Serotonin Signaling Modulates Health and Lifespan in *Drosophila melanogaster*

by

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DEDICATION

This thesis is dedicated to Paula Rosenwinkel.

Your strength, determination, and intelligence were a constant inspiration to me.

I know you would be proud.

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Thank you from the bottom of my heart; I love you all.

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LIST OF CONTRIBUTIONS

This section provides clarity regarding my individual contributions to the various sections in this thesis, as fellow lab members made significant experimental and/or intellectual contributions to the projects described here.

The overarching goal of this thesis is to understand how manipulations of serotonin signaling influence lifespan using *Drosophila* as the model system. All of the text in the introductory Chapter I was written by me and some portions of this text were included in a review article authored by myself and other lab members (Gendron, Chakraborty et al. 2020). Chapter II details the characterization of each serotonin receptor mutant in *Drosophila* health and lifespan. I collected all the data associated with Chapter II and wrote the entire text for this chapter. Chapters III and IV provide more detailed insight into mechanisms by which one serotonin receptor, 5-HT2A, influences lifespan. Chapter III contains my first-authored publication (Munneke, Chakraborty et al. 2022), for which I collected the majority of data with some contributions from co-authors, Dr. Tuhin Chakraborty and Saige Porter. The direction of this project was aided by my mentor, Dr. Scott Pletcher, and a fellow lab member who made significant intellectual contributions, Dr. Christi Gendron. Dr. Scott Pletcher also provided guidance on the written manuscript. Chapter IV is based on a co-authored publication from a project in which I generated data and contributed intellectual effort throughout the writing and revisions of the manuscript (Chakraborty, Gendron et al. 2019). Specifically, I generated the data presented in Fig. 4.6, assisted with many of the behavioral choice experiments (ex. Fig. 4.1), and assisted with the data collection for the sensory-modality sufficiency experiments. Finally, the conclusions presented in Chapter V are all original writing.

ABSTRACT

Sensory perception of environmental cues such as nutrients, mates, and threats, modulates aging across taxa. These effects on lifespan require the action of conserved neuromodulators, such as serotonin. Despite clear links between serotonin and lifespan in specific environmental contexts, little was known about the influence of serotoninergic signaling pathways on lifespan in normal environments. I therefore, aimed to understand how manipulation of serotonin signaling through each of its receptors influenced lifespan in *Drosophila.*

I first discovered that loss of specific serotonin receptors induces profound differences in lifespan. Of the five receptors for serotonin, loss of three led to a shortened lifespan in both males and females; however, loss of two receptors (5-HT2A and 5-HT7) caused a lifespan extension in female flies, with no effect on male lifespan. Behavioral characterization of these receptor mutants revealed most mutants do not display broad changes in sleep, activity, and feeding behavior. This 1) implies loss of individual receptors does lead to drastic negative health consequences, and 2) decouples the lifespan extension from any behavioral changes. This supports the notion that loss of receptor signaling pathways influence lifespan, independent of behavioral changes.

Of the changes in lifespan across the 5-HT receptor mutants, I was most interested in the manipulations that extended lifespan, loss of 5-HT2A or 5-HT7. I focused on the mechanisms underlying the lifespan extension in the *5-HT2A*$^{-/-}$ mutant in greater detail, as additional data in the lab revealed 5-HT2A was an important modulator of lifespan in two separate contexts. I was involved with one of these projects studying the mechanisms by which flies' perception of presumed threat cues in the environment influenced lifespan. Specifically, visual detection of dead conspecifics shortens lifespan, and this effect requires 5-HT2A signaling. Further, a

previous project identified that lifespan is shorted when flies must choose between sugar and protein consumption and this lifespan phenotype requires 5-HT2A.

Based on these data, I suspected 5-HT2A plays an important role in evaluation of the nutritional environment, and disruption of this by loss of 5-HT2A may influence lifespan. and I found that 5-HT2A plays an important role in determining a protein consumption target in the fruit fly. My findings indicate that 5-HT2A signaling is recruited to promote protein consumption, perhaps by establishing a heightened protein consumption target, and to enact a physiological state of higher protein utilization that subsequently accelerates aging. In the absence of this receptor, protein demand remains chronically unsatisfied, which leads to an adaptive lowering of protein utilization and metabolism, extending lifespan.

Together, the results from these projects support the notion that manipulating distinct aspects of serotonin signaling induces variable effects on lifespan. Closer examination of one receptor, 5-HT2A, revealed new insights into the ways in which organisms cope with nutrient stress and highlight how perception of nutrient demand influence lifespan. This work also opens the door for further investigations into the direct effects on lifespan that result from manipulations of serotonin signaling pathways.

CHAPTER I

Introduction

The Central Nervous System as a Modulator of Aging

For centuries, inquisitive minds have wondered why we age and how to slow the process. More than 1,500 years ago, the "Father of History," Herodotus, wrote of the Fountain of Youth, a pool which restored vitality to those who swam in its waters. Great heroes and explorers alike, from Alexander the Great to Juan Ponce de Leon, spent years searching the world for this elixir of life. Yet until the 20th century, little was known about ways in which to slow aging. Studies in the early 1900's demonstrated that experimental manipulation of diets significantly extended lifespan in two distinct model systems, fruit flies and rodents (Kopec 1928, McCay, Crowell et al. 1935). This suggested that specific interventions, such as dietary modulation, were conserved across species, offering hope that aging could also be influenced in humans.

Still, little was known about the molecular mechanisms underlying aging interventions. The biological study of aging gained substantial momentum when Cynthia Kenyon and colleagues identified that loss of specific genes in the nematode model, *C. elegans*, extended lifespan by up to 30% (Apfeld and Kenyon 1999). Interestingly, many of these genes were involved in processing sensory information, such as olfactory cues. This provided the first evidence that genetic manipulation of specific sensory pathways was sufficient to alter lifespan, regardless of environmental conditions, and this concept has served as the basis for my dissertation work.

It is no surprise that neuronal pathways influence lifespan; perception of environmental cues and execution of the appropriate responses is paramount to an organism's survival and reproduction, the keys to maximizing overall evolutionary fitness. Since Kenyon's seminal work, many additional studies have shown that sensory pathways impact health and aging. Manipulation of specific senses is sufficient to alter lifespan in fruit flies; genetically smell-blind flies are long-

lived (Libert, Zwiener et al. 2007), and olfactory perception of sex hormones shortens lifespan in both fruit flies (Gendron, Kuo et al. 2014) and *C. elegans* (Maures, Booth et al. 2014). In mammals, altering odor perception influences peripheral fat metabolism (Riera, Tsaousidou et al. 2017) and loss of pain signaling pathways extends lifespan (Riera, Huising et al. 2014), further underscoring the conserved role of the central nervous system as a modulator of lifespan.

Serotonin Acts as a Conserved Modulator of Lifespan

In many cases, the lifespan alteration by perceptive events is dependent on highly conserved neurotransmitters, such as serotonin (5-hydroxytryptamine, or 5-HT). First, some context on the conservation of serotonin receptors across phylogeny: serotonin is an incredibly ancient, and thus highly important, molecule found in several protozoan species and nearly all metazoans (Turlejski 1996). This single molecule accomplishes an amazing array of biological processes, ranging from mood and perception to body temperature and sleep (Berger, Gray et al. 2009). Its tissue-specific expression patterns and its binding to 15 different receptors in humans help specify its various roles. Serotonin receptors are categorized into seven families, named 5-HT1 through 5-HT7, and grouped based on their associated signaling pathways. G Protein-Coupled Receptors (GPCRs) comprise all but one of these seven classes, and as such, exert their functions by activating or inhibiting downstream signaling cascades, depending on the receptor (Saudou and Hen 1994). *Drosophila* possess five GPCR serotonin receptors named for the family they most closely resemble based on sequence alignment: *5-HT1A*, *5-HT1B*, *5-HT2A*, *5-HT2B*, and *5-HT7* (Blenau and Thamm 2011). *C. elegans* have three GPCR serotonin receptors; *ser-1* (homologous to mammalian *5-HT2B/5-HT2C)*, *ser-4* (homologous to mammalian *5-HT1*), and *ser-7* (homologous to mammalian *5-HT7*) (Tierney 2018). *C. elegans* also possess atypical receptors for serotonin. These include *mod-1*, which enables both serotonin-dependent GPCR activity and acts as an ion channel, and *ser-6*, which responds to octopamine, the invertebrate orthologue of norepinephrine (Axelrod and Saavedra 1977), but closely resembles the mammalian *5-HT5A* receptor (Tierney 2018).

Work from our lab and others have found that specific environmental manipulations influence lifespan in both *Drosophila* and *C. elegans* in a serotonin-dependent manner. When flies are presented with a choice between yeast and sugar, the two major calorie sources in the laboratory

diet, their lifespan differs from that of controls exposed to a homogenous mixture of these ingredients, independent of changes in individual nutrient consumption; this lifespan phenotype was dependent on neuronal signaling through the 5-HT2A receptor (Ro, Pak et al. 2016, Lyu, Weaver et al. 2021). In fruit flies, perception of presumed threat cues, such as the presence of dead conspecifics, alters lifespan in a visual and olfactory cue-dependent manner, which also requires serotonin signaling through the 5-HT2A receptor (Chakraborty, Gendron et al. 2019).

Despite clear links between serotonin and lifespan in specific contexts, little is known about the ways in which manipulation of serotonin signaling pathways influences lifespan under standard conditions. Some evidence from *C. elegans* indicates that loss of the *5-HT2* homologue, *ser-1*, extends lifespan by more than 30%; however, loss of the *5-HT1* homologue, *ser-4*, shortens lifespan (Murakami and Murakami 2007). In both cases, little is known about the mechanism by which loss of these receptors influence lifespan, aside from the possible involvement of insulin signaling in the *ser-1* mutant lifespan extension. A large drug screen in *C. elegans* also identified a broad serotonin receptor antagonist and atypical antidepressant, mianserin, as capable of extending lifespan (Rangaraju, Solis et al. 2015). Despite this, however, it still remains largely unclear how individual 5-HT receptors influence health and aging under standard conditions, and this question serves as the basis for the investigations in Chapter II. There are certain contexts in which serotonin, among other neurotransmitters and neuromodulators, has been implicated as an important modulator of lifespan. These are summarized in the following sections on nutrient perception and threat perception. These studies provided important insights into the mechanisms by which direct manipulations of serotonin signaling pathways may influence lifespan in standard conditions.

Nutrient Perception and Aging

Arguably the most important environmental input for an organism to process are nutrient cues, as they are constantly changing in natural habitats, and organisms must be able to sense this and appropriately adjust behavior and physiology to maximize fitness. Given this, it is not surprising that dietary restriction (DR) is the most well-studied intervention known to increase lifespan across diverse taxa, including worms (Lakowski and Hekimi 1998), dogs (Masoro 2002), and primates (Mattison, Colman et al. 2017, Pifferi, Terrien et al. 2018). Building upon these studies,

it is now known that even the way in which individual nutrients are presented may be important; simply separating the sugar and yeast components of the *Drosophila* diet, thereby allowing the animals to decide on the pattern and amount of nutrient uptake, modulates lifespan independent of the amount of each nutrient consumed (Ro, Pak et al. 2016). These studies provide compelling evidence that aging is influenced not only by caloric intake, but also by the type, quantity, consumption, and presentation of individual dietary components.

Perception of dietary components is sufficient to modulate aging and metabolic health independent of food intake in a variety of model systems, including *C. elegans* (Alcedo and Kenyon 2004), *Drosophila* (Ostojic, Boll et al. 2014), and mice (Riera, Tsaousidou et al. 2017). In *C. elegans*, food-derived odors were sufficient to increase insulin-like peptide 6 and decrease lifespan, effects that required sensory neurons that are lost in *tax-2*/*tax-4* mutants (Artan, Jeong et al. 2016). In flies, food odors partially reversed the longevity-extending benefits of dietary restriction, and olfactory-deficient flies lived up to 50% longer than control animals (Libert, Zwiener et al. 2007). Taste-blind flies lived longer than control flies, despite eating more (Ostojic, Boll et al. 2014). Ablation of mature olfactory sensory neurons in mice stimulated sympathetic nerve activity that resulted in β-adrenergic receptor activation in adipocytes to promote fat degradation, suggesting a possible neuronal mechanism by which mice were resistant to diet-induced obesity (Riera, Tsaousidou et al. 2017). The environmental cues that elicit effects on lifespan are often species specific and ecologically relevant. For example, olive flies (*Bactrocera oleae*) exposed to alpha-pinene, a common plant compound that is present in both olive fruit and leaves, exhibited increased lifespan and reproduction (Gerofotis, Ioannou et al. 2016).

These examples highlight a growing realization of the link that exists between dietary cues and specific neural or endocrine circuits that modulate healthy aging. There is a large established literature on the metabolic effects of protein intake and an emerging literature on the neuronal effects of protein sensing (Journel, Chaumontet et al. 2012). Many animals will seek and ingest a protein source when starved of it, suggesting they have dedicated protein sensors (Ro, Pak et al. 2016), although there is little known about the molecular details behind this perception. In mammals, the T1R1- and T1R3-type receptors are found on taste cells and form a complex that

is responsible for amino acid sensing. In invertebrates, the ionotropic receptor Ir76b was recently identified to mediate responses to amino acids (Croset, Schleyer et al. 2016). It is not currently known what role, if any, these receptors have in aging.

Most of what is known about the impact of neuronal protein sensing on aging comes from research using *Drosophila*. Flies deprived of amino acids or starved of all nutrients switch from a diet comprised primarily of sugar to one primarily of protein (Steck, Walker et al. 2018). This behavioral switch in feeding preference requires both serotonin signaling through the 5-HT2A receptor and plasticity of a dopaminergic circuit (Ro, Pak et al. 2016, Liu, Tabuchi et al. 2017). While it is known that tryptophan or glutamine supplementation suppresses the dopaminergic plasticity that occurs during starvation (Liu, Tabuchi et al. 2017), the precise molecular intermediate(s) that signal protein starvation as well as the mechanism through which serotonin and dopaminergic circuits drive starvation-induced behaviors are unknown. When the two primary macronutrients in the diet, sugar and protein, are presented separately to flies so that they behaviorally construct the composition of their own diet, they live shorter than when presented with a single, complete diet (Ro, Pak et al. 2016). This effect also requires serotonin signaling through the serotonin receptor 5-HT2A (Ro, Pak et al. 2016).

Nutrient Perception States Mediate Longevity

In humans, sensory perception influences motivation, arousal, drive, and emotion. These states are encoded in our brains, and they subsequently control, in a causal sense, behavior, physiology, and conscious experience. Given that sensory perception of nutrients is sufficient to alter lifespan, it seems reasonable to speculate that the related neural states, such as those of hunger or satiety, also modulate aging. Specifically, discrepancies between nutrient expectation and consumption, and the putative adaptive coping responses, seem to modulate lifespan in both *Drosophila* and *C. elegans*, and rely on serotonin signaling to exert these effects.

Activation of serotonergic hunger neurons induces increased feeding in flies, which restores to baseline after sustained activation for several weeks (Weaver, Holt et al. 2022), indicative of an adaptive response to perceived inability to satisfy long-term nutrient demand. These flies are also long-lived, suggesting that the altered nutrient set-point is potentially beneficial to lifespan

(Weaver, Holt et al. 2022). This may occur through the serotonin receptor 5-HT2A; in flies, *5-HT2A*$^{-/-}$ null mutants display a reduced protein feeding target relative to controls, which consume to a similar protein level across diets of different compositions (Munneke, Chakraborty et al. 2022). These *5-HT2A*$^{-/-}$ mutants are also long-lived, which recapitulates the pattern of increased longevity and lowered nutrient set-points seen upon activation of hunger neurons. Similarly, differences between nutrient expectation and consumption alter lifespan in *C. elegans*. Worms that can smell, but not access food, are short-lived relative to those on DR, and blocking serotonin or dopamine signaling prevents this shortening of lifespan (Zhang, Jun et al. 2021, Miller, Huang et al. 2022). Additionally, certain drugs that antagonize serotonin signaling do not further extend lifespan when worms are dietary restricted (Petrascheck, Ye et al. 2007), suggesting blocking aspects of serotonin signaling may mimic nutrition deprivation, even when organisms are fully fed, decoupling the effects on longevity from direct changes in consumption.

Other populations of neurons that respond to nutrient cues have yet to be directly linked to longevity; however, they represent promising candidates for future study. In *Drosophila*, a few different neuronal populations have been implicated in evaluating nutrient availability and influencing the hunger state. Inactivation of four GABAergic interneurons resulted in voracious eating regardless of prior feeding, indicating that they are involved in hunger or the motivation to feed (Pool, Kvello et al. 2014). An additional set of cholinergic interneurons increased their activity in response to sucrose during the starvation state (Yapici, Cohn et al. 2016), suggesting that they are part of a complex, multi-input circuitry. In line with this, 12 pairs of *SLC5A11*-expressing neurons in the central fly brain responded to starvation by increasing their excitability via regulation of a potassium channel (Park, Dus et al. 2016). Second-order sweet taste neurons became more sensitive to sucrose following starvation, indicating that they may also be hunger sensors (Kain and Dahanukar 2015). Other neurons that regulate the activity of dopaminergic protocerebral posterior lateral region 1 (PPL1) "punishment neurons" have been proposed to mediate motivational states, including appetitive memory (Krashes, DasGupta et al. 2009, White, Humphrey et al. 2010). Taotie neurons encode an apparent hunger-like state that is independent of energy state, and their activation or inhibition influences feeding behavior regardless of whether a fly is fully fed or starved (Albin, Kaun et al. 2015, Zhan, Liu et al. 2016). They also regulate insulin secretion (Zhan, Liu et al. 2016).

In a broader context, a range of species, including mollusks, nematodes, and insects, increased serotonin release generally promotes hunger, as evidenced by increased feeding or elongation of an already initiated meal. However, lack of serotonin does not necessarily indicate satiety, as its release is not required to initiate feeding in mollusks or nematodes. Therefore, the level of serotonin is likely reflective of nutrient demand, which is adaptive and malleable. In nature, organisms must cope with changes in the nutritional environment and optimize behavior and physiology to maximize overall fitness, with responses tailored towards both short-term and long-term adaptions (López-Maury, Marguerat et al. 2008). For instance, in some cases of limited food availability, it would be most adaptive to relocate to find a nutrient-dense food source and activate pathways that promote foraging behavior (Searle, Thompson Hobbs et al. 2005, Pretorius, de Boer et al. 2011). However, in situations of chronic nutrient stress animals must alter their feeding behavior and physiology to survive and reproduce in relatively harsh conditions (Rodgers, Lerin et al. 2008). We can infer that this long-term adaption occurs via changes to nutrient demand level, encoded by the level of serotonin release, and induces changes in nutrient mobilization and usage to maximize survival, which in turn, beneficially influences lifespan. This notion that altered nutrient set-points, encoded by serotonergic neurons, modulate lifespan serves as the basis for my investigations in Chapter III.

Threat Perception Influences Longevity

In addition to nutrient perception, a key adaptive capability found in every organism is the ability to detect and respond appropriately to potential threats for survival, including predators, parasites, disease, and even death. Ants, zebrafish, scrub jays, elephants, and nonhuman primates all behaviorally respond to sick or dead conspecifics (Anderson 2016). There is also evidence of behavioral effects associated with exposure to sick and dead conspecifics in humans, where stressful stimuli have been associated with depression, anxiety, and broader physiological issues that may potentially influence lifespan (Petrie, Milligan-Saville et al. 2018). In *C. elegans*, exposure to media containing excretions from starved, predatory *Pristionchus pacificus* elicited immediate avoidance behaviors that require ASI, ASJ, ASH, and ADL sensory neurons (Liu, Kariya et al. 2018). *C. elegans* also avoid environmental threats such as media containing homogenized worm extract; this response requires cGMP signaling in ASI and ASK sensory

neurons (Zhou, Loeza-Cabrera et al. 2017). Interestingly, many of these specific sensory neurons have also been implicated in the regulation of *C. elegans* lifespan. ASI neurons are required for the lifespan extension following dietary restriction (Bishop and Guarente 2007), ASJ neurons modulate lifespan in response to food and temperature cues (Artan, Jeong et al. 2016, Zhang, Gong et al. 2018), and G protein signaling in the nociceptive ASH/ADL neurons modulates lifespan (Alcedo and Kenyon 2004). It is therefore attractive to speculate that these neurons may also modulate lifespan in response to perceived environmental threats.

Links between potential threats, behavior, and lifespan have also been established in *Drosophila*. Flies are capable of socially transmitting information about perceived threats. When housed with a parasitic wasp species, female *Drosophila* reduced their egg laying, and they communicated threat presence to naïve flies using wing movements, who also suppressed their egg laying (Kacsoh, Bozler et al. 2015). Behavioral and physiological responses to threats may also have long-term effects, including changes in lifespan. For example, when healthy flies were exposed to dead conspecifics, they became leaner, experienced reduced climbing ability, were aversive to naïve flies, and had reduced lifespan. The effects of exposure to dead conspecifics on aversiveness and lifespan required visual and olfactory function in the exposed flies, and the sight of dead flies produced changes in the head metabolome. Genetic and pharmacologic attenuation of serotonergic signaling eliminated these effects, providing evidence for an evolutionarily conserved neural mechanism that links threat perception with changes in behavior and aging (Chakraborty, Gendron et al. 2019) and this served as the basis for the investigations in Chapter IV.

Drosophila melanogaster as a Model Organism

The fruit fly, *Drosophila melanogaster*, is an ideal model to investigate the links between serotonin signaling and lifespan for several reasons. The relatively simple nervous system in *Drosophila*, consisting of approximately 100,000 neurons, make it feasible to identify links between neurons and elucidate causal relationships with behavioral and physiological outcomes. Additionally, as a long-standing model organism, a diverse toolkit of genetic reagents exists to manipulate genes or neuronal activity, both globally and in a cell- or tissue-specific manner. Lastly, flies are ideal for aging experiments as their short generation time and lifespan are

approximately three months. Together, the combination of precise genetic control, a relatively simple nervous system, and short lifespan make *Drosophila* the ideal model for establishing connections between specific serotonergic pathways and health and lifespan outcomes.

Summary and Outstanding Questions

The ways in the central nervous system integrates and interprets information about the environment is a potent modulator of lifespan across taxa. Sensory detection of nutrients, potential mates, and possible threats in the environment have all been shown to influence aging (Gendron, Kuo et al. 2014, Ro, Pak et al. 2016, Chakraborty, Gendron et al. 2019). This processing of various environmental stimuli requires the action of conserved neuromodulators, such as serotonin, to ultimately influence broad organismal physiology and lifespan. This suggested that manipulation of serotonin signaling directly may be sufficient to alter lifespan; however, this has not been previously thoroughly examined. The first question addressed in this thesis is how manipulation of individual serotonin signaling pathways health and lifespan in *Drosophila* (Chapter II). I then sought to investigate how loss of one serotonin receptor, 5-HT2A extended lifespan, building upon links between this receptor and nutrient perception (Chapter III) and threat perception (Chapter IV) in the context of aging.

