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Disruption of dopamine homeostasis has sexually dimorphic effects on senescence characteristics of *Drosophila melanogaster*

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Abstract

The neurotransmitter dopamine (DA) is known to be involved in a multitude of physiological processes. We investigated sexually dimorphic effects of disruptions in DA homeostasis and its relationship to senescence using three different *Drosophila melanogaster* mutants namely *Catsup* (*Catsup*²⁶) with elevated DA levels, and *pale* (*ple*²), *Punch* (*Pu*^{Z22}) with depleted DA levels. In all genotypes including controls, DA levels were significantly lower in old (45-50 day old) flies compared to young (3-5 day old) in both sexes. Interestingly, females had lower DA content than males at young age whereas this difference was not observed in old age, suggesting that males had a larger decline in DA levels with age. Females, in general, were longer lived compared to males in all genotypes except *ple*² mutants with depleted DA levels. This phenotype was abolished in the *ple*² rescue flies. Interestingly, females also demonstrated marked age-related decline in circadian locomotor activity compared to males. Old *Catsup*²⁶ males with elevated DA levels accumulated significantly lower levels of lipid peroxidation product 4-hydroxy 2-nonenal (4-HNE) compared to age-matched wild type, *ple*² and *Pu*^{Z22} mutant males. In *Catsup*²⁶ revertant lines this phenomenon was absent. We also observed a sexually dimorphic response in the expression levels of key stress and aging associated and/or related transcription factor genes across genotypes with elevated or depleted DA levels which was reverted to wild type levels in specific rescue lines. Taken together,

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Author contributions

A.B. and N.K. conceived and designed the experiments. A.B., H.M. and K.R. performed the experiments. A.B., K.R., J.M.D. and N.K. analyzed the data. A.B., K.R. J.M.D. and N.K. drafted the paper.

Competing Interests

The authors declare that no competing interests exist.

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Graphical abstract

Sexually dimorphic effects of disruptions in dopamine (DA) homeostasis and its relationship to senescence was investigated in *Drosophila melanogaster*. *Catsup²⁶* with elevated DA levels, and *ple²* and *Pu^{Z22}* with depleted DA levels showed sex specific effects in longevity, activity, senescence and age associated transcription factors. These results reveal a novel sexually dimorphic involvement of DA in senescence characteristics of *D. melanogaster*.

Keywords

Aging; circadian rhythms; 4-hydroxynonenal; lifespan; protein carbonyl

Introduction

Aging of the neuroendocrine system has been incriminated in the development of various age-dependent pathologies (Rehman & Masson, 2001). Moreover, it is known that aging affects the endocrine system by altering endocrine cells, hormones produced by these cells, and the hormone receptors or post-receptor processes in target cells (Chahal & Drake, 2007).

Dysregulation of the neuroendocrine system and its influence on senescence in a sexually dimorphic manner has been little explored. Implications of such a dysregulation would accelerate the senescence process through changes in neural and endocrine functions crucial for (a) coordination and programming physiological responses to external environmental stimuli, and (b) maintenance of optimal functional status for reproduction and survival. Such changes selectively affect not only regulation of evolutionarily important functions such as reproduction, growth and development, but also influence survival by adaptation to stress.

In mammals, the neurotransmitter dopamine (DA) modulates movement, motivation, and cognition (Aarts *et al.*, 2011). In insects such as *Drosophila melanogaster*, DA is involved in locomotion (Pendleton *et al.*, 2002), sleep and arousal (Andretic *et al.*, 2005; Foltenyi *et al.*, 2007), courtship behavior (Liu *et al.*, 2008), inhibition of startle-induced hyper-excitability (Friggi-Grelin *et al.*, 2003) fitness traits (Gruntenko *et al.*, 2005; Rauschenbach *et al.*, 2015), longevity (De Luca *et al.*, 2003) and mediating response to stress (Gruntenko & Rauschenbach, 2008; Hanna *et al.*, 2015). It has been previously demonstrated that DA levels are reduced in aging flies and dopaminergic modulated behaviors are perturbed with increasing age (Neckameyer *et al.*, 2000). DA metabolism is also impacted in an age-specific manner by juvenile hormone and 20-hydroxyecdysone in *Drosophila* (Rauschenbach *et al.*, 2011). However, it is unclear if there is a sexually dimorphic response to perturbations in DA levels that can impact senescence characteristics. Although *Drosophila* and mammalian neuroanatomy differ considerably, many molecular elements of the aminergic neurotransmission are conserved (Martin & Krantz, 2014). Thus, the fruit fly *Drosophila* provides a powerful system to explore mechanisms by which perturbations in aminergic neurotransmission may impact senescence characteristics associated with age.

Several previous studies in mammalian systems have shown sexually dimorphic behaviors, neuroendocrine changes, and alterations in neurotransmitter release in response to stress (see Sullivan, 2004 for review). In *Drosophila*, DA levels are sexually dimorphic (Rauschenbach *et al.*, 1993; Neckameyer *et al.*, 2000) and the stress response circuitry (involving DA) also differs based on sex (Argue, 2012). We have previously demonstrated that perturbation in DA levels and transport in young flies has a distinct impact on response to oxidative stress and other physiological characteristics (Hanna *et al.*, 2015). Although increased susceptibility of older animals to various forms of stress has been documented, very little is known about the molecular basis underlying this change (Papaconstantinou *et al.*, 1996). There is also evidence to suggest that longevity and the ability to resist oxidative and metabolic stress are related processes (Edwards *et al.*, 2003). Importantly, it has been demonstrated that similar gene expression patterns characterize oxidative stress response and aging (Landis *et al.*, 2004). In this study, we cumulatively tested gene expression levels of specific transcription factors implicated in stress response and aging together with other parameters of senescence among DA mutants in a sex specific context to gain insights into the sexually dimorphic role of DA in senescence.

