and *daughterless-Gal4* (*DaGal4*) lines were provided by John Phillips. Other *Gal4* lines were from the following sources: *Mef2-Gal4* from Sunita Gupta Kramer, *Appl-Gal4* from Lawrence Goldstein, *D42-Gal4* from Jay Hirsh, *GMH5-Gal4* from R.J. Wessells, *24B-Gal4* and *elav-Gal4* from the Bloomington stock center and *91Y[CS]-Gal4* and *188Y[CS]-Gal4* were generated in-house.

2.2 SOD activity

Groups of 25 adult males (0-2 days old) per genotype were collected under brief CO_2 anesthesia and homogenized in extraction buffer (50 mM potassium phosphate/0.1 mM EDTA/2% Triton-X-100, pH 7.8) on ice. Samples were then probe sonicated for 20 sec and incubated at 4^{o}C for 45 mins to rupture mitochondria. Next, samples were centrifuged at 14,000 rpm for 15 mins at 4^{o}C and the resulting supernatant was harvested and stored at 4^{o}C until use. Protein concentration measurements were performed using the Lowry method (Bio-rad DC Protein Assay). Samples containing equal amounts of protein were electrophoresed using Discontinuous Native PAGE (4% stacking gel pH 6.8, 20% resolving gel pH 8.8) in sample buffer (0.5 M Tris-HCl/50% glycerol/0.01% bromophenol blue) at 80-100 V. SOD activity was measured colorimetrically using a modified version of an "in-gel" SOD assay previously described (Kirby et al., PNAS 2002). Briefly, gels were first soaked in a solution containing 2.5 mM nitroblue tetrazolium in 50 mM potassium phosphate buffer for 20 mins in the dark under gently agitation. Gels were next washed briefly in 50 mM phosphate buffer then transferred to a second solution (28 mM N,N,N',N'-tetramethylethylenediamine/28 μM riboflavin/50 mM potassium phosphate) for 15 mins in the dark under gentle agitation. After a second brief wash in 50 mM pottasium phosphate buffer, gels were placed on a light box and exposed to white light to allow color development for approximately 15 mins. Gels were imaged and SOD activity index was quantified by densitometry using Alpha Imager software (Alpha Innotech Corp., San Leandro, CA). Statistical analysis was performed using JMP (SAS, Cary, NC, USA).

2.3 Survival

200 adult flies of each genotype 0-3-days old were collected under brief CO_2 anesthesia and transferred to food vials at a density of 25 flies per vial. Surviving flies were be counted at each transfer to fresh food, every 3-4 days. All genotypes were aged in parallel at 25°C, 60% relative humidity under a 12 hour light/dark cycle. Statistical analysis to derive mean, median and maximum life span was done using JMP (SAS, Cary, NC, USA).

2.4 Negative Geotaxis

Groups of 25 male flies were collected under brief CO_2 anesthesia and allowed to recover at least 18 hours at 25°C and 60% relative humidity prior to assay. Flies were transferred to the RING apparatus (described in Gargano et al., Exp Gerontol. 2005) After a 1 minute rest, the apparatus was rapped sharply on a table three times in rapid succession to initiate negative geotaxis responses. The flies' positions in the tubes were captured in digital images taken 4 sec after initiating the behavior. This constituted one trial. Five trials separated by 30 sec inter-trial rest periods were performed in all experiments. After testing, flies were transferred to food vials and housed until the next test. Digital images of the flies were transferred to a PC and analyzed to determine the positions for each fly in each tube as an X-Y coordinates. The performance of flies in a single vial was calculated as the average of 5 consecutive trials to generate a single datum. Five vials of flies were tested per genotype to derive N=5.

2.5 Odor avoidance

All flies for behavioral tests were reared and aged at 25°C, 55% relative humidity under a 12 hour light/dark cycle. Avoidance of 4-methylcyclohexanol (MCH, Sigma Chemical Co. St. Louis, MO, USA, dilution factor 1:100) was measured as follows: One- to four-day-old adults were briefly anesthetized with CO_2, separated by sex, and males were transferred in groups of 25 to fresh food vials. Male flies at various ages were transferred to a T-maze. After one minute of rest, flies were allowed two minutes to choose between a maze arm containing an air stream with MCH and an opposing arm containing an air stream without an explicit odorant. After each two-minute choice test, flies were briefly anesthetized with CO_2. Flies that moved into the two arms of the T-maze were counted and (for longitudinal studies) transferred together into a fresh food vial for aging until the next assessment. Avoidance index scores were calculated as the percentage of flies that moved into the arm without odorant minus the percentage of flies that moved into the arm with odorant. These scores were normalized to the performance of 3-5 day old w[CS] control flies tested in parallel during each assessment. Six to ten vials of flies were tested for each genotype to derive N=6-10. Statistical analyses were performed with JMP (SAS, Cary, NC, USA).

2.6 Gal4 expression histology

Gal4 expression patterns were determined at regular intervals throughout the fly life span by assessing *UAS-lac Z* reporter gene expression in a *Sod2* knock-down background. A recombinant chromosome containing both *UAS-lac Z* and *UAS-Sod2IR* inserts was

generated and the presence of both UAS constructs was confirmed by PCR. Mating females containing this chromosome to *Gal4*-harboring males produced progeny with *Sod2* RNAi and reporter gene expression driven simultaneously in a spatially-restricted manner according to the *Gal4* driver present.

To assess *Gal4* expression patterns, flies were collected at the indicated ages under brief CO_2 anesthesia and fully submerged in Tissue-Tek O.C.T. embedding medium (Sakura Finetak USA, inc.) for approximately 1 hour at room temperature. Next, individual flies were transferred in embedding medium to a specimen holder and placed at -40^{o}C for 30 mins to freeze. Whole-body tissue sections (15μM) were cut using a Hacker Bright Cryostat model OTC5000 (GMI, Ramsey, MN) with the chamber set at -20^{o}C. Sections were transferred to slides, allowed to air dry for 1 hour at room temperature then fixed in 2% glutaraldehyde for 20 mins. Slides were then washed twice for 5 mins in PBS, and placed at 37^{o}C until dry. β-galactosidase activity was triggered by adding pre-warmed X-gal substrate to the slides for 30 mins. Slides were next washed twice for 5 mins in PBS and coverslips were mounted in 70% glycerol in PBS Digital images were obtained using a Zeiss Axioplan-2 microscope, Axiocam CCD camera, and Axiovision software (Carl Zeiss, Germany).

Quantitative assessment of *Gal4* expression was obtained by spectrophotometric assessment of β-galactosidase activity as previously described (Seroude Aging Cell 2002). Three adult males were homogenized in extraction buffer (50 mM potassium

phosphate, 1 mM MgCl2, 0.5 μg/ml leupeptin, 0.5 μg/ml aprotenin, 0.7 μg/ml pepstatin A, pH 7.2) at the indicated ages. Extracts were centrifuged at 14,000 r.p.m. for 5 mins and the supernatants were transferred. β-galactosidase activity was triggered by adding a portion of the sample to 100 μM chlorophenol red-β-D-galactopyranoside solution in a cuvette, and the change in absorbance at 562 nM over 5 mins was measured using a Pharmacia Biotech Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Piscataway, NJ). *Gal4* expression levels were expressed as the absorbance change per minute per milligram of sample protein. The same protocol was used to assess background activity of endogenous β-galactosidase in control flies containing both UAS constructs but no *Gal4* and was found to be negligible. Hence, background activity was not considered further in our analyses.

2.7 ATP levels

ATP levels were assessed using the ATP Bioluminescent Assay Kit (Sigma product FL-AA). Fly thoraces were dissected from 5 adult male flies under CO_2 anesthesia, homogenized in 6 M guanine hydrochloride and heated at 95^{o}C for 5 mins to eliminate endogenous ATPase activity. Homogenates were centrifuged at 14,000 rpm for 15 mins at room temperature, and the supernatant was diluted 10-fold in sample buffer (0.2 M glycine, 50 mM $MgCl_2$, 4 mM EDTA, pH 7.4). Sample luminescence was measured following addition of ATP Assay Kit reagents according to the manufacturer's instructions in a Wallac 1420 Victor V plate reader (Perkin-Elmer, Waltham, MA). ATP

concentrations of extracts were derived from calibration curves obtained using the ATP standards provided.

2.8 Transmission electron microscopy

Thoraces were dissected from adult male flies under anesthesia and immediately submerged in fixative solution (1% glutaraldehyde/2% paraformaldehyde/0.2 M sodium cacodylate pH 7.4) then fixed, osmicated (1% osmium tetroxide), stained (1% uranyl acetate) and dehydrated in a series of solutions with increasing ethanol concentration using a microwave-assisted protocol. Samples were next briefly incubated in propylene oxide then embedded using a transitional series of propylene oxide/Embed 812 resin mixes with higher proportion of resin used on each transfer until samples were in pure resin. Samples were then transferred to resin-filled moulds and placed at 60^{o}C for 18 hours to trigger resin polymerization. Tissue blocks were trimmed and oriented to enable longitudinal sectioning of the indirect flight muscles. Sections (80-100 nM) were cut and transferred to copper grids then visualized using a JEOL 1010 transmission electron microscope (JEOL USA Inc., Peabody, MA).

For mitochondrial quantitation, mitochondrial density calculations were precluded by an inability to distinguish and therefore count individual mitochondria in TEM images. Instead, mitochondrial content in representative images was assessed as the fraction of total image area occupied by mitochondria. 4 to 8 representative low magnification

images were analyzed for each assessment using Scion Image software (Scion Corporation, Frederick, MD) resulting in a total image field of 0.65-1.3 mM^2 analyzed.

2.9 Caspase activity (Laurent Seroude lab)

The caspase assay is based on the hydrolysis of the peptide substrate Ac-asp-glu-val-asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), This assay was performed essentially as described before (Zheng et al., 2005). Two thoraces or abdomens were homogenized in lysis buffer (50 mM Hepes, pH 7.5/100 mM NaCl/1 mM EDTA/0.1% CHAPS/10% sucrose/5 mM DTT/0.5% Triton X-100/4% glycerol) and centrifuged at 13,000 × *g* for 5 min at 4°C to collect supernatant. Ten microliters of thorax extracts (2 mg/ml protein) or 3 µl of abdomen extracts (3 mg/ml protein) were incubated for 1 h at 27°C with 25 mM Ac-DEVD-AMC in lysis buffer with a final reaction volume of 50 µl. The specificity of the detection was controlled in a duplicate reaction pretreated for 15 min at 22°C with 2.5 mM Ac-DEVD-CHO inhibitor. The fluorescence of this control reaction was subtracted from the test reaction. AMC fluorescence was determined by using a Spectra Max Fluorescent Microplate Reader (Molecular Devices, Sunnyvale, CA) with the excitation and emission set at 360 nm and 460 nm, respectively. The concentration of the AMC released was calculated by using an AMC standard curve Protein concentrations in the various extracts were measured using the Lowry method (Bio-Rad DC protein assay). Caspase activity was expressed as nanomoles of AMC per second per milligram of protein.

3. Results

3.1 Confirmation of ubiquitous Sod2 RNAi phenotype and assessment of functional senescence

Ubiquitous *Sod2* knock-down using the GAL4/UAS system has previously been reported to result in undetectable SOD2 activity, reduced life span and mitochondrial oxidative damage in *Drosophila* (Kirby et al., 2002). Before proceeding with extensive tissue-specific expression studies to identify possible key tissues mediating these effects, it was important to confirm that ubiquitous *Sod2* knock-down using the GAL4/UAS system resulted in a similar phenotype in our hands. It was also necessary to determine whether, as with the *Sod2* mutants we previously studied (see Chapter 2), that *Sod2* knock-down flies using this system had accelerated age-related functional declines. As reported before (Kirby et al., 2002), substantial loss of SOD2 activity was observed in two independent *Sod2* knock-down lines (Sod2IR24 and Sod2IR15, Fig. 16). Control groups consisted of flies harboring either a *Gal4* or *Sod2IR* transgene alone in a $Sod2^{+/+}$ background to control for any possible mutagenic effects caused by insertion of these transgenes that could cause the phenotypes observed in *Sod2* knock-down lines. Some detectable SOD2 activity remained in Sod2IR24 and Sod2IR15 lines which is consistent with the established priniciple that RNAi results in partial and not complete gene silencing in organisms (Matzke et al., 2001). In further agreement with prior studies, the mean life span of Sod2IR24 flies was reduced 78% relative to control groups highlighting the severe effect on longevity caused by substantial loss of SOD2 (Fig. 16B). To determine

whether *Sod2* RNAi resulted in behavioral phenotypes akin to those seen in *Sod2* mutants, negative geotaxis and odor avoidance performance were measured across age in Sod2IR24 flies (Fig. 16C and 16D). In both of these tests, 1-day-old Sod2IR24 adults performed indistinguishably from controls. By day three (negative geotaxis) or day six (odor avoidance), clear deficits in performance could be seen for SodIR24 flies relative to control groups which became even more pronounced at later assessment points. By day nine, cohort performance in both behavioral assays had declined dramatically to around 30% of initial scores for Sod2IR24 and further assessments were precluded by rapid Sod2IR24 mortality occurring thereafter.

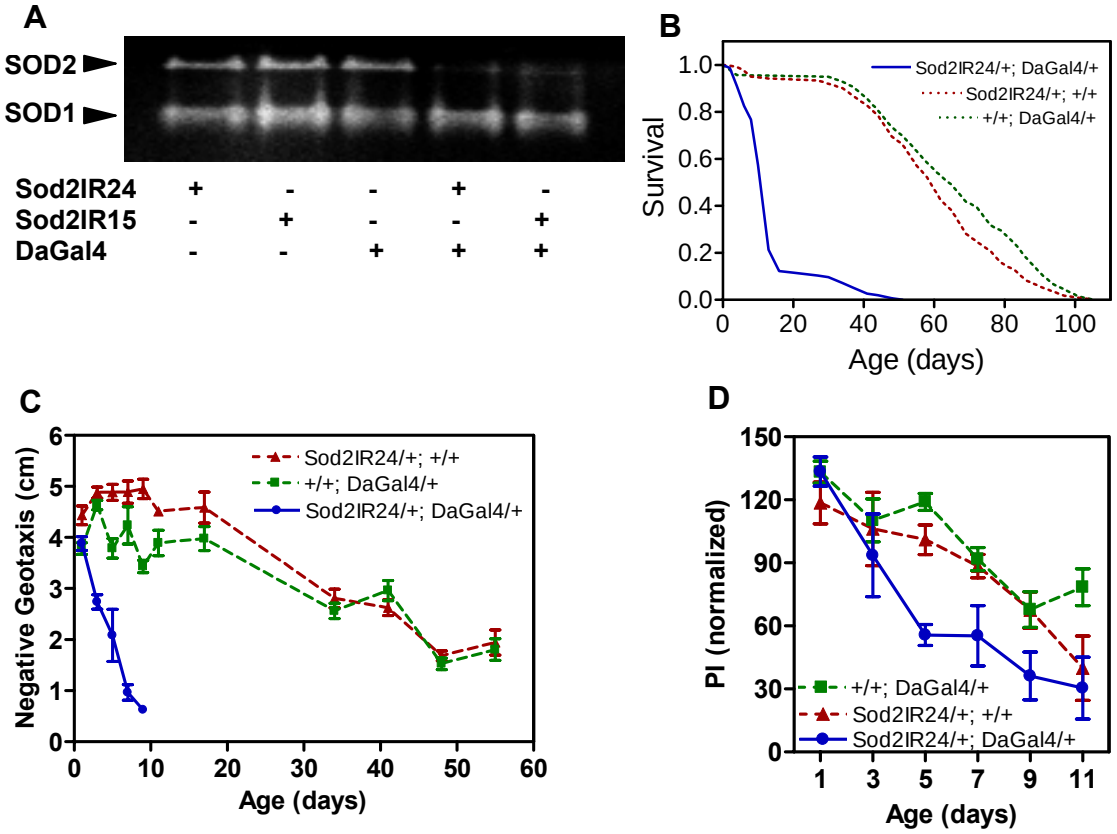
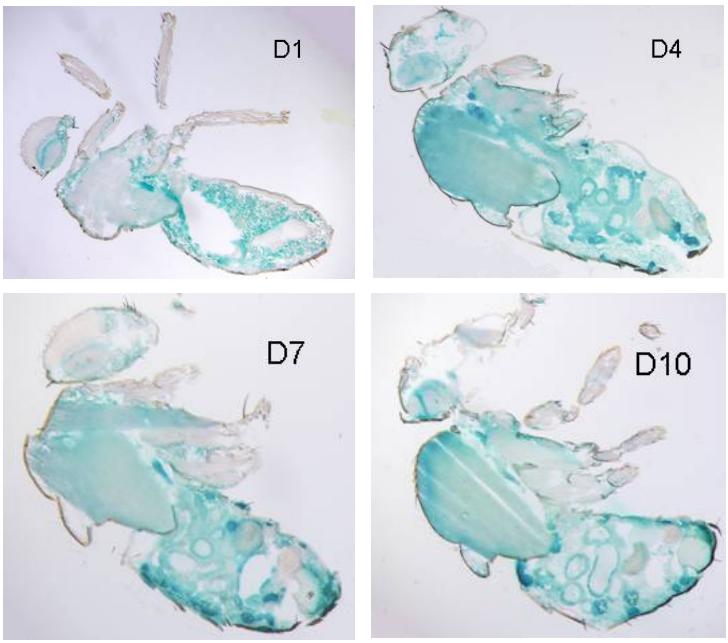


Figure 16. SOD activity, life span and behavioral senescence in flies with ubiquitous *Sod2* knock-down. (A) Extracts (each containing 45 µg protein) of adult males (0-2 days old) were electrophoresed on a native polyacrylamide gel and SOD activity was determined using an In-gel assay (see materials and methods). *Sod2* knock-down caused a clear reduction in SOD2 activity in both lines tested (B), Survival studies revealed a severe reduction in mean and median life span in *Sod2* knock-downs. The mean and median life spans (days) were as follows: *+/+; DaGal4/+* (mean = 65, median = 65), *Sod2IR24/+; +/+* (mean = 60, median = 62), *Sod2IR24/+; DaGal4/+* (mean = 14, median = 13). (C) There was a significant effect of age and genotype and an interaction between these factors on negative geotaxis behavior (two-way ANOVA, $p<0.0001$, $n=5$). Tukey's honestly significant difference (HSD) post-test revealed that *Sod2* knock-down lines performed significantly worse across age than both control groups. (D) There was a significant effect of *Sod2* knock-down on olfactory behavior decline (two-way ANOVA, $p<0.0001$, $n=8$). Again, Tukey's HSD post-test indicated that the *Sod2* knock-down line performed significantly worse across age than both control groups.