CHAPTER II

Characterization of Serotonin Receptors in Health and Lifespan

Abstract

Despite the importance of serotonin as a conserved modulator of lifespan in specific environmental contexts, little was known about the role of specific serotonin signaling pathways in modulating health and lifespan. Using null mutants for the five serotonin receptors in *Drosophila*, I found that loss of either 5-HT1A, 5-HT1B, or 5-HT2B induces a shortening of lifespan in both males and females, whereas loss of 5-HT2A or 5-HT7 causes a lifespan extension in females only. To better understand the effects of loss of each receptor in females, we performed a series of behavioral assays including sleep, activity, feeding behaviors, and starvation resistance. Across all mutants, we observed no changes in activity, and only a slight reduction in daytime sleep in *5-HT1A*$^{-/-}$ mutants with no changes in overall activity levels, indicative of fragmented sleep. 5-HT2B is potentially important for promoting sucrose feeding interactions, and all receptor mutants are starvation sensitive. As no trends emerged linking behavioral outcomes with lifespan phenotypes, this helps eliminate changes in behavior as causative for changes in lifespan, and highlights the need for closer examination of the mechanisms by which these receptors modulate aging, particularly the long-lived *5-HT2A*$^{-/-}$ *and* *5-HT7*$^{-/-}$ mutants.

Introduction

Given the importance of specific serotonin signaling pathways in mediating the effects of environmental inputs on longevity, we asked how manipulating different aspects of serotonin signaling influenced lifespan under standard conditions. *Drosophila* possess five serotonin (5-hydroxytryptamine, or 5-HT) receptors named based on the family of mammalian receptors they mostly closely resemble in terms of coupled downstream signaling mechanisms: *5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B,* and *5-HT7*. All are G protein-coupled receptors (GPCRs) coupled to either excitatory or inhibitory G protein complexes, that either increase or decrease the levels of second messengers, cyclic AMP (cAMP) or Calcium (Ca^{2+}) (Saudou and Hen 1994, Tierney 2018). Serotonin's binding to these receptors therefore influences neuronal firing by promoting (*5-HT7*) or inhibiting (*5-HT1*-type) cAMP production (Witz, Amlaiky et al. 1990, Saudou, Boschert et al. 1992) or activating Phospholipase C (PLC) signaling cascades (*5-HT2*-type), which increase the levels of intracellular Ca^{2+} (Blenau, Daniel et al. 2017).

All 5-HT receptors are expressed neuronally and published RNAseq data indicates that all but *5-HT2B* are also expressed in peripheral tissues and ganglia (Leader, Krause et al. 2017). *5-HT1A* and *5-HT1B* are expressed at low levels in the thoracico-abdominal ganglion in both sexes (Leader, Krause et al. 2017). *5-HT2A* and *5-HT7* are expressed at high and low levels, respectively, in the salivary gland and expressed at low levels in the thoracico-abdominal ganglion and crop of both males and females (Leader, Krause et al. 2017). Within the brain, these receptors also display distinct expression patterns (Gnerer, Venken et al. 2015). *5-HT1A* is expressed in the pars intercerebralis (PI, neuroendocrine center in the brain), antenna lobe (AL, olfactory sensory processing region), optic lobe (OL, visual perception region), suboesophageal ganglion (SOG, gustatory sensory processing region), and mushroom body α and β lobes (MB, olfactory learning and memory region) (Gnerer, Venken et al. 2015). *5-HT1B* displays a similar pattern as *5-HT1A* but is also expressed in the γ lobe of the MB (Gnerer, Venken et al. 2015). *5-HT2A* is strongly expressed in the OL, as well as the regions of the central brain, specifically the ellipsoid body (EB) and dorsal fan-shaped body (FSB) (Gnerer, Venken et al. 2015). The EB and FSB are part of the central complex (CC), a region of the brain previously thought to be almost exclusively involved in directed movement in response to visual stimuli (Pfeiffer and Homberg

2014); however, recently, the CC have been shown to mediate nutrient perception and decision-making (Musso, Junca et al. 2021, Sareen, McCurdy et al. 2021), as well as complex states induced by stressful environments (Gendron, Chakraborty et al. 2022). *5-HT2B* shares expression in the EB but is also strongly expressed in the PI (Gnerer, Venken et al. 2015). Lastly, *5-HT7* is also expressed in the EB and PI but displays some glial staining not seen in the other receptors (Gnerer, Venken et al. 2015). Additionally, these regions do not fully encompass the expression patterns, as most receptors also express in some cells not strongly associated with a particular region of the brain.

Serotonin signaling through at least some receptors is known to modulate lifespan. Perception of threats in the environment shortens lifespan and requires signaling through the 5-HT2A serotonin receptor (Chakraborty, Gendron et al. 2019). When flies are presented with the opportunity to construct their own diet as opposed to the standard homogenous nutrient mixture, lifespan is shortened in a manner dependent on neuronal signaling through 5-HT2A (Ro, Pak et al. 2016, Lyu, Weaver et al. 2021). In *C. elegans*, the lifespan extension via dietary restriction is reduced when worms can smell, but not access, food, and this effect requires both dopamine and serotonin signaling (Zhang, Jun et al. 2021, Miller, Huang et al. 2022). However, little was known about the influence of serotonin signaling pathways on lifespan in non-stressful conditions.

Results

5-HT2A$^{-/-}$ and *5-HT7*$^{-/-}$ mutant females are long-lived

We obtained null mutants for each receptor, generated by inserting a *GAL4* element into the first coding exon, rendering the receptor non-functional (Qian, Cao et al. 2017). To minimize any potential background effects on behavior or lifespan, we backcrossed each mutant to the laboratory control stock (w-;CS) for 11 generations. We first asked how loss of each receptor would influence lifespan in both males and females, as this had not never been investigated in detail, in *Drosophila*. We observed a striking level of variability in lifespan for each mutant, as well as sexually dimorphic effects (Fig. 2.1 and 2.2). In females, we found that *5-HT1A*$^{-/-}$, *5-HT1B*$^{-/-}$, and *5-HT2B*$^{-/-}$ mutants were all short-lived relative to controls (Fig. 2.1a,b,d). However,

5-HT2A$^{-/-}$ and *5-HT7*$^{-/-}$ mutant females were long-lived relative to controls (Fig. 2.1c,e). Interestingly, the short-lived phenotype of *5-HT1A*$^{-/-}$, *5-HT1B*$^{-/-}$, and *5-HT2B*$^{-/-}$ mutants were mimicked in males, but the lifespan of *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutant males was not significantly different from controls (Fig. 2.2).

For the purposes of this study, additional behavioral phenotyping of the receptor mutants focused primarily on females, as only females displayed a lifespan extension for certain receptor mutants (Fig. 2.1). To obtain a deeper understanding of the mechanisms underlying the lifespan extensions in the *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutant females, we performed a series of behavioral assays typically associated with overall health in *Drosophila*: sleep, activity, starvation resistance, and feeding behavior. An important point to note is that these assays are not used to determine whether altered sleep, for example, plays a causative role in altering lifespan. Rather, we can use these health metrics as additional readouts for metabolic processes and/or neural states that are altered upon loss of signaling through specific 5-HT receptors, which may also impact lifespan.

Loss of 5-HT1A leads to short and fragmented sleep

To evaluate sleep and activity, we used the *Drosophila* Activity Monitor (DAM) set-up, in which flies are housed individually in small tubes and their activity breaks an infrared beam in the center of the tube. Sleep is defined as five continuous minutes without a beam break (Hendricks, Finn et al. 2000, Shaw, Cirelli et al. 2000, Huber, Hill et al. 2004). When examining how loss of each receptor impacted activity, we observed no differences in any of the receptor mutants relative to the controls (Fig. 2.3). Despite no change in overall activity levels, *5-HT1A*$^{-/-}$ mutants displayed decreased daytime, but not nighttime, sleep (Fig. 2.4a). No large increase in activity counts, but a decrease in sleep bouts can only be explained by more frequent beam breaks during the day, enough to disrupt the lack of activity required to be measured as sleep, but not enough to significantly increase activity. This would be indicative of more fragmented sleep. This observation is consistent with the report that generated the null mutants used in these studies (Qian, Cao et al. 2017), as well as initial studies of sleep in *Drosophila* which examined the role of 5-HT1A, 5-HT1B, and 5-HT2, which refers to 5-HT2A as 5-HT2B had not yet been

discovered, nor had 5-HT7 (Yuan, Joiner et al. 2006). No other mutants displayed significant changes in sleep patterns (Fig. 2.4).

5-HT2B promotes sucrose feeding behaviors

We also asked how loss of each receptor influenced feeding behaviors. For these studies, we used the Fly Liquid-Food Interaction Counter (FLIC), which measures the precise timing and duration of fly feeding interactions through the closure of a circuit when a fly inserts its proboscis into a liquid food source (Ro, Harvanek et al. 2014). Using this method, we found that *5-HT2B*$^{-/-}$ mutants displayed decreased feeding interactions with a sucrose solution (Fig. 2.5d), which may be reflective of a lower motivation to feed. All other receptor mutants displayed behavior consistent with the controls (Fig. 2.5).

Loss of any 5-HT receptor leads to starvation sensitivity

Starvation resistance is also typically correlated with increased longevity (Johnson, Cypser et al. 2000, Lithgow and Walker 2002). Upon measuring the starvation resistance of all the receptor mutants, we found that all were significantly starvation sensitive (Fig. 2.6). This is somewhat surprising given that mutations that tend to increase lifespan, such as loss of 5-HT2A or 5-HT7, also make organisms more tolerant of other stressors; however, this is not always the case (Dues, Andrews et al. 2018). That said, a comprehensive analysis of oxidative, temperature, and heavy metal stressors, is a potential area for further study, and differences in stress tolerance across the receptor mutants may provide insight into specific stress pathways associated with increased longevity in *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutants.

Discussion

Investigation of mutants for the five serotonin receptors in *Drosophila* revealed a striking level of variability in the effects on lifespan. Loss of 5-HT1A, 5-HT1B, or 5-HT2B was detrimental to lifespan in males and females, but loss of 5-HT2A or 5-HT7 induced a female-specific lifespan extension (Fig. 2.1 and 2.2). The peripheral expression patterns of these receptors do not account for the variability in lifespan, suggesting the expression differences in regions of the brain may underlie the differences in lifespan across the receptor mutants. Within mutants, the sexually dimorphic effects seen in *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutants may be explained by sex-specific neural circuits. Based on large RNAseq data analysis (Janssens, Aibar et al. 2022), both *5-HT2A* and *5-HT7* co-express with the sex-specific transcription factor *fruitless* (*fru*) in the adult brain, and a limited population of neurons also seem to co-express all three genes. Neither *5-HT2A* nor *5-HT7* seem to co-express with another sex-specific transcription factor *doublesex* (*dsx*). Unfortunately, this dataset cannot provide insight into the specific populations or locations of the co-expressing genes. Antibodies do not exist for 5-HT2A and 5-HT7 in *Drosophila* currently; however, future studies could examine the precise *fru*$^{+}$ *5-HT2A*$^{+}$*/5-HT7*$^{+}$ neurons by driving expression of a reporter using *5-HT2A-GAL4* or *5-HT7-GAL4* and staining for Fru. This sexual dimorphism could be used as a tool to provide insight into the regions of the brain that influence aging, particularly if populations of *fru*$^{+}$*/5-HT2A*$^{+}$*/5-HT7*$^{+}$ exist. These populations could then be targeted using genetic reagents that divide the regulatory components of *GAL4* between two constructs, such that two genes must be expressed in the same cell for the *GAL4* to be functional (Luan, Peabody et al. 2006). These reagents could be used to drive expression of RNAi against *5-HT2A* or *5-HT7* in *fru*$^{+}$*/5-HT2A*$^{+}$or *fru*$^{+}$*/5-HT7*$^{+}$ and ask whether knock down of those receptors in *fru*$^{+}$ neurons is sufficient to extend lifespan.

Additionally, comparing the expression patterns of all 5-HT receptors in the brain, some trends emerge for regions associated with short vs. long life. Of the long-lived mutants, receptors 5-HT2A and 5-HT7, both receptors share expression in the EB and PI (Gnerer, Venken et al. 2015). However, PI expression may not be associated with long life, as 5-HT1A, 5-HT1B, and 5-HT2B are expressed in the PI (Gnerer, Venken et al. 2015). Therefore, the EB may be a promising candidate for exploring how the activity of this brain region modulates lifespan. The

EB may also be a good starting point for examination of sexually dimorphic *5-HT2A*$^{+}$ and *5-HT7*$^{+}$ circuits, and how those circuits influence lifespan.

Another potential reason for the differences in lifespan across mutants could arise from the different affinities of each receptor for serotonin, as global reductions in serotonin signaling increase lifespan in both *Drosophila* and *C. elegans* via loss of the rate-limiting enzyme in serotonin synthesis (Ro, Pak et al. 2016, Miller, Huang et al. 2022). *In vitro* experiments indicate the 5-HT2A and 5-HT7 receptors display approximately 10 to 100 times higher affinity, respectively, for serotonin than the least sensitive receptor, 5-HT1A (Gasque, Conway et al. 2013). 5-HT2A and 5-HT7 both induce excitatory responses when bound to serotonin (Tierney 2018), and perhaps the global decrease in excitatory signaling as result of loss of 5-HT2A or 5-HT7 is inherently beneficial to lifespan. Indeed, in humans, exceptional longevity is associated with a downregulation of genes related to neural excitation (Zullo, Drake et al. 2019). Further, global decrease of excitatory signaling by targeting glutamatergic or cholinergic signaling in *C. elegans* leads to increased longevity (Zullo, Drake et al. 2019). Although serotonin does not always elicit an excitatory response, these particular signaling pathways through 5-HT2A and 5-HT7 may induce a similar response, which ultimately mimics the global decrease in serotonin signaling shown to extend lifespan.

Characterization of the several behaviors of the 5-HT receptor mutants also revealed no clear association between lifespan and behavioral phenotypes. This is not surprising given that these assays were not conducted to ascertain whether loss of a specific receptor influenced lifespan via changes in behavior; rather, the goal was to identify how loss of specific receptors led to changes in behavior and/or lifespan, which would potentially provide insight into the neural state induced by loss of signaling through a given serotonin receptor. Aside from a known role for 5-HT1A in promoting proper sleep (Yuan, Joiner et al. 2006), it is worth noting that none of the other receptors mutants displayed changes in sleep and activity (Fig. 2.3 and 2.4), despite all receptor mutants showing either a lifespan extension or shortening. It is interesting that *5-HT2B*$^{-/-}$ mutants did not display a reduction in sleep as this was previously reported (Qian, Cao et al. 2017). This may be due to differences in diet, the age of the flies, and genetic background; as such, an experiment exactly mimicking the conditions used in the previous report would be beneficial.

Regardless, these data suggests that the serotonergic circuits regulating sleep and activity are largely independent of those that influence aging.

Examination of feeding behaviors revealed that 5-HT2B may play a role in promoting feeding motivation (Fig. 2.5d). 5-HT2B is one of the most recently discovered 5-HT receptors in *Drosophila*, and as such, little is known about its roles. Based on previous reports, both 5-HT2A and 5-HT2B may play important roles in regulating different aspects of feeding behavior. A broad serotonin receptor antagonist, metitepine, strongly suppresses feeding in *Drosophila* larvae, and this requires the 5-HT2A receptor (Gasque, Conway et al. 2013). This study differs from the FLIC assay, however, in the stage of development (larvae vs. adults) and the diets used. The FLIC assay contained only sucrose, but the metitepine studies used sucrose and yeast (primary source of protein in *Drosophila* diets). 5-HT2A is known to influence protein, but not sucrose, feeding behaviors (Ro, Pak et al. 2016), therefore, we would not have expected 5-HT2A feeding behavior to be significantly different from controls in the FLIC assay conducted in this study. Future studies could investigate the role of 5-HT2B in sucrose feeding motivation. Specifically, the feeding behavior of *5-HT2B*$^{-/-}$ mutants in the FLIC assay could be corroborated using a solid food assay that measures total consumption. Several diets using varying amounts of sucrose could be compared to evaluate whether *5-HT2B*$^{-/-}$ mutants show decreased consumption of sugar-containing food relative to back-crossed controls.

In summary, the 5-HT receptor mutants showed an expected high level of variability in effects on lifespan. In general, a lack of trends emerges between mutants that are long or short-lived and specific behavioral phenotypes. Rather, the sex-specific lifespan extension of *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutants may be used as a foothold for future studies investigating sex-specific neuronal circuits that modulate aging. Alternatively, these two receptor mutants may reflect a lifespan extension achieved by an overall reduction in excitatory signaling through serotonin receptors. Future studies could ask whether loss of both receptors is, thus, more beneficial than loss of one; this will also provide insight into potential epistasis between 5-HT2A and 5-HT7 in lifespan.

Methods

Fly Stocks

5-HT receptor mutants containing a *GAL4* element inserted into the first coding exon of the gene as previously described (Qian, Cao et al. 2017) were back-crossed to w-;CS for 11 generations prior to experimentation.

Sleep and Activity Measurements

Fly embryos were collected and washed with a 1% PBS solution and reared on standard CT fly media. Following eclosion, flies were mated for 2-3 days then separated by sex into 10% sucrose/10% yeast vials containing 20 flies and aged for one week. Flies were then sorted individually into small tubes containing SY10. The tubes were placed in Drosophila Activity Monitors (DAM), with each DAM containing a single receptor mutant and the *w-;CS* control (n=16/genotype). The DAMs were placed in an incubator with a 12:12 LD cycle and 60% RH, and allowed to acclimate to the conditions for 24 hours. Activity was assessed by measuring activity counts generated each time the fly crosses an infrared beam in the center of the tube, and sleep is defined as 5 minutes of continuous inactivity. Normalized activity represents the average activity of all flies in a given treatment normalized to the average activity per fly, and sleep patterns were analyzed using the Shiny-R software as previously described (Cichewicz and Hirsh 2018).

FLIC

Fly embryos were collected and washed with a 1% PBS solution and reared on standard CT fly media. Following eclosion, flies were mated for 2-3 days then separated by sex into SY10 vials containing 20 flies and aged for one week. Flies were tested on the Fly Liquid-Food Interaction Counter (FLIC) system as previously described to monitor feeding behaviors (Ro, Harvanek et al. 2014). Each liquid-food reservoir contained 5% (w/v) sucrose in 1% Tegosept (v/v) and 4 mg/l $MgCl_2$. Flies were anesthetized briefly on ice and manually aspirated into the *Drosophila* feeding monitors (DFMs). Each DFM was loaded with flies from at least two treatment groups to reduce technical bias from the DFM signals. Food interactions were analyzed using custom R code, which is available on GitHub at

https://github.com/PletcherLab/FLIC_R_Code. Default thresholds were used for analysis. Flies that had zero feeding events over the testing interval were removed from the analysis.

Starvation Resistance

Female flies were sorted on the third day after eclosion and maintained on SY10 media for 5 to 7 days before being used for experiments. Starvation resistance was measured using a high-throughput starvation survival assay that was developed in our laboratory and that takes advantage of the real-time activity recording by TriKinetics *Drosophila* activity monitors. Briefly, individual flies were placed into activity tubes with 2% agar and placed in DAM2 monitors (www.trikinetics.com). Activity counts were collected every 30 s; time of death was considered to be the last census time in which the activity count was greater than zero and was accompanied by at least two other periods of nonzero activity within a 6-hour window. This algorithm allowed an objective determination of death time and avoided artifacts caused by bumping or occasional false-positive measures from the DAM2. This assay was validated with visual observation. Analysis software written in the R programming language is freely available at https://github.com/PletcherLab/DAMSurvival.

Survival Assays

Lifespans were measured using established protocols (Linford, Bilgir et al. 2013). Unless otherwise noted, 10 replicate vials (~200 experimental flies) were established for each treatment. Flies were transferred to fresh media every 2-3 days, at which time dead flies were removed and recorded using the DLife system developed in the Pletcher Laboratory (Linford, Bilgir et al. 2013). Flies were kept in constant temperature (25°C) and humidity (60%) conditions with a 12:12 hr light:dark cycle.

Statistical Analysis

Differences in lifespan and starvation resistance curves were assessed using log-rank analysis. For comparisons of multiple mutants to a single genetic control, a One-way ANOVA w/ a Tukey Post-hoc test was used. When comparing differences between only two treatments in a single experiment, a two-sided t-test was used.

Figures

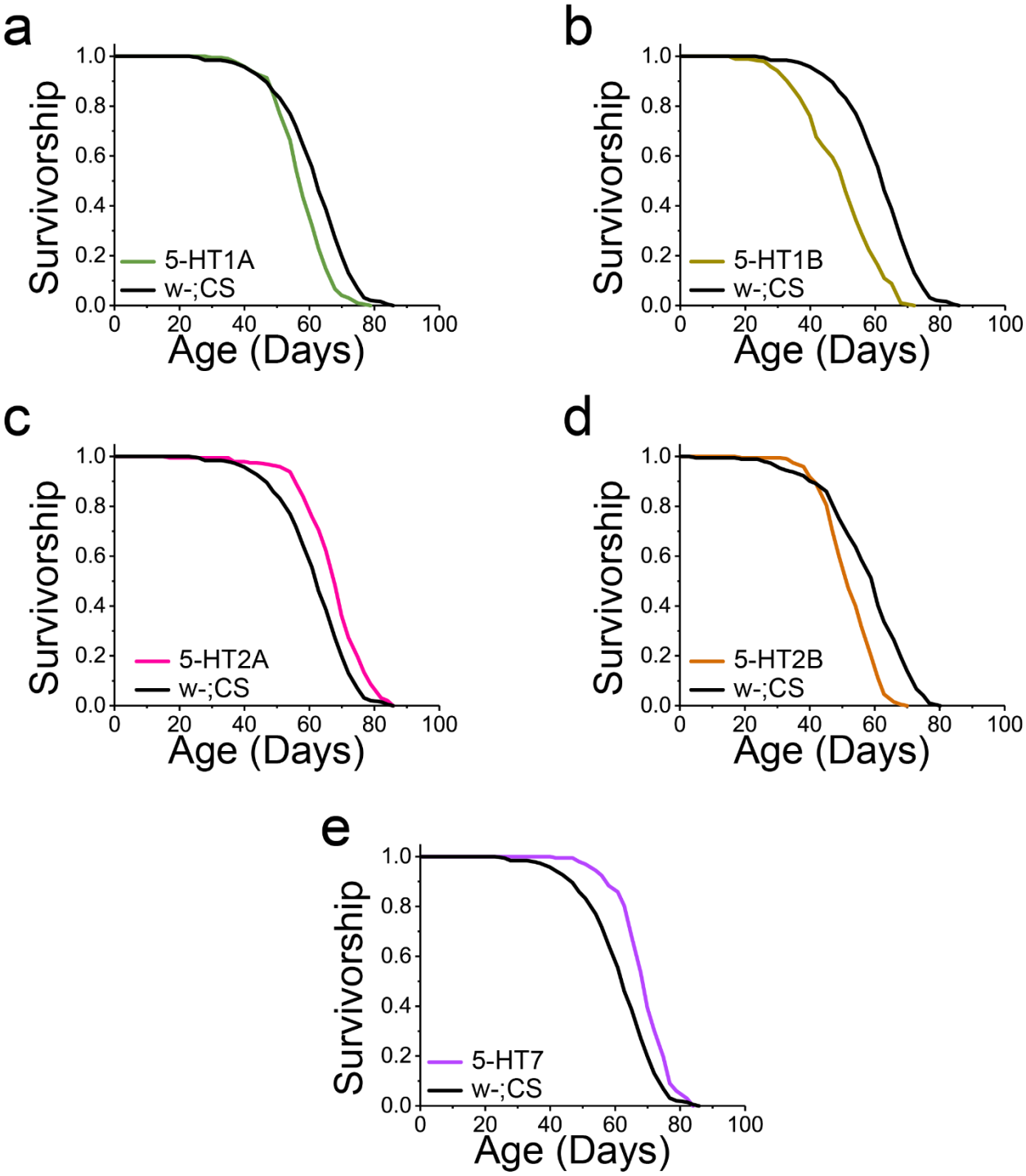


Figure 2.1. *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutant females display a lifespan extension. Mated female 5-HT receptor mutants were aged under standard lifespan conditions (N = 190-198; $p<0.001$ for all comparisons via log-rank analysis).