In recent years, the peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1) family of transcriptional co-activators have emerged as key regulators of energy metabolism and also implicated in aging at the tissue and organismal level (Rera *et al.*, 2011). Another sensor and regulator of energy balance is the 5'-AMP-activated protein kinase (AMPK)

which is responsive to hormonal and nutrient signals at the cellular level (Lage *et al.*, 2008) and shown to modulate tissue and organismal aging (Ulgherait *et al.*, 2014). AMPK is activated upon energy depletion and serves as an inhibitor of the target of rapamycin (TOR) – a key protein kinase that regulates cell growth and metabolism. Inhibition of TOR prolongs lifespan and increases quality of life (Xu *et al.*, 2013). On the other hand, insulin and insulin-like growth factors (In/IGF) are major TOR activators that operate through phosphoinositide3-kinase and the protein kinase B (Akt) (Lee *et al.*, 2010). The interplay between In/IGF on DA metabolism revealed that suppression of insulin-like receptor (InR) in corpus allatum of *Drosophila* increases DA levels and affects neuroendocrine stress reactions (Rauschenbach *et al.*, 2014). Phosphorylation of Akt in response to In/IGF signaling negatively regulates the forkhead box O (FoxO) family of transcription factors that play an important role in functions such as differentiation, metabolism, proliferation and survival (Bedná ová *et al.*, 2015). FoxO can induce transcriptional activation of sestrins (highly conserved anti-aging stress responsive proteins) which activate AMPK leading to inhibition of TOR and has a role in determining longevity. We hypothesized that disruption of DA homeostasis would impact these transcription factors in a sexually dimorphic manner and affect senescence characteristics. In the present study, we have examined longevity, dampening of circadian locomotor activity rhythms (as a read-out of senescence), age-associated oxidative protein and lipid damage, as well as the expression of key transcription factor genes involved in both stress response and aging in male and female *Drosophila* with perturbed DA levels at young and old age. This is important since many age-related pathologies exhibit sexual dimorphism. Insights into links between the dopaminergic system and its impact on senescence in a sexually dimorphic manner could eventually lead to novel gender-based therapeutic strategies.

Material and Methods

Drosophila stocks and husbandry

D. melanogaster were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25 °C in 12 h light:12 h dark (LD 12:12) cycles (with an average light intensity of ~2000 lux). Fly lines *w¹¹¹⁸* and Canton-S were used as the control strains for the catecholaminergic pathway mutations employed in this study. Comparison of the two controls established that they were virtually identical in all parameters studied for each sex (data not shown), hence, the data was combined and represented as wildtype. Three mutant fly lines were used in this study namely *Catsup²⁶*, *ple²* and *Pu^{Z22}*. *Catsup²⁶/CyO* is a 600 bp deletion extending immediately upstream of the start codon produced no detectable protein and was derived from the mobilization of a 5' P-element insertion in *Catsup^{KO5042}* (Stathakis *et al.*, 1999). Loss of *Catsup* function also results in impaired Notch signaling (Groth *et al.*, 2013). Since *Catsup* mutant alleles are homozygous lethal, all experiments in the study were performed using heterozygous strains. The homozygous lethal *ple²* is a loss-of-function allele recovered in an ethylmethane sulfonate (EMS) mutagenesis screen and the heterozygous mutant *w; ple²/TM3 Sb e* was used (Neckameyer & White, 1993). The *Pu* mutant allele employed in this study was generated in an EMS screen and the genotype is *dp cn Pu^{Z22} a px sp/SM1*. Genetic characteristics of this strain are reported elsewhere (Mackay *et al.*, 1985; Reynolds and O'Donnell, 1988). All mutants were isogenized with their

respective wild type controls by out-crossing for 6 generations and selection on the basis of balancers. All behavioral studies were conducted on mutant heterozygotes crossed into the appropriate wild type background to eliminate balancers. To revert DA levels to wild-type levels in a *Catsup*²⁶ background, transgenic expression of a wild-type epitope-tagged *Catsup-V5* construct expressed under a *daughterless* gene regulatory control was employed (Groth *et al.*, 2013). Hence, we crossed *Catsup*²⁶/*CyO*;*da-Gal4* and *Catsup*²⁶/*CyO*;*UAS-Catsup-V5*. Non-CyO progeny (*Catsup*²⁶; *da Gal-4/UAS-Catsup-V5*) were used for behavioral assays. To generate the *ple*² revertant the *ple* mutation was rescued using the native *DTHg* construct as described previously (Riemensperger *et al.*, 2011) by crossing *w*; *Ddc-GAL4*; *TH-GAL4*; *ple*^{2cl}/TM6B(Tb), e with *w*; *UAS-DTHg*^{wt}; *ple*^{2cl}/TM6B(Tb), e (kindly provided by Prof. Serge Birman, Brain Plasticity Unit, CNRS, PSL University, Paris, France). These flies have been designated as *DTHg*; *ple*. Both male and female flies were examined separately within this study to observe sexual dimorphism in parameters tested.

2.2 DA content quantitation

DA neurons in the brain were visualized by TH-Gal4 driven Green fluorescent protein (GFP) expression and identification of DA neurons by GFP fluorescence. Whole mounts of fly brains of young (3-5 days) or old (45-50 days old) males and females of different genotypes were dissected out separately and observed using a Zeiss LSM 510 Axiovert 200M Laser Scanning Confocal Microscope. Medial and lateral dopaminergic neurons scored. There is one major paired dopaminergic cluster on the anterior side, protocerebral anterolateral (PAL), and five on the posterior side: protocerebral posterior medial -PPM1 (unpaired), PPM2 (paired), PPM3 (paired) and protocerebral posterolateral-PPL1 and PPL2 (paired). For quantitative assessment, we measured DA levels in whole body homogenates of flies. The levels of DA in whole bodies of young (3-5 days) and old (45-50 days) *Drosophila* were measured by collecting 10 flies of each sex and genotype separately and homogenizing them in 250 µl of ice-cold 0.1 M perchloric acid containing 25 ng of 3,4-dihydroxybenzylamine. After centrifugation of the homogenate at 17,000 g for 30 min at 4°C the supernatant was collected. DA was measured using a C18 reverse phase column in a Hitachi 655A HPLC autosampler with a Coulochem M 5100A electrochemical detector (Newland *et al.*, 2015). To control for dietary DA, we tried to detect DA in fly diet but the levels were below detection thresholds.