One limitation with the use of available *Gal4* drivers is that their expression patterns typically have not been characterized across the life span and as previously shown, can vary in intensity and location with age (Seroude, 2002). To determine whether *DaGal4* expressed ubiquitously over the life span of Sod2IR24 flies and was therefore driving *Sod2* knock-down throughout the entire *Drosophila* body, histological staining of *Gal4* expression was carried out in flies expressing *DaGal4*-driven *lac Z* reporter gene expression. The fairly short half-life of β-galactosidase in flies (1 day) allows reasonably accurate assessment of GAL4 expression over age (Kalb et al., 1993). Indeed, *Gal4* expression was detected throughout the body at all ages assessed (Fig. 17) confirming that *Sod2* was silenced ubiquitously. Quantification of *DaGal4* expression in whole-body homogenates revealed comparable levels in larval and pupal stages and that expression levels varied significantly over the adult life span but were robust throughout.



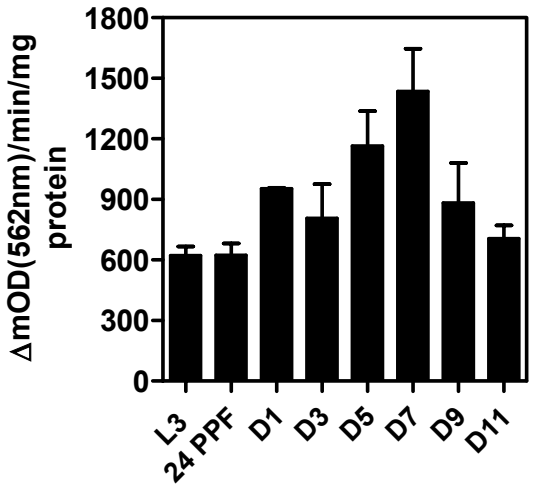


Figure 17. *DaGal4* expression pattern in *Sod2* knock-down flies. Longitudinal sections through the whole body of males were taken at the adult ages indicated as described in materials and methods. *DaGal4* is expressed ubiquitously at all ages tested. *Gal4* expression levels were quantified in whole-body extracts of 3rd instar larvae (L3), pupae 24 hrs following puparium formation (24 PPF) and at the adult ages indicated. There was a significant effect of age on *Gal4* expression levels (ANOVA, $p<0.0001$, n =3). O.D. data are mean ± S.D.

3.2 Sod2 RNAi in the nervous system has no measurable effect on SOD2 activity and a modest effect on functional senescence and survival

Complete loss of SOD2 leads to neurodegenerative phenotypes in both flies and mice (Paul et al., 2007; Melov et al., 1999). To determine the effects of pan-neuronal *Sod2* knock-down on flies, SOD2 activity, negative geotaxis and survival studies were carried out. Additionally, components of the nervous system thought to be crucial for the negative geotaxis response were chosen for selective *Sod2* knock-down in order to determine whether this had any effect on negative geotaxis performance in young or aging flies. Surprisingly, pan-neuronal *Sod2* knock-down did not reduce SOD2 activity relative to both controls in measurements taken from the heads of young flies (Fig. 18).

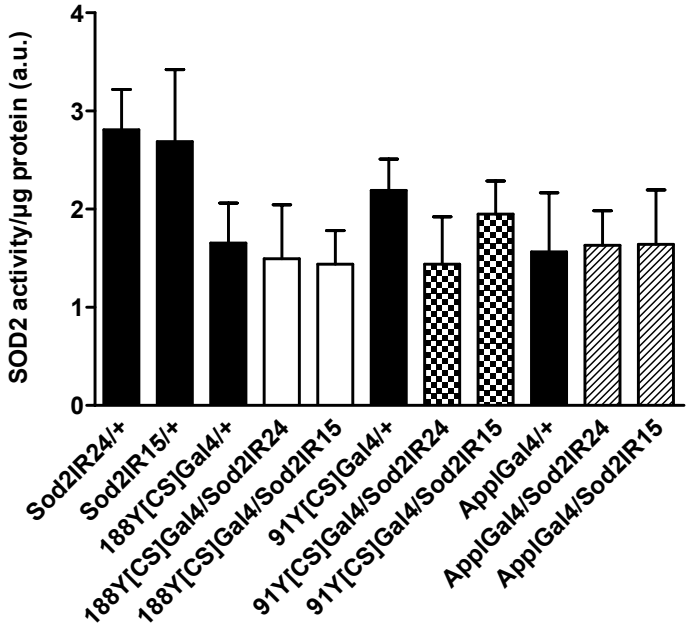
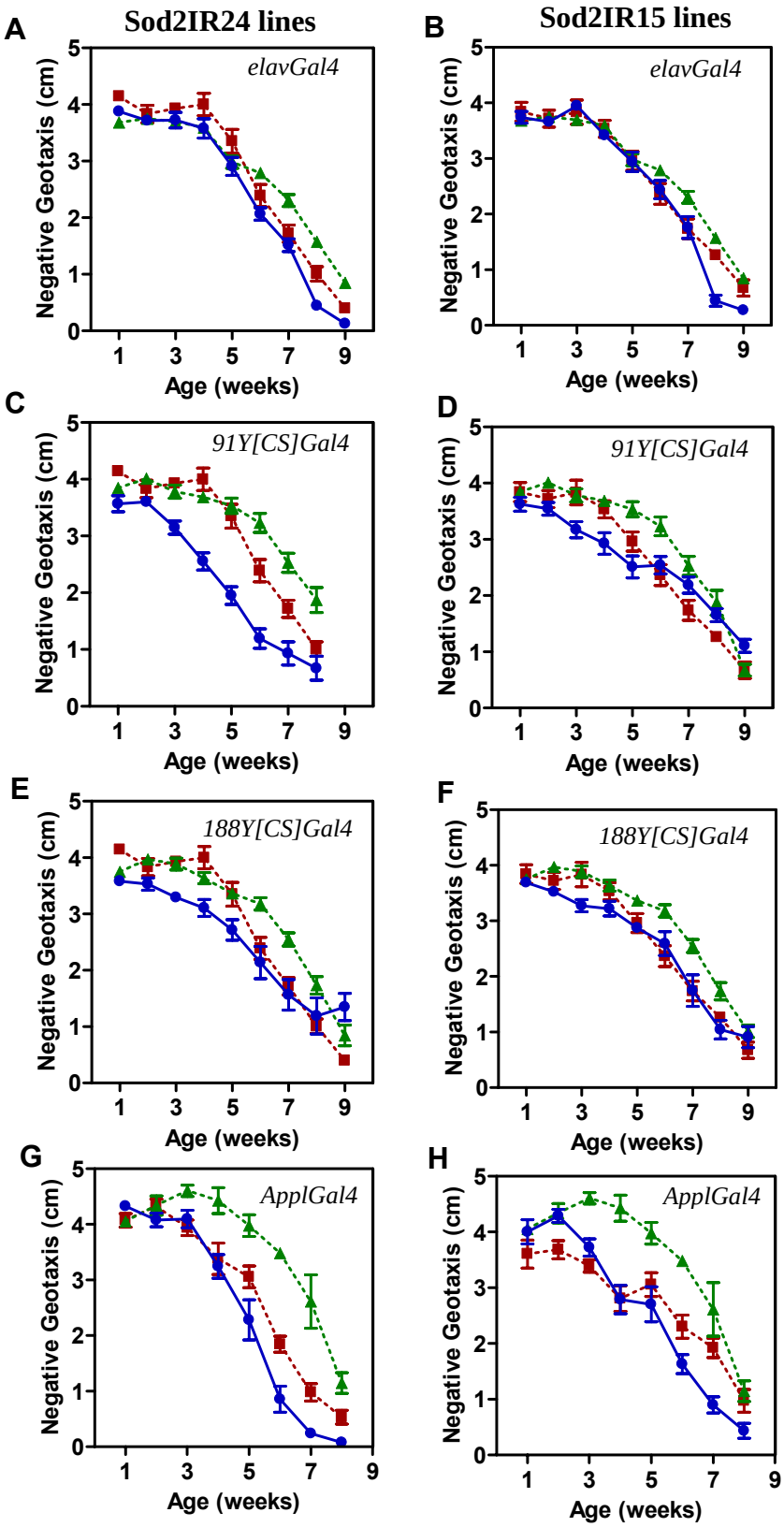


Figure 18. SOD2 activity in pan-neuronal *Sod2* knock-down flies. SOD2 activity was measured in extracts (containing 45 µg protein) from adult males (0-2 days old) as described in materials and methods. None of the pan-neuronal *Sod2* knock-down lines exhibited significantly less SOD2 activity than both *Gal4/+* and *Sod2IR/+* controls when compared using Tukey's HSD post-test following individual two-way ANOVAs for each knock-down plus controls. Data are mean ± S.D (n =3). For clarity, chromosomal designations of transgenes are omitted.

The data for Sod2IR24 and Sod2IR15 lines activated by three independent pan-neuronal *Gal4* drivers (a total of six knock-down lines generated) showed that SOD2 activities were essentially unchanged in the *Sod2* knock-down lines except for one line (*91Y[CS]Gal4/Sod2IR24*) that exhibited a modest reduction in SOD2 activity compared to controls that didn't reach statistical significance. Phenotypic studies on negative geotaxis behavior showed that pan-neuronal *Sod2* knock-down did confer significant effects on negative geotaxis declines across age for all pan-neuronal Sod2IR24 lines but not for any pan-neuronal Sod2IR15 lines tested (Fig 19). Likewise, *Sod2* knock-down specifically in motorneurons or dopaminergic neurons thought to be critical for negative geotaxis behavior yielded significant effects on age-related decline in this behavior in the case of Sod2IR24 lines (Fig 20). Survival tests on pan-neuronal *Sod2* knock-down flies revealed life span reductions in the range of 16-25% seen in the Sod2IR24 lines tested whereas only one of the Sod2IR15 lines had a reduced life span (Fig. 21).

Figure 19 (overleaf). Negative geotaxis senescence following pan-neuronal *Sod2* knock-down. Negative geotaxis assays were carried out as described in materials and methods. Sod2IR24 (A,C,E,G) and Sod2IR15 (B,D,F,H) knock-down lines (●) with *Sod2* RNAi driven by the *Gal4* drivers indicated were compared to controls containing *Gal4* (▲) or *Sod2IR* (■) transgenes alone within each data set. There were significant effects of age and genotype on all data sets (individual two-way ANOVAs, $p<0.0001$, n =5-10) except for the data set with the *188Y[CS]Gal4/Sod2IR15* line which did not exhibit an effect of genotype when compared to its controls (two-way ANOVA, n =5-10). Tukey's HSD post-test on each two-way ANOVA revealed that all *Sod2IR24* knock-down lines performed significantly worse across age than both controls ($p<0.05$) whereas none the *Sod2IR15* knock-down lines did. Data (mean ± S.E.M.) are compiled from two independent experiments except in (G) and (H) which are from one experiment.


A
Sod2IR24 lines
elavGal4
B
Sod2IR15 lines
elavGal4
C
91Y[CS]Gal4
D
91Y[CS]Gal4
E
188Y[CS]Gal4
F
188Y[CS]Gal4
G
ApplGal4
H
ApplGal4
Negative Geotaxis (cm)
Age (weeks)
0
1
2
3
4
5
1
3
5
7
9

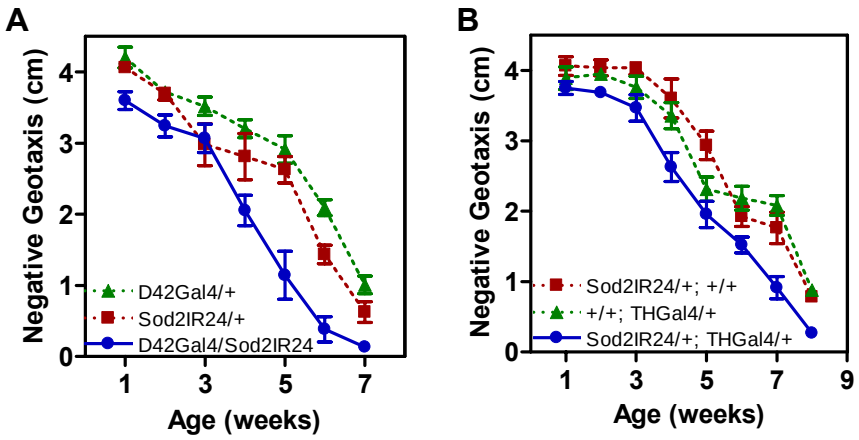


Figure 20. Locomotor senescence in flies with *Sod2* knock-down in motor or dopaminergic neurons. In (A), there were significant effects of age and genotype on behavior and an interaction between these factors (two-way ANOVA, $p<0.0001$ for age and genotype, $p = 0.0143$ for interaction, $n = 5$). Tukey's HSD post-test revealed that motor neuron-specific *Sod2* knock-down caused significantly worse performance than seen in both controls ($p<0.05$). (B) Two-way ANOVA indicated effects of age and genotype ($p<0.0001$, $n = 5$). Tukey's HSD post-test revealed that *Sod2* knock-down flies performed significantly worse than both control groups ($p<0.05$). Data are mean ± S.E.M.

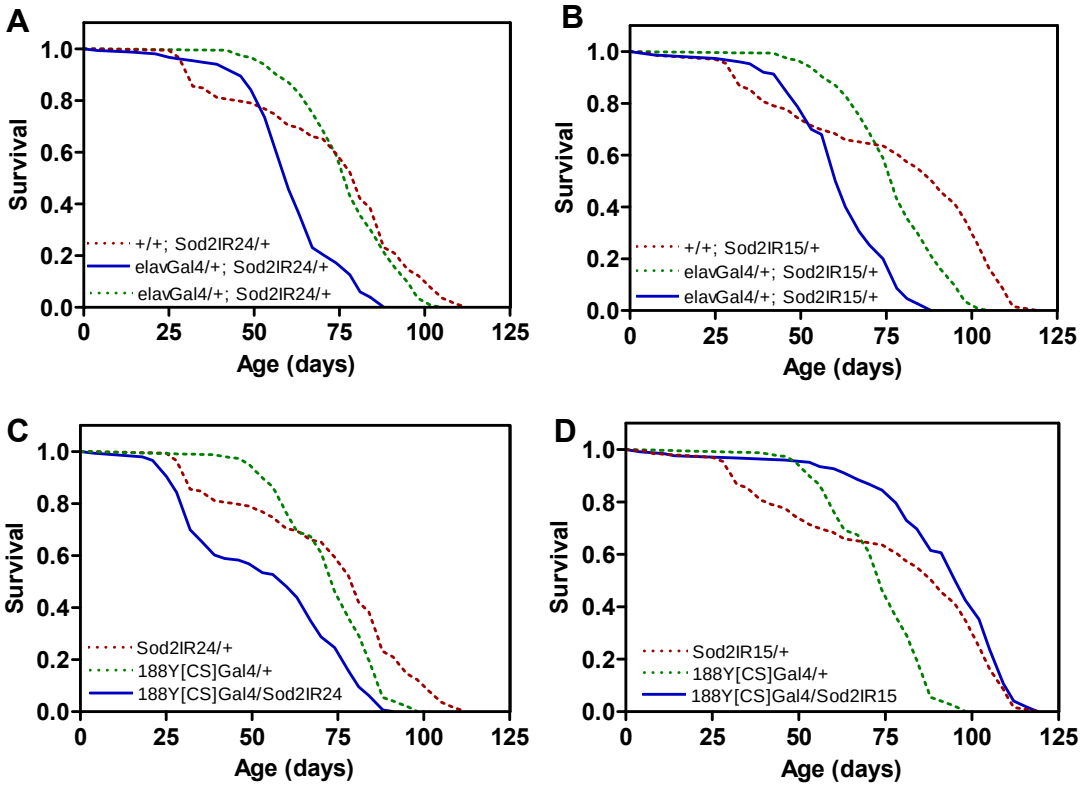


Figure 21. Survival of pan-neuronal *Sod2* knock-down flies. Survival studies were carried out as described in materials and methods. *Sod2* RNAi via the *elavGal4* driver (A,B) resulted in life span reductions for both Sod2IR24 and Sod2IR15 lines. Sod2 knock-down via *188Y[CS]Gal4* resulted in life span truncation in the Sod2IR24 line only. The mean and median life span (days) were as follows: *+/+; Sod2IR24/+* (mean =73, median =81), *+/+; Sod2IR15/+* (mean =78, median =91), *elavGal4/+; +/+* (mean =78, median =78), *elavGal4/+; Sod2IR24/+* (mean =61, median =60), *elavGal4/+; Sod2IR15/+* (mean =62, median =63). *188Y[CS]Gal4/+* (mean =73, median =74), *188Y[CS]Gal4/Sod2IR24* (mean =55, median =60), *188Y[CS]Gal4/Sod2IR15* (mean =92, median =95).