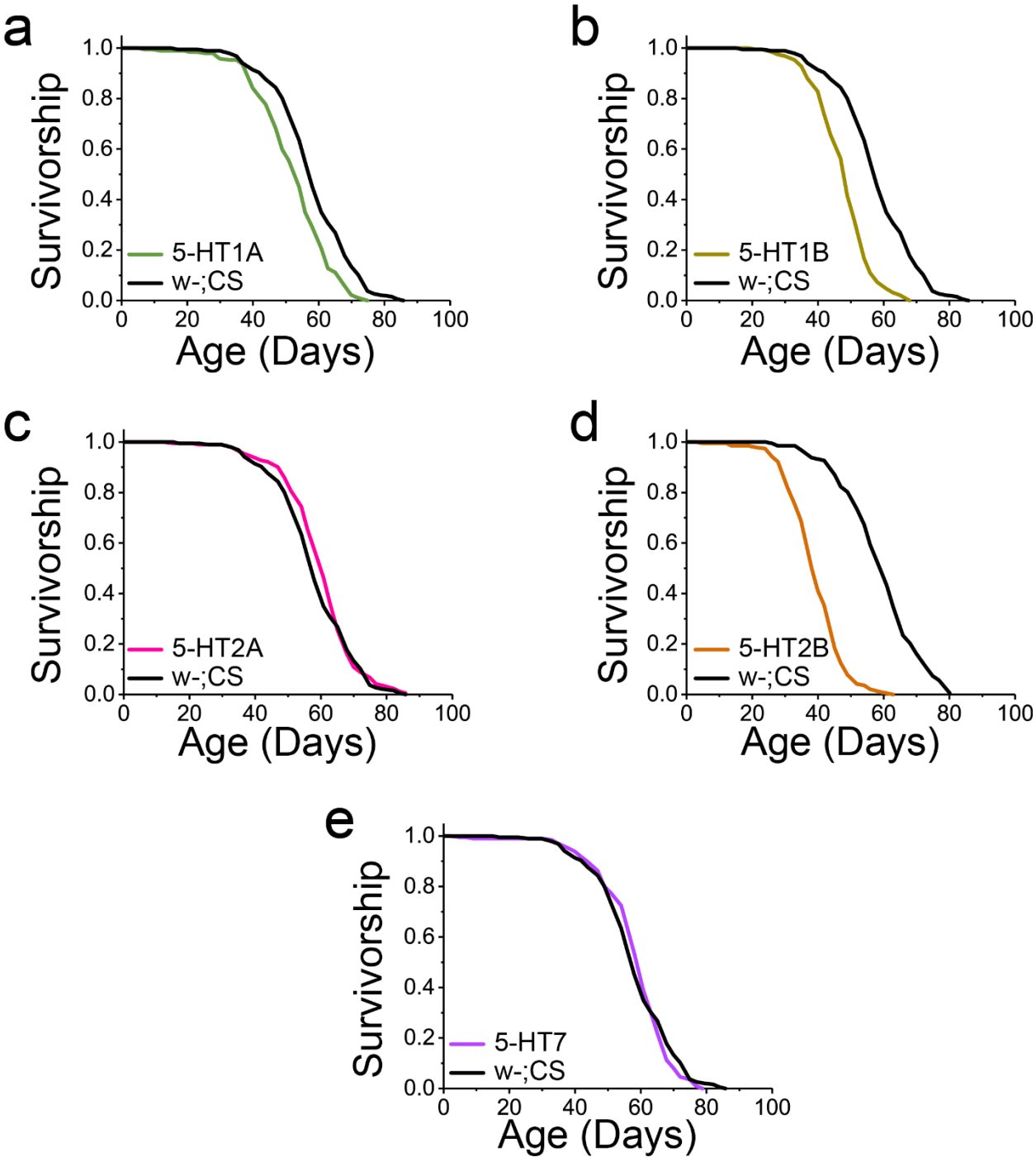


Figure 2.2. 5-HT receptor mutant males show varying effects on lifespan. All 5-HT receptor mutant males are short-lived or not significantly different from w-;CS controls (N = 186-196; *5-HT1A*$^{-/-}$ P < 0.001, *5-HT1B*$^{-/-}$ P < 0.001, *5-HT2A*$^{-/-}$ P = 0.237, *5-HT2B*$^{-/-}$ P < 0.001, and *5-HT7*$^{-/-}$ P = 0.591 via log-rank analysis).

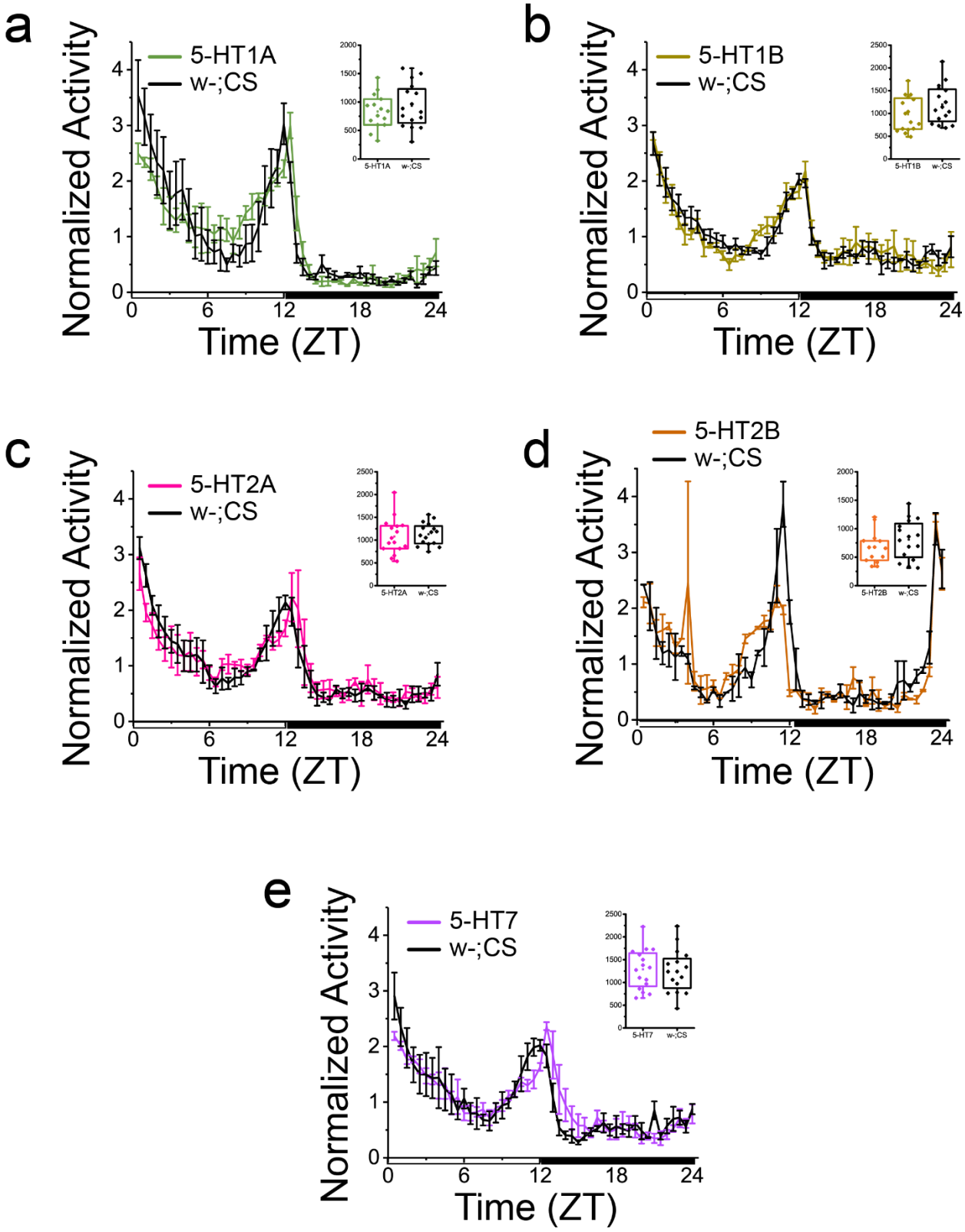


Figure 2.3. Female 5-HT receptors mutants do not exhibit altered activity. Activity was measured using the Drosophila Activity Monitor (DAM) system. Plots show the 24hr binned

activity averaged over at least 2 days in LD, and plot insets quantify the average daily activity count of each fly (N = 15-16; *5-HT1A*$^{-/-}$ P = 0.428, *5-HT1B*$^{-/-}$ P = 0.138, *5-HT2A*$^{-/-}$ P = 0.662, *5-HT2B*$^{-/-}$ P = 0.221, and *5-HT7*$^{-/-}$ P = 0.813 via two-sided t-test).

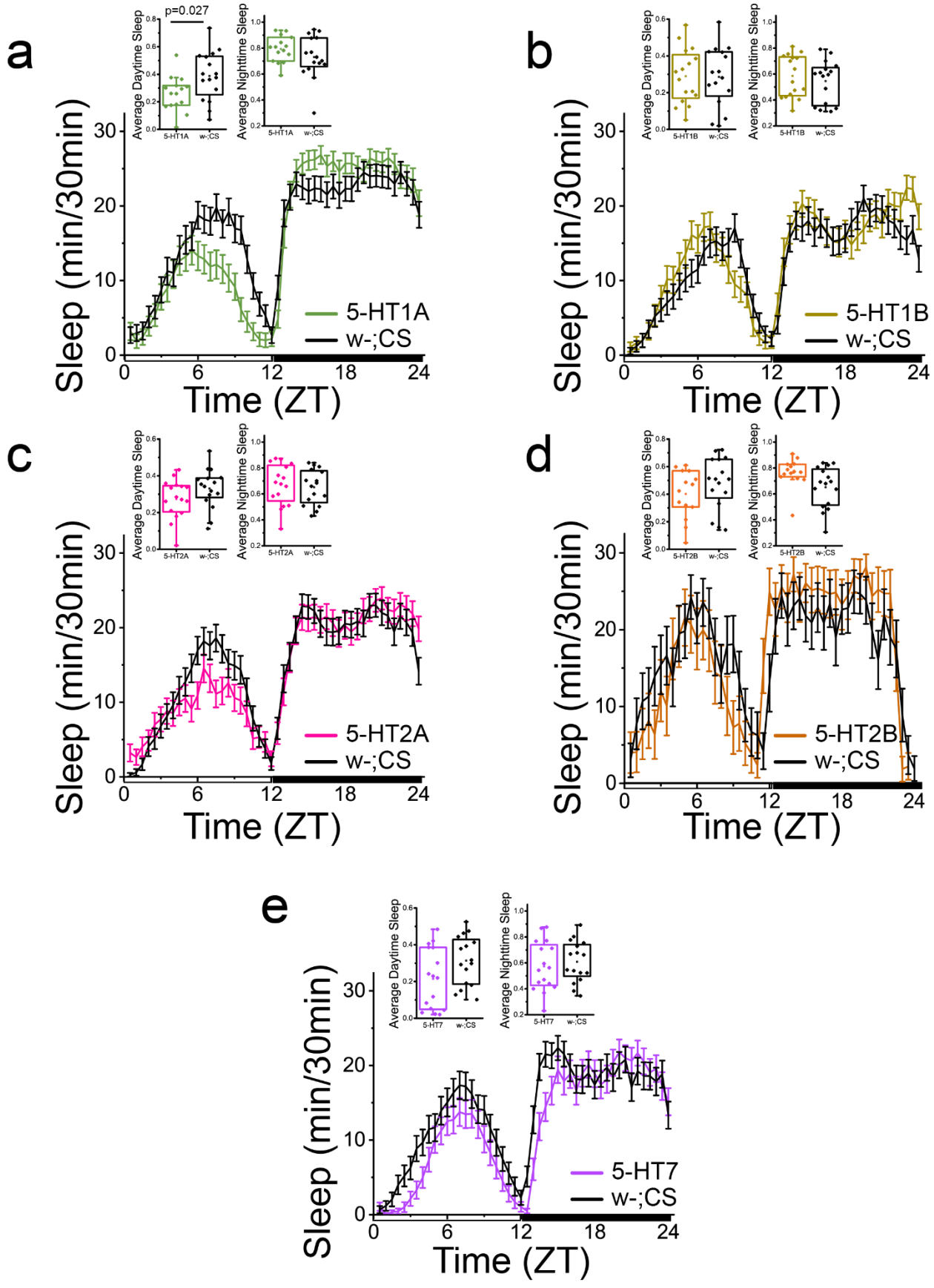

a
p=0.027
Average Daytime Sleep
Average Nighttime Sleep
5-HT1A
w-;CS
Sleep (min/30min)
Time (ZT)
5-HT1A
w-;CS
b
Average Daytime Sleep
Average Nighttime Sleep
5-HT1B
w-;CS
Sleep (min/30min)
Time (ZT)
5-HT1B
w-;CS
c
Average Daytime Sleep
Average Nighttime Sleep
5-HT2A
w-;CS
Sleep (min/30min)
Time (ZT)
5-HT2A
w-;CS
d
Average Daytime Sleep
Average Nighttime Sleep
5-HT2B
w-;CS
Sleep (min/30min)
Time (ZT)
5-HT2B
w-;CS
e
Average Daytime Sleep
Average Nighttime Sleep
5-HT7
w-;CS
Sleep (min/30min)
Time (ZT)
5-HT7
w-;CS

Figure 2.4. Female *5-HT1A*$^{-/-}$ mutants display decreased daytime but not nighttime sleep. Sleep was measured using the Drosophila Activity Monitor (DAM) system. (N = 15-16; *5-HT1A* P = 0.027 via two-sided t-test). Plots show the 24hr binned sleep averaged over 3 days in LD, and plot insets quantify the percent of time each fly spent sleeping (1=sleep, 0=active) (N = 15-16; Daytime Sleep: *5-HT1A*$^{-/-}$ P = 0.027, *5-HT1B*$^{-/-}$ P = 0.946, *5-HT2A*$^{-/-}$ P = 0.142, *5-HT2B*$^{-/-}$ P = 0.271, and *5-HT7*$^{-/-}$ P = 0.076 via two-sided t-test; Nighttime Sleep: *5-HT1A*$^{-/-}$ P = 0.216, *5-HT1B*$^{-/-}$ P = 0.498, *5-HT2A*$^{-/-}$ P = 0.712, *5-HT2B*$^{-/-}$ P = 0.035, and *5-HT7*$^{-/-}$ P = 0.580 via two-sided t-test).

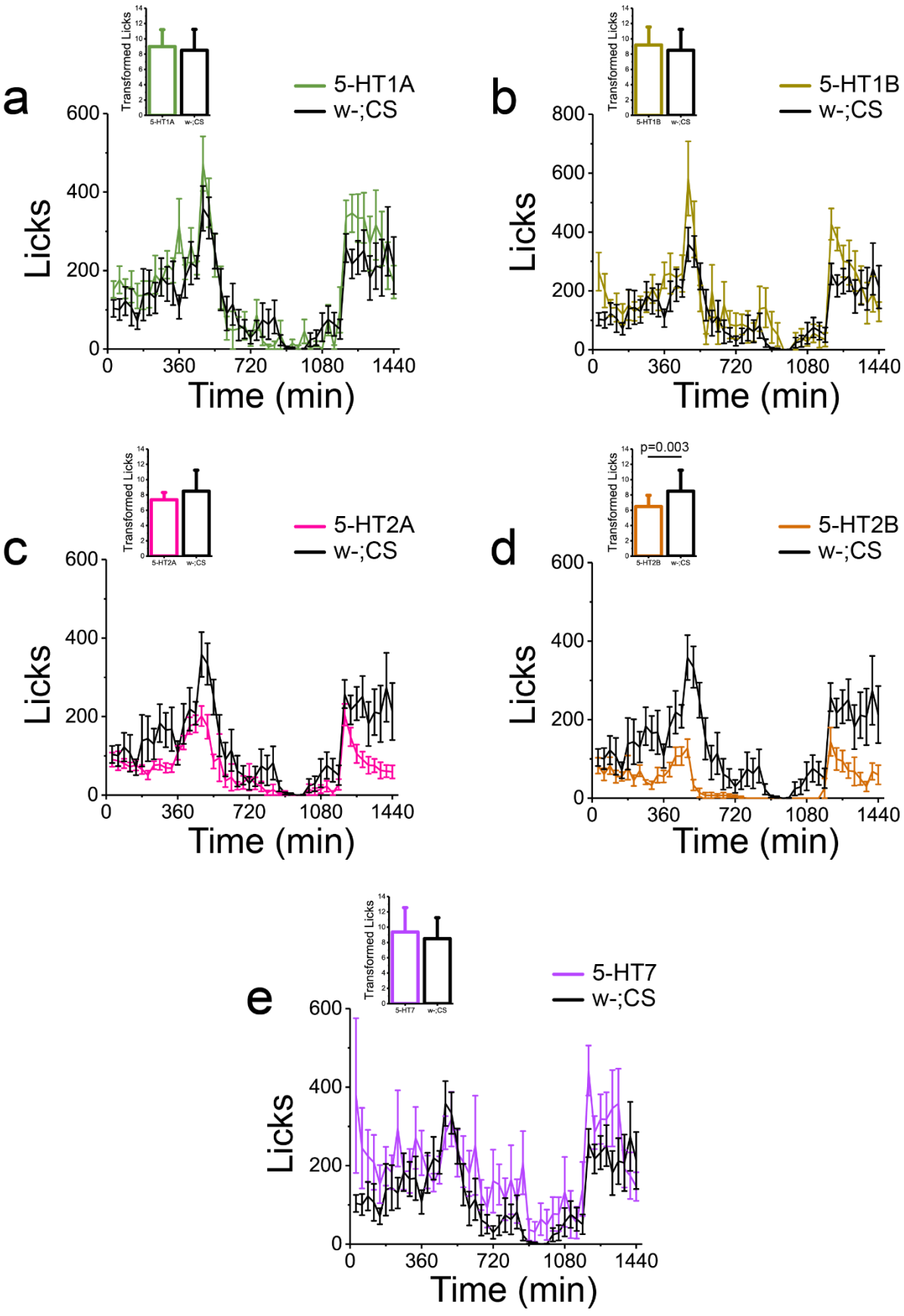

a
5-HT1A
w-;CS
Licks
Time (min)
Transformed Licks
5-HT1A
w-;CS
b
5-HT1B
w-;CS
Licks
Time (min)
Transformed Licks
5-HT1B
w-;CS
c
5-HT2A
w-;CS
Licks
Time (min)
Transformed Licks
5-HT2A
w-;CS
d
5-HT2B
w-;CS
Licks
Time (min)
p=0.003
Transformed Licks
5-HT2B
w-;CS
e
5-HT7
w-;CS
Licks
Time (min)
Transformed Licks
5-HT7
w-;CS

Figure 2.5. Female *5-HT2B* mutants display significantly fewer feeding interactions over a 24hr period. The Fly Liquid-Food Interaction Counter (FLIC) was used to quantify feeding interactions of each mutant over a 24hr period (N = 11-16; *5-HT1A*$^{-/-}$ P = 0.901, *5-HT1B*$^{-/-}$ P = 0.749, *5-HT2A*$^{-/-}$ P = 0.186, *5-HT2B*$^{-/-}$ P = 0.003, and *5-HT7*$^{-/-}$ P = 0.485 via One-way ANOVA w/ Tukey Post-Hoc test).

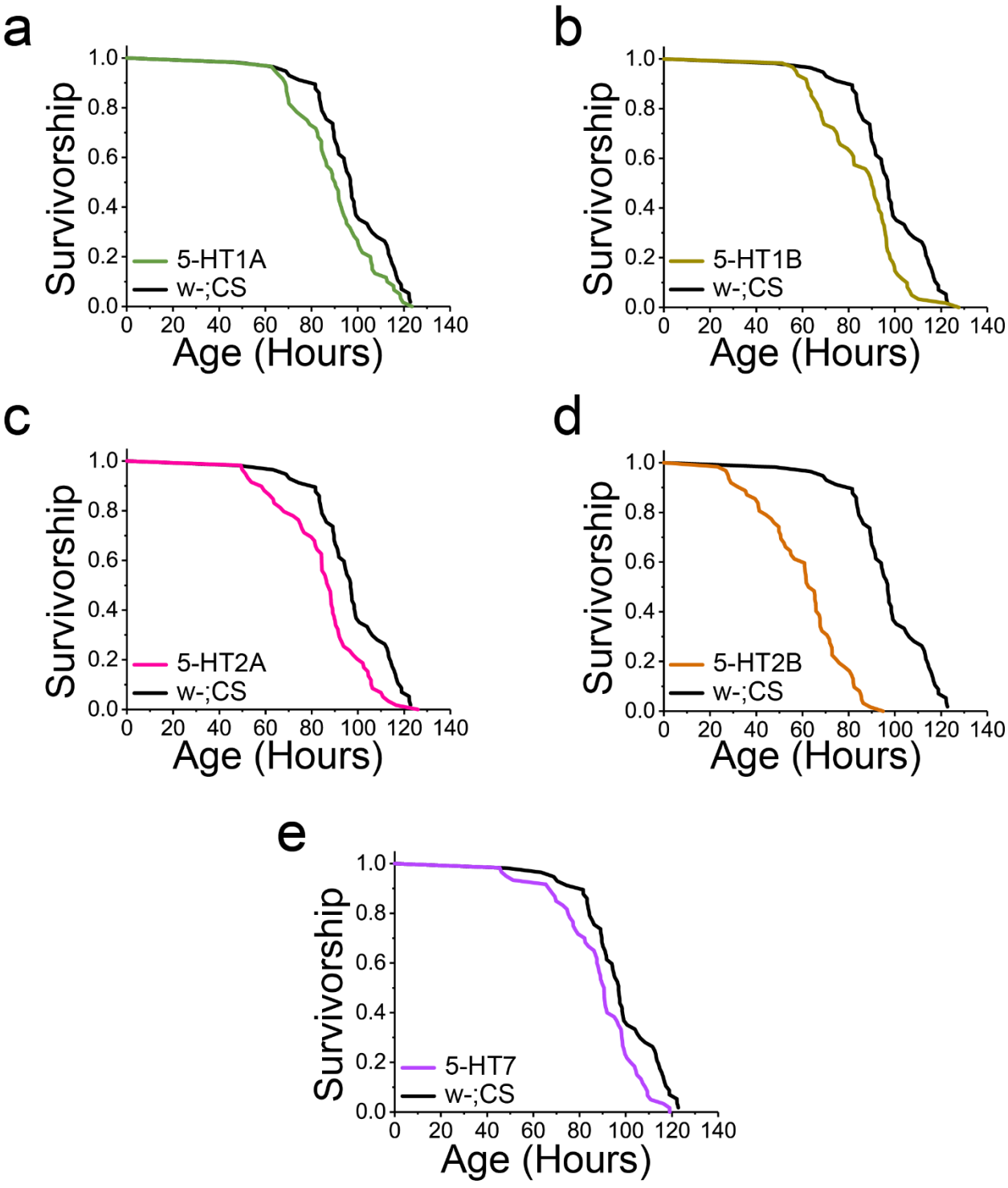


Figure 2.6. Female 5-HT receptor mutants are starvation sensitive. Flies were fed 2% agar and the Drosophila Activity Monitor (DAM) system was used to detect when activity ceased as a readout for death (N = 57-62, $P < 0.001$ for all comparisons via log-rank analysis).