2.3 Lifespan measurements

For measuring lifespan, 3 cohorts (replicates) of approximately 80 mated male and 80 mated female flies of each genotype (n=240) were housed separately in 8 oz round bottom polypropylene bottles (Genesee Scientific, San Diego, CA, USA) inverted over 60 mm Falcon Primaria tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA) containing 15 mL of diet. Diet dishes were replaced on alternate days without anesthesia (CO₂) after tapping flies to the bottom of the bottle. Mortality was recorded daily and mortality calculations and Gompertz-Makeham maximum likelihood estimates done using WinModest (v.1.0.2) as described previously (Krishnan *et al.*, 2009).

2.4 Circadian locomotor activity analysis

Flies of each sex, genotype and age were entrained in LD 12:12 at 25 °C for 3 days to acclimatize them to the activity monitoring tubes of the *Drosophila* activity monitor. Locomotor activity of young (3-5 days) and old (45-50 days) male and female flies of each genotype were recorded for 3 d in LD 12:12, followed by 10 d in constant darkness (DD) using the Trikinetics locomotor activity monitor (Waltham, MA, USA) as described (Pfeiffenberger *et al.*, 2010). Compared to young flies of both sexes there were more deaths recorded among old flies in all genotypes studied and so individuals who were surviving among the old groups were selected for locomotor activity analysis. There was also some mortality recorded among old flies of all genotypes in activity tubes and these data were eliminated from final analysis. While selecting individuals who survived in old groups may likely have been the healthiest among the population, it was felt that they would still be representative of phenotypic effects of DA perturbations at that age. Locomotor activity was counted by number of infrared beam crossings of the individual flies collected in 15 min bins (actograms). For daily activity profiles, the number of beam crossings in LD cycles was averaged for the 3 days in LD for all flies of a particular sex and genotype. For a quantitative measure of circadian rhythmicity in DD, Fast Fourier Transform (FFT) analysis was conducted using CLOCKLAB software (Actimetrics; Coulbourn Instruments, Whitehall, PA, USA). Flies with FFT values <0.04 were classified as arrhythmic, 0.04-0.08 as weakly rhythmic, whereas >0.08 were considered strongly rhythmic. Flies with both weak and strong rhythms that showed single peak in the periodogram were included for calculations of free-running period using the CLOCKLAB software.

2.5 Total protein carbonyl content assay

Protein carbonyls were quantified in whole body homogenates (25 flies in each replicate in three bioreplicates) of young (3-5 days) and old (45-50 days) flies of each sex and genotype. Samples were derivatized after reaction with 2,4 dinitrophenylhydrazine (DNPH) as previously described (Krishnan *et al.*, 2007). Results were calculated as nmol mg⁻¹ protein using an extinction coefficient of 22,000 M⁻¹ cm⁻¹ at absorbance maxima of 370 nm in H1M Synergy plate reader (BioTek, Winooski, VT, USA). Bovine serum albumin (BSA) standard curve was used for protein concentrations in guanidine solutions (Abs 280 nm). Protein carbonyl values were corrected for interfering substances by subtracting the Abs 370 nm mg⁻¹ protein measured in control samples. Relative fold change for different sex, age and genotype was calculated with respect to pooled data of young male wild type *w¹¹¹⁸*, and wild type Canton-S flies whose level of protein carbonyls was set as 1.

2.6 Lipid peroxidation product 4-Hydroxynonenal assay

4-hydroxy-2-nonenal (4-HNE) was assayed as a marker of lipid peroxidation in whole body homogenates (25 flies in each replicate in three bioreplicates) of young (3-5 days) and old (45-50 days) flies of each sex and genotype using a competitive enzyme-linked immunosorbent assay as described previously (Bedná ová *et al.*, 2013). In brief, free hydroxynonenal (4-HNE, Alpha Diagnostic, San Antonio, TX, USA) was conjugated to glyceraldehyde-3-phosphate (GAPDH) protein. The wells in a 96-well high binding plate (Costar) were coated with 500 ng of HNE-GAPDH protein for 2 h at 4°C, washed in PBS-

Tween (PBST) and blocked with 1% BSA. Standard curve was prepared from serial dilutions of HNE-GAPDH with polyclonal anti-HNE antibody (1:1000 Alpha Diagnostics). Flies were homogenized in 50 mM Na-PO₄ buffer (pH 7.2) using a Kimble-Kontes motorized homogenizer followed by sonication for 30 s. Samples were centrifuged at 16,000 g for 15 min, at 4°C. The protein content of the supernatant was estimated using bicinchoninic acid (BCA). 50 µg of protein lysate from each sample was mixed with 1:1000 polyclonal rabbit anti-HNE antibody and added to wells in triplicate. Plates were incubated for 1 h, washed with PBST, incubated with 1:5000 secondary anti-rabbit antibody conjugated with horseradish peroxidase, washed, mixed with detection buffer TMB (Alpha Diagnostics) and the reaction stopped with Stop solution (Alpha Diagnostic), and absorbance read at 450 nm in the H1M Synergy plate reader (BioTek). 4-HNE values from samples were obtained from the interpolation of values in the standard calibration curve and calculated as pmol of 4-HNE per mg of protein. Relative fold change for different sex, age and genotype was calculated with respect to pooled data of young male wild type *w¹¹¹⁸* and Canton-S flies whose 4-HNE level was set as 1.