The effects on locomotor function and survival in pan-neuronal Sod2IR24 lines indicated that *Sod2* expression was likely reduced in the nervous system to some extent, despite the lack of measurable effect on SOD2 activity. To determine whether clearer reductions in SOD2 activity might manifest at a later age, SOD2 activity was measured in 4-week-old flies, where locomotor deficits were the most pronounced. When assessed at this age, SOD2 activity was again indistinguishable from control flies (Fig. 22). Taken together, the SOD2 activity data suggest that *Sod2* expression was not robustly reduced across age in pan-neuronal lines.

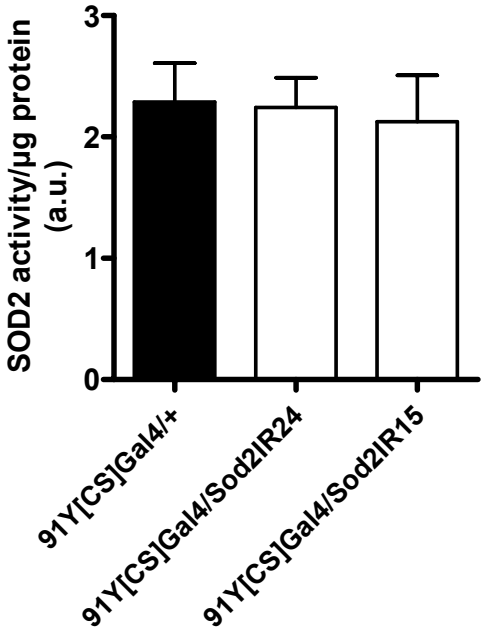


Figure 22. Measurement of SOD2 activity in aged pan-neuronal *Sod2* knock-down flies. There was no significant effect of *Sod2* knock-down on SOD2 activity in 91Y[CS]Gal4/Sod2IR24 or 91Y[CS]Gal4/Sod2IR15 lines (individual t-tests, n =3).

3.3 Muscle-specific Sod2 knock-down severely affects locomotion and life span

The consequences of muscle-specific *Sod2* knock-down on SOD activity were examined. Two separate *Gal4* drivers were each used to generate Sod2IR24 and Sod2IR15 lines with *Sod2* knock-down throughout the fly musculature. SOD activity assays revealed statistically signifant reductions in whole-body SOD2 activity for all lines generated, ranging from 51-64% (Fig. 23A and 23B). SOD1 activity was unaffected by *Sod2* knock-down (Fig. 23C) suggesting that there was no compensatory upregulation of *Sod1* expression.

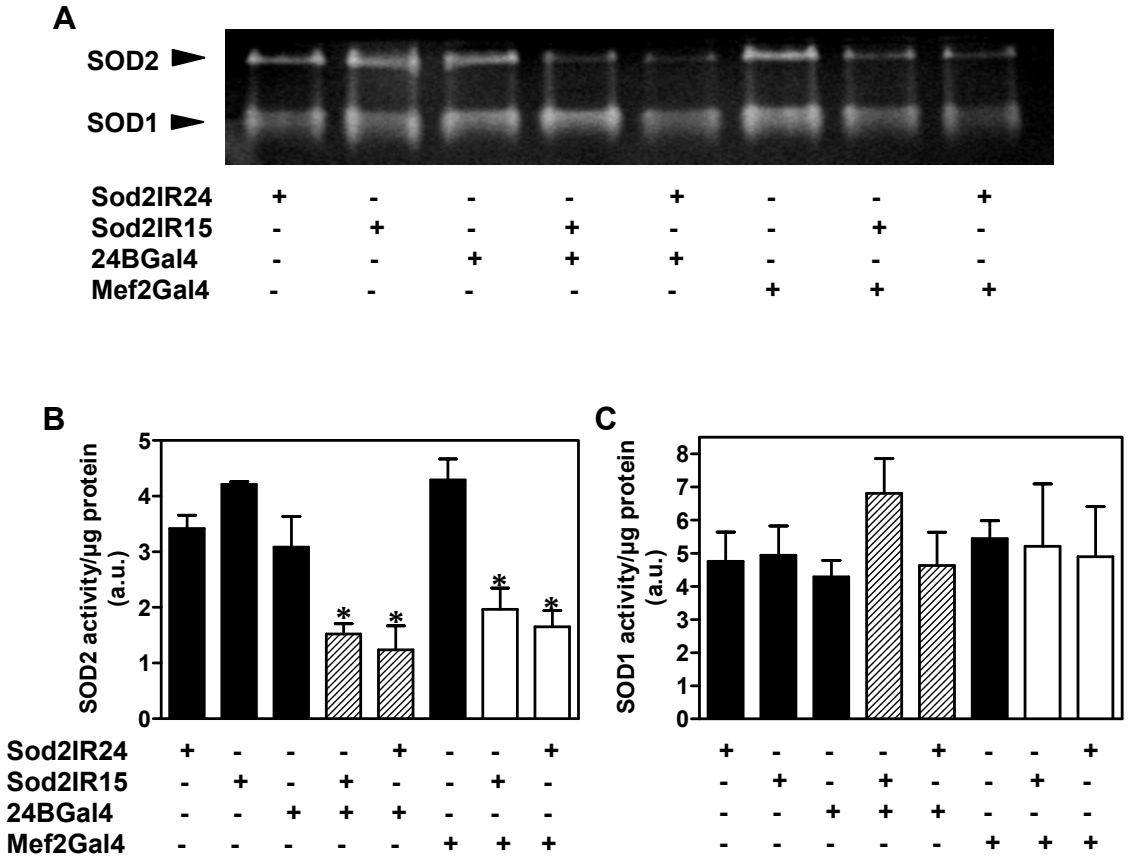
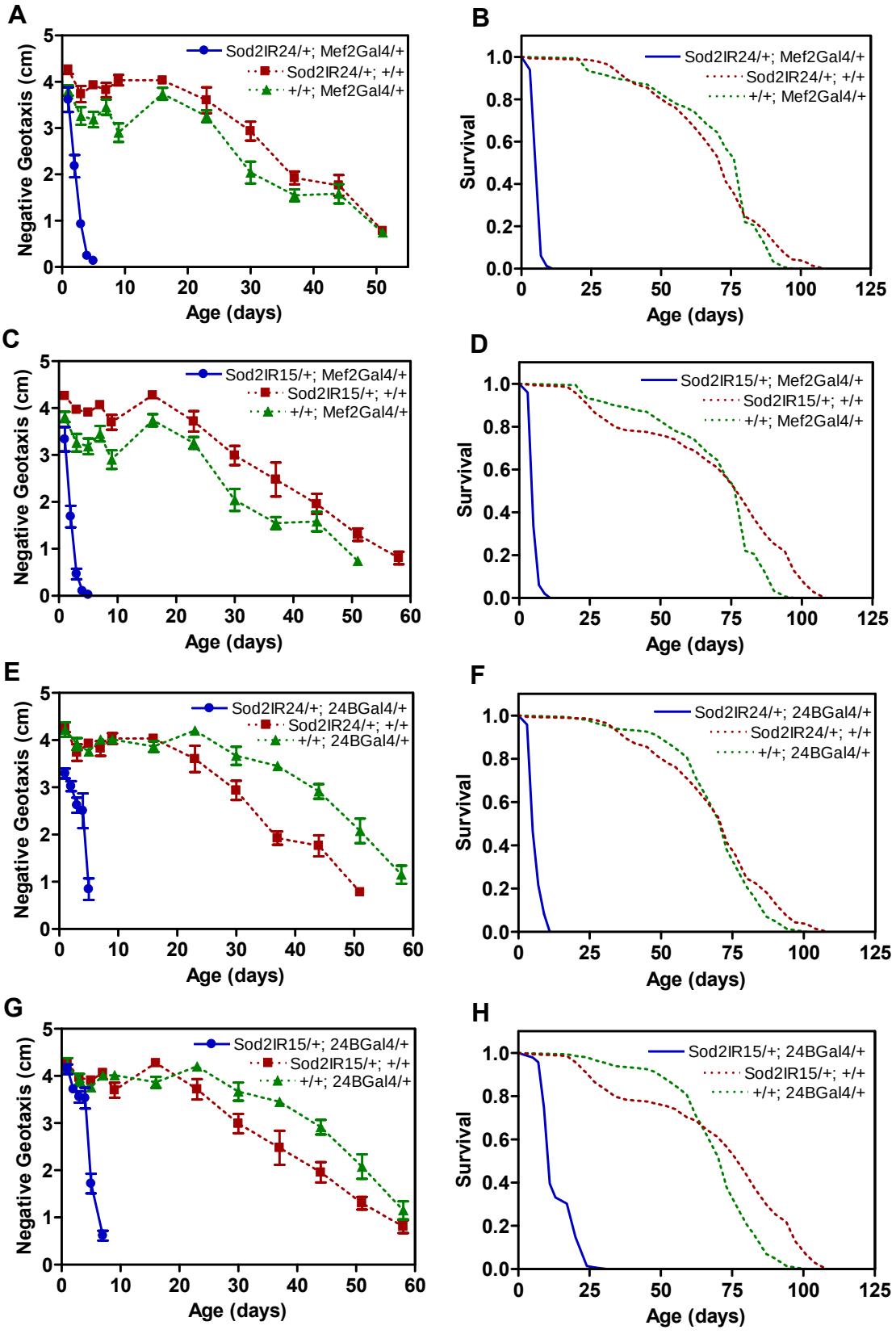


Figure 23. SOD activity in muscle-specific *Sod2* knock-down flies. (A) In-gel SOD activity assay of whole-body extracts (containing 45 µg protein) from flies expressing *Sod2IR24* or *Sod2IR15* transgenes via two independent muscle *Gal4* drivers (*Mef2Gal4* or *24BGal4*). *Sod2* knock-down flies and controls were generated as described in materials and methods. Densitometric analysis revealed a significant effect of genotype on SOD2 activity for all four *Sod2* knock-down lines compared to their respective controls. (individual ANOVAs, $p \leq 0.0126$, $n = 3$). Tukey's HSD post-test indicated that all *Sod2* knock-down lines had significantly less SOD2 activity than both of their relevant control groups (*, $p<0.05$). There was no significant effect of *Sod2* knock-down on SOD1 activity (individual ANOVAs). Densitometry data are mean ± S.D., $n = 3$.

Negative geotaxis assessment across age revealed that while young muscle *Sod2* knock-down flies perform comparably to controls, they experience a dramatic decline in locomotor performance over the first week of adult life to the point where negative geotaxis behavior could no longer be measured in flies aged ~6 days. All controls exhibited normal age-related declines in negative geotaxis occurring over a period of ~8 weeks. Additionally, reduced muscle SOD2 activity lead to a striking reduction in mean life span, between 91-94% for Sod2IR24 lines and 80-92% for Sod2IR15 lines.

Figure 24 (overleaf). Locomotor senescence and survival following loss of muscle SOD2 activity. Newly-emerged flies with muscle-specific *Sod2* knock-down performed comparably to control groups in negative geotaxis assessments (A,C,E,G), but thereafter exhibited a rapid decline in this behavior which was virtually absent in 7-day-old flies. All individual two-way ANOVAs revealed effects of age and genotype on behavior ($p<0.0001$, n =5). Survival (B,D,F,H) was also dramatically reduced in muscle-specific *Sod2* knock-down lines tested. Mean and median life span (days) were as follows: *Sod2IR24/+; +/+* (mean =69, median =73), *Sod2IR15/+; +/+* (mean =71, median =80), *+/+; Mef2Gal4/+* (mean =70, median =80), *+/+; 24BGal4/+* (mean =70, median =73), *Sod2IR24/+; 24BGal4/+* (mean =6, median =5), *Sod2IR15/+; 24BGal4/+* (mean =14, median =11), *Sod2IR24/+; Mef2Gal4/+* (mean =6, median =5), *Sod2IR15/+; Mef2Gal4/+* (mean =6, median =5). Negative geotaxis data are mean ± S.E.M. All data sets are representative of two independent experiments.


A
Negative Geotaxis (cm)
Age (days)
Sod2IR24/+; Mef2Gal4/+
Sod2IR24/+; +/+
+/+; Mef2Gal4/+
B
Survival
Age (days)
Sod2IR24/+; Mef2Gal4/+
Sod2IR24/+; +/+
+/+; Mef2Gal4/+
C
Negative Geotaxis (cm)
Age (days)
Sod2IR15/+; Mef2Gal4/+
Sod2IR15/+; +/+
+/+; Mef2Gal4/+
D
Survival
Age (days)
Sod2IR15/+; Mef2Gal4/+
Sod2IR15/+; +/+
+/+; Mef2Gal4/+
E
Negative Geotaxis (cm)
Age (days)
Sod2IR24/+; 24BGal4/+
Sod2IR24/+; +/+
+/+; 24BGal4/+
F
Survival
Age (days)
Sod2IR24/+; 24BGal4/+
Sod2IR24/+; +/+
+/+; 24BGal4/+
G
Negative Geotaxis (cm)
Age (days)
Sod2IR15/+; 24BGal4/+
Sod2IR15/+; +/+
+/+; 24BGal4/+
H
Survival
Age (days)
Sod2IR15/+; 24BGal4/+
Sod2IR15/+; +/+
+/+; 24BGal4/+

To confirm that the *Gal4* drivers used in this study were in fact muscle-specific, expression patterns were profiled for each *Gal4* line by histological assessment of *lac Z* reporter gene expression. This revealed strong expression of *Mef2-Gal4* in thoracic muscle and weaker expression in other muscles (Fig. 25A). *24B-Gal4* expression was localized both to muscle and fat body (Fig. 25B), and hence the common area of overlap in the expression of *Mef2-Gal4*, *24B-Gal4* and *Da-Gal4* is muscle. Since *Gal4* expression patterns can exhibit age-related changes, it was deemed necessary to profile the expression of *Mef2-* and *24B-GAL4* drivers across age. To recapitulate the expression patterns of these drivers as accurately as possible, they were assessed in a *Sod2* knock-down background. Generation of a recombinant chromosome containing *Sod2IR* and *lac Z* (see materials and methods) allowed us to use *Mef2-* and *24B-Gal4* lines to drive *Sod2* knock-down and reporter gene expression simultaneously. Assessment of expression patterns at intervals throughout the life span of *Sod2* knock-downs led to the conclusion that both *Gal4* drivers expressed in a spatially-consistent manner across age (Fig. 25). Quantitative analysis of *Mef2-* and *24B-Gal4* expression levels across age demonstrated temporal variations in expression intensity across the adult life span. In both cases, adult expression levels were generally higher than those in larval and pupal stages of development (Fig. 25) and tended to increase across the life span.

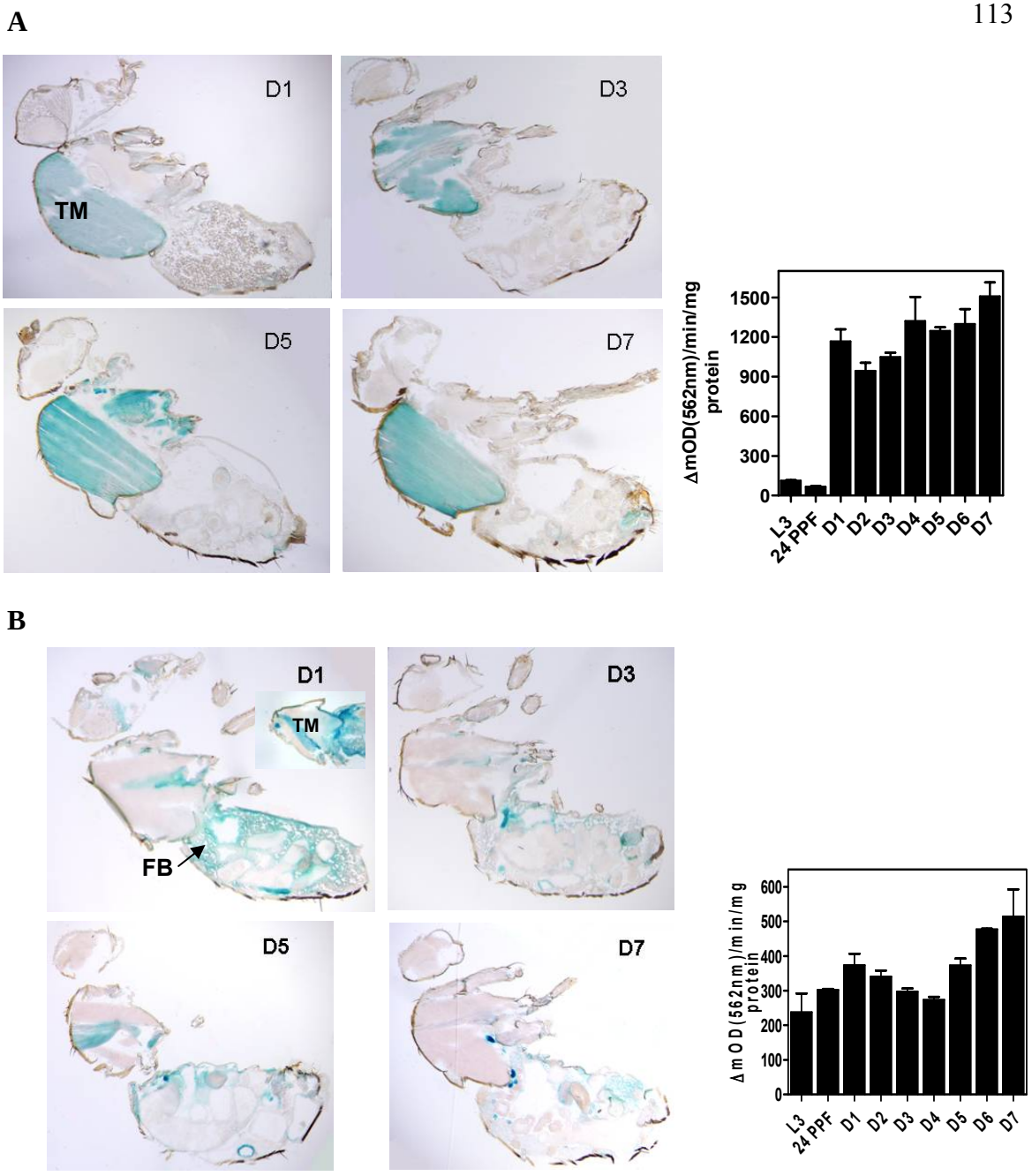


Figure 25. *Mef2-* and *24B-Gal4* expression patterns across age in *Sod2* knock-down flies. Longitudinal sections through the whole body of males were taken at the adult ages indicated as described in materials and methods. *Mef2Gal4* expressed strongly in the thoracic muscle (TM) and to a lesser extent in other muscle tissue throughout adulthood. *24BGal4* expression was found in throughout the adult muscle and fat body (FB). *24BGal4* expression was much weaker overall at all ages tested as confirmed by quantitative assessment across age. Stronger *24BGal4* staining in the TM was obtained upon inbubating sections in X-gal substrate for longer than the normal protocol used (D1 panel inset).