CHAPTER III

The Serotonin Receptor 5-HT2A Modulates Lifespan and Protein Feeding[1]

Abstract

The conserved neurotransmitter serotonin is an important modulator of lifespan in specific nutritional contexts; however, it remained unclear how serotonin signaling influences lifespan under normal conditions. Here, we show that serotonin signaling through the 5-HT2A receptor influences lifespan, behavior, and physiology in *Drosophila*. Loss of the 5-HT2A receptor extends lifespan and induces a resistance to changes in dietary protein that are normally detrimental to lifespan. *5-HT2A*$^{-/-}$ null mutant flies also display decreased protein feeding and protein content in the body. Therefore, serotonin signaling through receptor 5-HT2A is likely recruited to promote motivation for protein intake, and chronic reduction of protein-drive through loss of 5-HT2A signaling leads to a lower protein set-point adaptation, which influences physiology, decreases feeding, and increases lifespan. Our findings reveal insights into the mechanisms by which organisms physiologically adapt in response to perceived inability to satisfy demand.

[1] This chapter is based on a publication under review: **Munneke, A. S.**, Chakraborty, T. S., Porter, S. S., Gendron, C. M., & Pletcher, S. D. (2022). The serotonin receptor 5-HT2A modulates lifespan and protein feeding in *Drosophila melanogaster*. Submitted: *PLOS Genetics.* Individual contributions to this manuscript are detailed on Page xi.

Introduction

It has long been known that manipulating an organism's diet can profoundly impact its behavior, physiology, and most notably, its lifespan. Since the early 1900s, studies in both rodents (McCay, Crowell et al. 1935, Weindruch and Walford 1982, Weindruch, Walford et al. 1986) and fruit flies (Chippindale, Leroi et al. 1993) have shown that reductions in total calorie consumption significantly extend lifespan. Since then, this method of dietary restriction has also been shown to extend lifespan of worms (Lakowski and Hekimi 1998), fish (Masoro 2002), rodents (Masoro 2002), dogs (Masoro 2002), and non-human primates (Mattison, Colman et al. 2017, Pifferi, Terrien et al. 2018), indicating highly conserved roles for nutrients as modulators of aging.

Additional studies have since found that reduction in specific macronutrients (i.e., protein or carbohydrates) is also sufficient to extend lifespan in many model systems. Many animals, including primates (Raubenheimer, Machovsky-Capuska et al. 2015, Uwimbabazi, Raubenheimer et al. 2021), consume nutrient ratios that maximize overall fitness. Low protein diets extend lifespan in a wide range of taxa (Mair, Piper et al. 2005, Reddiex, Gosden et al. 2013); and notably, geometric frameworks for nutrition studies have shown that low protein-high carbohydrate diets maximize lifespan in flies (Lee Kwang, Simpson Stephen et al. 2008, Bruce, Hoxha et al. 2013) and mice (Solon-Biet, McMahon et al. 2014) compared to calorically equivalent high protein-low carbohydrate diets, emphasizing the importance of specific nutrients, rather than total calories, in the context of aging.

Much of the literature on the mechanisms by which calorie or nutrient restriction influences health and longevity has focused on major metabolic integrator systems in peripheral tissues, such as mTOR (mammalian target of rapamycin) (Selman, Lingard et al. 2008, Harrison, Strong et al. 2009), insulin signaling (Clancy, Gems et al. 2001, Hwangbo, Gersham et al. 2004), and AMPK (Anisimov, Berstein et al. 2005, Burkewitz, Morantte et al. 2015). However, many methods of dietary manipulations influence lifespan independent of changes in consumption (Mair, Piper et al. 2005, Linford, Kuo et al. 2011), suggesting that the integration and interpretation of nutrient sensing in the brain are also important modulators of lifespan.

Indeed, a growing body of evidence suggests that perception of environmental cues by the nervous system plays an important role in healthy aging, and manipulation of sensory pathways is a robust modulator of lifespan. Ablation of specific sensory neurons is sufficient to extend lifespan in *C. elegans* and sensing hypoxic conditions and cold temperature do so as well (Apfeld and Kenyon 1999, Xiao, Zhang et al. 2013, Leiser, Miller et al. 2015). In flies, loss of olfactory and taste perception extends lifespan (Libert, Zwiener et al. 2007, Ostojic, Boll et al. 2014, Waterson, Chung et al. 2014), and in mice, loss of pain perception increases lifespan, as does loss of olfactory perception of same-sex pheromones (Riera, Huising et al. 2014, Riera, Tsaousidou et al. 2017), suggesting a role for the central nervous system as a global regulator of overall physiology and lifespan is highly conserved.

In at least some cases, the mechanisms by which environmental perception influences aging rely on evolutionarily conserved neuromodulators, such as serotonin, particularly in regard to nutrient perception. When flies are presented with the opportunity to construct their own diet as opposed to the standard homogenous nutrient mixture, lifespan is shortened in a manner dependent on neuronal signaling through the 5-HT2A serotonin receptor (Ro, Pak et al. 2016, Lyu, Weaver et al. 2021). In *C. elegans*, the lifespan extension via dietary restriction is reduced when worms can smell, but not access, food, and this effect requires both dopamine and serotonin signaling (Zhang, Jun et al. 2021, Miller, Huang et al. 2022).

Despite the clear links between 5-HT2A and aging in different nutritional contexts, little is known about whether it alters lifespan in non-stressful or nutrient-replete conditions, and, if so, the mechanisms by which this occurs. Here, we report that loss of 5-HT2A extends lifespan in protein replete conditions and induces a resistance to changes in dietary protein levels that normally modulate lifespan, suggesting that 5-HT2A is important for mediating behavioral and physiological adaptations to protein availability in the environment. We further show that *5-HT2A*$^{-/-}$ null mutant flies exhibit consistently lower protein consumption and that activation of specific *5-HT2A*$^{+}$ neurons induces protein feeding. Therefore, serotonin signaling through receptor 5-HT2A is likely recruited in response to physiological protein demand to promote motivation for protein intake, and chronic reduction of protein-drive through loss of 5-HT2A signaling leads to a lower physiological protein target which alters protein metabolism and

utilization in a way that is favorable for lifespan. Future studies are aimed towards further understanding the relationship between serotonin signaling and feeding motivation adaptions in the context of aging.

Results

Loss of the serotonin receptor 5-HT2A extends lifespan

To better understand the role of serotonin receptor 5-HT2A in aging, we studied flies homozygous for a putative null mutation (*5-HT2A*$^{-/-}$), which contained a *GAL4* element in place of the first coding exon (Qian, Cao et al. 2017). We first asked whether *5-HT2A*$^{-/-}$ mutant flies were long-lived when aged on a standard laboratory diet consisting of 10% sucrose and 10% yeast (w/v, SY10) relative to a laboratory control strain (Canton-S) to which it had been extensively backcrossed. We found that *5-HT2A*$^{-/-}$ mutant females lived significantly longer than control flies (Fig. 3.1a). Loss of 5-HT2A did not have a significant effect on male lifespan (Fig. 3.1b), leading us to focus on females for the majority of the study. Previous experiments had shown that 5-HT2A is required for modulating fly lifespan when the major dietary components of sucrose and yeast are presented separately, compared to when they are mixed and presented as a single, homogeneous mixture (Ro, Pak et al. 2016). We therefore wondered whether altering the levels of sucrose or yeast in our fixed diet would influence the magnitude of the mutant lifespan phenotype. To examine this, we measured the lifespans of *5-HT2A*$^{-/-}$ mutant and control females on both yeast- and sucrose-rich diets, consisting of 15% yeast/5% sucrose or 5% yeast/15% sucrose, respectively. We observed that *5-HT2A*$^{-/-}$ mutants were only long-lived on the yeast-rich diet (Fig. 3.1c-d), suggesting that either a low sugar or high yeast environment promotes extended lifespan in *5-HT2A*$^{-/-}$ mutant flies.

Dietary protein as yeast is particularly impactful in fly aging, as it largely drives the dietary restriction (DR) lifespan extension phenotype (Chippindale, Leroi et al. 2004, Min and Tatar 2006). Decreasing the levels of protein typically increases lifespan in a dose-dependent manner, up to a point where protein levels are presumably too low to support essential metabolism (Min and Tatar 2006), and we hypothesized that loss of 5-HT2A might alter the magnitude of this response. Holding sucrose levels constant, we therefore titrated the amount of protein in the diet and measured the lifespan of *5-HT2A*$^{-/-}$ mutant and control flies. Not unexpectedly, the mean lifespan of control animals increased significantly as dietary protein levels decreased (Fig. 3.1e). The mean lifespan of *5-HT2A*$^{-/-}$ mutant flies, however, was less affected by dietary protein and remained high even in protein-rich conditions (Fig. 3.1e). There was also a statistically

significant interaction between genotype and diet (P=0.03), supporting the conclusion that the mean lifespans of the two genotypes respond differently to diet. This suggests that loss of 5-HT2A promotes a long-lived state that renders flies resistant to the modulation of lifespan by dietary protein. Similar to the phenotype on SY10 (Fig. 3.1b), male *5-HT2A*$^{-/-}$ mutants showed no differences in lifespan on 5% or 15% yeast diets (Supplementary Fig. 3.1a-b).

5-HT2A influences yeast consumption

To further examine the interaction between the effects of 5-HT2A loss and dietary yeast, we investigated its role in feeding behavior. We titrated levels of yeast in fly diets and estimated food consumption in *5-HT2A*$^{-/-}$ mutant and background control flies by spiking diets with a non-metabolizable blue dye (FD&C Blue 1) and used the amount of excreted dye as an estimate of consumption (i.e., the ConEx assay (Shell, Schmitt et al. 2018)). Using the standard 10% sugar/yeast laboratory medium as a reference diet, we measured consumption on diets containing 10% sucrose and ranging from 5-20% yeast (w/v). In flies from both genotypes, we observed that the volume of food consumed decreased as the amount of yeast in the diet increased (Fig. 3.2a). When presenting these data in terms of the mass of yeast consumed, we observed a significant effect of genotype and no significant effect of dietary yeast concentration, supporting the notion that flies have a protein consumption target that drives their feeding behavior in fixed diets (Fig. 3.2b) (Lee Kwang, Simpson Stephen et al. 2008). The genotype effect was indicative of a consistent reduction of protein consumption in *5-HT2A*$^{-/-}$ mutant flies, suggesting the possibility that they exhibit a lower protein consumption target. Differences in the mass of sucrose consumed matched the pattern of total consumption because sucrose was held constant in these diets (Supplementary Fig. 3.2a). Similar to lifespan, loss of *5-HT2A*$^{-/-}$ did not significantly affect feeding in males. Both mutant and control genotypes responded similarly to increased dietary protein by consuming less, and there was no significant effect of diet or genotype on the mass of yeast consumed (Supplementary Fig. 3.2b-c). Similar to females, sucrose mass consumption decreased as dietary protein increased, but there was no effect of genotype in males (Supplementary Fig. 3.2d).

A similar experiment in which feeding was measured on diets ranging from 5-20% sucrose (w/v) only (to avoid confounding effects from yeast) revealed that female flies of both genotypes

decreased total volume consumed as the concentration of dietary sucrose increased (Fig. 3.2c). However, when scaled to present the mass of sucrose consumed, female flies of both genotypes consumed a higher mass of sucrose as concentration increased (Fig. 3.2d). Unlike the experiments in which we manipulated dietary yeast, however, we observed no significant difference in pattern of consumption between *5-HT2A*$^{-/-}$ mutant and control flies on sucrose diets (Fig. 3.2c-d).

5-HT2A modulates protein body content

A reduced protein consumption target in *5-HT2A*$^{-/-}$ mutants might be reflected in lower protein content in the body. Indeed, we found that *5-HT2A*$^{-/-}$ mutant females exhibited decreased whole-body protein content (Fig. 3.3a). To evaluate if nutrient absorption was involved in female body composition differences, we measured protein content in their excretion and observed no differences between *5-HT2A*$^{-/-}$ mutant and control females (Fig. 3.3b). We also asked whether changes in body composition in *5-HT2A*$^{-/-}$ mutant females was specific to protein or whether it extended to other forms of nutrient storage. In *Drosophila*, excess carbohydrates are converted and stored primarily as triglycerides (TAG), and we found no differences in female TAG levels (Fig. 3.3c). To determine whether the reduction in total protein influenced overall body mass, we measured both wet and dry mass and observed a significant reduction in both measures in *5-HT2A*$^{-/-}$ mutant females (Fig. 3.3d-e). The reduction in body mass is likely due to lower protein levels in *5-HT2A*$^{-/-}$ mutants, with the lack of differences in TAG suggesting fat storage levels and carbohydrate usage are similar.

Loss of 5-HT2A mimics lifespan extension in virgin flies

Our behavioral and physiological data led us to speculate that the absence of 5-HT2A receptor results in a chronic state of perceived protein limitation, independent of its availability in the diet. This situation might be expected to stimulate adaptive processes that reduce protein utilization, which would manifest as increased lifespan, particularly when dietary protein is replete. In this view, *5-HT2A*$^{-/-}$ mutant flies would exhibit lifespan phenotypes that are associated with physiological states of low protein utilization and would be resistant to manipulations that subsequently increase it. Female reproductive status is associated with such a state; virgin female flies lack developing embryos, exhibit a reduced drive for protein consumption, and are long-

lived (Ro, Pak et al. 2016). Mating increases reproductive output and drives protein feeding. We found that as little as three days of mating following eclosion (following our typical lifespan measurement protocol) significantly reduced the lifespan of control flies fed our standard 10% sugar-yeast diet (Fig. 3.3f). *5-HT2A*$^{-/-}$ mutant flies, however, were long-lived regardless of mating status; average mutant lifespan was not influenced by mating and was not significantly different from that of the control genotype virgin flies (Fig. 3.3f). Interestingly, as postulated by Ro et al (Ro, Pak et al. 2016), extended lifespan in mutant females is likely due to differences in responses to perceived protein availability rather than to availability itself because feeding differences did not depend on mating status (Supplementary Fig. 3.3a) and because mutant females showed no differences in egg-laying relative to control flies (Supplementary Fig. 3.3b).

Activation of *5-HT2A*$^{+}$ neurons promotes interaction with protein

We next sought to investigate whether 5-HT2A signaling influences motivational or reward circuitry related to protein consumption, which would affect responses to perceived availability. Published data suggest that 5-HT2A signaling is not involved in specifying protein demand but instead influences protein feeding by stimulating its consumption or by acting subsequent to consumption to reinforce feeding (Ro, Pak et al. 2016). The first model predicts that activation of 5-HT2A neurons would precede protein feeding and that neuronal activation would promote protein, but not carbohydrate, feeding and would not be generally rewarding. On the other hand, if 5-HT2A were acting to reinforce protein feeding, we might expect that activation of 5-HT2A neurons would follow protein feeding and that their activation would reinforce behavior when closely paired with feeding events.

To evaluate when and where *5-HT2A*$^{+}$ neuronal activity is responsive to protein availability in food, we used *5-HT2A-GAL4* (Gnerer, Venken et al. 2015) to express a transcriptional reporter system involving an NFAT-based tracing method (CaLexA) through which sustained neural activity drives expression of GFP (Masuyama, Zhang et al. 2012). We then quantified fluorescent intensity to identify populations of neurons that showed increased activity upon protein manipulation. Specifically, *5-HT2A>NFAT* flies were provided 2% agar coated with 200mM sucrose +/- 2.5x complete amino acid mix (Piper, Blanc et al. 2014). Flies fed amino acids exhibited decreased GFP intensity, relative to flies fed sucrose alone, in a population of *5-*

HT2A^{+} neurons in the supramedial protocerebrum (SMP; Fig. 3.4a), indicating these neurons were responsive to dietary amino acid concentration.

We next determined whether acute activation or inhibition of *5-HT2A*$^{+}$ neurons influence how the animals interact with different nutrients. For these studies, we used the Fly Liquid-Food Interaction Counter (FLIC), which measures the precise timing and duration of fly feeding behaviors through the closure of a circuit when a fly inserts its proboscis into a liquid food source (Ro, Harvanek et al. 2014). For neuronal activation, we coupled the FLIC technology to optogenetic stimulation to drive expression of a red light-sensitive channelrhodopsin protein (*CsChrimson*) in all *5-HT2A*$^{+}$ neurons. To accomplish this, we installed red LED lights on top of individual FLIC feeding chambers and executed an open-loop design, whereby light was illuminated for 12 seconds (40Hz and 32% duty cycle) every five minutes, activating neurons regardless of the fly's behavior. We compared feeding interactions between *5-HT2A>CsChrimson* flies and their genotypic controls (see Methods for strain details) exposed to either sucrose or yeast food and in the presence or absence of red-light stimulation. When putatively all *5-HT2A*$^{+}$ neurons were activated in this open-loop configuration, we observed a rapid, significant increase in interactions with a protein-containing solution in *5-HT2A>CsChrimson* flies relative to controls, but not with a sucrose-only solution (Fig. 3.4b-c). When these same experiments were conducted in the absence of light, no differences were observed between 5-HT2A-neuronal activated flies and controls in the presence of either protein or sucrose (Supplementary Fig. 3.4a-b). For inhibition, we expressed an inhibitory anion channelrhodopsin (*GtACR1*) in all *5-HT2A*$^{+}$ neurons and substituted green LEDs in the same open-loop configuration. This manipulation did not significantly impact interactions with a protein-containing solution (Fig. 3.4d), showing that the activity of *5-HT2A*$^{+}$ neurons does not bi-directionally control protein feeding and suggesting that 5-HT2A signaling is specifically recruited to promote protein feeding behaviors.

To determine whether activation of *5-HT2A*$^{+}$ neurons is sufficient to reinforce behavior regardless of the presence of protein, perhaps as a result of it conveying a more general sense of reward, we exposed flies to a denatonium solution (100μM), which is bitter and aversive, and activated *5-HT2A*$^{+}$ neurons in response to individual flies' interaction with it (i.e., a closed-loop

configuration). Unlike the robust increase in interactions observed upon activation of putative reward neurons that express the neuropeptide NPF (Shao, Saver et al. 2017), we observed only a modest increase in interactions with the bitter solution when *5-HT2A*$^+$ neurons were activated (Fig. 3.4e), suggesting *5-HT2A*$^+$ neuronal activation alone does not provide a potent reward.

Finally, we also asked whether activation or inhibition of *5-HT2A*$^+$ neurons impacted lifespan. We observed that both manipulations significantly shortened lifespan (Supplementary Fig. 3.4c-d). We hesitate to interpret these results in the context of our model because *5-HT2A*$^+$ neurons express many receptors and signaling peptides, all of which would be affected by optogenetic manipulations. These effects on lifespan may, therefore, be non-specific and due to the pleiotropic effects of activating or inhibiting a large group of neurons.

In summary, increased neuronal activity was observed when flies were amino acid deprived (Fig. 3.4a), not when they had fed on protein, and *5-HT2A*$^+$ neuronal activation was sufficient to drive protein consumption (Fig. 3.4b) but was not generally sufficient to reinforce feeding behavior (Fig. 3.4e). Taken together, therefore, these data support the notion that 5-HT2A signaling is recruited to promote protein consumption, perhaps by establishing a heightened protein consumption target, and to enact a physiological state of higher protein utilization that subsequently accelerates aging.

Activation of *5-HT2A*$^+$ neurons in the fan-shaped body promotes protein consumption

We next aimed to narrow the population of *5-HT2A*$^+$ neurons that influence protein feeding behaviors with the hope of obtaining a better understanding of the relationship between the consumption set point and lifespan. Driving GFP expression with the broad *5-HT2A-GAL4* marks diverse populations of nearly 80 neurons in the *Drosophila* brain (Davie, Janssens et al. 2018) (Fig. 3.5a). Our neural activity data (Fig. 3.4a) indicated that *5-HT2A*$^+$ SMP neurons might influence feeding behavior because the increase in activity upon amino acid deprivation suggested they are responsive to protein demand. We therefore sought to determine whether manipulation of their activity alone might phenocopy the results that we observed following broader 5-HT2A neuronal activation. The FlyLight collection contains thousands of *GAL4* lines that contain fragments of gene-specific promotors (enhancer-trap, or et-*GAL4*), thus, labeling

smaller populations of cells that express that gene. One such *GAL4* line for 5-HT2A (R50D04, BDSC #38744) specifically labels SMP neurons and recapitulates the expression of *5-HT2A*$^{+}$ neurons with altered activity in the NFAT data. We used this genetic reagent to express *CsChrimson* and to ask whether optogenetic stimulation was sufficient to increase behavioral interactions with dietary protein. Using a similar open-loop configuration with slightly longer activation periods to account for the possibility that a single et-*GAL4* line may not encompass all *5-HT2A*$^{+}$ neurons involved in promoting feeding behaviors, we observed that activation of R50D04^{+} neurons in the FLIC resulted in a significant increase in interactions with a protein-containing solution relative to controls (Fig. 3.5b), recapitulating the broad 5-HT2A-neuronal activation phenotype. The increase in feeding behaviors was not as rapid as the broad 5-HT2A-neuronal activation phenotype, suggesting the R50D04 line may not provide full coverage over *5-HT2A*$^{+}$ neurons that promote protein feeding behaviors. This difference between genotypes was not observed in the absence of light activation (Fig. 3.5c).

We also asked whether activation of these R50D04^{+} neurons would impact feeding in the same nutritional context as the standard lifespan conditions: a solid food diet consisting of 10% sucrose and 10% yeast (w/v). To test whether activation of *5-HT2A*$^{+}$ SMP neurons also led to increased consumption of solid food, we coupled optogenetic approaches with the ConEx assay, allowing us to activate specific neurons during solid food consumption. In this assay, flies expressing *CsChrimson* in R50D04^{+} neurons were provided with a blue dye spiked diet of 10% sucrose and yeast and housed in vials that were exposed to high-intensity red LEDs. When R50D04^{+} neurons were activated (at 40Hz and 32% duty cycle) periodically for three minutes every fifteen minutes over 24 hours, we saw a significant increase in total consumption relative to the genetic controls, which restored to baseline in a subsequent 24-hour dark period (Fig. 3.5d). Both inhibition and activation of these neurons had no effect on lifespan (Fig. 3.5e-f), suggesting protein consumption and lifespan are controlled through, at least partially, distinct mechanisms. Together, these data indicate that this limited subset of *5-HT2A*$^{+}$ neurons in the SMP are critical to promote protein feeding behaviors, and thus, may help encode a protein drive that affects protein feeding behaviors; however, additional *5-HT2A*$^{+}$ neurons may determine how these protein-dependent states impact lifespan.