2.7 Quantitative real-time polymerase chain reaction

Three independent biological replicates of young (3-5 days) and old (45-50 days) males and females of each genotype was collected separately. Total RNA was extracted from whole body homogenates (25 flies) using Tri Reagent (Sigma, St. Louis, MO, USA). Samples were treated with Takara Recombinant DNase I (Clontech Laboratories Inc., Mountain View, CA, USA) followed by cDNA synthesis with iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed on the Eppendorf realplex² Mastercycler (Eppendorf, Hauppauge, NY, USA) under default thermal cycling conditions, with a dissociation curve step. Every reaction contained Power SYBR Green (Applied Biosystems, Carlsbad, CA, USA), 10 ng cDNA, and 400 nM primers. Primer sequences are provided in Supplemental Table S1. Data were analyzed using the 2^{-CT} method with mRNA levels normalized to the housekeeping gene *rp49* (Hanna *et al.*, 2015). Relative mRNA expression was calculated with respect to pooled data from young male wild type *w¹¹¹⁸* and Canton-S flies set as 1.

2.8 Statistical analyses

DA content, locomotor activity data, protein carbonyl levels, 4-HNE levels and gene expression analysis was subjected to one or two-way ANOVA with **Bonferroni's** post-hoc tests as appropriate (IBM SPSS Statistics v.24, **GraphPad Prism v 6.07**). Analysis of longevity and mortality characteristics was conducted in WinModest (v. 1.0.2), followed by two-way ANOVA with Tukey's post hoc tests in GraphPad Instat v 3.0 (GraphPad Software Inc. La Jolla, CA). Sex and genotype were the two factors whereas average survival and other Gompertz-Makeham estimates were the parameters evaluated. Graphs were generated using GraphPad Prism 6 v 6.07 (GraphPad Software, La Jolla California USA, www.graphpad.com).

Results

Age related decline in DA content

In insects, DA plays a dual role as a neurotransmitter in the central nervous system, and a cuticle hardening and pigmenting factor in the epidermis. Dopaminergic neurons were visualized in *Drosophila* brain using the *TH-Gal4* driver crossed to *UAS-GFP* responder (Figure 1A). One major cluster in anterior (PAL), and five clusters in posterior – medial (PPM1, PPM2 and PPM3) and lateral (PPL1 and PPL2) dopaminergic neurons (Fig. 1A) scored as mentioned in the Methods section. Since visualization of DA neurons in different genotypes, sex and age did not reveal marked differences (data not shown), we quantified DA in whole body homogenates of various genotypes in both sexes at young and old age. In general, there is a marked and significant depletion of DA levels in old age across genotypes (Figure 1B). As expected, young *Catsup*²⁶ males and females revealed significantly higher DA levels compared to either wild type flies, *ple*² or *Pu*^{Z22} mutants of the same sex and age. This was reversed in the *Catsup*²⁶-revertant flies where DA levels were similar to wild-type flies. DA levels of young male and female flies of *ple*² mutants were significantly lower than wild type and *Catsup*²⁶ mutants but similar to *Pu*^{Z22} mutants. In *DTHg; ple* rescue lines the DA levels were not significantly different from their wild-type counterparts. In general, females had significantly lesser DA levels than male counterparts in wild type and *Catsup*²⁶ flies except in *ple*² and *Pu*^{Z22} mutants where there was no difference in DA content between males and females of a particular age. Also, the marked sexual dimorphism in DA content between males and females of all genotypes (except *ple*² and *Pu*^{Z22} mutants) at young age was abolished in old flies (Figure 1B).

Lifespan characteristics

Assessment for lifespan indicated differences between genotypes as well as between males and females of each genotype (Table 1). While no significant difference in longevity was observed between wild type controls (mean longevity 53.9 ± 1.3 days) or *Catsup*²⁶ mutants (54.6 ± 1.6), among males there was a marginal albeit significant reduction in mean longevity in *ple*² mutants (51.4 ± 1.4 days). *Pu*^{Z22} male mutants on the other hand showed least longevity (37.8 ± 1.2) (Table 1). No difference was observed in the longevity of either *Catsup*²⁶-revertant or the *DTHg; ple* (*ple* rescue) flies compared to wild type flies. Among females, wild type controls (*w*¹¹¹⁸ and Canton-S) exhibited highest mean longevity (61.5 ± 0.9 days) followed by *Catsup*²⁶ mutants (57.9 ± 1.1 days). Female *ple*² mutants with decreased DA levels showed reduced longevity compared to either wild type controls or *Catsup*²⁶ mutant females. *Pu*^{Z22} females were longer lived than *ple*² mutants but short lived compared to wild type and *Catsup*²⁶ mutants (Table 1).

When males and females of a particular genotype were compared, we found sex specific differences in the mean longevity (Table 1). In general, female wild type and *Catsup*²⁶ and *Pu*^{Z22} mutants were significantly longer lived than their male counterparts. In contrast, male *ple*² mutant lived longer than their female counterparts. Longevity of females and the sexually dimorphic longevity pattern in *Catsup*²⁶ revertant and *DTHg; ple* were similar to wild type flies. These male- or female-specific mortality characteristics were also reflected

in the mortality parameters derived from the Gompertz-Makeham model and maximum likelihood estimates (MLE) (Supplemental Table S2).

Age-related senescence of circadian locomotor activity

Substantial changes in circadian locomotor activity rhythms were recorded across all genotypes with age in both sexes (Table 2). Male wild type flies exhibited decline in rhythmicity from 100% in young to 63% in old. In females, such a decline was significant with only 46% of old flies remaining rhythmic. In *Catsup*²⁶ mutants with elevated DA, there was only a marginal decline in rhythmicity in males from 70% in young to 63% in old; however, in old females, there were only 12% rhythmic individuals. The *Pu*^{Z22} mutant line also exhibited a significant decline in rhythmicity with age across sexes whereas the *ple*² mutant showed such decline only in females (Table 2).