Comparison of phenotypes caused by tissue-specific knock-down to those caused by whole-body knock-down are informative in identifying key tissues susceptible to *Sod2* knock-down. In both ubiquitous and muscle-specific *Sod2* knock-down lines, locomotor dysfunction occurs progressively over comparable periods. Likewise, the extent of life span reduction is similar following ubiquitous or muscle-specific *Sod2* knock-down. These comparisons strongly suggest that muscle is a key tissue underlying the devastating phenotype observed upon ubiquituos *Sod2* knock-down. Interestingly, visual inspection of flies with ubiquitous or muscle-specific *Sod2* knock-down reveals a clear pattern in which flies progressively lose all locomotor function resulting in an apparent paralysis that closely preceeds death.

3.4 Loss of SOD2 in cardiac tissue causes moderate effects on locomotor decline and survival

One muscle type critical for verterbrate survival is cardiac muscle which also functions in flies to circulate blood throughout the circulatory system. One feature of *Sod2* null mice thought to be causal in their neonatal lethality is a dilated cardiomyopathy along with a whole spectrum of degenerative changes to the myocardium (Li et al., 1995). To test the possibility that loss of SOD2 in cardiac muscle might underlie the locomotor and mortality phenotypes accompanying *Sod2* knock-down, flies with cardiac-specific silencing of *Sod2* were generated and assessed for negative geotaxis behavior across age and survival. Age-associated negative geotaxis declines were significantly accelerated by loss of cardiac SOD2 (Fig. 26). This decline, however, was much less rapid than that

observed with pan-muscle *Sod2* knock-down. Similarly, cardiac-specific *Sod2* knock-down resulted in a modest decrease in life span (10-15%) that did not match the severe mortality effects seen in pan-muscle lines (Fig. 24). These data indicate that loss of SOD2 activity in cardiac muscle may only partially contribute to the phenotypes observed upon *Sod2* knock-down throughout the musculature.

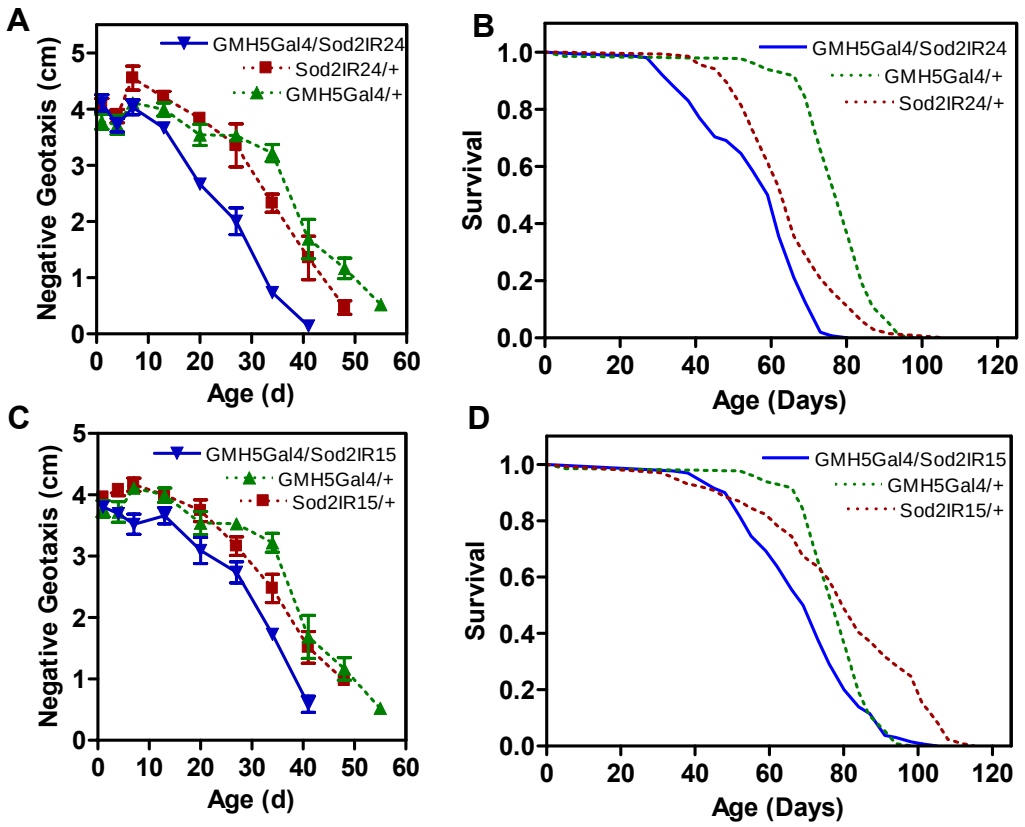


Figure 26. Cardiac *Sod2* knock-down effect on locomotor senescence and survival. There were significant effects of age and genotype on negative geotaxis behavior (A,C) in cardiac-specific Sod2IR24 and Sod2IR15 lines (individual two-way ANOVAs, $p<0.0001$, n =5) and Tukey's HSD test revealed that these lines were both significantly different to each of their control groups ($p<0.05$). Loss of cardiac SOD2 resulted in reductions in life span (B,D). Mean and median life spans (days) were as follows: *Sod2IR24/+* (mean = 65, median =66), *Sod2IR15/+* (mean =79, median =80), *GMH5Gal4/+* (mean =78, median =80), *GMHGal4/Sod2IR24* (mean =56, median =59), *GMH5Gal4/Sod2IR15* (mean =69, median =73). Negative geotaxis data are mean ± S.E.M. All data sets are representative of two independent experiments.

3.5 Sod2 overexpression in the musculature does not attenuate age-related locomotor decline or extend life span

The effects of silencing muscle *Sod2* on function and survival illustrates the degree of toxic ROS generation in energetically demanding tissues such as muscle and the critical protective role played by antioxidants in these tissues. Normal levels of muscle SOD2, however, still permit the age-dependent accumulation of oxidative damage thought to underlie locomotor senescence (Das et al., 2001). One question that arises from these observations is whether normal levels of muscle SOD2 are limiting for locomotor function span. To address this, we tested the hypothesis that adding extra SOD2 to muscle by expression of a *UAS-Sod2* transgene may provide greater protection against the accrual of oxidative damage and thereby ameliorate locomotor declines seen in normal aging. Additionally, since oxidative damage, locomotor senescence and life span determination have been linked in a number of studies (Arking and Wells, 1990; Ruan et al., 2002; Ku et al., 1993; Barja, 1998, Sohal et al., 1995; Barja and Herrero, 2000), mitigating age-associated locomotor decline could in turn lead to an extension in life span. Accordingly, negative geotaxis and survival studies were carried out in flies overexpressing *Sod2*. Interestingly, muscle-specific *UAS-Sod2* overexpression did not benefit locomotor function decline or survival (Fig. 27). In fact, *Mef2-Gal4*-driven *Sod2* overexpression (the stronger of the two *Gal4* drivers) resulted in significantly accelerated locomotor decline (Fig. 27C) and a slight decrease in the pre-mortality phase of population survival (Fig. 27D). This data taken together with the *Sod2* knock-down results suggest that the normal levels of SOD2 activity native to *Drosophila* muscle are

in abundance and that manipulating these levels in either direction can lead to negative consequences on fly locomotor senescence and survival.

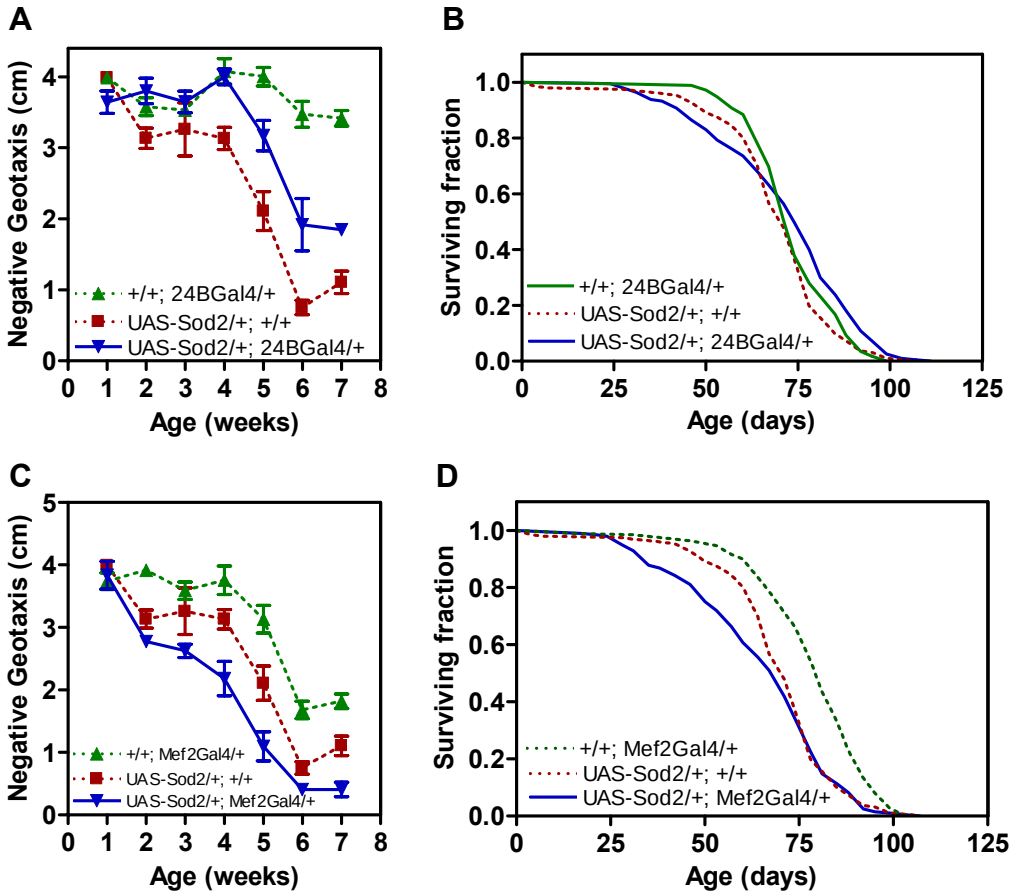


Figure 27. Locomotor senescence and survival in muscle-specific *Sod2* overexpressing flies. Negative geotaxis behavior (A,C) was significantly affected by age and genotype (individual two-way ANOVAs, $p<0.0001$, $n=5$). Tukey's HSD post-test revealed that in (A), *24BGal4*-driven *Sod2* overexpressing flies performed statistically worse than *+/+; 24BGal4/+* controls ($p<0.05$) and in (B) that *Mef2Gal4*-driven *Sod2* overexpressing flies performed significantly worse than both control groups over age ($p<0.05$). There was no substantive effect on life span when *Sod2* was overexpressed via either *Gal4* driver. Negative geotaxis data are mean ± S.E.M. All data sets are representative of two independent experiments.

3.6 Loss of muscle SOD2 leads to impaired ATP production and mitochondrial pathology

To gain insight into the mechanism driving the loss of locomotor function and mortality in flies with loss of muscle SOD2 activity, several molecular-cellular studies were carried out. Oxidative damage-mediated impairment in the activity of mitochondrial macromolecules important for energy metabolism has been reported by us (Paul et al., 2007) and other investigators (Kirby et al., 2002; Melov et al., 1999) following reduced *Sod2* expression. The effects of muscle-specific *Sod2* knock-down on energy production in fly muscle were examined by measuring thoracic ATP levels. There was no significant change in thoracic ATP levels in 1-day-old pan-muscle Sod2IR24 flies (Fig. 28) consistent with the normal locomotor performance in flies at this age.

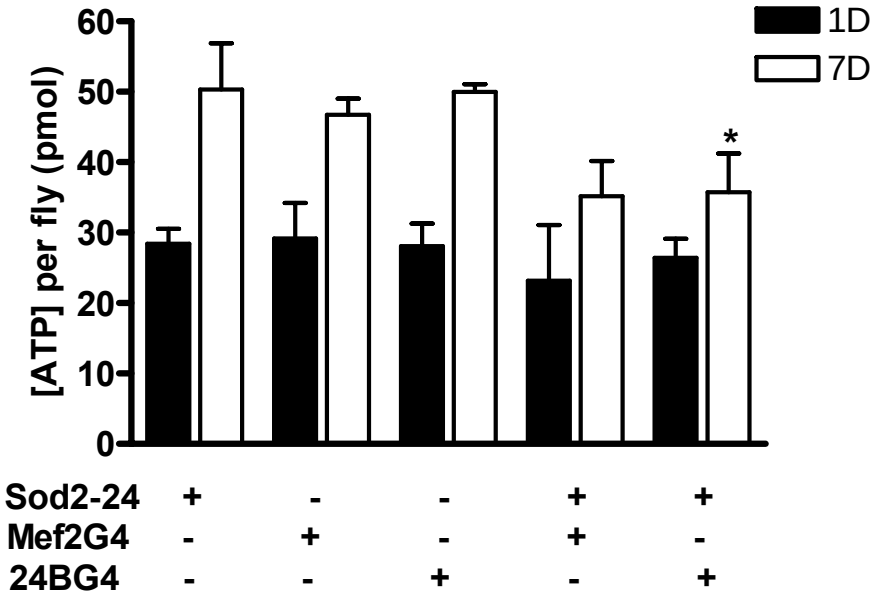
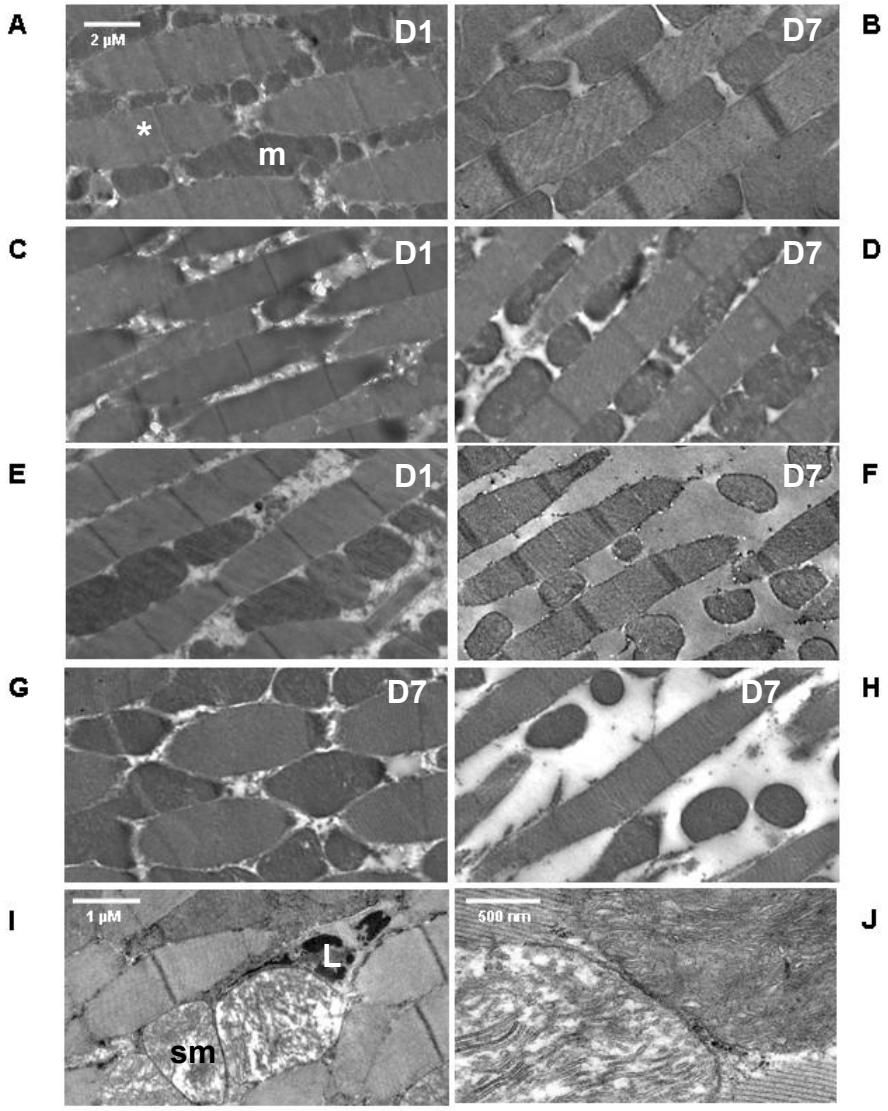


Figure 28. Thoracic ATP levels following muscle-specific *Sod2* knock-down. ATP levels were measured from thoracic extracts at the indicated ages as described in materials and methods. Comparison of *Mef2*- or *24B-Gal4*-driven Sod2IR24 lines with the respective controls indicated no effect of genotype in 1-day-old flies (individual ANOVAs, n =3) but significant effects for both Sod2IR24 lines at 7-days of adulthood (individual ANOVAs, $p<0.02$, n =3). Tukey's HSD test revealed that 7-day-old *Sod2IR24/+; 24BGal4/+* flies had significantly lower ATP levels than both of its control groups, whereas *Sod2IR24/+; Mef2Gal4/+* had significantly lower ATP than *Sod2IR24/+; +/+* controls but not *+/+; Mef2Gal4/+* controls. The age-related increase in ATP levels was also observed in whole-body extracts. Data are mean ± S.D., n=3.