Discussion

Our findings support the notion that flies consume to protein targets and that 5-HT2A signaling plays an important role in this. We observed that specific *5-HT2A*$^{+}$ neurons are activated in response to protein deprivation (Fig. 3.4a) and that activation of these neurons promotes protein, but not sucrose, feeding behaviors (Fig. 3.4b-c). This suggests that 5-HT2A signaling does not encode protein demand; rather, upstream serotonergic circuit(s) set demand, and 5-HT2A is recruited to promote consumption of protein. Specific regions of the *Drosophila* brain are strong candidates for circuits in which nutrient demand signals to *5-HT2A*$^{+}$ neurons to promote consumption. The protein deprivation-responsive *5-HT2A*$^{+}$ neurons are found in the superior medial protocerebrum (SMP) and appear to project to a region of the brain known as the fan-shaped body (FSB). The FSB is emerging as a key hub for integrating nutrient cues and information on internal state to make feeding decisions. As a major integration center, it is likely that several upstream circuits that evaluate protein availability converge on the SMP, and may signal through 5-HT2A to satisfy this protein demand. Several neuropeptides (AstA, NPF, and DH44) have been shown to directly inhibit FSB neurons to shift food preferences and dopaminergic neurons indirectly modulate FSB activity through these neuropeptidergic neurons (Sareen, McCurdy et al. 2021). It seems plausible that activity of SMP dopaminergic neurons is regulated by 5-HT2A or that multiple neuromodulator-expressing neurons in the SMP converge on the FSB to influence feeding behaviors. Furthermore, FSB neurons integrate information about internal glucose and fructose levels to induce prolonged feeding when energy levels are low. The activity of these FSB neurons is regulated by glutamatergic signaling via neurons in the superior lateral protocerebrum (SLP), which is adjacent to the SMP; however, the inputs for these SLP neurons remain unknown (Musso, Junca et al. 2021). As such, it is attractive to speculate that 5-HT2A signaling could also modulate the activity of these glutamatergic neurons, which ultimately converge on the FSB.

Our results also suggest that nutrient set-points are potentially altered by long-term inability to satisfy demand. Long-term discrepancies between nutrient expectation and consumption have been shown to modulate global physiology and lifespan, also in a serotonin-dependent manner. Chronic activation of serotoninergic hunger circuits extends lifespan in flies and leads to a short-

term increase in food consumption, which eventually dissipates, putatively as an adaptation to sustained hunger (Weaver, Holt et al. 2022). In *C. elegans*, the perception of food cues blunts lifespan extension by dietary restriction, and blocking serotonin signaling abrogates this effect (Zhang, Jun et al. 2021, Miller, Huang et al. 2022). This work suggests serotonin-dependent neural states modulate lifespan in response to evaluation of the nutritional environment, and the inability to satisfy protein demand, leading to a long-term reduction in protein motivation, may be one mechanism through which these effects arise.

We note that there is a need for a focused dissection of the populations of *5-HT2A*$^+$ neurons that regulate feeding behavior and lifespan. We identified a group of *5-HT2A*$^+$ neurons in the SMP that modulate protein feeding behavior (Fig. 3.4a, Fig. 3.5b,d); however, manipulation of the activity of these neurons does not alter lifespan (Fig. 3.5e-f) and is less potent at promoting protein feeding than the population as whole. It remains unknown whether some or all the neurons that modulate feeding also influence lifespan. Additional studies in flies have also implicated 5-HT2A signaling in the modulation of lifespan via environmental factors such as exposure to chronic nutrient choice (Ro, Pak et al. 2016) or perception of dead conspecifics (see Chapter IV) (Chakraborty, Gendron et al. 2019), suggesting some still unknown population of *5-HT2A*$^+$ neurons may act as important longevity regulators. Examination of neuronal populations that are activated in these scenarios (but not protein deprivation) may help differentiate *5-HT2A*$^+$ neurons involved in feeding vs. lifespan.

In nature, organisms must cope with changes in the nutritional environment and optimize behavior and physiology to maximize overall fitness, with responses tailored towards both short-term and long-term adaptions (López-Maury, Marguerat et al. 2008). For instance, in some cases of limited food availability, it would be most adaptive to relocate to find a nutrient-dense food source and activate pathways that promote foraging behavior (Searle, Thompson Hobbs et al. 2005, Pretorius, de Boer et al. 2011). However, in situations of chronic nutrient stress animals must alter their feeding behavior and physiology to survive and reproduce in relatively harsh conditions (Rodgers, Lerin et al. 2008). Given these fitness consequences, it is likely that adaptive responses to low protein, or perceived low protein, could also apply to other species, even humans. Indeed, specific polymorphisms of the *HTR2A* gene (homologous to *Drosophila 5-*

HT2A), are associated with altered nutrient preferences (Prado-Lima, Cruz et al. 2006), metabolic diseases (Halder, Muldoon et al. 2007), and eating disorders (Genis-Mendoza, Ruiz-Ramos et al. 2019, Yan, Gao et al. 2021). They are also associated with longevity in some populations (Jobim, Prado-Lima et al. 2008), which raises the interesting possibility that altered 5-HT2A signaling in humans may modulate lifespan, which would make it a potential therapeutic target for aging interventions.

Methods

Fly Stocks

The w-;CS stock was established by mixing a population of w^{1118} flies with standard Canton-S (CS) flies for more than 10 generations and re-isolating white-eyed flies. The *5-HT2A*$^{-/-}$ null mutants containing a *GAL4* element in place of the first coding exon (Qian, Cao et al. 2017) were generously donated by Yi Rao, Peking University. The broad-expressing *5-HT2A-GAL4* was created by replacing a MiMIC insertion in the *5-HT2A* locus with a *GAL4* element (Gnerer, Venken et al. 2015) and was kindly shared by Herman Dierick, Baylor College of Medicine. The restricted 5-HT2A-*GAL4* expressing in the superior medial protocerebrum (BDSC #38744) contains a *GAL4* element in the promoter region of *5-HT2A*. *Npf-GAL4* contains a *GAL4* element fused to the regulatory sequence region for *npf* (BDSC# 25682). Neuronal activation experiments were performed using *;UAS-CsChrimson* (BDSC #55135), which was back-crossed to the w-;CS control stock for 10 generations. Neuronal inhibition experiments were performed using the anion channelrhodopsin (*GtACR1*) fused to a *UAS* element (Mauss, Busch et al. 2017), which was generously shared by Monica Dus, University of Michigan and back-crossed to the w-;CS control stock for 10 generations.

Husbandry

All fly stocks were maintained on a standard cornmeal-based larval growth medium (produced by LabScientific Inc and purchased from Fisher Scientific) in a constant environment (25 °C, 60% humidity) with a 12:12 hr light:dark cycle. We controlled the developmental larval density by aliquoting 32 µl of collected eggs into individual bottles containing 25 mL of food. Following eclosion, mixed-sex flies were kept on SY10 (10% [w/v] sucrose and 10% [w/v] yeast) medium for 2–3 days until they were used for experiments. Pioneer table sugar (purchased from Gordon Food Service, MI) and MP Biomedicals Brewer's Yeast (purchased from Fisher Scientific) were used in our study.

Metabolic Assays

After the flies were aged on SY10 for 10-14 days (food was changed every 2–3 days), experimental flies were quickly frozen, collected into groups of five, and then homogenized in 200 µl of ice-cold phosphate-buffered saline containing 0.1% Triton X-100 for 30 s at 30 Hz

using a QIAGEN TissueLyser. For triacylglycerol (TAG) quantification, the homogenate (20 µl) was added into 200 µl of Infinity Triglyceride Reagent (Thermo Electron Corp.) and incubated at 37°C for 10 min with constant agitation. TAG concentrations were determined by measuring the absorbance at 520 nm and estimated by a known triglyceride standard. For protein measurement, 5 µl of fly homogenate was incubated with 200 µl of (1:50) 4% (w/v) cupric sulfate/bicinchoninic acid solution (Novagen) at room temperature for 30 min. For protein excretion measurements, flies were fed SY10 food for 72 hours (20 flies per vial, 7-8 replicates for each group). The food was then removed from the vials and 1 mL of Milli-Q water was used to wash the excrement from the vials. This solution was then passed through a dechorionation sieve to remove any eggs and 10 µl of the wash volume was used to measure protein concentration. Protein concentrations were calculated by measuring the absorbance at 562 nm through the comparison with bovine serum albumin standards for all protein assays. For mass measurements, flies were aged on SY10 for 10-14 days, then separated into Eppendorf tubes for weight measurements. The tubes were kept at -20°C for 30 min to ensure the flies were not moving prior to wet mass measurements. The tubes were then placed in a 37°C oven for 3 days before measuring dry mass. Average weight, TAG, and protein values were based on at least 10 independent biological replicates from multiple vials.

Reproduction Assays

Virgin flies of each genotype were collected and mated to one week-old Canton-S males in a 1:1 male to female ratio (five females and five males were co-housed in SY10 vials with ten vials per treatment). Exposure to males began 48 hours prior to the start of the experiment. Vials were flipped daily, and the total number of eggs were counted each day then summed for each vial over seven days.

Consumption-Excretion (ConEx) Assays

We used the protocol described previously (Shell, Schmitt et al. 2018). Briefly, experimental flies were co-housed in vials (10 flies per vial, 8–10 replicates for each group) for 2–3 days following eclosion and then sorted into individual sex cohorts on SY10 food. After 10-14 days, the flies were transferred onto diets with 1% (w/v) FD and C Blue No. 1 in varying percentages from 5-20% of Yeast or Sucrose (w/v) based on the experiment (see table below under Diet

Compositions for details). Vials were discarded if one or more dead flies were observed after the 24 hour feeding period. For ConEx experiments coupled to optogenetics, 3 minutes of 40Hz red light was supplied every 15 minutes to minimize depolarization block (Shao, Saver et al. 2017). Excreted dye (ExVial) was collected by adding 3 ml of Milli-Q water to each vial followed by vortexing for 10 seconds. The concentration of the ExVial dye in water extracts was determined by reading the absorbance at 630 nm, which was used to infer macronutrient consumption.

OptoFLIC

Flies were tested on the Fly Liquid-Food Interaction Counter (FLIC) system as previously described to monitor feeding behaviors (Ro, Harvanek et al. 2014) coupled to optogenetic activation technology. Specifically, custom lids were produced containing one LED for each well in the FLIC chamber, allowing precise control over the dynamics of the light timing, duration, and frequency. Easycargo Aluminium Radiator Cooler Heat Sinks were used to dissipate any heat generated by the LEDs [DigiKey manufacturer numbers: LXM2-PD01-0050 (red), LXML-PM01-0100 (green)]. Each liquid-food reservoir contained either 2% (w/v) yeast extract + 1% (w/v) sucrose or 1% (w/v) sucrose in 1% Tegosept (v/v) and 4 mg/l $MgCl_2$. For closed-loop experiments, 100µM denatonium in 1% Tegosept (v/v) and 4 mg/l $MgCl_2$ was used. Flies were anesthetized briefly on ice and manually aspirated into the *Drosophila* feeding monitors (DFMs). Each DFM was loaded with flies from at least two treatment groups to reduce technical bias from the DFM signals. LED lights were always pulsed at 40Hz with a 32% duty cycle. In the open-loop activation experiments, red light was provided 12 seconds every six minutes for experiments using the broad 5-HT2A-GAL4 driver and for three minutes every fifteen minutes for the more restricted 5-HT2A-GAL4 (BDSC #38744). For open-loop inhibition experiments, green light was supplied for three minutes every fifteen minutes. Red light was pulsed when flies interacted with the liquid food in closed-loop experiments. All LEDs were pulsed at 40Hz to minimize depolarization block (Shao, Saver et al. 2017). For closed-loop experiments, red light was pulsed at 40Hz upon interaction with the liquid food well and included a one second decay after food interaction ceased. Food interactions were analyzed using custom R code, which is available on GitHub at https://github.com/PletcherLab/FLIC_R_Code. Default thresholds were used for analysis. Flies that had zero feeding events over the testing interval were removed from the analysis.

Neuronal Activity Experiments

Flies emerged and were collected after 48 hours to allow for mating. Female flies were then separated from males and placed in individual vials during nutrient exposure to minimize potential neuronal signals from social cues. Vials were prepared containing 2% agar coated with 200mM sucrose +/- 2.5x complete amino acid solution, prepared as previously described (Piper, Blanc et al. 2014) Nutrient exposure time was 48 hours, after which the female brains were dissected and imaged as described below using a wavelength of 488nm to visualize the NFAT GFP signal.

Brain Dissection

Four days post-eclosion brains from adult females exposed to 200mM sucrose +/- 2.5x complete amino acid solution conditions were dissected in ice-cold phosphate buffer saline (PBS) using sharp tweezers and fixed in PBS containing 4% paraformaldehyde for 60 min at room temperature. The brains were washed thoroughly with 1 ml of PBS with 0.1% Triton-X (PBS-T), then moved using a wide-bore pipet tip to a glass slide with Vectashield (Vector Laboratories) and sealed using a coverslip and clear nail polish for immediate imaging.

Imaging and Analysis

Imaging was carried out using an Olympus FLUOVIEW FV3000 confocal microscope. The brains were brought into focus with 10x (0.40 NA) objective lens before switching to 20x (0.75 NA). Extra care was taken in order not to saturate the image. Images were acquired at 1024 X1024 pixels with a step size of 3.0 micron. The laser power and the parameters for image acquisition was kept similar between control and treatment groups.

For data analysis, the imaging files were analyzed in the publicly available imaging software, Fiji. Selective slices were combined and collapsed into a single image using SUM slices. Brightness and contrast were adjusted manually when required for better visualization of the image. Background was calculated from the brain region adjacent to the ROI and subtracted. Representative images are displayed in mpl-inferno pseudo color.

Survival Assays

Lifespans were measured using established protocols (Linford, Bilgir et al. 2013). Unless otherwise noted, 10 replicate vials (~200 experimental flies) were established for each treatment. Flies were transferred to fresh media every 2-3 days, at which time dead flies were removed and recorded using the DLife system developed in the Pletcher Laboratory (Linford, Bilgir et al. 2013). Flies were kept in constant temperature (25°C) and humidity (60%) conditions with a 12:12 hr light:dark cycle. For optogenetic lifespans, flies were kept in specially designed rigs containing red or green LEDs, to activate or inhibit neurons, respectively. Controls were kept in a dark box in the same incubator and flipped under dim red light. Red LEDs were pulsed at 2Hz for activation experiments and green LEDs were pulsed at 40Hz for inhibition experiments.

Statistics

Unless otherwise indicated, pairwise comparisons between different treatment survivorship curves were carried out using the statistical package R within DLife (Linford, Bilgir et al. 2013). Each p-value was obtained using a Log-Rank analysis. For testing the interaction between genotypes and diets, we used Cox-regression analysis to report p-value for the interaction term. To test the effects of diet and genotype involved in food consumption, we performed Two-way ANOVA followed by a Bonferroni post-hoc significance test or ANCOVA. For OptoFLIC data, total licks for a given time period were fourth-root transformed, which was empirically found to normalize the majority of FLIC data. Significance was determined using a one-sided t-test. Significance for metabolic assays was measured using a two-sided t-test. For imaging experiments, a one-sided t-test was used to determine significance.

Acknowledgements

The authors would like to acknowledge the members of the Pletcher laboratory for their comments on the experimental design and analysis. This research was supported by the US National Institute of Health, National Institute on Aging through R01 AG051649, R01 AG030593, R01 AG063371, and R61 AG078428 (S.D.P.) and the Glenn Medical Foundation (S.D.P.), as well as training grant T32-GM007315 (A.S.M) though the National Institute of General Medicine Sciences and training grant T32-AG000114 (A.S.M.) through the National Institute on Aging. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Figures

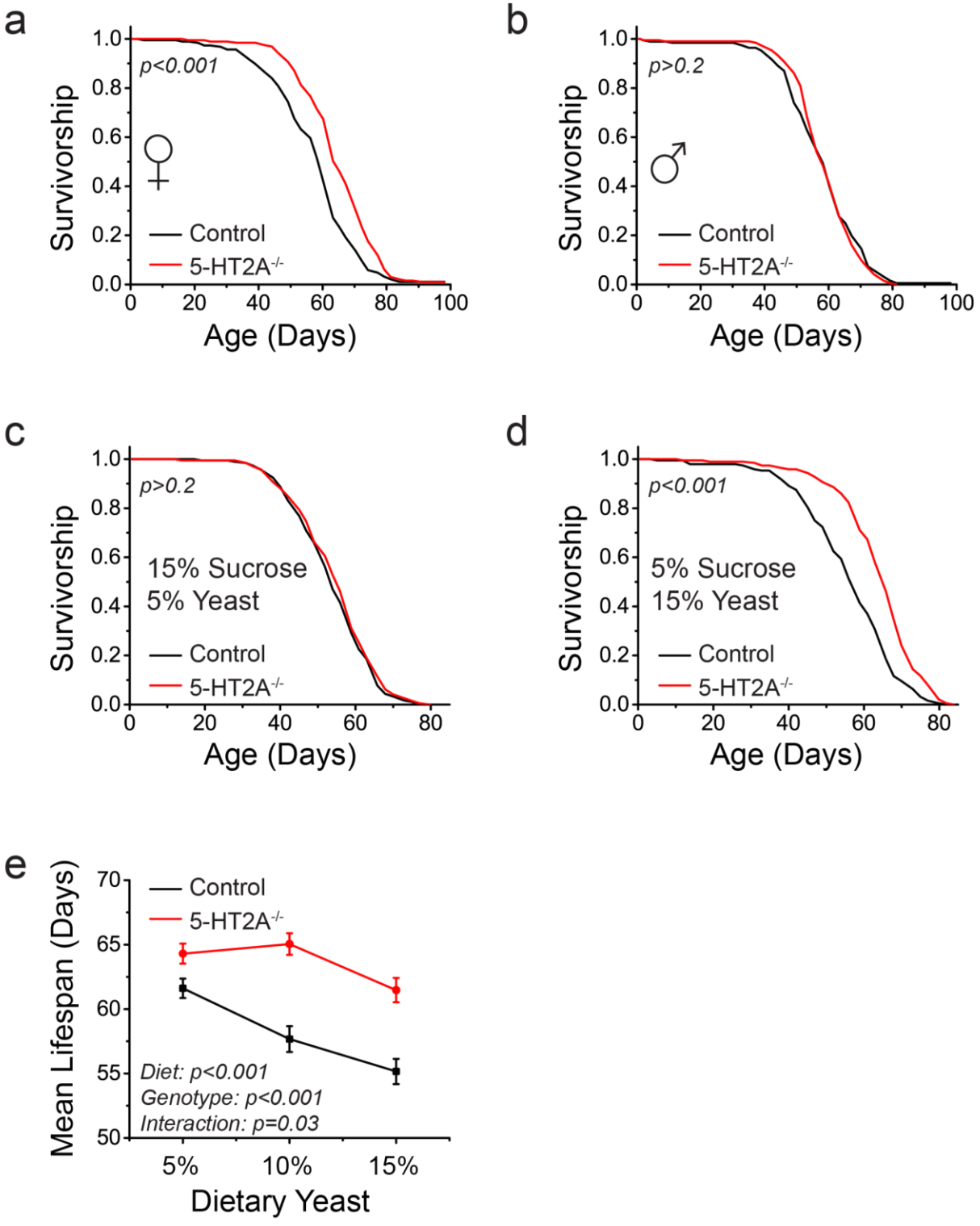


Figure 3.1. 5-HT2A interacts with protein levels to modulate lifespan. a Female *5-HT2A*$^{-/-}$ mutants are long-lived relative to white-eyed Canton-S (w-;CS) controls on a standard laboratory

diet consisting of 10% sucrose and 10% yeast (n=193 and 188, log-rank analysis $P < 0.001$). **b** Male *5-HT2A*$^{-/-}$ mutants are not long-lived relative to w-;CS controls on a 10% sucrose and 10% yeast diet (n=194 and 192, log-rank analysis $P = 0.71$). **c** *5-HT2A*$^{-/-}$ mutant females do not show a lifespan extension on a 15% sucrose/5% yeast diet (n=193 and 185, $P = 0.4$). **d** *5-HT2A*$^{-/-}$ mutant females are long-lived on a 5% sucrose/15% yeast diet (n=195 and 193, $P < 0.001$). **e** The mean lifespan of w-;CS control females decreases significantly as dietary protein increases (n=187-195, One-way ANOVA Diet: $P < 0.001$), and dietary protein slightly significantly affects lifespan in *5-HT2A*$^{-/-}$ mutant females (n=187-195, One-way ANOVA Diet: $P = 0.01$). The *5-HT2A*$^{-/-}$ mutant lifespan is significantly different from that of controls across diets containing 5-15% yeast (n=187-195, ANCOVA Diet: $P < 0.001$ Genotype: $P < 0.001$ Interaction: $P = 0.03$). Censored observations were ignored for the analysis of mean longevity.

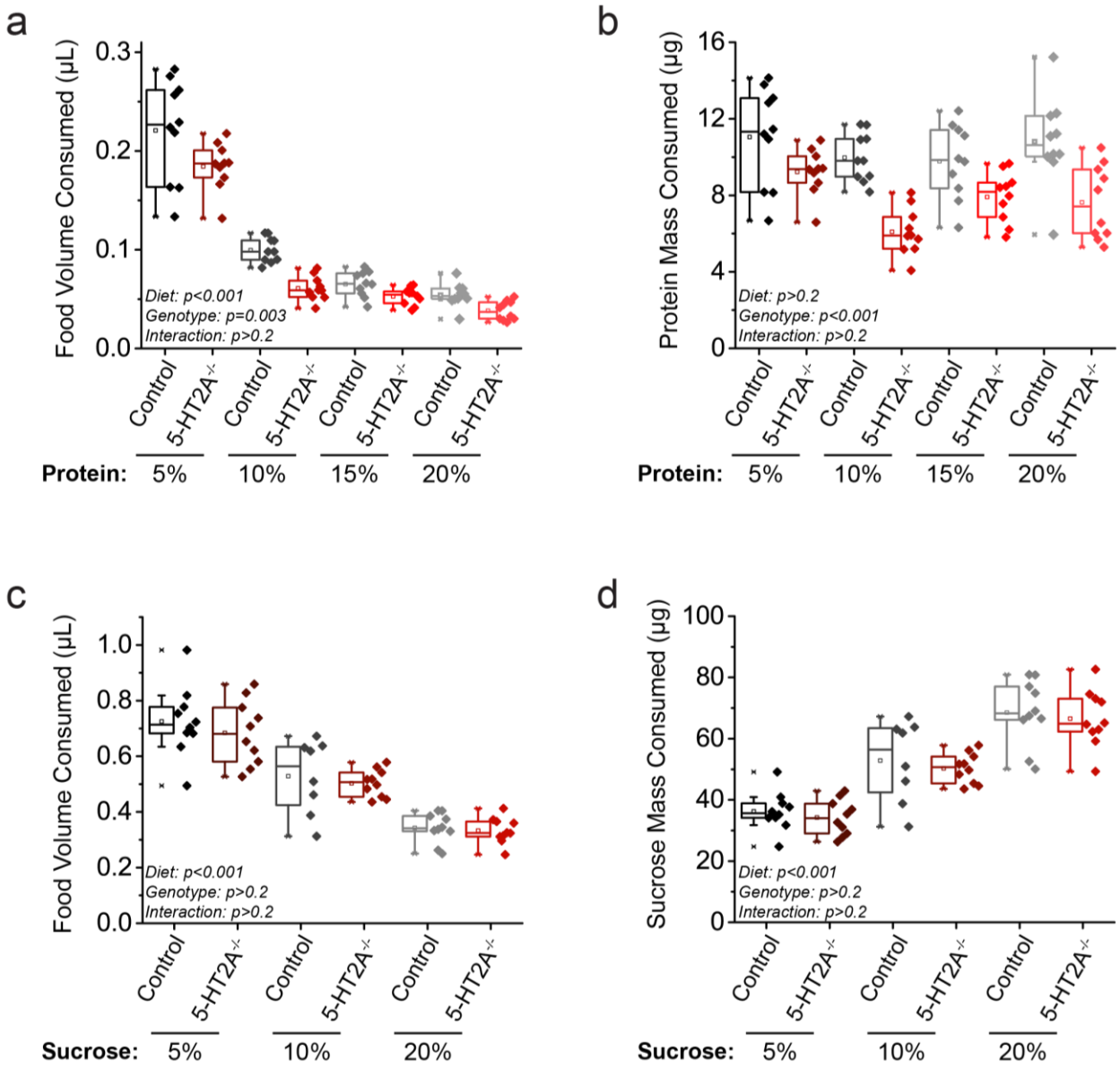


Figure 3.2. 5-HT2A modulates the protein consumption set-point. a As dietary yeast increases, both *5-HT2A*$^{-/-}$ mutants and w-;CS controls consume less (N = 10 where each replicate is comprised of 10 flies, ANCOVA Diet: P < 0.001 Genotype: P = 0.002 Interaction: P = 0.242), and when these data are scaled to represent the mass of protein consumed across diets, **(b)** *5-HT2A*$^{-/-}$ mutants show a decreased protein consumption target across diets (N = 10 where each replicate is comprised of 10 flies, ANCOVA Diet: P = 0.322 Genotype: P < 0.001 Interaction: P = 0.605). Relative to controls, *5-HT2A*$^{-/-}$ mutants show no differences from controls in the **(c)** total volume (N = 8-10 where each replicate is comprised of 10 flies, ANCOVA Diet: P < 0.001 Genotype: P = 0.263 Interaction: P = 0.594) or **(d)** mass of sucrose consumed across three different concentrations of a sucrose-only diet (N = 8-10 where each replicate is comprised of 10 flies, ANCOVA Diet: P < 0.001 Genotype: P = 0.409 Interaction: P = 0.996). All experiments were conducted in females.