It is interesting to note that the average daily activity in males declined in old age across all genotypes (except *ple*²) with the most drastic decline in *Pu*^{Z22} mutants. However, this was quite the opposite in females with old flies exhibiting either comparable activity levels or even elevated activity compared to young (Figure 2A). In females, *ple*² and *Pu*^{Z22} mutants with depleted DA levels exhibited a slight non-significant decline in average daily activity in old age. On the contrary, activity declined in old age in males across genotypes. *Catsup*²⁶ mutants generally showed higher daily average activity in both males and females compared to their wild type or *ple*² counterparts. Across genotypes, females of a genotype showed higher average daily activity compared to males (Figure 2A). Both the *Catsup*²⁶ revertant and *DTHg; ple* flies mirrored the activity patterns of wild type flies across sex and age.

The strength of rhythms revealed a tendency to decline in old age among both males and females across genotypes (Figure 2B). Interestingly, rhythm strength was the least in young male *Catsup*²⁶ mutants when compared to wild type or *ple*² mutant males of similar age. In both wild type and *Catsup*²⁶ mutants, old females revealed drastic reduction in rhythm strength, whereas in *ple*² mutants such decline was not significant (Figure 2B).

Accumulation of damaged protein and lipids with age

Studies have found evidence of oxidative damage to macromolecules (DNA, lipids, protein), and data in transgenic *D. melanogaster* support the hypothesis that oxidative injury might directly cause the aging process (Fukagawa, 1999). However, while oxidative damage is the cause of aging or is consequent to aging is still debatable, oxidative damage to proteins (protein carbonylation in particular) and lipid peroxidative damage is closely associated with the aging process. Hence, biomarkers of protein and lipid damage were targeted in this study. There was a significant accumulation of protein carbonyls in old flies compared to young in both males and females of each genotype (Figure 3). However, no sex-specific differences were observed in young flies across all genotypes. Old females accumulated significantly lower carbonyls compared to their male counterparts within a genotype. Old *Catsup*²⁶ females had lower carbonyls compared to old *ple*² and *Pu*^{Z22} females (Figure 3). In contrast, old males did not show any differences in carbonyl levels across all genotypes (Figure 3). Overall, it could be stated that males accumulated more carbonyls across genotypes compared to females in old age except in *Pu*^{Z22} mutants where carbonyl content

was not significantly different between old males and females. No significant changes in *Catsup*²⁶-revertant and *DTHg; ple* flies were observed compared to sex and matched age wild type (data not shown).

The lipid peroxidation product 4-HNE levels also showed tendency to increase with old age in general across genotypes. Young male *ple*² mutants with reduced DA had significantly more 4-HNE content than comparable wild-type and *Catsup*²⁶ males (Figure 4). Old *Catsup*²⁶ males accumulated the least amount of 4-HNE compared to its counterparts in wild type and *ple*² and *Pu*^{Z22} mutant flies suggesting that elevated DA levels leads to lower lipid peroxidation and vice versa. Also, old male female *ple*² and *Pu*^{Z22} mutants had significantly higher levels of 4-HNE compared to wild type or *Catsup*²⁶ counterparts (Figure 4). This phenomenon was absent in *DTHg; ple* flies which showed similar carbonyl levels to wild type. Moreover, the *Catsup*²⁶-revertant also exhibited similar carbonyl accumulation levels to sex and age-matched wild type flies. Within a genotype, young females showed tendency to have higher 4-HNE levels compared to young males with the exception of *ple*² mutants where the levels were similar between young males and females.

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

A sexually dimorphic as well as age-dependent response of some key genes involved in stress response and aging was observed (Figure 5). *dPGC-1* expression was dramatically up-regulated in all young female fly lines of various genotypes compared to age-matched males (Figure 5). The highest *dPGC-1* expression was observed in young wild type females followed by *Catsup*²⁶, *ple*² and *Pu*^{Z22} females (the latter two not significantly different from each other) of same age. Young males had substantially lower *dPGC-1* expression than females across genotypes. It is interesting to note that both sexes of young *ple*² and *Pu*^{Z22} mutants with depleted DA levels had lower *dPGC-1* expression compared to the other genotypes. This was reversed in *DTHg; ple* flies which had similar expression levels to wild type flies (data not shown). Also, there is a general reduction in *dPGC-1* expression in old age across genotypes and sex (Figure 5).

Expression of the gene encoding energy sensor protein AMPK was similar in both wild type and *Catsup*²⁶ mutant flies when males and females of a particular age were compared (Figure 5). On the other hand, *ple*² and *Pu*^{Z22} mutants (both males and females) showed significantly lower expression of AMPK compared to wild type and *Catsup*²⁶ mutant flies of respective sex. Both the *Catsup*²⁶-revertant and *DTHg; ple* rescue flies showed AMPK expression levels comparable to wild type flies (data not shown). In general, a marked reduction in AMPK expression levels was recorded in old age across all genotypes similar to *dPGC-1* (Figure 5).

The gene encoding for the target of rapamycin (*dTor*) was significantly elevated in young females of all genotypes compared to age-matched males (Figure 5). Interestingly, the highest *dTor* expression was observed in young wild type females followed by age-matched *ple*² females. Young *Catsup*²⁶ females, on the other hand, had significantly lower expression of *dTor* when compared to wild type or *ple*² mutants of same age and sex (Figure 5). This was reverted to wild type levels in the *Catsup*²⁶-revertant lines (data not shown). However, *dTor* expression in young males did not differ significantly different from each other across

genotypes. Age associated decline in *dTor* expression was recorded in all genotypes and genders.

The expression of serine threonine kinase *Akt* followed very similar pattern to *dTor* (Figure 5). On the other hand, *dFoxO* expression pattern was a complete reversal of *dTor* and *Akt*. Thus, young *Catsup*²⁶ mutants had the highest and young *ple*² mutants had the lowest expression of *dFoxO* in both males and females (Figure 5). Again, an age-related decline in expression of *dFoxO* was recorded across genotypes; however unlike the young, there was no difference in the expression of *dFoxO* when old flies of either sex were compared across genotype (Figure 5).