ATP levels increased in the first week of adulthood (Fig. 28) as previously reported in *Drosophila* (Vernace et al., 2007) and other organisms (Dillin et al., 2002; Drew et al., 2003). By 7-days of age, however, there was a 25-30% reduction in thoracic ATP levels in *Sod2* knock-down flies relative to controls indicating that energy production was impaired at this age. Since muscle contraction requires a constant and abundant supply of ATP generated by mitochondrial oxidative phosphorylation, it is concievable that reductions in ATP levels resulting from mitochondrial oxidative damage could contribute to impaired muscle function in flies with loss of muscle SOD2 activity. To investigate the consequences of *Sod2* silencing on sub-cellular integrity, thoracic indirect flight muscle (the largest muscle in *Drosophila*) was assessed by electron microscopy. On the whole, muscle cell ultrastructure was normal relative to controls in 1-day-old flies, with regular arrangement of myofibrils interspersed by rows of densely-packed mitochondria (Fig. 29A,C and E). In contrast with control myocytes, however, there were focal regions across the cell containing clusters of swollen mitochondria (Fig. 29I).

Figure 29 (overleaf). Thoracic mitochondrial content and total protein levels. Transmission electron micrographs show longitudinal or oblique sections through the indirect flight muscle, in which interdigitating rows of myofibrils (*) and mitochondria (m) can be seen. There were focal areas of swollen mitochondria (sm) in 1-day-old *Sod2* knock-down flies (high magnification images I and J) often associated with lysosomes (L) and a widespread reduction in mitochondrial content by day 7 (F and H) which was confirmed by quantifying mitochondrial content in representative images (K). There were significant effects of age and genotype on both mitochondrial content (two-way ANOVA, $p<0.003$, $n=4$-8) and total protein levels (L) (two-way ANOVA, $p<0.0005$, $n=3$). Bonferroni's post-test indicated significant differences between 1- and 7-day-old *Sod2* knock-down lines ($p<0.05$) for both measures whereas controls were unchanged. Genotypes are as follows: A, I and J, *+/+; 24BGal4/+* (1d), B, *+/+; 24BGal4/+* (7d), C, *Sod2IR24/+; +/+* (1d), D, *Sod2IR24/+; +/+* (7d), E, *Sod2IR24/+; 24BGal4/+* (1d), F, *Sod2IR24/+; 24BGal4/+* (7d), G, *+/+; Mef2Gal4/+* (7d), H, *Sod2IR24/+; Mef2Gal4/+* (7d). Mitochondrial content and protein level data are mean ± S.D.


A
2 μM
D1
*
m
B
D7
C
D1
D
D7
E
D1
F
D7
G
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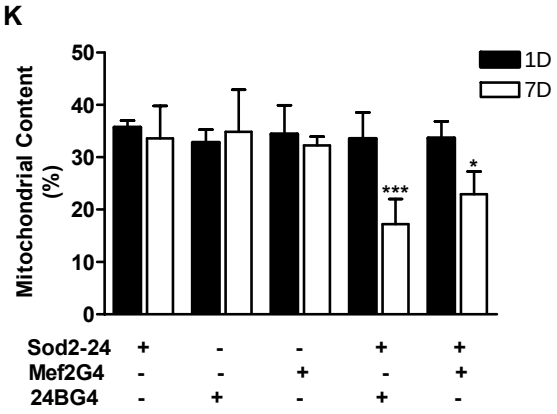
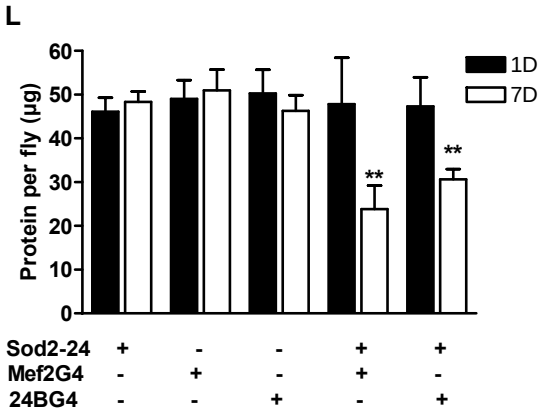
H
D7
I
1 μM
L
sm
J
500 nm


K
Mitochondrial Content (%)
50
40
30
20
10
0
1D
7D
***
*
Sod2-24 + - - + +
Mef2G4 - - + - +
24BG4 - + - + -


L
Protein per fly (μg)
60
50
40
30
20
10
0
1D
7D
**
**
Sod2-24 + - - + +
Mef2G4 - + - + -
24BG4 - - + - +

The matrix of these swollen mitochondria exhibited a rarified cristal arrangement that often appeared fragmented and in a state of degeneration (Fig. 29J). Interestingly, a number of these mitochondria were engulfed in autophagolysosomes, indicating that they were damaged and undergoing removal by the cell. Visual assessment of muscle ultrastructure in 7-day-old *Sod2* knock-down flies revealed a normal arrangement of myofibrils but a striking paucity of mitochondria (Fig. 29F and H). Quantitative assessment confirmed that mitochondrial content (percent area of assessment field occupied by mitochondria) was significantly reduced by 32-49% in 7-day old *Sod2* knock-down flies relative to 1-day-old adults and unchanged in controls (Fig. 29K). Calculating mitochondrial density from micrograph images was made impossible by an inability to distinguish and count individual mitochondria in cells from all young animals and in 7-day-old controls. Collectively, these data suggest a progressive pathology in which oxidatively-damaged mitochondria undergo osmotic swelling (a hallmark of mitochondrial permeability transition) and then are removed at a rate that surpasses mitochondrial biogenesis, leading to an overall attrition of mitochondria. Interestingly, the reduced mitochondrial content observed in 7-day-old thoracic muscle corresponded to an equivalent (35-50%) drop in total thoracic protein at this age (Fig. 29L), highlighting the muscle atrophy experienced due to loss SOD2.

3.7 Muscle-specific silencing of Sod2 causes apoptotic cell death

Partial SOD2 deficiency in *Sod2*$^{+/-}$ mice was shown to result in elevated induction of stress-induced apoptosis in old animals (Van Remmen et al., 2001). It seemed likely that

the oxidative damage, reduced ATP levels and mitochondrial pathology displayed upon eliminating SOD2 in *Drosophila* would have consequences on fly muscle cell viability. To determine whether these events ultimately lead to cell death, caspase activity was measured as a marker of apoptotic cell death. Thoracic caspase activity was significantly elevated in 1-day-old *Sod2* knock-down flies and continued to increase between 1 and 4 days of adulthood while controls remained essentially unchanged across this period (Fig. 30A and B). In the abdomen, 1-day-old *Sod2* knock-down flies exhibited normal caspase activity which showed a marked increase over the first 4 days of adulthood (Fig. 30C and D). The earlier effects on cell death levels in the thorax may be due to the fact that Mef2Gal4 expresses stronger in the thorax than abdomen (Fig. 25). The elevated thoracic cell death apparent even in 1-day-old *Sod2* knock-down flies suggests that the subtle morphological changes evident in mitochondria and normal ATP levels observed at this age may mask the extent of cellular damage already caused by mitochondrial oxidative stress.

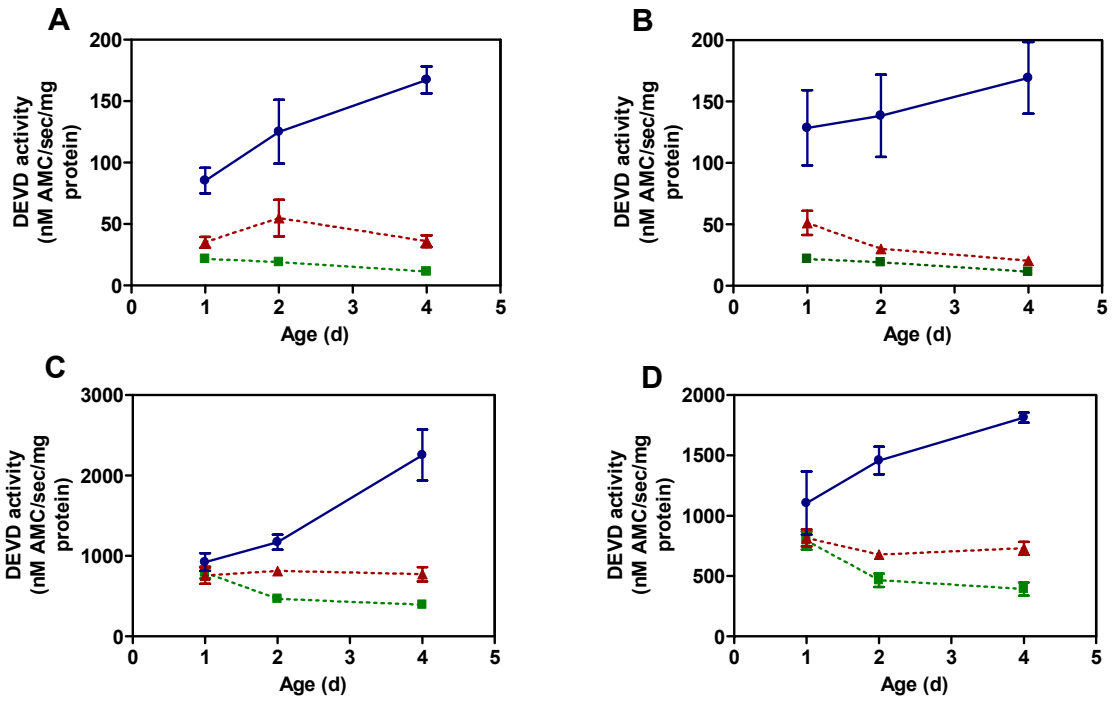


Figure 30. Caspase activity in muscle-specific *Sod2* knock-down flies. Caspase-mediated Ac-DEVD-CHO hydrolysis was monitored in thoraces (A and B) and abdomens (C and D) to determine caspase activity at the ages indicated. *Mef2Gal4* was used to drive *Sod2* knock-down in Sod2IR24 (A and C) and Sod2IR15 (B and D) lines (●). There was a significant effect of genotype on caspase activity in the thorax and abdomen of both Sod2IR24 and Sod2IR15 lines compared to controls carrying non-activated *Sod2IR* (▲) or *Mef2Gal4* (■) transgenes alone (two-way ANOVA, $p<0.0001$, $n=4$). Bonferroni's post-tests revealed that caspase activity was significantly elevated in the thorax of *Sod2* knock-down flies at all ages assessed ($p<0.05$), whereas in the abdomen, significant differences in both Sod2IR24 and Sod2IR15 lines were seen only in 4-day-old flies. Experiments were performed in Laurent Seroude's lab.

4. Discussion

Taken together, these data illustrate the importance of SOD2 in protecting *Drosophila* muscle against oxidative damage. *Sod2* knock-down throughout the fly musculature resulted in a progressive degenerative cellular and locomotor phenotype which culminated in death. The data suggest a model (Fig. 31) in which oxidative damage results in mitochondrial pathology, manifested initially as abnormal mitochondrial morphology and subsequently as a striking reduction in muscle mitochondrial content. Mitochondrial dysfunction was seen to cause a substantial deficit in ATP levels which likely contributed to the progressive cell death and locomotor dysfunction which culminated in organism death.

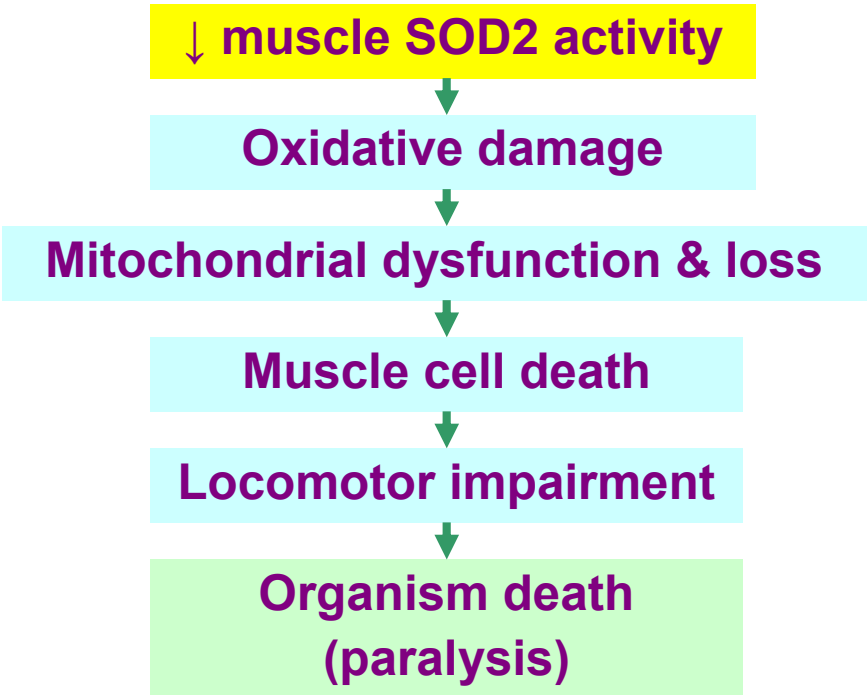


Figure 31. Schematic representation of a model linking loss of muscle SOD2 to fly death. See text for details.

It is not surprising that muscle is particularly prone to oxidative damage in the absence of SOD2 due to its high rate of ROS generation. The severe consequences incurred by reducing muscle SOD2, however, reveal the devastating nature of oxidative damage to this tissue on *Drosophila* function and survival. This strong association raised the possibility that normal muscle SOD2 levels might be limiting on ordinary functional status and survival. This hypothesis was tested by targeting *Sod2* overexpression to muscle, which did not provide any benefitial effect on age-related functional impairment or survival (Fig. 27). Hence, it is likely that endogenous SOD2 is present at optimal levels in *Drosophila* muscle. The life span-extending effect of ubiquitous *Sod2* overexpression (Sun et al., 2002), is therefore not likely due to increased SOD2 levels in muscle. Another question asked was whether loss of SOD2 in cardiac muscle was responsible for the phenotype observed in flies with pan-muscle Sod2 knock-down. This query was based on evidence that Sod2 null mice exhibit substantial cardiomyopathy which was thought to be instrumental in the death of these animals. Consistent with this observation, selective *Sod2* knock-down in cardiac tissue accelerated age-related functional decline and shortened life span (Fig. 26). The extent of these changes, however, were not comparable to those observed through pan-muscle *Sod2* silencing suggesting that loss of cardiac muscle SOD2 was not primarily responsible for the pan-muscle phenotype.

Interestingly, the changes in mitochondrial morphology and content observed here were not found in *Sod2* null mice which were reported to have normal skeletal muscle

ultrastructure (Li et al., 1995). This dissimilarity suggests that although loss of SOD2 devastates function and survival in both species, there may be a partial separation in the organ systems failing in each case. The cause of this difference is unknown, but could include differences in levels of ROS generation or compensatory antioxidant function in the muscles of these species.

The almost complete lack of effect that neuronal *Sod2* RNAi had on SOD2 activity, negative geotaxis or survival in Sod2IR15 lines and lack of clear SOD2 activity reductions in Sod2IR24 lines tested could be due to several explanations. To probe the possibility that *Sod2* gene silencing was dysfunctional in the nervous system, SOD2 activity was measured in the heads of flies with ubiquitous *Sod2* knock-down. Assuming that nervous system SOD2 activity comprises a major portion of total head SOD2 activity, any remaining SOD2 activity would mostly be attributable to the nervous system. The results showed that SOD2 activity was reduced by 82-84% in heads of flies with ubiquitous *Sod2* knock-down (Fig. 32). Since RNAi results in incomplete gene silencing (Matzke et al., 2001), however, it was not possible to know whether the remaining SOD2 activity was primarily located in the nervous system or just the result of incomplete gene silencing throughout the head.

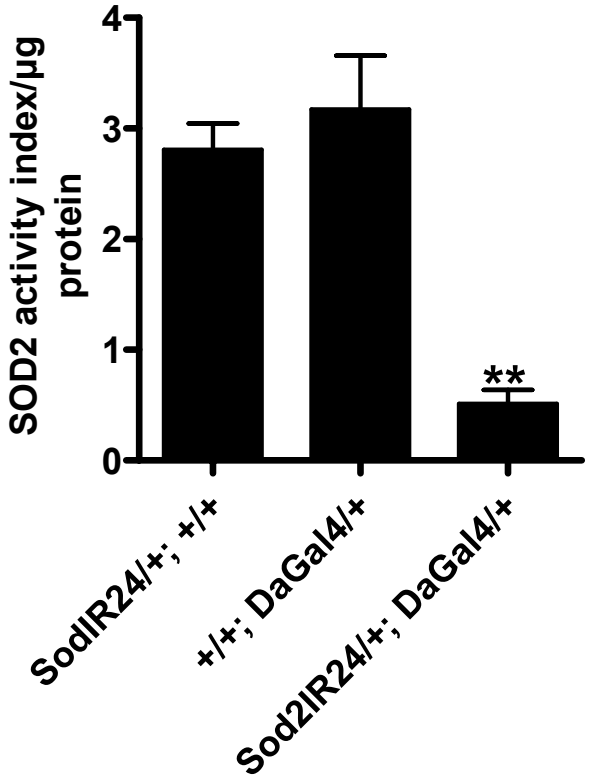


Figure 32. SOD2 activity in fly heads following ubiquitous Sod2 knock-down. There was a significant reduction in SOD2 activity compared to both control groups (ANOVA, $p=0.0022$, $n=3$, followed by Tukey's HSD post-test, $p<0.05$).