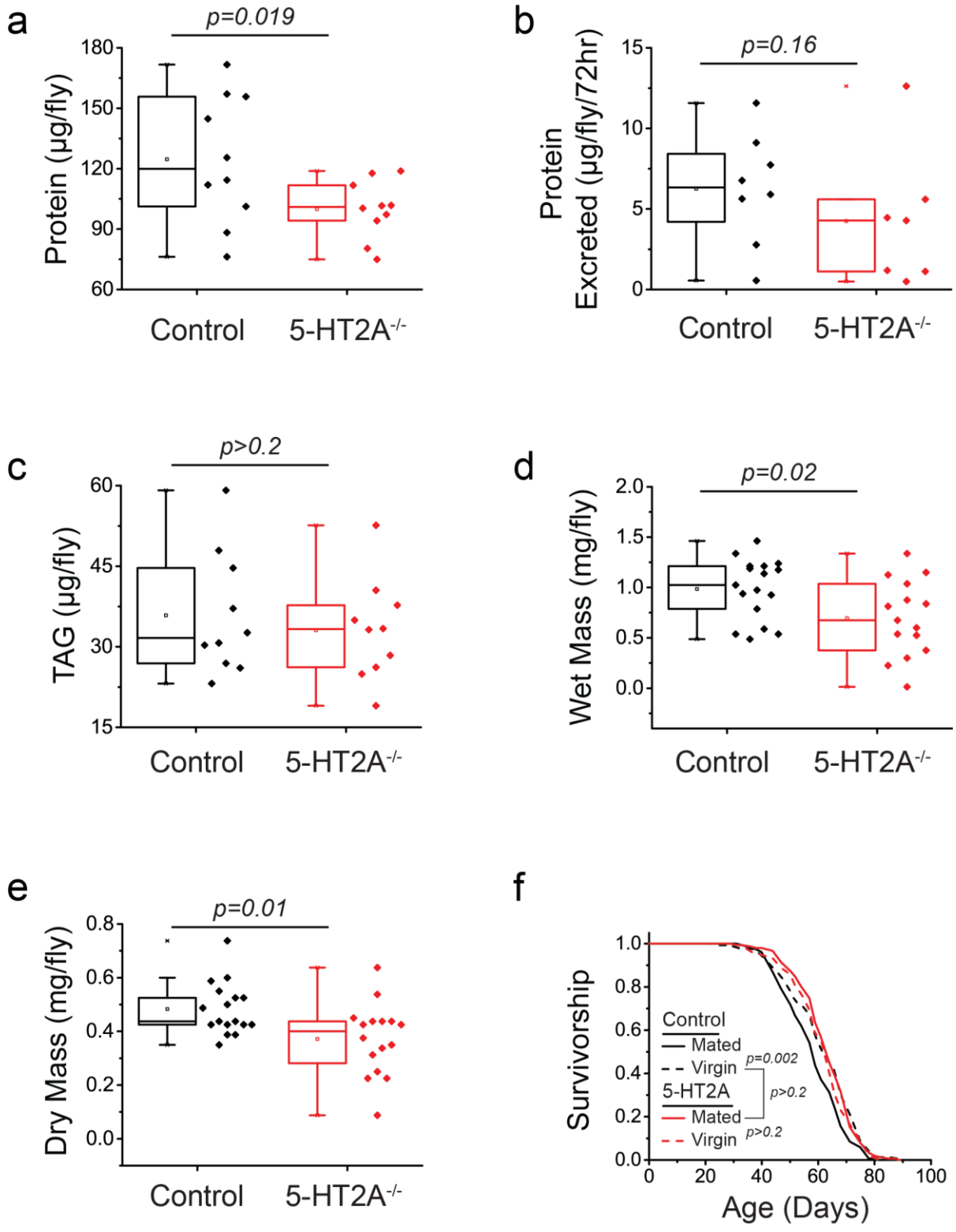


Figure 3.3. 5-HT2A modulates protein body content. a *5-HT2A*$^{-/-}$ mutants show reduced protein levels relative to controls, as measured by BCA (N = 10 where each replicate is

comprised of 5 flies, one-sided t-test P = 0.019) **(b)** but excrete the same levels of protein as controls (N = 7 and 8 where each replicate is comprised of 20 flies, one-sided t-test P = 0.16). **c** *5-HT2A*$^{-/-}$ mutants show no differences in triglyceride (TAG) levels relative to controls (N = 10 where each replicate is comprised of 5 flies, two-sided t-test P = 0.56). *5-HT2A*$^{-/-}$ mutants show a significant reduction in **(d)** wet mass (N = 15 and 17 where each replicate is comprised of 8 flies, two-sided t-test P = 0.02) or **(e)** dry mass relative to controls (two-sided t-test P = 0.01). **f** Lifespan of control flies is shortened by mating (N = 153 and 146, log-rank analysis P = 0.002), while the lifespan of *5-HT2A*$^{-/-}$ mutants is not (N = 151 and 134, log-rank analysis P = 0.3) and is not significantly different from control virgin lifespan (N = 151 and 146, log-rank analysis P = 0.9). All experiments were conducted in females.

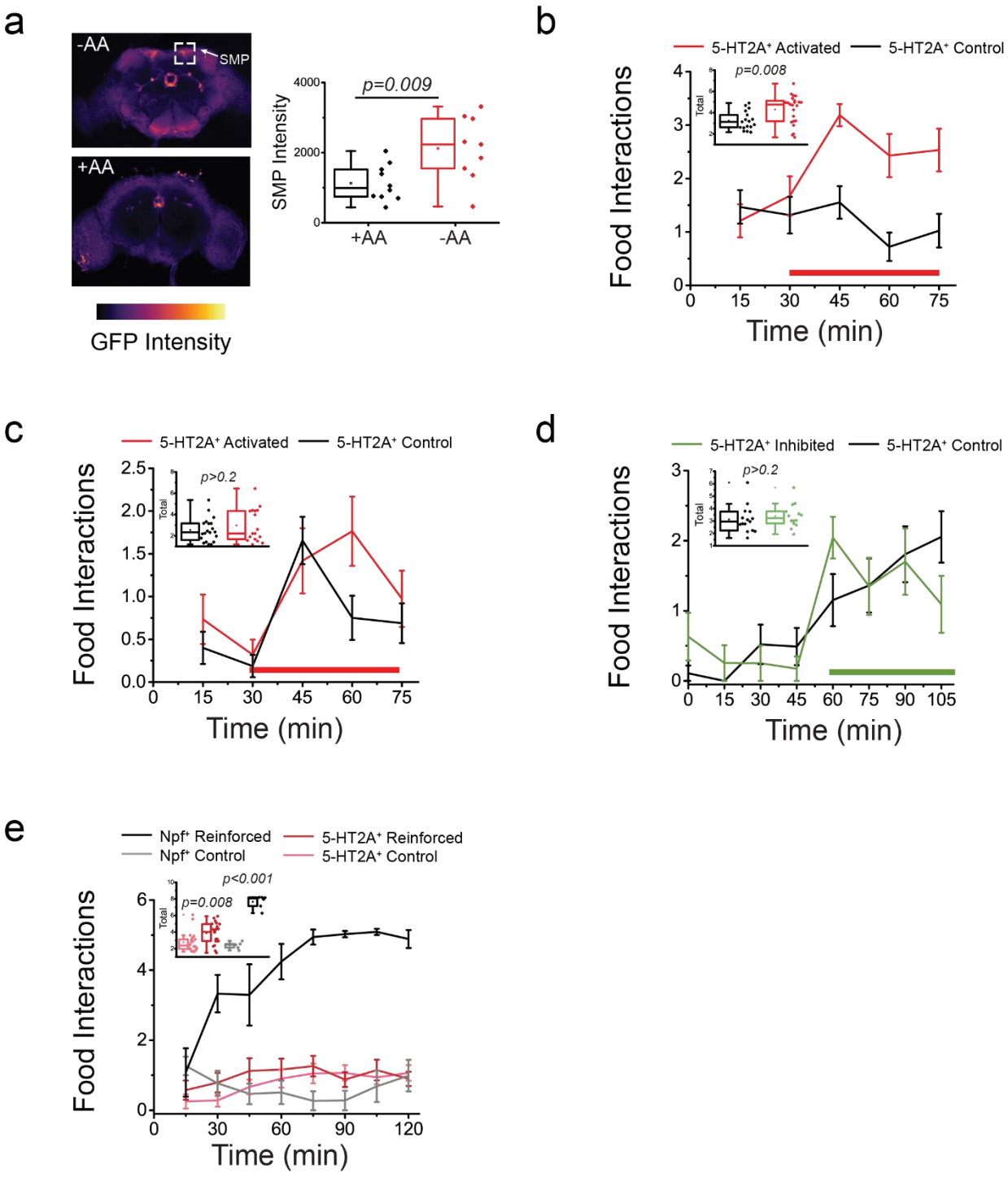


Figure 3.4. Activation of *5-HT2A*$^+$ neurons promotes protein feeding behaviors. a Amino acid (AA) deprivation activates *5-HT2A*$^+$ neurons in the superior medial protocerebrum (SMP, top) relative to amino acid replete controls (bottom). Quantification of SMP intensity (N = 10 and 9, two-sided t-test P = 0.009. **b** Activation of *5-HT2A*$^+$ neurons (red bar indicates red light activation period) promotes acute interaction with a protein solution. Inset: Quantification of

total interactions during the light period (N = 15 and 22, one-sided t-test P = 0.008). **c** Activation of *5-HT2A*$^{+}$ neurons (red bar indicates red light activation period) does not significantly alter interactions with a sucrose-only solution. Inset: Quantification of total interactions during the light period (N = 18 and 15, two-sided t-test P = 0.32). **d** Inhibition of *5-HT2A*$^{+}$ neurons (green bar indicates green light inhibition period) does not alter interactions with a protein solution. Inset: Quantification of total interactions during the light period (N = 15 and 17, two-sided t-test P = 0.5). **e** Closed-loop activation (red bar indicates red light reinforcement period) of *5-HT2A*$^{+}$ neurons modestly increases interactions with a denatonium solution compared to activation of *Npf*$^{+}$ neurons. Inset: Quantification of total interactions for closed-loop activation of *Npf*$^{+}$ neurons (N = 6, two-sided t-test P < 0.001) and *5-HT2A*$^{+}$ neurons (N = 24, two-sided t-test P = 0.008). All experiments were conducted in females.

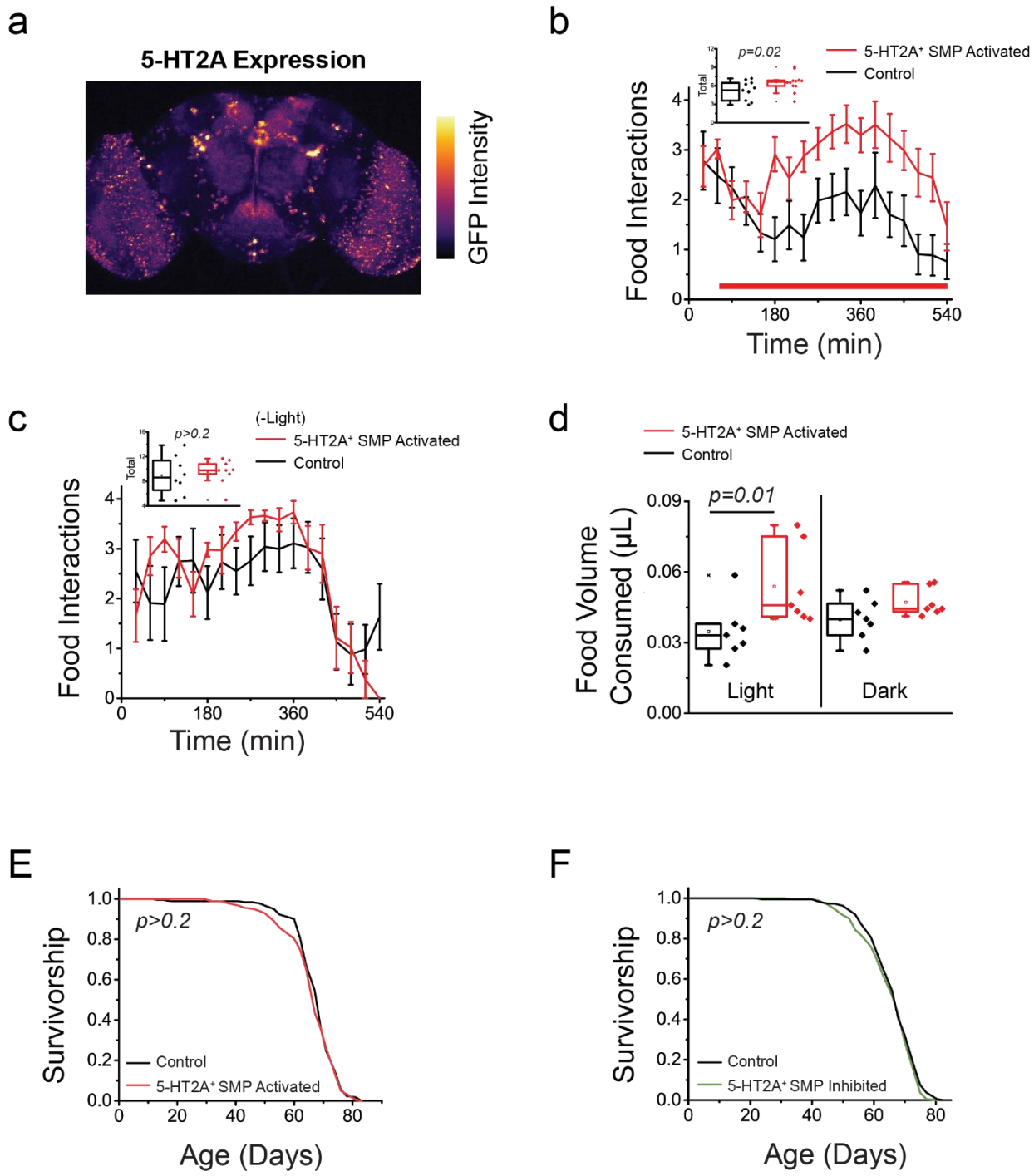
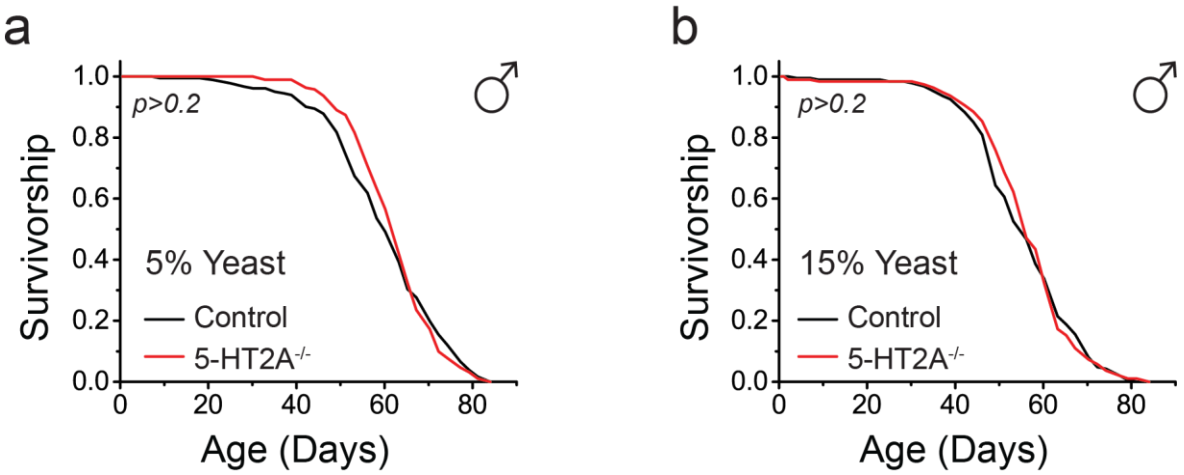
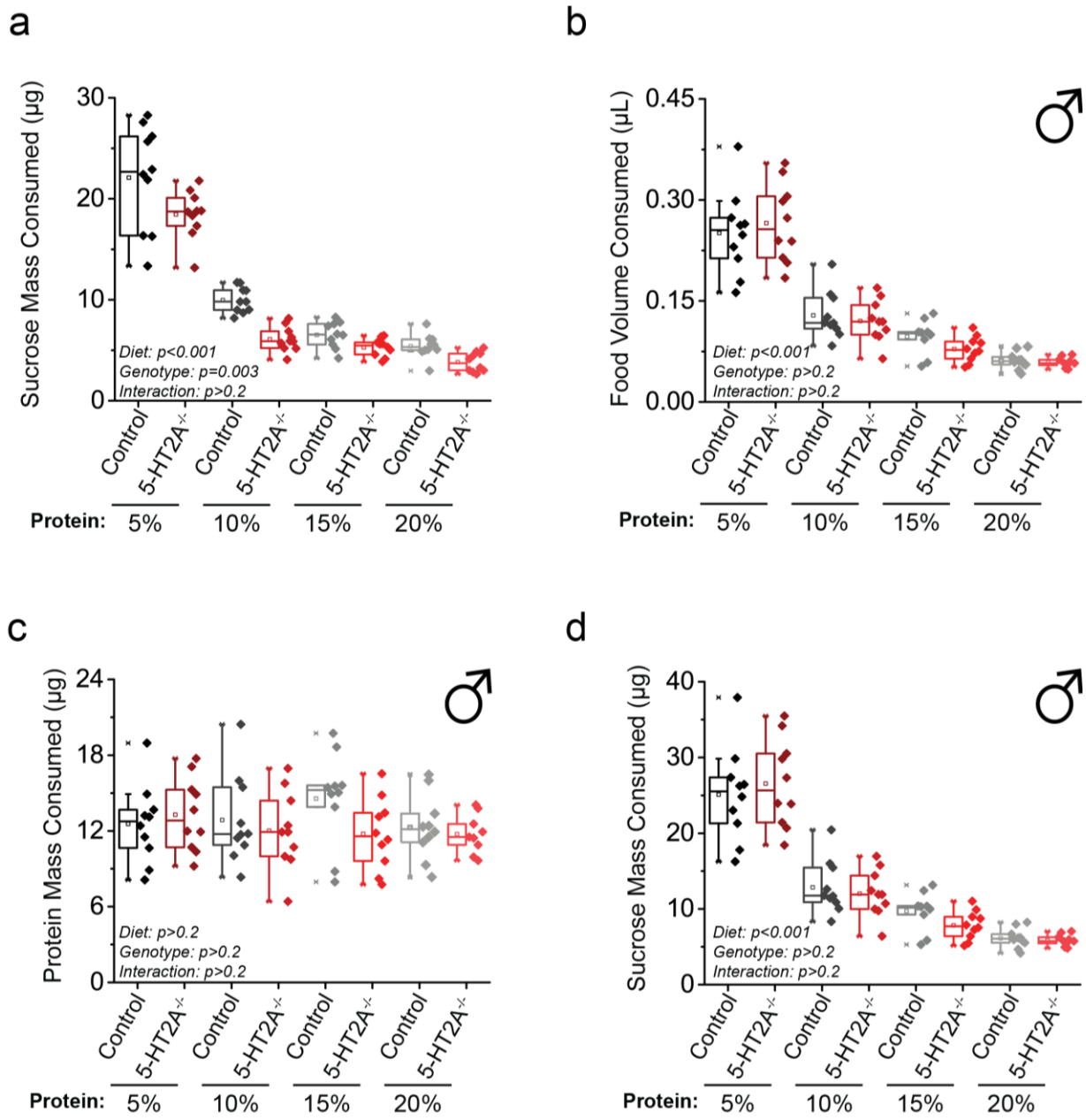


Figure 3.5. Activation of *5-HT2A*$^+$ SMP neurons promotes protein feeding behaviors. a GFP labeling of neurons driven by *5-HT2A-GAL4* in the brain (image is a max projection). **b** Activation of *5-HT2A*$^+$ SMP neurons (red bar indicates red light activation period) promotes acute interaction with a protein solution. Inset: Quantification of total interactions during the light period (N = 11 and 12, one-sided t-test P = 0.02). **c** Flies expressing *CsChrimson* in *5-HT2A*$^+$ SMP neurons do not show differences in interactions with a protein solution in the absence of red light activation (N = 8 and 9, two-sided t-test P = 0.68). **d** Activation of *5-HT2A*$^+$

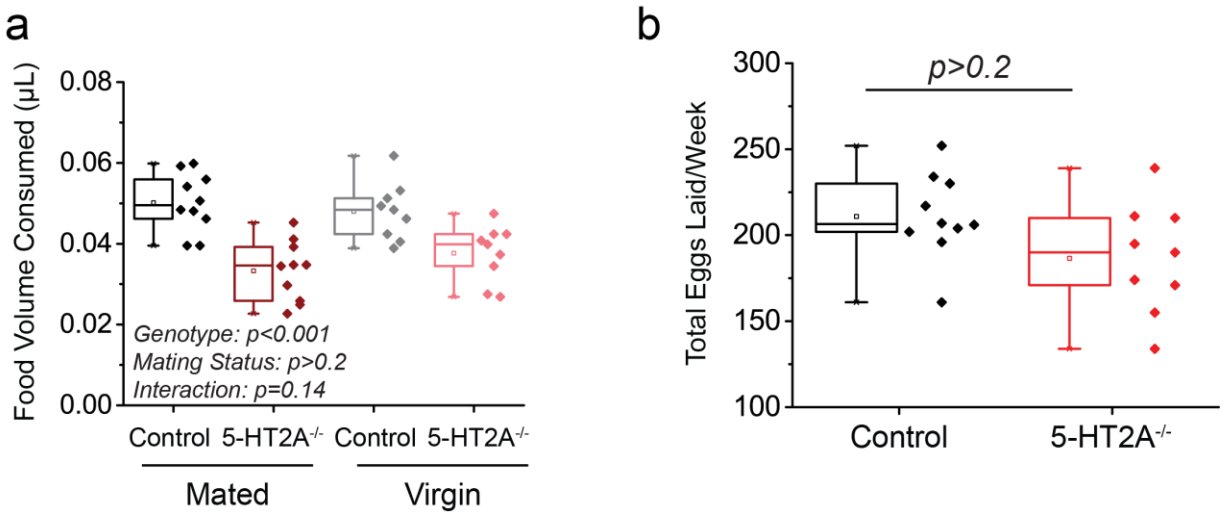
SMP neurons promotes consumption of high protein solid food (N = 7 where each replicate consists of 10 flies, one-sided t-test P = 0.01) and flies expressing *CsChrimson* in *5-HT2A*$^{+}$ SMP neurons do not show differences in solid food consumption in the absence of red light activation (N = 7 where each replicate is comprised of 10 flies, one-sided t-test P = 0.09). **e** Activation (N = 182 and 185, log-rank analysis P = 0.5) and **(f)** inhibition (N = 186 and 191, log-rank analysis P = 0.2) of *5-HT2A*$^{+}$ SMP neurons have no effect on lifespan relative to genotypic controls maintained in constant darkness. All experiments were conducted in females.