Interestingly, the expression of *dSesn* encoding the stress inducible protein sestrin was markedly high in young females of all genotypes compared to age-matched males (Figure 5), although no difference was recorded in their expression level among genotypes. In contrast, there was no difference in *dSesn* expression levels among age-matched males across genotypes (Figure 5). As in case of other genes, a marked decline in expression of *dSesn* was recorded in both sexes across all genotypes. Importantly, the specific effects of manipulation of DA levels on expression of all the above genes were reverted to wild type levels with specific rescue lines (data not shown).

Discussion

The results of this study strongly indicate the possible involvement of DA in the process of senescence, manifested in a sexually dimorphic manner. On one hand, male and female *ple*² and *Pu*^{Z22} mutants with impaired DA synthesis showed significantly reduced longevity compared to controls, on the other hand, *Catsup*²⁶ mutant males with elevated DA levels did not live significantly longer than wild type controls. In fact, female *Catsup*²⁶ even had markedly lower lifespan than wild type females. Thus, increased DA levels did not enhance longevity of flies of either sex, but depleted DA levels had a negative impact on their longevity in agreement with previously reported results (Vermeulen *et al.*, 2006; Hanna *et al.*, 2015). The impaired longevity in *ple*² mutants was rescued in the *ple* rescue fly lines *DTHg; ple*. It has also been reported that there are behavioral consequences to DA deficiency in the adult brain, but this does not impact their longevity significantly compared to wild-type flies (Riemensperger *et al.*, 2011). The apparent discrepancy observed between their results and our present study could be genotype specific.

A sexually dimorphic decline in whole body DA levels with age has been reported earlier (Neckameyer *et al.*, 2000); however, brain DA levels and DA neurons do not get impacted with age (White *et al.*, 2010) as observed in the present study. A marked decline in whole body DA levels with age was also recorded in our study, supporting the hypothesis that quantification of DA in brain alone would not yield the correct profile of DA levels with age. Also, whole body DA levels were substantially lower in females compared to males in a genotype as previously reported (Neckameyer *et al.*, 2000). To negate the influence of dietary DA, we measured DA levels in the diet but this was below threshold detection values. However, the contribution of dietary tyrosine levels cannot be negated, but these would be in peptide form rather than free tyrosine and would need to be hydrolyzed after

ingestion to release free tyrosine for DA synthesis. While we did not account for food consumption differences in females and males in this study, it has been reported previously that female *Drosophila* feed more frequently than males (Wong *et al.*, 2009). This probably indicates that food consumption *per se* did not translate to DA levels observed.

We also report that perturbed DA levels have significant impacts on both longevity and senescence characteristics in a sexually dimorphic manner. Elevated DA levels in *Catsup*²⁶ mutants resulted in enhanced walking activity in females compared to age-matched males and females among all genotypes. This was reverted to wild type levels in the *Catsup*²⁶ revertant flies. Only aged females showed enhanced walking activity in wild type and *Catsup*²⁶ flies compared to young females. This enhanced walking activity was not observed in old males across genotypes, or in old *ple*² or *Pu*^{Z22} mutant females. Enhanced walking activity in aged *Drosophila* has been previously demonstrated (White *et al.*, 2010), which is very different from startle-induced locomotion and negative geotaxis. A rapid decline in rhythmicity was observed with age across genotypes, more so in females. Age-related changes in behavior as well as circadian rhythms have been reported in numerous studies across different animal species including *Drosophila* (Iliadi & Boulianne, 2010; Rakshit *et al.*, 2012; Nakamura *et al.*, 2016). It has been suggested that changes in circadian rhythms can alter metabolic processes associated with circadian timing, eventually resulting in accelerated senescence (Grosbellet *et al.*, 2015). Flies with depleted DA levels (*ple*² and *Pu*^{Z22} mutants) show significant shortening of lifespan compared to wild type and *Catsup*²⁶ flies with elevated DA levels. Although *Catsup*²⁶ flies do not have substantial enhancement of lifespan compared to wild type, elevated DA levels might still have a stress-protective function (Hanna *et al.*, 2015), which may help maintain lifespan compared to mutants with impaired DA transport.

Oxidative damage to proteins (assessed by levels of protein carbonyls) showed a tendency to accumulate lesser in old females compared to age-matched males across all genotypes, correlating with enhanced longevity observed in females compared to males except in *ple*² and *Pu*^{Z22} mutants. This strengthens the previously reported links between protein oxidative damage and aging process (de Graff *et al.*, 2016). However, correlation cannot be taken as causation. Thus, introduction of a stress paradigm would be needed to tease apart the link between stress-induced damage and lifespan (Hanna *et al.*, 2015). The link between oxidative lipid damage and longevity was also borne out by the pattern revealed in 4-HNE levels across genotypes and sex.

According to several theories of aging, lifespan is causally related to the ability to withstand extrinsic or intrinsic stresses (Vermeulen & Loeschcke, 2007). Hence, longevity should positively correlate with the ability to resist stress (Kirkwood & Austad, 2000). Therefore, genes involved in stress response should be relevant candidates for lifespan determination at the mechanistic level. To explore this aspect, we targeted several candidate genes involved in both stress response and aging pathways. The dramatic up-regulation of *dPGC-1* in age-matched females compared to males across genotypes suggests a strong link with aging and lifespan; however, this upregulation could also be due to the specific role of *dPGC-1* in oogenesis (Mukherjee *et al.*, 2014). The PGC-1 family of transcriptional co-activators plays a central role in regulation of mitochondrial biogenesis and function (Scarpulla, 2008). The