The efficacy of RNAi function in the *Drosophila* nervous system has previously been demonstrated (Bhandari et al., 2006), and we are unaware of any mechanisms that might act to suppress *Sod2* knock-down specifically in the nervous system. Nonetheless, this remains a possibility and if true, would preclude any accurate interpretation of the behavioral and life span data obtained. Another possibility is that the nervous system may simply contain low levels of SOD2 activity under normal conditions. In this scenario, changes in neuronal SOD2 activity may be obscured in measurements of total head SOD2 activity if it comprises only a minor portion of total head SOD2 activity. There are no previous reports describing the relative SOD2 activity levels in the nervous system and other tissues of *Drosophila* although levels of SOD1 were reported to be barely detectable in nervous tissue (Klichko et al., 1999). A lack of neuronal SOD2 is hard to reconcile with the high energy requirements of this tissue that would presumably necessitate a large mitochondrial content. Due to practical considerations, isolating the nervous system to measure SOD2 acitivty levels under normal and *Sod2* RNAi conditions was beyond the scope of this investigation. Since our attempts to identify key tissues involved in the dramatic phenotype resulting from whole-body *Sod2* knock-down depend on successful knock-down in each tissue of interest, we are currently unable to determine the contribution of nervous system *Sod2* knock-down to this phenotype.

The *Sod2* RNAi model presented here and elsewhere (Kirby et al., 2002) is now a well-characterized model for the effects of elevated endogenous mitochondrial oxidative damage on mitochondrial integrity, cell death, organism function and survival in flies.

One use of this model currently underway in our lab is in the search for suppressors of oxidative damage and its sequelae in *Sod2* knock-down flies. One part of this approach involves overexpressing a number of candidate suppressor transgenes in *Sod2* knock-down flies and screening for manipulations that result in a rescue of life span. The candidate suppressors have been selected based on their potential protective effects against oxidative damage or its downstream effects, and include genes that encode antioxidants, heat shock proteins and apoptosis inhibitors. A second approach involves the administration of antioxidant compounds (by food supplementation) to *Sod2* knock-down flies and again screening for compounds that have a rescue effect on life span. A robust rescue of life span in any of these studies would indicate that some of the deleterious effects of silencing *Sod2* at the molecular-cellular level have been mitigated and would warrant further investigation into which features of the *Sod2* knock-down phenotype have been affected. These studies will hopefully shed light on successful approaches to suppress mitochondrial oxidative damage and if so may ultimately hold promise in the treatment of diseases in which pathogenesis is known to involve elevated mitochondrial oxidative damage.

Research Chapter 4.

Characterization of Functional Senescence Data Sets

Part (i) A Proposed Set of Descriptors

1. Introduction

Declines in biological function are common manifestations of aging in many phyla (Arking, 1998). As functional senescence is thought to drive the increasing risk of death with age, understanding functional senescence is important for understanding aging. Experimental investigation of functional senescence requires one to quantitate and compare age-dependent declines in function between cohorts. Such quantitation and comparison is often difficult owing to complexities in functional senescence data sets. Here, I discuss issues related to describing and contrasting age-related declines in function. Functional senescence data were parameterized in simple ways to generate descriptors for (1) the rate of functional decline, (2) the time to onset of functional decline and (3) total function. To illustrate how these descriptors can be used, a hypothetical data set and one of our previously published data sets (Goddeeris et al., 2003) were analyzed. We found that no one descriptor alone sufficiently characterizes functional senescence. Useful distinctions between functional senescence in different cohorts can be made, however, when multiple descriptors are used in an integrated fashion.

2. Results and discussion

Four conceptual data sets are presented to illustrate some of the complexities in interpreting functional senescence data (Fig. 33A).

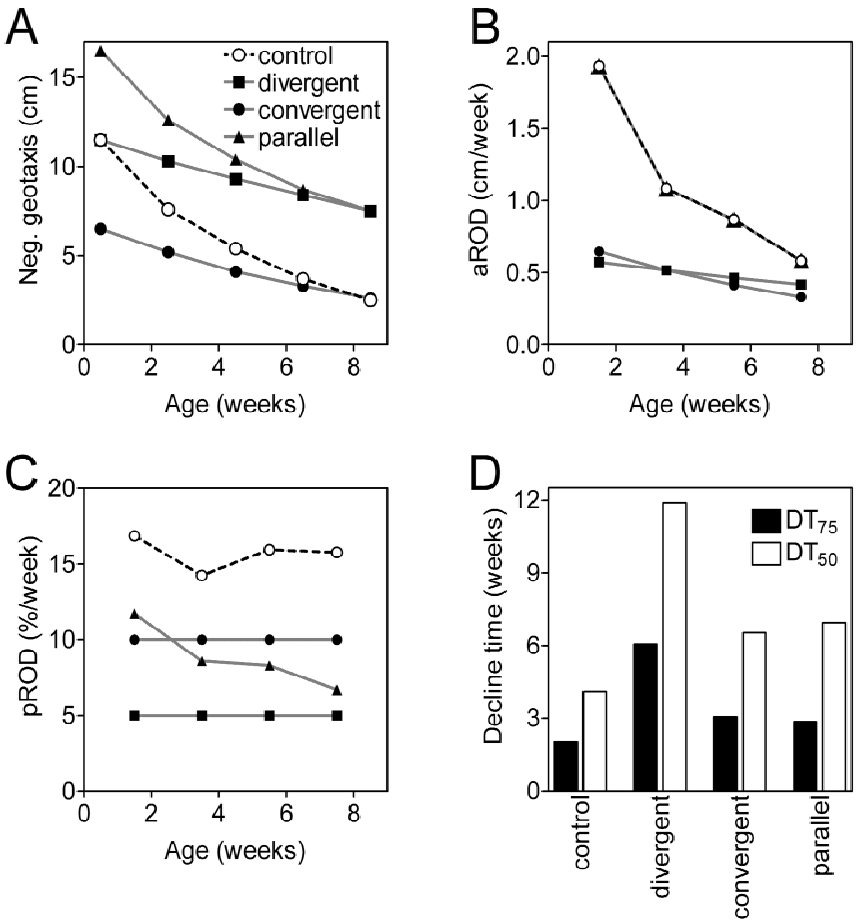


Figure 33. Descriptors for age-related declines in function. (A) Control data are from 3 experiments with *w[cs]* flies (Goddeeris et al. 2003). Divergent data had the same peak value as control, but declined 10% during each interval. The convergent data had a peak value 5 cm less than control that declined 20% during each interval. The parallel data were 5 cm greater than control at all assessments. Symbols in B and C are the same as in A. Theoretical data were derived through simple mathematical operations and are not related to biological manipulations. (B) aROD, (C) pROD and (D) Decline-times from panel A.

The control data are derived from previously published studies from this lab on senescence of negative geotaxis in wild-type flies (Goddeeris et al., 2003). The divergent, convergent and parallel data sets are hypothetical; they were designed to represent three major types of actual results that might be consistent with ameliorated functional senescence as compared with the control (Fig. 33A). The divergent group had the same peak function as control, but declined more slowly. The convergent group had a lower peak value than control, but declined so that its function at the last assessment was indistinguishable from control. The parallel group had elevated negative geotaxis scores at all ages so that its curve was parallel to control. Although statistical tests such as analysis of variance (ANOVA) can be used to compare overall function across age in data sets like these (e.g Goddeeris et al., 2003), this approach leaves several questions unanswered, including: (1) Does the rate of functional decline differ between the data sets? (2) Is the time to onset of functional decline changed? (3) Is total function altered? Several summary statistics (descriptors) were calculated from each of these hypothetical data sets toward addressing these questions.

First, we considered descriptors for the rate of functional decline. As a starting point, we calculated the absolute rate of decline (aROD, negative value of slope) from the hypothetical data (Fig. 33A). This descriptor depicts the absolute change in function per unit time determined from each assessment interval. Following the assumptions in our example data sets, the aRODs were lower overall for the divergent and convergent data sets relative to control, whereas this descriptor was identical in the parallel and control

data (Fig. 33B). We also determined the proportional rate of decline (pROD) on the example data (Fig. 33A) using the formula $((F_o-F_i)/F_o)\times100\%$ where F_o is the functional value at the beginning of each interval and F_i is the value at the end of each interval. This descriptor represents the proportion of a function that is lost during each interval. As designed, pROD was constant in the divergent and convergent sets with the divergent set being lower overall (Fig. 33C). Additionally, pROD was lower in the parallel set than in control (Fig. 33C).

aROD and pROD naturally have different constraints based on their mathematical definitions. The maximum potential value of aROD is directly proportional to the magnitude of the function being investigated. Thus, large values for this descriptor might be artifactual in cohorts with peak function greater than control and small values might be artifactual in cohorts with peak function lower than control. From the perspective that a reduction in aROD could be interpreted as a reduction in the rate of functional senescence, this descriptor is conservative forgroups with peak function equal to or greater than control and less conservative in groups with peak function lower than control. pROD, by contrast, varies inversely with the absolute magnitude of the function. Given groups with similar aRODs, pROD is higher in those with lower function (compare control with parallel and convergent with divergent, Fig. 33C). From the viewpoint that a reduction in pROD could be interpreted as a reduction in the rate of functional senescence, pROD is a conservative descriptor in groups with peak function

similar to or lower than control but is less conservative in groups with peak function greater than control.

Second, we considered a descriptor for the time to onset of functional decline. Arking & Wells (1990) defined a loss-of-function constant (here called decline time, DT) as the time required for function to decline to 50% of its peak value. To explore the usefulness of this descriptor further, we determined the DT_{75} and DT_{50} (time required for function to decline to 75% and 50% of its peak value, respectively) on the data in Fig. 33(A). Values for DT_{75} and DT_{50} were interpolated from second-order polynomial curve fits (the least complicated curve that fits all the data). As expected, both DT_{75} and DT_{50} were increased for the divergent set relative to control (Fig. 33D). These two measures were also increased in the convergent and parallel sets, although not as robustly as in the divergent set (Fig. 33D). Groups with increased DT_{50} and DT_{75} as in the divergent, convergent and parallel sets would be good candidates for having extended periods during which function remains high relative to peak function for each cohort.

Finally, we considered a descriptor for total function throughout an experiment. As expected, total function (calculated as the area under the curve) was decreased (28%) in the convergent group and increased in the divergent (58%) and parallel(84%) groups relative to control. Groups such as the divergent and parallel sets could be interpreted to have increased total function, whereas groups such as the convergent set could be interpreted to have decreased total function.

When used together, our descriptors should provide a robust characterization of functional senescence data sets (Table 6). In contrast, comparing functional senescence between cohorts by using any single descriptor in isolation might be misleading because some descriptors might change while others do not and certain descriptors might exhibit contradictory changes.

Table 6. Summary of analyses on theoretical data. Data in Fig. 33 were analyzed as described in the text. Downward arrows indicate reductions in the descriptor; upward arrows indicate increases. Major interpretations are listed for each of the groups.

Group	aROD	pROD	DTs	Total Function	Interpretations
divergent	↓	↓	↑	↑	slows functional senescence and enhances total function
convergent	↓	↓	↑	↓	slows functional senescence at the cost of total function
parallel	unchanged	↓	↑	↑	Hyperfunctional at all assessments, positively impacts most descriptors of function across age

We would be confident that functional senescence has been slowed without obvious trade-offs in cohorts with reduced absolute and proportional rates of decline, extended decline times, and enhanced total function (e.g. divergent set). This is a straightforward example in which all of the descriptors have been enhanced. A more complicated case is the parallel set. In this case, pROD is reduced while aROD remains unchanged. If pROD exclusively were considered, one would conclude that the rate of functional senescence is slowed; if aROD exclusively were considered, the interpretation would be that the rate of functional senescence is unchanged. Neither of these interpretations is adequate because both ignore other information. By viewing all four descriptors together, one could reach a more satisfactory conclusion: although it remains ambiguous whether the rate of decline is altered, the parallel set exhibits extended decline times and enhanced total function that likely stem from an overall elevation in function. Thus, the parallel set would have meaningful positive changes in functional status within the context of aging. A final example is the convergent set. This example has decreased absolute and proportional rates of decline and extended decline times, but reduced total function. Such results would suggest that rate of functional senescence is slowed, but at the cost of reduced total functional capacity. This would indicate that an important trade-off has occurred in this group.

We recently reported that reduced expression of βPS, the *mys* gene product, ameliorates age-dependent senescence of negative geotaxis in *Drosophila* (Goddeeris et al., 2003). Here we report values for each of the descriptors calculated from these data. aROD in

mys^{xG}/+ and *mys*^{nj}/+ flies (Fig. 34C) was reduced relative to control during the first two intervals and converged with control during later intervals. Similarly, pROD was initially reduced in *mys*^{xG}/+ and *mys*^{nj}/+ animals (Fig. 34D), but converged with control at later intervals. The consistent changes in aROD and pROD in all three *mys* hypomorphs suggest that the rate of functional decline is reduced by mutations in *mys*. Additionally, all three *mys* mutants had significantly

increased DT_{75} and DT_{50} values (Fig. 34E) as well as elevated total negative geotaxis (Fig. 34F).

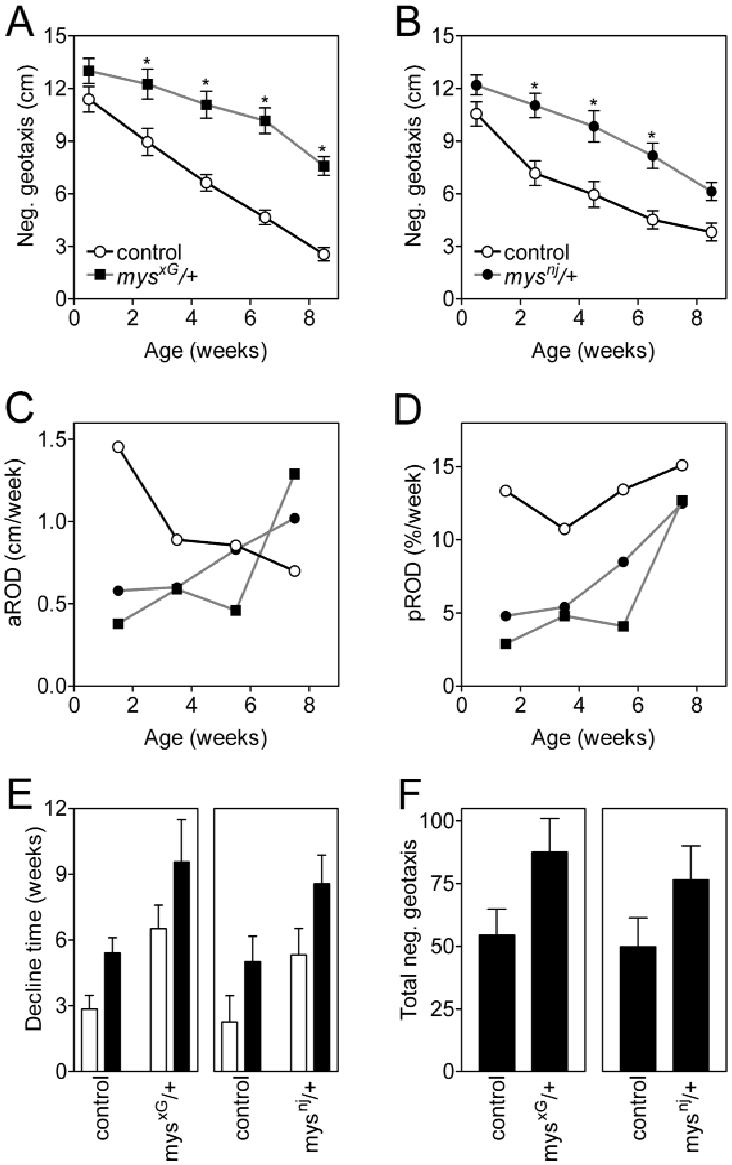


Figure 34. Analysis of negative geotaxis senescence in *mys* mutants. (A and B) Overall, negative geotaxis was greater in mys^{xG}/+ (A) and mys^{nj}/+ (B) mutants than in controls (two-way ANOVAs, P<0.0001). Data (mean ± S.E.M.) are from (Goddeeris et al. 2003). aROD (C) and pROD (D) from the data in panels A and B. Since the experiments in A and B used the same *w[cs]* control strain, the values for these groups were averaged in C and D. Data are individual determinations for the *mys* mutants. S.E.M. (not shown) ranged 0.64-1.15 cm/week for aROD (C) and 8-18% per week for pROD (D). Symbols are the same as in panels A and B. (E) The DT_{75} and DT_{50} are extended in mys^{xG}/+ (left panel) and mys^{nj}/+ (right panel). Data are mean ± 95% C.I.. (F) Total negative geotaxis (area under the curve) was greater in mys^{xG}/+ (left panel) and mys^{nj}/+ (right panel) flies than in controls (resampling analysis (Lunneborg 2000), p<0.0001). Data (mean ± S.E.) are derived from resampling analyses with 10,000 iterations (Rani and Padh 2000).

It is interesting that the beneficial effects of *mys* mutations on the rates of functional senescence appeared during the first few weeks of adult life. Although this change in the rate of functional senescence occurred only for a relatively short time, the resulting favorable effects on negative geotaxis were evident for a considerable time thereafter. Importantly, the *mys* mutants have increased total negative geotaxis, confirming that reduced expression of βPS has positive consequences on total negative geotaxis function during the first eight weeks of life. Together, the reduced rates of functional decline, extended periods of high function and enhanced total function indicate that *mys* mutations confer large beneficial effects on senescence of negative geotaxis. Our proposed descriptors provide a framework to characterize age-related declines in many functions (Arking & Wells, 1990; Le Bourg & Minois, 1999; Cook-Wiens & Grotewiel, 2002). It is possible that a multitude of treatments will change at least one of the descriptors in a positive way. We suggest that manipulations conferring the greatest beneficial effects can be identified when they decrease absolute and proportional rates of functional decline, extend decline times and enhance total function. Such determinations can be made only by evaluating multiple descriptors in an integrated fashion.