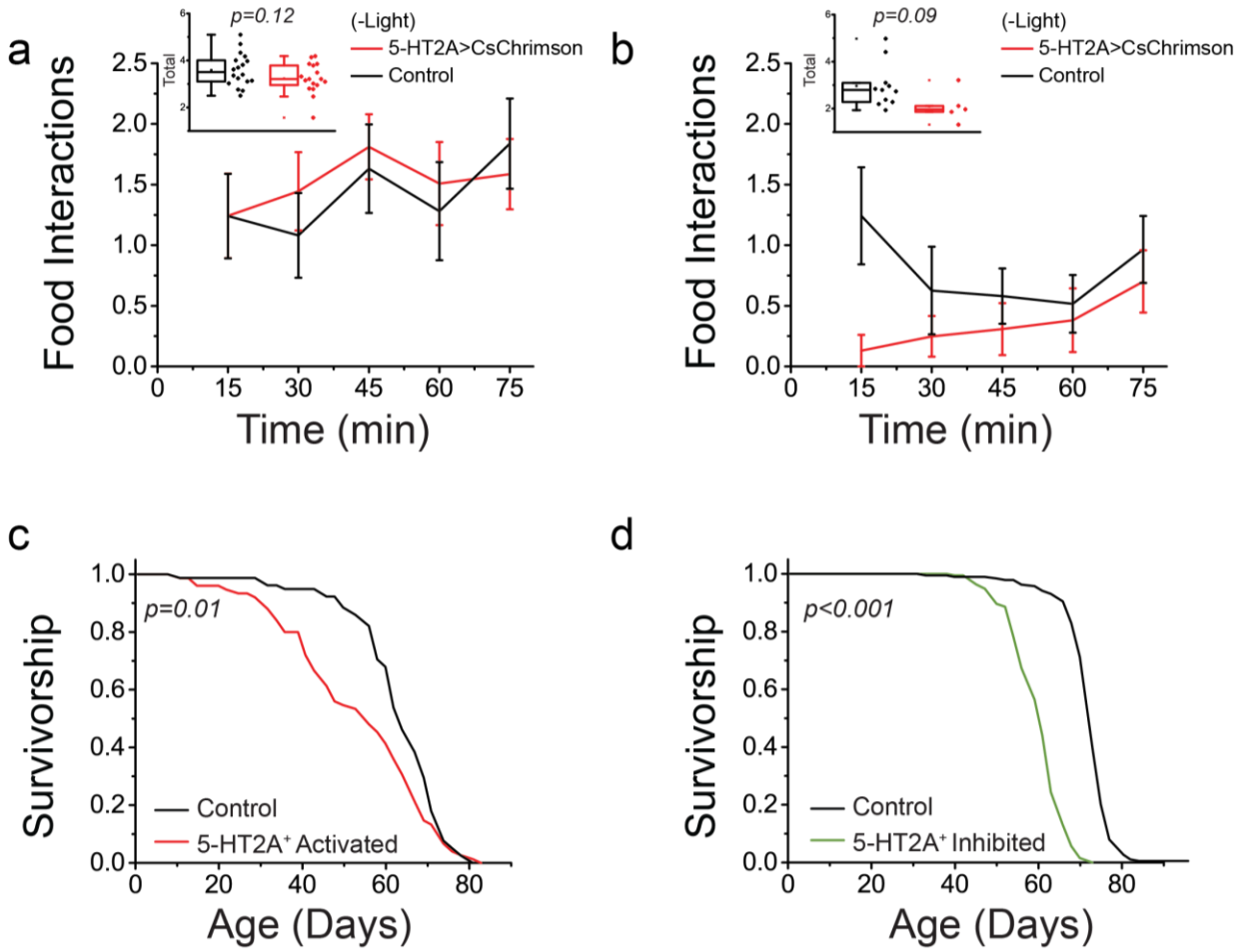
Supplementary Figure 3.1. The *5-HT2A*$^{-/-}$ mutant male lifespan is not diet-dependent. *5-HT2A*$^{-/-}$ mutant males are not long-lived on **(a)** 5% yeast (N = 181 and 192, log-rank analysis P = 0.9) or **(b)** 15% yeast diets (N = 191 and 188, log-rank analysis P = 0.8).



Supplementary Figure 3.2. The 5-HT2A-dependent changes in protein set-point are sexually dimorphic. a Female *5-HT2A*$^{-/-}$ mutant flies show differences in sucrose consumption when 10% sucrose is mixed with varying levels of yeast (N = 10 where each replicate is comprised of 10 flies, ANCOVA Diet: P < 0.001 Genotype: P = 0.002 Interaction: P = 0.242). Relative to controls, male *5-HT2A*$^{-/-}$ mutants show no differences in **(b)** total consumption (N = 9-10 where each replicate is comprised of 10 flies, ANCOVA Diet: P < 0.001 Genotype: P = 0.675 Interaction: P = 0.451) or **(c)** mass of yeast consumed across diets (N = 9-10 where each replicate is comprised of 10 flies, ANCOVA Diet: P = 0.515 Genotype P = 0.206 Interaction: P = 0.338). **d** Male *5-HT2A*$^{-/-}$ mutant flies show differences from controls in sucrose consumption when 10% sucrose is mixed with varying levels of yeast (N = 9-10 where each replicate is comprised of 10 flies, ANCOVA Diet: P < 0.001 Genotype: P = 0.675 Interaction: P = 0.451).



Supplementary Figure 3.3. **The changes in protein set-point are not influenced by mating status or reproduction. a** Mating status does not affect the 5-HT2A-dependent reduction in yeast consumption (N = 9-10 where each replicate is comprised of 10 flies, Two-way ANOVA Genotype: P < 0.001 Mating Status P = 0.64 Interaction P = 0.14), and **(b)** loss of 5-HT2A does not alter total reproduction, measured as eggs laid over seven days (N = 9 and 10 where each replicate is comprised of 5 flies, two-sided t-test P = 0.23). All experiments were conducted in females.



Supplementary Figure 3.4. 5-HT2A$^+$ neuronal activation requires light and neuronal manipulations influence lifespan. Flies expressing *CsChrimson* in *5-HT2A*$^+$ neurons do not show differences in interactions with a **(a)** protein solution (inset: quantification of total interactions, N = 23 and 25, two-sided t-test P = 0.12) or **(b)** sucrose-only solution in the absence of red light activation (inset: quantification of total interactions, N = 12 and 18, two-sided t-test P = 0.09). **c** Activation (N = 78 and 79, log-rank analysis P = 0.01) and **(d)** inhibition of 5-HT2A$^+$ neurons shorten lifespan relative to controls maintained in constant darkness (N = 188 and 194, log-rank analysis P < 0.001). All experiments were conducted in females.

	SY10	S15Y5	S5Y15	S10Y5	S10Y15	S10Y20	S5	S10	S20
Agar (g)	20	20	20	20	20	20	20	20	20
Sucrose (g)	100	150	50	100	100	100	50	100	200
Yeast (g)	100	50	150	50	150	200			
Tegosept (20%)	15	15	15	15	15	15			
Propionic Acid (mL)	3	3	3	3	3	3			

Table 3.1 Diets used in lifespan and/or ConEx experiments. Values are based on 1L total volume.

CHAPTER IV

Sensory Perception of Dead Conspecifics Modulates Lifespan through 5-HT2A Signaling[2]

Preface

The overarching goal of this thesis was to understand the roles of serotonin signaling pathways in health and lifespan. Upon discovering a high level of variability in the effects on lifespan through manipulation of distinct signaling pathways (see Chapter II), my attention was drawn to one serotonin receptor in particular, 5-HT2A, for its numerous roles in lifespan. As I was uncovering the potential mechanisms by which 5-HT2A signaling modulates lifespan in response to perception of the nutritional environment (see Chapter III), I was simultaneously part of a project focused on understanding the mechanisms by which sensory perception of other dead flies influences lifespan in a 5-HT2A signaling-dependent fashion. In this study, detailed in this chapter, we observed that fly lifespan is shortened upon visual and olfactory perception of dead conspecifics. Additionally, when flies are exposed to dead conspecifics, they become aversive to other flies. When 5-HT2A signaling is disrupted via pharmacological and genetic manipulations, this blocks the lifespan shortening and aversion induced by death perception. This study was led by two lab members, Drs. Tuhin Chakraborty and Christi Gendron, who initiated this project prior to my tenure in the lab. Upon working on the project, I quickly became interested in the requirement of 5-HT2A signaling in this context. In support of 5-HT2A signaling requirement in death perception, I discovered that chronic activation of 5-HT2A-expressing neurons is sufficient to both shorten fly lifespan and make flies aversive to other flies, the opposite effect of loss of 5-HT2A. This is also consistent with my findings in Chapters II and III, which suggest that less 5-

[2] This chapter is based on a co-authored published manuscript: Chakraborty, T. S., Gendron, C. M., Lyu, Y., **Munneke, A. S.,** DeMarco, M. N., Hoisington, Z. W., & Pletcher, S. D. (2019). Sensory perception of dead conspecifics induces aversive cues and modulates lifespan through serotonin in Drosophila. *Nature communications*, *10*(1), 2365. https://doi.org/10.1038/s41467-019-10285-y. Individual contributions to this manuscript are detailed on Page xi.

HT2A signaling is more beneficial to lifespan in other contexts, as well. The findings detailed in this chapter, together with the results from the pervious chapter, provide insight into the various scenarios in which 5-HT2A signaling acts as an important modulator of lifespan.

Abstract

Sensory perception modulates health and aging across taxa. Understanding the nature of relevant cues and the mechanisms underlying their action may lead to novel interventions that improve the length and quality of life. We found that in the vinegar fly, *Drosophila melanogaster*, exposure to dead conspecifics in the environment induced cues that were aversive to other flies, modulated physiology, and impaired longevity. The effects of exposure to dead conspecifics on aversiveness and lifespan required visual and olfactory function in the exposed flies. Furthermore, the sight of dead flies was sufficient to produce aversive cues and to induce changes in the head metabolome. Genetic and pharmacologic attenuation of serotonergic signaling eliminated the effects of exposure on aversiveness and lifespan. Our results indicate that *Drosophila* have an ability to perceive dead conspecifics in their environment and suggest conserved mechanistic links between neural state, health, and aging; the roots of which might be unearthed using invertebrate model systems.

Introduction

Sensory perception influences energy homeostasis, tissue physiology, and organism aging through neuronal circuits that emanate from sensory tissues and that interface with deeper regions of the central nervous system. The molecular nature of these relationships was first described in the nematode, *Caenorhabditis elegans* and sensory effects on aging have been observed across the phylogeny of vertebrate and invertebrate animals (Apfeld and Kenyon 1999, Libert, Zwiener et al. 2007, Smedal, Brynem et al. 2009, Riera, Huising et al. 2014, Waterson, Chung et al. 2014, Fletcher and Kim 2017). Sensory inputs relate information about nutrient availability and reproductive opportunity to rapidly initiate physiological changes that occur in coordination with known behavioral outcomes, suggesting similarities in the underlying circuitry. Conserved neuromodulators, including biogenic amines and neuropeptides, that influence responses to food and mates are known to also modulate lifespan in a state-dependent manner (Libert, Zwiener et al. 2007, Gendron, Kuo et al. 2014, Maures, Booth et al. 2014, Shi and Murphy 2014, Waterson, Chung et al. 2014, Ro, Pak et al. 2016).

The ability to perceive dead individuals is not exceptional in the animal kingdom, as individuals from a range of species respond to dead conspecifics with a variety of different effects. Social insects, including ants and honey bees, exhibit necrophoresis in which dead colony members are systematically removed from the nest to promote hygienic conditions (Choe, Millar et al. 2009). Dead zebrafish scents provoke defensive behavior in live individuals (Oliveira, Koakoski et al. 2014), and the sight of a dead conspecific induces alarm calling in scrub-jays (Iglesias, McElreath et al. 2012), suggesting that dead individuals may indicate danger. Elephants and nonhuman primates exhibit stereotypical behaviors toward dead individuals associated with permanent loss of a group member (Anderson 2016). In humans, the effects of experiences with death include emotional dysregulation and depression as well as physical effects, such as headaches, fatigue, and cardiovascular disease (Marmar, McCaslin et al. 2006, Alexander and Klein 2009, Keyes, Pratt et al. 2014).

In some cases, the sensory mechanisms through which individuals perceive dead conspecifics are known (e.g., (Choe, Millar et al. 2009, Wisman and Shrira 2015, McAfee, Chapman et al.

2018)), but to our knowledge, the extent to which these experiences influence aging and, if so, the degree to which the effects are shared across species are yet to be determined. In this study, we provide evidence that exposure of *Drosophila melanogaster* to dead conspecifics (*i*) induces cues in the exposed flies that are aversive to other non-exposed flies, (*ii*) modulates several physiological parameters including the abundance of stored lipid, respiration rate, and climbing ability, and (*iii*) reduces lifespan. These behavioral and physiological effects are likely mediated by sensory perception because our observed phenotypes required visual cues and were modulated by olfactory cues. Furthermore, the sight of dead flies, but not their smell or taste, was sufficient to induce the production of aversive cues. The negative effects of exposure to dead conspecifics were reversed by targeted pharmacologic and genetic attenuation of serotonin signaling, suggesting the possibility that such effects are conserved in other taxa.

Results

Drosophila exposed to dead conspecifics induce aversive cues

While investigating whether adult *Drosophila* behaviorally respond to diseased individuals in their environment, we discovered that flies show an aversive response after exposure to dead conspecifics. In our initial experiments, we established a binary choice assay (T-maze) in which flies that were previously infected with the lethal pathogen *Pseudomonas Aeruginosa PLCS* were placed behind a screen in one side of a T-maze and healthy flies were placed in the opposite side. When naïve choosers were loaded into the T-maze, we found that they sorted non-randomly, in that they avoided the side of the T-maze containing a group of flies that had been infected 24-48 hours previously (Supplementary Fig. 4.1a). We consistently failed to observe avoidance in naïve choosers to groups of flies that had been infected for less than 24 hours. Flies began dying from our *Pseudomonas* infection roughly 24 hours post-infection, suggesting that the appearance of dead flies rather than infection might be the cause for the aversion. We therefore asked whether dead flies alone were sufficient to create an aversive stimulus. We found that they were not (Supplementary Fig. 4.1b). When comparing preference between only healthy live flies vs. only dead flies, naïve choosers preferred dead flies (Supplementary Fig. 4.1c) presumably due to CO_2 emitted from live animals, which is a known repulsive stimulus (Suh, Wong et al. 2004), establishing that the dead animals themselves were not intrinsically aversive. We therefore asked

whether a mixture of dead flies with healthy live flies was aversive compared to healthy live flies alone. We observed a strong preference of naïve flies choosing the side of the T-maze without dead animals (Supplementary Fig. 4.1d). Finally, healthy flies from different laboratory strains that had been pre-exposed to dead conspecifics for 48 hours (the dead flies were removed immediately prior to the choice assay) exhibited aversive qualities (Fig. 4.1a and b), establishing that the presence of dead flies in the T-maze was not required for aversion. Together these data indicated that dead fly exposure triggered changes in healthy live individuals that repelled naïve choosers.

Control experiments ruled out positional artifacts and biases in our technical apparatus as causes for the preferred segregation of naïve choosers away from flies exposed to dead animals. Naïve choosing flies segregated randomly when both sides of the T-maze were empty or when both sides contained equal numbers of live, unexposed flies (Supplementary Fig. 4.2a-b). Aversive cues were not induced when flies were mock-exposed to dead animals using black beads that are roughly the size of a fly, and the presence of dead flies did not affect feeding over 24 hours (Supplementary Fig. 4.2c-d). This suggests that aversive cues in exposed flies are not triggered by structured environments or by changes in food accessibility. We also tested whether exposed flies were exhibiting a response to perceived increases in population density by augmenting the number of live flies in the unexposed treatment so that the total number of flies in the pre-choice environments was equal. We observed no induction of aversive cues, thus ruling out density effects (Supplementary Fig. 4.2e).

Cause of death, duration of exposure, and evolutionary relatedness affect the aversive response
Subsequent experiments using only healthy animals pre-exposed to dead individuals before behavioral testing revealed that the effects of dead fly exposure are reproducible and are influenced by the characteristics and or/reason for death of the dead flies. Exposed flies lost their aversive characteristics approximately ten minutes after dead flies were removed (Fig. 4.1c), which is approximately the time-span of short-term memory in *Drosophila* (Keene and Waddell 2007), indicating that the aversive effect is persistent but short-lived. The aversiveness of exposed flies was affected by the number of dead flies included in the environment and the duration of exposure: exposed flies became more aversive as the time of exposure and number of

dead in the environment increased, although in standard rearing vials the magnitude of the effect saturated at roughly 48 hours and 10 dead animals, respectively (Fig. 4.1d-e). Flies exposed for 48 hours to flies that died from starvation or from normal aging triggered aversive cues, while a similar exposure to flies killed by immersion in liquid nitrogen did not (Fig. 4.1f). Flies that had died 46 days prior to testing also failed to induce aversive cues in exposed animals (Fig. 4.1g).

We tested whether the effects of exposure to dead animals would be influenced by the evolutionary relatedness between the dead and live animals by exposing *Drosophila melanogaster* to dead individuals from one of three related species (*Drosophila virilis*, *Drosophila simulans*, and *Drosophila erecta*). We found that exposure to dead animals from the two closely related species (*D. simulans* and *D. erecta*) were able to induce aversive cues in *D. melanogaster* to a similar extent as did exposure to their conspecifics, while exposure to the evolutionarily more distant *D. virilis* did not (Fig. 4.2a).

Exposure to dead conspecifics alters physiology and lifespan

Having observed that exposure of healthy flies to dead conspecifics consistently resulted in the production of aversive cues that repelled naïve choosers, we next sought to investigate whether this treatment affected physiology and longevity in the exposed flies. We found that short-term exposure of *D. melanogaster* to dead conspecifics compromised starvation survival and reduced levels of triacylglycerol (TAG), which is the primary storage lipid in flies (Fig. 4.2b-c). It also resulted in a moderate but significant reduction in CO_2 production, indicative of an altered metabolic rate (Fig. 4.2d). Exposed flies were capable of normal levels of spontaneous activity and exploration (Fig. 4.2e), but they showed impaired motivated climbing ability (Fig. 4.2f). Finally, chronic exposure to dead animals significantly reduced lifespan (Fig. 4.2g), which was robust to experimental strain (Supplementary Fig. 4.3a), was sex-specific in its magnitude (Supplementary Fig. 3b), was reduced in isolation (Supplementary Fig. 4.3c), and was not caused by population density (Supplementary Fig. 3d) or by environmental structure (Supplementary Fig. 4.3e).

Sight of dead is necessary and sufficient for aversive cues

We hypothesized that the effects of exposure to dead animals relied on one or more sensory modalities in the healthy exposed flies. This is supported by the fact that gustatory and olfactory circuits have previously been shown to influence aging and physiology in *Drosophila* (Libert, Zwiener et al. 2007, Gendron, Kuo et al. 2014, Ostojic, Boll et al. 2014, Waterson, Chung et al. 2014). We therefore asked which sensory modalities were necessary for aversive cues to be triggered upon exposure to dead animals. We found that naïve choosers exhibited no behavioral preference in the T-maze when the exposure to dead flies had taken place in the dark (Fig. 4.3a). To further demonstrate that the response is visually mediated and to assess potential interaction between light and dead flies, we repeated these experiments in our standard 12h:12h light:dark conditions using *norpA* mutant flies, which are blind. We observed no evidence of aversive cues from *norpA* mutants following exposure to dead flies (Fig. 4.3b).

Orco^{2} mutant flies are broadly anosmic, and they exhibited a significant, but not complete, loss of the aversive cues (Fig. 4.3c). On the other hand, flies lacking the *ionotropic receptor 76b* (Zhang, Li et al. 2013), which is involved in chemosensory detection of amino acids and salt, exhibited normal aversion following dead exposure, as did flies lacking both *Ir8a* and *Ir25a* ionotropic co-receptors, which are required for multiple sensory functions (Abuin, Bargeton et al. 2011) (Supplementary Fig. 4.4a-b). Flies carrying a *poxn* mutant allele, which have impairment in taste perception, exhibited a similar response to death exposure as genetically homogenous control flies (Fig. 4.3d).

We next asked whether different sensory properties of the dead flies as stimuli were sufficient to induce aversiveness in healthy flies. Using a chamber that was designed to allow flies to see dead flies but remain physically separated from them (Supplementary Fig 4.5a), we found that the sight of starvation-killed flies was sufficient to induced aversive cues to the same extent as direct exposure (Fig. 4.3e). Using this chamber, the sight of flies that had been killed by immersion in liquid nitrogen had no effect (Fig. 4.3e), thus replicating our earlier results that the type of death is important for the induction of aversive cues (Fig. 4.1f, Supplementary Fig. 4.6a), and that flies killed by freezing lack one or more key visual characteristics that are present in flies that died from starvation. The ability of flies to distinguish differences by sight may also explain why live

D. melanogaster do not convey aversive signals when exposed to dead *D. virilis*, as these flies are darker and larger than *D. melanogaster* themselves (Supplementary Fig. 4.6b). Interestingly, however, repeated exposure to flies killed by freezing over a 20 day (but not 10 day) period was sufficient to induce aversiveness cues indicating that flies may eventually learn to recognize these as dead or to respond to alternative cues (Supplementary Fig. 4.5b-c). A second specialized chamber was used to investigate if olfactory cues (Supplementary Fig. 4.5d) were sufficient to induce aversion. An aversive effect was not seen in flies that were exposed to volatile odors from flies that died of starvation indicating that olfactory cues alone were not sufficient to induce aversion (Fig. 3f). Olfactory cues from starved animals were also incapable of gating otherwise insufficient visual cues from animals that had died by freezing (Fig. 4.3g). Finally, aversiveness cues were not induced in flies by direct exposure to extracts from homogenized flies that had died by starvation, suggesting gustatory cues are also not sufficient to induce aversiveness (Fig. 4.3h).