Drosophila homolog is Spargel/dPGC-1, and shares certain functional similarities and divergences with the mammalian PGC-1 protein (Mukherjee *et al.*, 2014). While Spargel/dPGC-1 apparently does not influence antioxidant enzymes, it plays an important role in growth, longevity and aging because of its involvement in energy homeostasis (Rera *et al.*, 2011; Mukherjee *et al.*, 2014). The AMP-activated protein kinase (AMPK) is involved in the adaptive response to energy deficit. Activation of AMPK induces PGC-1. While we did not record any difference in *AMPK* expression among age-matched males or females of wild type or *Catsup*²⁶ mutants, it was substantially lower in both sexes of *ple*² and *Pu*^{Z22} mutants with depleted DA levels. With aging, there is a blunted metabolic response to AMPK activation (Reznick *et al.*, 2007), which is also observed in our study. AMPK is involved in PGC-1 autoregulation, such that direct phosphorylation by AMPK promotes PGC-1 dependent induction at the PGC-1 promoter (Jager *et al.*, 2007). In addition to activating PGC-1, AMPK also inhibits TOR (Gwinn *et al.*, 2008), a nutrient activated factor that regulates PGC-1 gene expression (Cunningham *et al.*, 2007). In this study, *dTor* expression levels were significantly downregulated especially in young males compared to age-matched females across all genotypes. Inhibition of TOR has been shown to extend lifespan in yeast, worms and flies (Anderson & Weindruch, 2007). However, *dTor* expression levels alone might not be a determinant of longevity phenotype. dTOR is also a repressor of dFoxO. The serine threonine kinase AKT is a negative regulator of PGC-1 (Anderson & Prolla, 2009). *Akt* expression was also downregulated in young males compared to age-matched females. AKT has also been demonstrated to have a repressive effect on dFoxO by phosphorylating it at specific sites (Brunet *et al.*, 1999). Both *dFoxO* and *dSesn* expression levels were upregulated in young females compared to age-matched males across genotypes. The FoxO transcription factors are involved in several physiological and pathological processes, including aging, cancer, and neurological diseases (Greer & Brunet, 2008). It's important to note that enhanced *dFoxO* expression alone does not necessarily correlate with enhanced longevity since *Catsup*²⁶ females did not live longer than wild type females despite enhanced *dFoxO* expression. Moreover, DA depleted young *ple*² mutant females with higher expression of *dFoxO* than age-matched males did not have enhanced longevity. This could be due to the fact that in old flies, there was no difference in *dFoxO* expression across sexes and genotypes. Sestrins encoded by *dSesn* are a family of stress inducible proteins that can function as antioxidants and inhibitors of TOR and play key roles in stress response and aging (Lee *et al.*, 2010). *dSesn* expression declined with age in both sexes across genotypes but did not reveal any significant differences when compared between genotypes either in young or old age. This again reveals the importance of a stress paradigm to bring out the effects of such signaling molecules.

Taken together, our data strongly suggests that there is a sexually dimorphic role for DA in senescence. However, the precise role played by DA is not yet very clear-cut and this is compounded by the fact that loss of function mutations in DA synthesis result in lethality. Future studies will have to focus on elucidating the precise links between DA and senescence using the tools such as RNA interference, spatial and temporal expression of rate limiting enzymes and cofactors involved in DA synthesis and mutants with impaired DA transporters generated using state of the art genome editing technologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Dopaminergic neurons in a representative 5 day old wildtype (Canton-S) *Drosophila* brain (w; *TH-Gal4*; *UAS-GFP*). Posterior view of the brain showing green fluorescent protein (GFP) labeled DA neurons. PPL: Paired posterior lateral; PPM: Paired posterior medial DA neuronal clusters (see Methods for details). (B) Bar graph showing quantification of DA content in male and female whole fly bodies at young (3-5 day) and old (45-50 day) age. Every data point represents mean $\pm$ SEM of 10 independent individual measurements. Statistical analysis was conducted using two-way ANOVA with Bonferroni's multiple comparisons post-test (**Supplemental Table S3**). Bars with different letters are significantly different at $p < 0.05$ (Statistics for comparison among genotypes: $F=578.32$, $DFn=5$; For comparison across genotypes and age $F=297.56$, $DFn=3$; For interaction between genotype and sex: $F=54.19$, $DFn=15$).

Figure 2.

Graphical representation of the average daily activity per fly (**A**), and strength of the rhythm exhibited by rhythmic flies (**B**). Data for wild type flies is pooled from *w¹¹¹⁸* and Canton-S flies which showed similar circadian behavioral patterns. Average daily activity was computed as beam crossings in 15 min bins over 3 days of LD cycles (see Material and Methods Section 2.4 for more details). Comparisons for various parameters were made between male (young vs old), female (young vs old) and young (male vs female) and old (male vs female) for each genotype as well as among genotypes. Bars with different superscripts are significantly different at $p < 0.05$ (two-way ANOVA with Bonferroni's multiple comparisons post-test, **Supplemental Table S4 and S5**). Data are represented as mean $\pm$ SEM. (Statistics for Activity: For comparison among genotypes: $F=1014.64$,

DFn=5; For comparison across sex and age $F=2034.81$, DFn=3; For interaction between genotype and sex: $F=72.92$, DFn=15; Statistics for strength of rhythm: For comparison among genotypes: $F=219.55$, DFn=5; For comparison across sex and age: $F=2009.56$, DFn=3; For interaction between genotype and sex: $F=107.70$, DFn=15).

Figure 3.

Accumulation of total protein carbonyls with age. Protein carbonyl levels (nmol.mg⁻¹ protein) were converted to fold change, setting the levels of young wild-type males (*w¹¹¹⁸* and Canton-S) as 1 and expressing all other data relative to it. Data are mean $\pm$ SEM of assays from three independent bio-replicates. Bars with different superscripts are significantly different at $p < 0.05$ using two-way ANOVA with Bonferroni's multiple comparisons post-test (**Supplemental Table S6**; Statistics for comparison among genotypes: $F=2.52$, $DFn=3$, $P = 0.0725$; For comparison across sex and age $F=367.09$, $DFn=3$; For interaction between genotype and sex: $F=0.31$, $DFn=9$, $P=0.96$).