Part (ii) Use of Functional Senescence Descriptors to Reveal Distinct Genetic Influences on Locomotor Senescence

1. Introduction

Senescence of physiological function (functional senescence) is believed to drive the decrease in quality of life and the increase in mortality associated with aging (Grotewiel et al., 2005). Accordingly, understanding functional senescence is crucial to understanding the biology of aging. To facilitate the investigation of functional senescence, we previously designed a series of metrical analyses for quantifying and comparing age-associated functional impairments between cohorts (Martin et al., 2005). These metrics describe the rate of functional decline, the age at which function declines to 75%, 50% and 25% of initial performance and total function across the assessment period. In the current study, we used these metrics to rigorously evaluate senescence of negative geotaxis, a locomotor behavior in *Drosophila*. Age-related decline in this behavior is sensitive to genetic background and alleles of chico and Indy that extend life span (Gargano et al., 2005). Here, using a series of detailed metrical analyses, we show (1) that negative geotaxis declines more rapidly in Canton-S and Oregon-R than in Samarkand and Lausanne-S flies and (2) that mutation of *chico* has significantly more pronounced beneficial effects on locomotor senescence than does mutation of *Indy*.

2. Materials and methods

2.1. Fly stocks and husbandry

Canton-S, Oregon-R, Samarkand and Lausanne-S strains flies were used as representative genetic backgrounds. The Canton-S strain was provided by Ron Davis (Baylor College of Medicine, Houston, TX, USA). The Oregon-R, Samarkand and Lausanne-S strains were obtained from the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN, USA). The $Indy^{206}$ and $chico^{1}$ mutants were provided by Stephen Helfand and Marc Tatar (Brown University, Providence, RI, USA). Flies were housed, collected and tested as previously described (Gargano et al., 2005).

2.2. Negative geotaxis

Rapid iterative negative geotaxis (RING) assays were performed as previously described (Gargano et al., 2005). Ten vials initially containing 25 adults each were assessed for each genotype and genetic background in longitudinal studies at weekly intervals. The performance of each vial was treated as a single datum at each age.

2.3. Metrical analyses and statistical tests

Metrical analyses described previously (Martin et al., 2005) were used on previously published senescence of negative geotaxis data sets (Gargano et al., 2005). The absolute rate of decline (aROD) was calculated as the change in negative geotaxis scores between

each assessment interval. The proportional rate of decline (pROD) was calculated as the proportion of a function lost during each interval. Decline time$_{75}$ (DT_{75}), DT_{50} and DT_{25}, representing the time required for function to decline to 75%, 50% and 25% of initial values, respectively, were interpolated or extrapolated from second-order curve fits. Total negative geotaxis during each experiment was determined as the area under the curve. Metrics were calculated from each vial individually. Data are reported as mean ± SEM for the 10 vials constituting each genotype or genetic background. Since the effects of genetic background on senescence of negative geotaxis were similar in males and females and the effects of the *Indy*^{206} and *Indy*^{302} alleles on senescence of negative geotaxis were similar, data from only males in the genetic backgrounds and only the *Indy*^{206} allele are reported here for brevity. Data from females for chico^{1} mutants and controls were analyzed since loss of function in chico has more pronounced effects on aging in this sex (Tu et al., 2002). Parametric tests in Prism (GraphPad Software, San Diego, CA, USA) were used to assess statistical significance ($p < 0.05$).

3. Results

3.1. Effect of genetic background on senescence of locomotor function

Age-dependent loss of negative geotaxis varies with genetic background (Fig. 35A, (Gargano et al., 2005)). Toward a better understanding of how genetic background can impact senescence of negative geotaxis, we performed a series of metrical analyses as previously described (Martin et al., 2005) on these data. To determine whether the pace

of senescence in negative geotaxis was influenced by genetic background, we calculated the absolute rate of decline (aROD) and proportional rate of decline (pROD) (Martin et al., 2005) from the data in Fig. 35A.

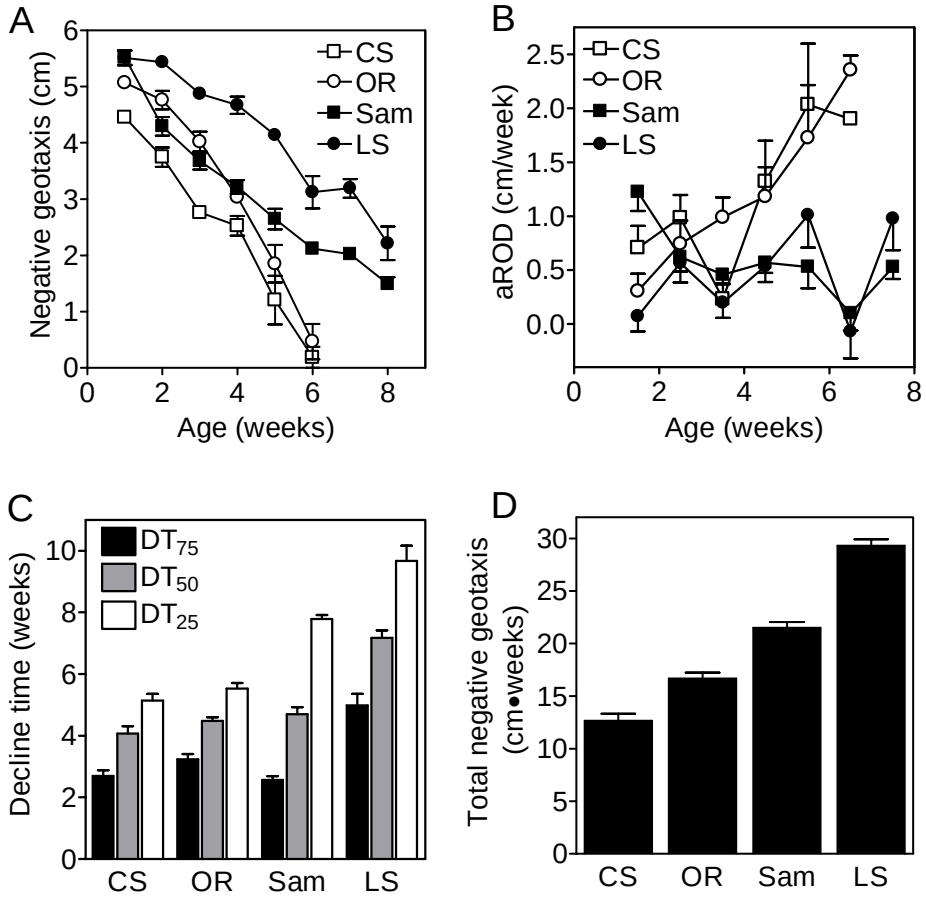


Figure 35. Negative geotaxis senescence in different genetic backgrounds. (A) Negative geotaxis in Canton-S (CS), Oregon-R (OR) Lausanne-S (LS) and Samarkand (Sam) males. There was a significant effect of age and genetic background on negative geotaxis (two-way ANOVA, $p<0.0001$ for both factors) and significant interactions between age and genetic background ($p<0.0001$). Data (mean ± S.E.M.) are taken from Gargano et al. (2005) and are from two experiments with 10 vials of up to 25 flies each. (B) There was a significant effect of age and genetic background as well as significant interactions between age and genetic background on aROD (two-way ANOVA, $p<0.0001$ for all factors). (C) There was a significant effect of genetic background on decline to 75%, 50% and 25% of initial negative geotaxis (individual ANOVAs, $p<0.0001$). Bonferroni's *post-hoc* tests revealed that DT_{75}, DT_{50} and DT_{25} for Lausanne-S were significantly different than all other backgrounds ($p<0.001$) and that DT_{25} for Samarkand was significantly different from that of Oregon-R and Canton-S ($p<0.001$). (D) There was a significant effect of genetic background on total negative geotaxis (ANOVA, $P<0.0001$). Bonferroni's post-hoc tests revealed that total negative geotaxis was different in all genetic backgrounds ($p<0.001$).

Genetic background and age had significant effects on the aROD of negative geotaxis (Fig. 35B). aROD was higher overall in Canton-S and Oregon-R than in Samarkand and Lausanne-S. Statistical interactions between age and genetic background indicate that the effects of age on aROD were not uniform across the different genetic backgrounds tested. Interestingly, Canton-S, Oregon-R and Lausanne-S all exhibited a significant increase in aROD with age whereas Samarkand displayed an age-associated decrease in this measure (Fig. 35B). The age-related changes in aROD in Lausanne-S and Samarkand, however, were quite small. Similarly, the pROD of negative geotaxis was affected by age and genetic background, and was higher overall in Canton-S and Oregon-R than in Samarkand and Lausanne-S (data not shown). These analyses establish that the rate of negative geotaxis senescence was influenced by genetic background.

To quantify the locomotor declines in Fig. 33A further, we derived the DT_{75}, DT_{50} and DT_{25}, which represent the time required for function to decline to 75%, 50% and 25% of initial values, respectively (Martin et al., 2005). Genetic background had significant effects on these measures (Fig. 35C). Specifically, decline times in Lausanne-S were consistently longer than in the other three backgrounds. Interestingly, DT_{25} in Samarkand was longer than in Canton-S and Oregon-R, although DT_{75} and DT_{50} were indistinguishable in these three genetic backgrounds. This suggests that Samarkand males exhibited a pattern of decline in negative geotaxis distinct from Canton-S and Oregon-R. These data demonstrate that the time at which negative geotaxis declined by quartile percent amounts was sensitive to genetic background.

We calculated area under the curve to assess the effect of genetic background on total negative geotaxis throughout our experiments. Total negative geotaxis was different in all four genetic backgrounds (Fig. 35D). Lausanne-S had the highest total negative geotaxis followed in descending order by Samarkand, Oregon-R and Canton-S. These data reveal that total negative geotaxis can vary between different genetic backgrounds by more than twofold. This metric illustrates a marked difference in the total functionality of these four genetic backgrounds.

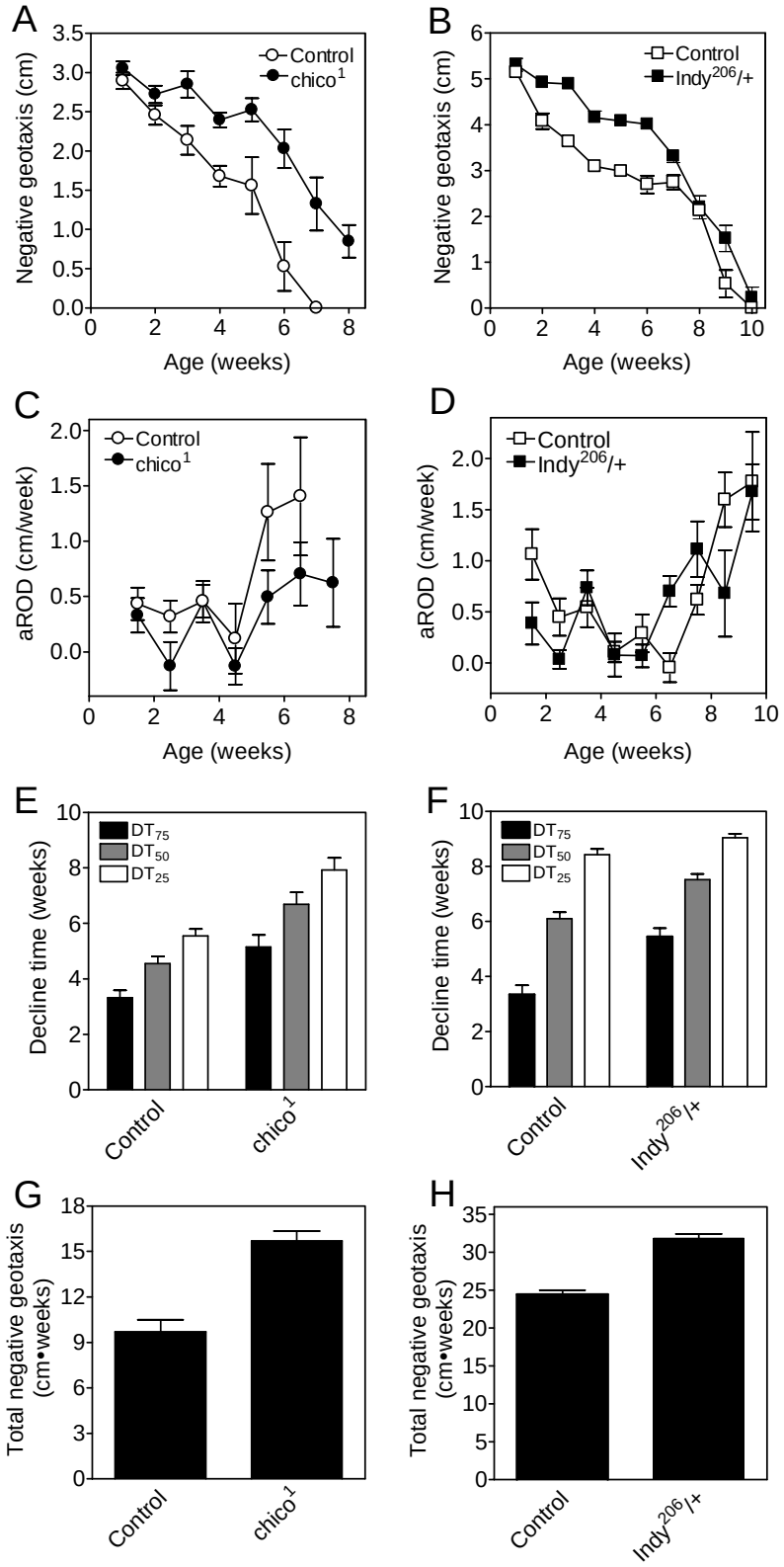
3.2. Effects of chico and Indy mutations on senescence of locomotor function

Mutations in *chico* (Clancy et al., 2001 and Tu et al., 2002) and *Indy* (Rogina et al., 2000) extend life span in *Drosophila*. We previously reported that these mutations also alter senescence of negative geotaxis (36A and B, (Gargano et al., 2005)). To quantify and compare age-related loss of negative geotaxis behavior in $chico^{1}$ homozygous and $Indy^{206}$ heterozygous mutants, we assessed rate of decline, decline times and total negative geotaxis from the data in Figs. 36A and B.

There was a significant effect of age on aROD for both $chico^{1}$ and $Indy^{206}$ mutants and their respective controls (Figs. 36C and D). Interestingly, all strains tested in these studies exhibited a trend towards an increase in aROD in the second half of the assessment period. Overall, $chico^{1}$ mutants had lower aROD values than control flies (Fig. 36C). In contrast, overall aROD values in $Indy^{206}$ mutants were not statistically distinguishable from controls (Fig. 36D). $chico^{1}$ mutants appeared to have reduced aRODs at most

assessments whereas *Indy*^{206} flies did not. This suggests that while the beneficial effects of *chico*^{1} on functional senescence manifested throughout the experiment, for *Indy*^{206}

Figure 36 (overleaf). Negative geotaxis senescence in *chico*^{1} and *Indy*^{206} mutants. RING assays were performed at the indicated ages on *chico*^{1} females (A) and *Indy*^{206} heterozygous males (B). Age and genotype significantly affected negative geotaxis (individual two-way ANOVAs, $p<0.0001$ for both factors). There were also significant interactions between these two variables ($p\leq0.017$ in *chico*^{1} and *Indy*^{206} flies). Data (mean ± S.E.M.) are taken from Gargano et al. (2005). (C) There was a significant effect of age and genotype on aROD (two-way ANOVA, effect of age, $p = 0.0005$, effect of genotype, $p = 0.0121$) for *chico*^{1} mutants. (D) There was an effect of age but no effect of genotype on aROD for *Indy*^{206} mutants (two-way ANOVA, $p<0.0001$). (E) There was a significant effect of genotype on decline to 75%, 50% and 25% of initial function for *chico*^{1} mutants (ANOVA, $p<0.0001$, followed by Bonferroni's *post-hoc* tests, $p\leq0.01$). (F) There was also a significant effect of genotype on decline times for *Indy*^{206} mutants (ANOVA, $p<0.0001$) although only the differences in DT_{75} and DT_{50} reached statistical significance by Bonferroni's *post-hoc* tests ($p<0.001$). Genotype significantly affected total negative geotaxis over the assessment period for *chico*^{1} (G) and *Indy*^{206} (H) mutants (individual student's t-tests, $p<0.0001$).


A
Negative geotaxis (cm)
Age (weeks)
Control
chico1
B
Negative geotaxis (cm)
Age (weeks)
Control
Indy206/+
C
aROD (cm/week)
Age (weeks)
Control
chico1
D
aROD (cm/week)
Age (weeks)
Control
Indy206/+
E
Decline time (weeks)
DT75
DT50
DT25
Control
chico1
F
Decline time (weeks)
DT75
DT50
DT25
Control
Indy206/+
G
Total negative geotaxis (cm•weeks)
Control
chico1
H
Total negative geotaxis (cm•weeks)
Control
Indy206/+

mutants these effects were restricted to the first few weeks of life. The pROD in negative geotaxis in *chico*^{1} and *Indy*^{206} mutants followed a pattern similar to that of aROD; there were significant effects of age and genotype on pROD in *chico*^{1} flies, but in *Indy*^{206} mutants there was a significant effect of age only (data not shown).

All three DTs for *chico*^{1} mutants were significantly increased (Fig. 36E). Hence, a lower rate of functional decline resulted in a delay in the decline time to 75%, 50% and 25% of initial function. This further supports the idea that *chico*^{1} delayed senescence of negative geotaxis throughout the function span. DTs for the *Indy*^{206} allele indicate a significant delay in the DT_{75} and DT_{50} (Fig. 36F). No difference was found, however, for the DT_{25}. This is consistent with the positive effects of *Indy* mutations on senescence of negative geotaxis occurring mainly during the first few weeks of life.

Total negative geotaxis during the experiments in *chico*^{1} mutants was increased by 62% relative to controls (Fig. 36G). Although *chico*^{1} did not bestow any increase in peak locomotor function in young flies (Fig. 36A), the attenuated pattern of senescence in *chico*^{1} animals conferred a substantial increase in their total locomotor functionality. *Indy* mutants also exhibited an increase in total negative geotaxis (Fig. 36H), although the effect was not as large as in *chico*^{1} flies. The larger increase in total negative geotaxis in *chico*^{1} mutants is consistent with the idea that *chico*^{1} loss of function had a greater impact on aging of negative geotaxis throughout the life span than did mutations in *Indy*.

4. Discussion

Although most multicellular species experience some form of functional senescence during the post-reproductive phase of life, the nature of this senescence varies between different species and between individuals of the same species (Arking, 1998). Variability among individuals of the same species depends both on external factors related to the environment and internal factors including genetic composition (Stadtman, 2002, Grotewiel et al., 2005 and Martin and Grotewiel, 2006). We previously reported that senescence of negative geotaxis was sensitive to genetic background and single gene mutations that extend life span in *Drosophila* (Gargano et al., 2005). Here, we applied a series of detailed metrical analyses to these previously published data to better define genetic influences on senescence of behavior.

Our studies demonstrate that many aspects of senescence of negative geotaxis are dependent on genetic background. The rates of decline were different in the four genetic backgrounds as were the age-dependent changes in this metric. Both aROD and pROD increased with age in Canton-S, Oregon-R and Lausanne-S during the first 8 weeks of life. In contrast, Samarkand showed a decrease in aROD with age and no significant change in pROD for the same period. This indicates that altered senescence of negative geotaxis can result from distinct changes in the age-dependent dynamics of aROD and pROD. All three of the DT metrics were longer in Lausanne-S than the other genetic backgrounds, reflecting the lower rate of locomotor decline in Lausanne-S. DT_{25} for

Samarkand was longer than that of both Canton-S and Oregon-R due to the increase in locomotor decline with age in these latter two backgrounds. Finally, total negative geotaxis was different in all four genetic backgrounds and was lowest in Canton-S and Oregon-R, the two strains that exhibited the fastest pace of locomotor decline. Together, these data demonstrate that senescence of negative geotaxis in Canton-S and Oregon-R flies proceeds more rapidly than it does in Lausanne-S and Samarkand flies. These studies illustrate that senescence of negative geotaxis proceeds via patterns of decline that are characteristic of each genetic background, highlighting the need to control for genetic background in behavioral aging studies.

Both *chico*^{1} and *Indy*^{206} mutants have altered senescence of locomotor function (Gargano et al., 2005). Detailed analysis of these age-related declines using our series of metrics revealed subtle, but important, differences in the way functional senescence is modified in these two mutants. The rates of decline for *chico*^{1} mutants were reduced throughout most of the assessment period. This resulted in a significant increase in all decline time measures in addition to a large increase in total negative geotaxis function in *chico*^{1} flies. For *Indy*^{206} mutants, reductions in the rate of decline were rather marginal overall and restricted to the early stages of senescence. Interestingly, early reductions in decline rates appeared to be followed by increases at greater ages. Nevertheless a small initial deceleration in the rate of decline in *Indy*^{206} flies manifested as extensions in DT_{75} and DT_{50} in addition to increased total negative geotaxis activity. Since mutation of chico had greater effects than mutation of *Indy* on the rates of functional decline, the time required

for function to decline and total function, our analyses indicate that the *chico*^{1} mutation exerts a larger benefit on the biological systems that support locomotor function throughout the *Drosophila* life span.

The aROD, decline time and total function descriptors we applied here to analyze senescence of negative geotaxis should be useful for characterizing age-related decline in many other functions across age (Martin et al., 2005). When function is highest in the youngest groups and declines with age (as we typically find for negative geotaxis), the use of the descriptors is fairly straightforward. Although, we have not extensively investigated the use of these descriptors on data sets in which peak function initially increases with age and then declines (e.g., female fecundity in various species (Novoseltsev et al., 2003)), we predict that they will provide important insights even in these somewhat more complicated scenarios when used in an integrated fashion. It seems likely, however, that additional descriptors will be required to provide a comprehensive analysis of more complex data sets. For example, a measure of time to peak function might be informative in such cases.

Discussion

Over the last half century, numerous theories have been put forth that attempt to explain why we age and how this process occurs (Kirkwood and Austad, 2000). This growth in attention toward aging research has been driven principally by the substantial lengthening of human life span which has led to a growing percentage of elderly in the human population and a growing burden on national health expenditures toward the elderly (Weinert and Timiras, 2003). Theories exploring the reasons of why organisms age are mainly based on evolutionary concepts that after reaching reproductive fitness, there is a decline in force of natural selection. If evolutionary theories hold true, then longevity would only be selected for if it improved reproductive fitness. Although theoretically this idea is feasible, in reality most animals including humans die via extrinsic causes of mortality (due to predation or diseases) before aging can occur (Kirkwood and Austad, 2000). Consequently, organisms that die from extrinsic causes of mortality will evolve a life span that optimizes reproductive success in their own environment. Imposing artificial selection by selecting *Drosophila* flies that are the offspring of older adults results in the generation of long-lived fly strains (Partridge et al., 1999). This did, however, result in reduced fecundity in the long-lived flies supporting the idea that a trade-off between survival and fertility occurred (Partridge et al., 1999). This has led to the notion that longevity requires somatic maintenance that diverts resources away from reproductive fitness. Since longevity is prevented in most natural environments by

extrinsic mortality as mentioned above, it is likely that this loss of reproductive fitness would be selected against. This is the central tenet behind the 'disposable soma' theory which proposes that somatic function is maintained at high levels only for reproductive success and that afterwards it is disposable (Weinert and Timiras, 2003). A similar hypothesis based on the idea of optimization of reproductive fitness underlies the 'antagonistic pleiotropy' theory which posits that pleiotropic genes with beneficial effects in younger animals up to reproductive age would be favorable for selection even if negative effects of these genes occurred at more advances ages (Kirkwood and Austad, 2000).

While evolutionary theories attempt to explain why we age, mechanistic insight into the aging process has been provided by numerous molecular/cellular/systemic theories of aging. It has been proposed that any mechanistic theory of aging should be able to explain (i) the loss of physiological function over time, (ii) the variability in emergence and progression of these losses in individual organisms and between species and (iii) the ability to achieve life span extensions by dietary, pharmacologic and genetic manipulations (Sohal *et al.* 2002). An in-depth examination of these theories has been provided elsewhere (Weinert and Timiras 2003) and the key principles are briefly summarized here (Table 7). Examination of these theories reveals that many of them are not mutually exclusive and that a number of them overlap to a certain degree. An increasing wealth of data from several model organisms is beginning to test the validity

of these theories and has led to the current widely held belief that aging is a complex multifactorial process that cannot be attributed to one single mechanism alone.

Table 7. Summary of main theories of aging

Theory classification	Theory name	Description
Molecular	Gene regulation	Changes in gene expression profiles with age
	Somatic mutation	↑ nucleic acid and protein damage with age
	Error catastrophe	↓ gene expression fidelity with age
	Oxidative damage	↑ oxidative damage to macromolecules with age
Cellular	Cell senescence-telomere	↑ cellular senescence due to telomere shortening (replicative senescence) or cell stress (stress-induced senescence) with age
	Wear-and-tear	Accrual of normal injury with age
	Apoptosis	↑ programmed cell death with age
System	Neuroendocrine	Change in neuroendocrine function with age
	Immunologic	↓ immune system function with age
	Rate-of-living	Metabolic potential of each organism is fixed and life span determined by rate of metabolism
Evolutionary	Disposable soma	Body is optimized for reproductive success, becomes disposable after reproductive peak
	Antagonistic pleiotropy	Genes advantageous to young organism become deleterious following reproductive peak but are not selected against
	Mutation accumulation	Genetic mutations impacting health of individuals past reproductive peak are not selected against

The free radical theory of aging was conceived by Denham Harman in the mid 20th century following the observation that reactive oxygen species are formed *in vivo* after radiation exposure and are responsible for the ensuing damage, some of which resembled an accelerated form of aging (Harman, 1956). Although initially met with a deal of skepticism, the free radical theory gained credibility following the demonstration of H_2O_2 *in vivo* by Chance (Chance et al., 1979) and the discovery of superoxide dismutase by McCord and Fridovich (McCord and Fridovich, 1969). The free radical theory was later modified to the oxidative damage theory of aging upon the discovery that many reactive species that cause oxidative damage to cells are not actually free radicals (Sohal et al., 2002). The oxidative damage hypothesis has been under intense investigation over the past 50 years. This testing has provided several lines of supporting evidence, e.g. oxidative damage to cell macromolecules increases with age and is often found to correlate with organismal longevity, and genetic or pharmacological manipulations of antioxidant levels have been shown to frequently result in exacerbation or amelioration of aging phenotypes as would be predicted by the oxidative damage hypothesis (Sohal et al., 2002). Although these findings indicate a role for oxidative damage in life span determination and age-related phenotypes, they do not offer insight into the mechanism by which oxidative damage impacts organismal aging. Oxidative damage is known to have detrimental consequences on the function of key cellular macromolecules, especially proteins (Stadtman et al., 2005) which could concievably underly its impact in aging. Whether this is due to oxidative damage to a few key molecules or a more global

spread of oxidative damage remains to be determined and will be crucial to a proper understanding of the link between oxidative damage and organismal aging.

A key prediction from the oxidative damage theory is that manipulating the levels of cellular antioxidant defenses will alter levels of ROS and thereby affect molecular oxidative damage and potentially impact the course of aging. In one scenario, a decrease in antioxidant levels might lead to an increase in ROS levels and in turn, promote oxidative damage and result in an accelerated manifestation of age-related phenotypes. Conversely, augmenting antioxidant capacity might lower pro-oxidant levels, mitigate oxidative damage and potentially delay or attenuate the appearance of age-associated phenotypes. This theory was directly tested in this investigation by manipulating the expression levels of the key cellular antioxidant superoxide dismutase (SOD). Extending the results of earlier work demonstrating an increase in life span following overexpression of *Sod1*, we found that this also lead to an attenuated decline in age-related function in odor avoidance assays. Tissue-specific *Sod1* overexpression in neuronal or muscle tissues failed to reproduce the delayed functional senescence or life span extending effects suggesting that these phenotypes were due to either (i) augmented SOD1 levels in a tissue not tested in our tissue-specific studies or (ii) combined *Sod1* overexpression in multiple tissues simultaneously.

In a separate study, a series of mutants with graded reductions in SOD2 levels were created in order to test the possibility that this may confer accelerated age-related phenotypes. Loss of SOD2 was shown to result in oxidative damage to aconitase and life span studies revealed that longevity was reduced in proportion to the loss of SOD2, strongly supporting the role of ROS in regulation of life span. Importantly, population survival studies showed that this life span shortening of each *Sod2* mutant was not due to an affect on the pre-mortality phase of survival curves but due to an accelerated decline in survival during the mortality phase of survival plots. This finding is consistent with *Sod2* mutants experiencing reduced longevity due to accelerated aging as opposed to being perpetually sick since conception. In further support of this, behavioral aging was accelerated in mutants with greater than 50% reduction in SOD2, and apart from the pronounced locomotor defects observed in *Sod2* null homozygotes at the youngest age tested, performance of newly-emerged *Sod2* mutants in functional assays was comparable to that of controls. Tissue-targeted knock-down of Sod2 expression using RNAi revealed that muscle was a key tissue underlying the accelerated aging phenotypes found following loss of SOD2. Loss of muscle SOD2 lead to several degenerative phenotypes in thoracic muscle including reduced mitochondrial content and ATP levels, elevated cell death and progressive loss of locomotor function. Knock-down of *Sod2* specifically in cardiac muscle resulted in significant effects on locomotor senescence and life span although these phenotypes were not comparable to those following pan-muscle *Sod2* knock-down indicating that cardiac-specific loss of SOD2 was not primarily responsible

for these phenotypes. Muscle-specific *Sod2* overexpression did not attenuate functional senescence or extend life span suggesting that it had no beneficial effects on aging and that muscle SOD2 levels are ordinarily in abundance. Finally, pan-neuronal *Sod2* knock-down resulted in modest acceleration of age-related locomotor function decline and subtle reduction in life span, although there remains a possibility that RNAi-mediated *Sod2* knock-down was not functioning properly in the nervous system which would preclude an accurate interpretation of these results.

Collectively, the results from this investigation support the oxidative damage theory of aging and illustrate a crucial role for SOD2 in fly muscle in protecting against oxidative damage which leads to a devastating effect on fly functionality and survival.

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Curriculum Vitae

Ian Martin

11-050 Sanger Hall
Medical College of Virginia Campus, Virginia Commonwealth University
1101 East Marshall Street
Richmond, VA 23298
Tel.: (804) 828 9632 ext. 157
E-mail: martini3@vcu.edu

Biographical Information

Born 18th March 1981, London, England
United Kingdom Citizen

Education

2003-2008 *Ph.D. Human Genetics with a concentration in Neuroscience*
Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA
Dissertation: The effects of altered superoxide dismutase expression on age-related functional declines and survival in *Drosophila*
Advisor: Michael S. Grotewiel, Ph.D.

1999-2003 *B.Sc. Biochemistry (1st class degree with honors)*
University of London at King's College, London.

Prior Research Experience

2001-2002 University of Pittsburgh, Pittsburgh, PA.
Pre-doctoral Fellow in the lab of Valerian Kagan, Ph.D., Department of Health and Toxicology.
I contributed to experiments assessing the role of oxidative damage in (i) the pathogenesis of esophagitis caused by lung cancer radiotherapy and (ii) a cell-based model of etoposide-induced acute myelogenous leukemia. We showed that manganese-superoxide dismutase overexpression and ascorbate can protect 2C6 cells from irradiation damage by scavenging

reactive oxygen species generated. We also developed a sensitive method for the detection of glutathionyl radicals and used this method to show that glutathionyl radicals propagate cytotoxicity in etoposide-treated HL-60 cells.

Honors and Awards

2007	Graduate Student's Association Travel Award, VCU
2007	Graduate School Travel Grant, VCU
2006	Roscoe D. Hughes Fellowship for excellence in graduate studies and research, VCU
2006	Outstanding presentation award, 34th John C. Forbes Graduate Student Honors Colloquium, VCU
2006	Who's Who among Students in American Universities and Colleges, VCU
2005-2006	Graduate Research Grant, VCU
2003	William Robson Memorial Prize for outstanding B.S. Biochemistry graduate, King's College, London
2001-2002	Pre-doctoral Fellowship, University of Pittsburgh.

Articles

Rhodenizer, D., **Martin, I.,** Bhandari, P. and Grotewiel, M.S. (2007). Genetic and environmental factors impact age-related impairment of negative geotaxis in *Drosophila* by altering age-dependent climbing speed. *Experimental Gerontology*, In Press

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Abstracts

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Martin, I. and Grotewiel, M.S. The effects of superoxide dismutase overexpression on age-related functional declines in *Drosophila melanogaster*. Graduate Research Symposium, VCU, April 2006.

Martin, I., Warner-Gargano, J., Bhandari, P. and Grotewiel, M.S. Effect of genetic background and life span-extending mutations on locomotor activity senescence. *Drosophila* Research Conference, San Diego, CA, April 2005.

Martin, I., LaPrairie, J.L. and Lonstein, J.S. Effects of dopamine D1 and D2 receptor antagonists in the preoptic area on maternal behavior in lactating rats. Society for Neuroscience, San Diego, CA, October 2004.

Grotewiel, M.S., Warner-Gargano, J., Bhandari, P. and **Martin, I.** Rapid iterative geotaxis (RIG), a new method for assessing locomotor behavior. *Drosophila* Research Conference, Washington DC, March 2004.

Borisenko, G., **Martin, I.,** Zhao, Q., Jiang, J.F. and Kagan, V. Specific detection of GS• generated by peroxidase metabolism of phenolic compounds in live cells using acridine-tempo. Oxygen Society, San Antonio, TX, November 2002.

Epperly, M.W., Osipov, A.N., **Martin, I.,** Kawai, K.K., Borisenko, G.G., Tyurina Y.Y., Jefferson, M., Bernarding, M., Greenberger J.S. and Kagan, V.E. Ascorbate as a“redox-sensor” and protector against γ-irradiation-induced oxidative stress: EPR evidence in 32 D cl 3 and MnSOD-transfected 32D 2C6 cells. Radiation Research Society, Reno, NV, April 2002.

Jiang, J., Borisenko, G.G., Osipov, A., **Martin, I.,** Chen, R., Graham, S.H. and Kagan, V.E. Arachidonic acid-induced carbon-centered radicals and phospholipid peroxidation in cyclo-oxygenase-2-transfected PC12 cells. Society of Toxicology, Nashville, TN, March 2002.

Teaching

2001	Teaching Assistant: Biological Effects of Radiation-Generated Free Radicals lecture, EOH2130 Survey of Radiation Health course, University of Pittsburgh.

Student Memberships

2007-2008	Genetics Society of America
2005	School of Medicine representative Graduate Student's Association of Virginia Commonwealth University.
2005	Travel Grants Committee member, Graduate Student's Association of Virginia Commonwealth University.
2003-2004	Graduate Treasurer and Co-founder, International Student's Association of Michigan State University.