Figure 4.

Accumulation of lipid peroxidation product 4-hydroxynonenal with age. 4-HNE levels (pmol.mg⁻¹ protein) were converted to fold change, setting the levels of young wild-type males (*w¹¹¹⁸* and Canton-S) as 1 and expressing all other data relative to it. Data are mean ± SEM of assays from three independent bio-replicates. Bars with different superscripts are significantly different at $p < 0.001$ using two-way ANOVA with Bonferroni's multiple comparison post-test (**Supplemental Table S7**; Statistics for comparison among genotypes: $F=3091.46$, $DFn=5$; For comparison across sex and age $F=26094.04$, $DFn=3$; For interaction between genotype and sex: $F=1256.92$, $DFn=15$).

Figure 5.

Gene (qPCR) expression of key signaling members involved in stress response and aging.

Expression levels of *dPGC-1*, *AMPK*, *dTOR*, *Akt*, *dFoxO*, *dSesn* genes in male and female flies at young and old ages from different fly lines with mutations in synthesis or transport of DA. Relative mRNA expression of wild type controls (*w*¹¹¹⁸ and Canton-S) was set as a reference (=1). Values represent mean $\pm$ SEM of three independent bio-replicates. Two-way ANOVA with Bonferroni's multiple comparisons post-hoc test was conducted to separate out the means (**Supplemental Tables S8-S13**). Values with different superscripts are significantly different at $p < 0.05$.

Table 1

Mean longevity (in days) of male and female flies with mutations in DA synthesis. Values are mean $\pm$ SD. Values in parenthesis are total number of individuals studied. In each row (Male vs Female, for a particular genotype) mean values followed by different alphabetic superscripts are significantly different at $p < 0.0001$. In each column, mean values followed by different symbols in superscripts are significantly different at $p < 0.001$.

Genotypes	Males	Females
Wild type (<i>w¹¹¹⁸</i> or Canton-S)	53.9 ^a $\Psi \pm 1.3$ (n=237)	61.5 ^b $\Psi \pm 0.9$ (n=230)
<i>Catsup</i> ²⁶	54.6 ^a $\Psi \pm 1.6$ (n=291)	57.9 ^b $\ddagger \pm 1.1$ (n=253)
<i>ple</i> ²	51.4 ^a $\ddagger \pm 1.4$ (n=291)	48.7 ^b $\& \pm 1.0$ (n=179)
<i>Pu</i> ^{Z22}	37.8 ^a $\& \pm 1.2$ (n=285)	50.5 ^b $\& \pm 1.0$ (n=140)
<i>Catsup</i> ²⁶ -revertant	55.1 ^a $\Psi \pm 2.2$ (n=248)	60.2 ^b $\Psi \pm 1.2$ (n=256)
<i>DTHg; ple</i>	53.6 ^a $\Psi \pm 1.7$ (n=247)	59.8 ^b $\Psi \pm 1.4$ (n= 214)

Table 2

Data representing the percentage of rhythmic flies (based on number of rhythmic individuals out of total number of flies tested for each genotype and sex), the period of rhythm (in constant darkness –DD) and the number of individuals of a genotype tested. Flies of each sex (male or female) and age (young or old) from each genotype were tested separately. Data for wild type flies is pooled data of *w¹¹¹⁸* and Canton-S flies which showed similar circadian locomotor behavior patterns. Period of rhythm is represented as mean (hours) $\pm$ SD.

Sex/Age	Genotype	% Rhythmic	Period of rhythm in DD (Hrs)	Sample size (n)
Male (young) 3-5 days	wild type	100	23.7 $\pm$ 0.2	32
	<i>Catsup²⁶</i>	70	23.4 $\pm$ 0.2	32
	<i>ple⁻²</i>	67	23.5 $\pm$ 0.2	48
	<i>Pu^{Z22}</i>	94	23.0 $\pm$ 0.1	48
	<i>Catsup²⁶-revertant</i>	100	23.6 $\pm$ 0.2	32
	<i>DTHg; ple</i>	98	23.5 $\pm$ 0.1	32
Male (old) ~ 45 days	wild type	63	24.3 $\pm$ 7.7	48
	<i>Catsup²⁶</i>	63	26.0 $\pm$ 7.0	40
	<i>ple⁻²</i>	69	26.6 $\pm$ 3.9	32
	<i>Pu^{Z22}</i>	25	20.3 $\pm$ 8.2	32
	<i>Catsup²⁶-revertant</i>	67	24.7 $\pm$ 6.2	48
	<i>DTHg; ple</i>	62	24.1 $\pm$ 2.2	32
Female (young) 3-5 days	wild type	100	23.9 $\pm$ 0.2	32
	<i>Catsup²⁶</i>	94	23.7 $\pm$ 0.1	32
	<i>ple⁻²</i>	75	23.6 $\pm$ 0.1	32
	<i>Pu^{Z22}</i>	100	23.8 $\pm$ 0.3	48
	<i>Catsup²⁶-revertant</i>	97	23.4 $\pm$ 0.2	32
	<i>DTHg; ple</i>	100	24.0 $\pm$ 0.1	48
Female (old) ~ 45 days	wild type	46	24.7 $\pm$ 7.1	26
	<i>Catsup²⁶</i>	12	24.5 $\pm$ 6.0	32
	<i>ple⁻²</i>	56	26.3 $\pm$ 5.3	36
	<i>Pu^{Z22}</i>	63	23.1 $\pm$ 7.1	32
	<i>Catsup²⁶-revertant</i>	48	24.6 $\pm$ 6.2	32
	<i>DTHg; ple</i>	52	24.4 $\pm$ 4.3	32