Vision is necessary for impaired lifespan in exposed flies

The significant reduction in lifespan that resulted from chronic exposure to dead animals was absent when flies were aged in constant darkness (Fig. 4.4a; Supplementary Fig. 4.7a). Unexposed control flies were longer-lived in constant darkness, which is consistent with an effect of death perception in cohorts aging normally in light-dark cycles. Blind *norpA* mutants also exhibited significantly reduced effects of exposure to dead flies on lifespan (Fig. 4.4b, Supplementary Fig. 4.7c). Long-term exposure to flies killed by freezing reduced lifespan (Supplementary Fig. 4.7b). Largely anosmic $Orco^{2}$ mutant flies exhibited a partial decrease of the effect of exposure to dead flies on lifespan (Fig. 4.4c), while flies lacking *Ir76b* or flies lacking both *Ir8a* and *Ir25a* exhibited a decreased lifespan following exposure to dead flies to the same extent as genetically homogenous control animals (Supplementary Fig. 4.4c-d). Finally, *poxn* mutants also exhibited a similar decrease in lifespan in response to exposure to dead flies as control flies (Fig. 4.4d, Supplementary Fig. 4.7c).

Our findings suggest the effects of exposure to dead animals are not caused by infection, bacterial proliferation, or changes in the gut biota in either dead or exposed animals. Similar levels of aversion were induced when axenic flies were used as both exposed and dead flies,

establishing that these factors do not play a significant role in the aversive response (Supplementary Fig. 4.8). It remains possible that the effects of death exposure on aversion and lifespan are, at least in part, mechanistically distinct, and we were unable to formally test the latter because lifespan exposure experiments comprised entirely of axenic animals is technically unfeasible. To date we know of no evidence that the disparate environmental and genetic manipulations used here to reduce or eliminate the effects of exposure to dead conspecifics on lifespan affect the microbiota, but nonetheless we cannot rule this out as a potential confound. Together, these data indicate that there is an essential perceptual component associated with the physiological and health effects of exposure to dead conspecifics. Sight of naturally dead conspecifics is both necessary and sufficient to induce physiological effects, suggesting a model in which visual cues serve as the primary way in which *D. melanogaster* distinguish dead flies. While gustatory cues are not involved in the effects that we observe, the role of olfaction is less clear. Smell-deficient flies respond less strongly to dead conspecifics, but odors from dead flies are not sufficient to induce changes in aversiveness. The aversive cues emitted by flies following exposure to dead individuals do have a significant olfactory component: when $Orco^2$ mutants were used as naïve choosers in the T-maze assay (e.g., described in Fig. 4.1a), they assorted randomly between the two arms (Supplementary Fig. 4.9). Similar results were observed when naïve choosers carried a loss of function mutation for *Gr63a*, an essential component of the *Drosophila* CO_2 receptor (Supplementary Fig. 4.9). We therefore currently favor a model in which olfaction mediates a social cue among exposed animals as a result of visual perception of dead flies.

Changes in the head metabolome follow exposure to dead flies

We next asked whether we might identify a signature of this putative perceptive event by comparing the metabolome of the homogenized heads of experimental flies following 48 hours of dead exposure to that of unexposed animals. *norpA* mutant flies, which were treated identically but which lacked the ability to see dead flies, were analyzed simultaneously to account for temporal effects and to isolate potential causal metabolites. Targeted metabolite analyses identified 119 metabolites present in treatments for positive and negative modes (see Supplementary file for raw data). Using a randomization procedure together with principal component analysis (PCA), we identified a single principal component (PC10) that significantly

distinguished the neuro-metabolomes of exposed and unexposed flies but was unchanged by exposure in *norpA* mutant flies (Fig. 4.5a and Supplementary Fig. 4.10a). The multivariate analysis revealed that five of the top ten metabolites associated with the effects of exposure to dead flies (i.e., those strongly loaded on PC10) have been implicated in models of anxiety, depression, and/or mood disorders in mammals (specifically lactate (Ernst, Hock et al. 2017), quinolinate (Myint 2012), sorbitol (Liu, Zheng et al. 2014), 3-hydroxybutyric acid (Yin, Maalouf et al. 2016), and sarcosine (Woo, Chun et al. 2015)). In addition to our systems biology analysis, we also asked whether any individual metabolites were statistically correlated with exposure to dead individuals in experimental but not *norpA* mutant flies (i.e., exhibited a statistically significant interaction between treatments). We found only one, glyceraldehyde (Fig. 4.5b), likely due to limited statistical power for this question.

Serotonin signaling mediates the effects of exposure to dead

Following the metabolomic analysis, we focused on pharmacologic compounds with anti-depressant or anti-anxiety effects with the goal of identifying molecular targets that are required for the health consequences of exposure to dead flies (Supplementary Table 4.1). For T-maze assays, flies were treated with each drug prior to exposure to dead individuals, while for lifespan assays flies were treated throughout life. Of those compounds examined, we found one, pirenperone, which abrogated the effects of death perception on both aversive cues and lifespan (Fig. 4.5c-d, Supplementary Table 4.1). Pirenperone is a putative antagonist of the serotonin 5-HT2 receptor in mammals, although it may interact with other biogenic amine receptors at high concentrations (Pawłowski, Siwanowicz et al. 1985). We therefore tested whether loss of the 5-HT2A receptor recapitulated the effects of pirenperone and abrogated the effects of death perception on aversion and lifespan in *Drosophila*. We found that it did (Fig. 4.5e-f, Supplementary Fig. 4.10b-c), suggesting that serotonin signaling through the 5-HT2A branch is required to modulate health and lifespan in response to this perceptive experience.

Finally, we asked whether activation of *5-HT2A*$^{+}$ neurons was sufficient to recapitulate the aversion and lifespan phenotypes we observed following death perception. We ectopically expressed the thermosensitive cation channel Transient Receptor Potential A1 (TRPA1) in cells that putatively produce 5-HT2A (using *5-HT2A-GAL4*). The *Drosophila* TrpA1 channel

promotes neuron depolarization only at elevated temperatures (>25°C), thereby allowing temporal control over cell activation (Hamada, Rosenzweig et al. 2008). Transgenic flies (*5-HT2A-GAL4>UAS-dTrpA1*) and their genetic control strain (*5-HT2A-GAL4;+*) were raised and maintained for 10 days after eclosion at 18°C (non-activating conditions). They were then placed at 29°C for 48 hours to activate the neurons, over a time frame similar to the death perception assays. We found that after activation, transgenic flies were aversive relative to the genetic control (Fig. 4.6a; $P < 0.001$). Chronic activation of *5HT2A*$^{+}$ neurons throughout life also decreased longevity consistent with our previous data (Fig. 4.6b).

Discussion

Sensory perception has emerged as a potent modulator of aging and physiology across taxa. Exposure of *Drosophila* and *C. elegans* to food-based odorants limits the beneficial effects of dietary restriction (Libert, Zwiener et al. 2007, Smith, Kaeberlein et al. 2008), while perception of the opposite sex modulates lifespan through neural circuits that utilize conserved neuropeptides to establish motivation and reward (Gendron, Kuo et al. 2014, Maures, Booth et al. 2014, Waterson, Chung et al. 2014). Loss of specific olfactory and gustatory neurons modulates lifespan and physiology and influences measures of healthy aging, including sleep and daily activity patterns (Alcedo and Kenyon 2004, Linford, Kuo et al. 2011, Waterson, Chung et al. 2014, Linford, Ro et al. 2015). Our data are consistent with an additional perceptive influence that affects longevity in *Drosophila*, exposure to dead conspecifics, which depends on visual and olfactory function. Not all dead flies induced such effects (flies that died by freezing or flies that were long dead or were of a distantly-related species failed to do so) suggesting flies have the perceptive ability to distinguish differences in these carcasses. These results are consistent with reports documenting sufficient visual acuity in flies to distinguish different ecologically-relevant cues in their environment, such as parasites and competitors (Kacsoh, Lynch et al. 2013, de la Flor, Chen et al. 2017, Schneider, Murali et al. 2018). Additional experiments designed to identify the precise cues that convey information about dead conspecifics to exposed flies, and on to non-exposed counterparts, will be required

This report adds to growing evidence that serotonin is an important component of how different sensory experiences modulate aging and aging-related disease across taxa. Serotonin modulates sensory integration in mammals (Hanson and Hurley 2014) and has been linked to the longevity effects associated with sensory perception of food (Chao, Komatsu et al. 2004, Ro, Pak et al. 2016) and hypoxia (Petrascheck, Ye et al. 2007, Petrascheck, Ye et al. 2009, Leiser, Miller et al. 2015), In *C. elegans*, a global, cell non-autonomous response to heat is triggered by thermo-sensory neurons in a serotonin dependent manner, which is also capable of extending lifespan (Tatum, Ooi et al. 2015). Lifespan extension by activation of the hypoxic response also requires specific components of serotonin signaling in sensory neurons (Leiser, Miller et al. 2015). In *Drosophila*, serotonin is required for protein perception, and loss of receptor *5-HT2A* increases

fly lifespan in complex and potentially stressful nutritional environments (Ro, Pak et al. 2016). Whether serotonin is merely permissive for changes that affect lifespan or whether it directly modulates aging remains unclear. There is evidence for a direct role in *C. elegans*, where feeding worms serotonin receptor antagonists is sufficient to extend lifespan by putatively mimicking dietary restriction (Petrascheck, Ye et al. 2007). We present evidence that activation of 5-HT2A neurons is sufficient to modulate aging, but these results should be interpreted with caution; both aversiveness and short lifespan might instead be reflective of unrelated molecular changes that result in less healthy animals.

Although death perception is known to occur in several species throughout the animal kingdom (Anderson 2016), this is, to our knowledge, the first indication that such an ability may be present in an invertebrate laboratory model system. We suggest a model in which dead conspecifics serve as a threat cue that results in negative consequences on metabolism, physical condition, and aging (Curran and Chalasani 2012, Mohammad, Aryal et al. 2016). Neural states akin to fear, anxiety, and depression have recently been described in *Drosophila*, with phenotypic manifestations and molecular causes that are consistent with those observed in mammals (O'Kane 2011, Mohammad, Aryal et al. 2016, Jiang, Zheng et al. 2017, Ries, Hermanns et al. 2017). Moreover, associations linking human morbidity and aging-related disease are prevalent in the demographic and epidemiological literatures (Dello Buono, Urciuoli et al. 1998, Kato, Zweig et al. 2016, Zaninotto, Wardle et al. 2016). Invertebrate models systems allow us to study the mechanisms underlying how aging and other health metrics are determined by neuronal circuits that emanate from sensory tissues and that influence conserved neuroendocrine processes. These may have evolved to modulate physiology in response to the perception of environmental conditions that affect evolutionary fitness, such as the presence of food, mates, or danger (Anderson and Adolphs 2014).

Methods

Experimental Model and Subject Details

The laboratory stocks w^{1118}, Canton-S, *UAS-dTrpA1*, *norpA*, and $Ir76b^{-/-}$ [BL51309] *Drosophila* lines were obtained from the Bloomington Stock Center. $Poxn^{\Delta M22\text{-}B5\Delta XB}$ and $Poxn^{Full1}$ were provided by J. Alcedo (Boll and Noll 2002). $Orco^{2}$ mutant flies were a generous gift from L. Vosshall (Benton, Sachse et al. 2006). $Gr63a^{1}$ mutant flies were a gift from A. Ray. *5-HT2A*^{PL00052} mutant and *5-HT2A-GAL4* (*3299-GAL4*) flies were graciously provided by H. Dierick. $Ir8a^{-/-}/Ir25a^{-/-}$ mutant flies were kindly provided by R. Benton. Three species of *Drosophila (D. simulans, D. erecta* and *D. virilis)* were generously provided by P. Wittkopp. All of these strains were maintained on standard food at 25°C and 60% relative humidity in a 12:12 h light:dark cycle.

Generation of dead flies

Unless otherwise noted, dead flies were generated by starvation. One- to two-week old Canton-S female flies were separated using CO_2 anesthesia and transferred to vials containing 2% agar. Flies were transferred to fresh agar vials every ~3 days and dead flies were collected within three days of death. Vials in which dead flies stuck to the agar were not used. For the data in Fig 1F, flies died from natural causes or were killed by rapid freezing in liquid nitrogen. Age-matched Canton-S females were used for rapid freezing.

Short-term exposure to dead flies

Twenty 2-week-old mated female flies were collected under light CO_2 anesthesia and exposed for 48 hours to 14 freshly dead female flies in standard food, where they freely interacted with the dead flies. During the 48 hour exposure period, flies were maintained in a 12:12 hour light:dark cycle, except for those involving exposure in 100% darkness, which took place in a closed incubator. In both cases, flies were maintained at 25°C and 60% relative humidity. To test species specificity, experimental female flies (*D. melanogaster*) were exposed to three different species of dead *Drosophila*; we used 14 freshly dead female flies of *D. melanogaster*, *D. simulans,* and *D. erecta,* and 8 freshly dead *D virilis* due to their larger size.

Behavioral preference assays

To generate naïve choosers for preference assays, newly eclosed virgin female flies (< 7 hours old) were collected and transferred to standard food vials with 3 male flies per 20 females. Flies were kept at 25°C and 60% humidity, with a 12:12 hour light: dark cycle for 2 days, after which they were briefly anaesthetized to remove the male flies. Mated female flies were then transferred to fresh vials for one day to recover. On day four post-eclosion, the flies were placed into vials containing moist tissue paper for 4 hours prior to their introduction into the T-maze for behavioral monitoring. Choice was measured using binary traps made from commercially available T connectors (McMaster-Carr Part Number 5372K615) with 200µl pipette tips, which were trimmed, attached to opposite ends of the T connectors to form one-way doors that end in small collection chambers. Experimental flies that were pre-exposed to dead flies were loaded into a collection chamber with moist tissue paper in one arm of the T-connector while unexposed flies were loaded into the opposite chamber that also contained moist tissue paper. Unless otherwise noted, 20 live flies were exposed to 14 dead animals. Modifications were made to the apparatus to test specific sensory modalities:

- **Vision-only:** Canton-S mated female flies were exposed to flies that were dead either due to starvation or to liquid nitrogen-immersion where the dead were kept beneath an acrylic floor on a thin layer of agar. This apparatus ensures that only visual cues are transferred. As a control treatment, a group of flies were directly exposed to starvation-induced dead for 48 hours in the absence of the acrylic barrier.
- **Olfaction-only:** Mated Canton-S female flies were co-housed with starvation-killed flies, the latter of which were physically separated from exposed animals by the presence of a fine mesh screen that prevented physical interaction between the live and dead flies.
- **Gustatory-only:** Starvation-induced dead flies were homogenized and spread on top of the food prior to exposure. Mated Canton-S female flies were then exposed to either intact or grounded dead flies for 2 days.

Twenty naïve choosing flies were introduced into the central arm of the maze, and the number of flies trapped in each arm were counted at regular intervals. Behavioral preference was measured in a dark room under dim 660 nm red light at 24°C, and behavior was observed at 3, 6, 9, and 12 mins. A Preference Index (PI) at each time point was computed as follows: (Number of flies in

exposed arm (N_E)-Number of flies in unexposed arms (N_C))/(N_C+N_E). The fraction of flies that participated in the experiments was calculated as: (Nc + N_E)/20. Average PI values are weighted mean values among replicates with weights proportional to the number of animals that made a choice. Participation rates for all of the T-maze assays were > 50%. Experiments were replicated at least 2 independent times. Beads used for mock dead flies were obtained from Cospheric innovation in Microtechnology (Catalog number: CAS-BK 1.5mm).

Starvation experiments

At day 4 post-eclosion, 10 mated female flies were separated in 10 vials/treatment containing 2% agar. Flies were kept in constant temperature and humidity conditions with a 12:12 hour light: dark cycle. A census of live flies was taken every 6-8 hours. For exposed flies, the dead flies were left in each vial throughout the experiment. For control flies, dead flies were removed at each census point. Flies were transferred to fresh agar vials every 6 hours.

TAG assays

Four-day old, adult Canton-S female flies were collected and subsequently handled using our standard short-term exposure protocol (see above). Following the 48 hour exposure to dead animals, live experimental flies were removed and homogenized in groups of 10 in 150 µl PBS/0.05% Triton X. Unexposed flies were collected simultaneously. The amount of TAG in each sample was measured using the Infinity Triglyceride reagent (Thermo Electron Corp.) according to the manufacturer's instructions. Eight independent biological replicates (of 10 flies each) were obtained for treatment and control cohorts.

Negative geotaxis assay

Four-day old, adult Canton-S female flies were collected and subsequently subjected to our standard short-term exposure protocol (see above). Following the 48 hour exposure to dead animals, live experimental flies and their corresponding unexposed controls were removed and transferred to climbing chambers by aspiration. Negative geotaxis was measured using DDrop, an automated machine developed in the Pletcher laboratory that drops flies from 24" and then tracks upward movement of individual flies through a video tracking algorithm. For each fly, we

calculated both the total distance travelled and the time required to reach individual quadrants of the chamber.

CO_2 measurement

Four-day old, adult Canton-S female flies were collected and subsequently subjected to our standard short-term exposure protocol (see above). Following the 48 hour exposure to dead animals, live experimental flies and their corresponding unexposed controls were removed, and CO_2 production was measured from groups of five female experimental flies alongside their corresponding unexposed controls at 25°C. We used a Sable Systems Respirometry System, including a LiCor LI-7000 carbon dioxide analyzer, a Mass Flow Controllers (MFC2), and a UI-2 analog signal unit. Immediately prior to analysis, flies were transferred without anesthesia into glass, cylindrical respirometry chambers. Flies were allowed to acclimate to the new environment for 8 min before CO_2 collection began. Six chambers were analyzed simultaneously using stop-flow analysis and the Sable Systems multiplexer. Incoming atmospheric air flow was dried, scrubbed of CO_2, and then rehydrated before entering the respirometry chambers via the multiplexer. For each group, we collected 3 measures of CO_2 production over a period of 20 min that were averaged to determine a final, single estimate of CO_2 production per group. CO_2 production values were obtained using the EXPDATA software from Sable Systems, following adjustment using a proportional baseline.

Feeding analysis

Feeding behavior was measured using the Fly Liquid Interaction Counter (FLIC) as described previously (Ro, Harvanek et al. 2014). Following our standard 48 hour exposure treatment, individual Canton-S female flies were placed into a single FLIC chamber with two food wells, each containing a 10% sucrose solution. Two independent experimental blocks were conducted using 15 dead exposed and 15 unexposed flies per experiment, providing a total of 30 flies per treatment. The experiments were performed at constant temperature (25°C) with 12:12 hour light: dark cycle. Throughout the experiment, three dead flies were kept in the chambers assigned to the dead exposed condition. Feeding interactions with the food were measured for 24 hours continuously using the FLIC reservoir system (see http://www.wikiflic.com). Data were analyzed using the FLIC Analysis R Source Code (available from wikiflic.com). Relevant

feeding measures included the number of total interactions with the food, the total time spent interacting with the food, mean duration of each putative feeding event, and mean time between feeding events. These data were determined to be normally distributed, and a t-test was used to determine whether statistically significant differences were observed after noting the absence of significant block effects.

Axenic Fly Culturing

Canton S flies were placed into cages with purple grape agar and yeast paste for approximately 18 hrs. Embryos were then collected with 10ml PBS and moved into a sterile hood, where they were treated with 10ml 1:10 sterile bleach solution (3x washes) and then washed with sterile water. Using sterile technique, 8ul of embryos were aliquoted into sterile 50ml falcon tubes that contained 8ml sterile standard fly media. Embryos were allowed to develop in a humidified incubator at 25°C with 12hr:12hr light:dark cycles. After 12 days, axenic flies were collected in a sterile hood into fresh, sterile 50ml falcon tubes containing 8ml sterile standard fly media. Flies were aged for 2 weeks, during which time fresh, sterile media was provided every 2-3 days. After 2 weeks, the flies were split into 2 groups with half of the group given fresh, sterile agar (to generate sterile dead flies) and the other half given fresh, sterile standard fly media. Sterile dead exposures occurred in the sterile hood using sterile tools and sterile technique. Control, conventionally-reared flies (non-sterile) were handled in an identical manner, with the exception of bleach washing. The sterility of the axenic flies was verified by plating the supernatant from total fly extracts onto brain heart infusion agar plates; colonies grew from the extracts of traditionally-reared flies but never from the extracts of sterile flies.

Metabolomic analysis

Following 48 hours of dead fly exposure, experimental flies were quickly frozen in a dry ice bath, and stored at -80°C overnight. Heads were removed via vortexing and manually separated from the body parts. Forty heads were then homogenized for 20 sec in 200µl of a 1:4 (v:v) water:MeOH solvent mixture using the Fast Prep 24 (MP Biomedicals). Following the addition of 800µl of methanol, the samples were incubated for 30 mins on dry ice, then homogenized again. The mixture was spun at 13000 RPM for 5 mins at 4°C, and the soluble extract was collected into vials. This extract was then dried in a speedvac at 30°C for approximately 3 hours.

Using a LC-QQQ-MS machine in the MRM mode, we targeted ~200 metabolites in 25 important metabolic pathways, in both positive and negative MS modes. After removing any metabolites missing from more than 5 out of 32 samples (15%), we were left with 119 metabolites. Metabolite abundance for remaining missing values in this data set were log-transformed and imputed using the k-Nearest Neighbor (KNN) algorithm with the impute package of R Bioconductor (www.bioconductor.org). We then normalized the data to the standard normal distribution ($\mu=0$, $\sigma^2=1$). Principal Component Analysis (PCA) was performed using the made4 package of R Bioconductor. We used permutation tests (n=10,000) to select PCs that significantly separate between different treatments (genotype and/or exposure to dead flies). For each permutation, we randomly distributed the treatments to the real abundance of each metabolite. PC analysis was done for both randomized and real data. The degree of separation for each PC can be measured by analyzing between- and within-group variance based on the projection of samples on that PC, which is indicated by the Z-score:

$$Z = \frac{Variance\ between\ groups}{Variance\ within\ groups},$$

Variance between groups = $\sum_{k=1}^{N} n_k \times (Mean_{total} - Mean_k)^2$, where N indicate the number of groups and n_k indicates the number of samples in Group k. The distribution of Z-score was obtained from 10,000 randomized datasets. PCs that significantly deviated from this randomized distribution were considered as a significant separation of groups.

To identify individual metabolites of interest that are likely to be associated with death exposure, we sorted them per loadings on PC10 and selected the Top 10. For these candidates, we looked for metabolites that were (*i*) significantly different between control flies exposed to dead conspecifics versus those not exposed and that were (*ii*) less affected in *norpA* mutant flies. We found glyceraldehyde significantly up-regulated upon death exposure in Canton-S flies (one-sided Student's t-test, P = 0.007), whereas such differences were not significant in *norpA* flies (one-sided Student's t-test, P = 0.07).

Survival experiments

For lifespan experiments, experimental and control flies were reared under controlled larval density and collected as adults within 24 hours of emergence onto standard food where they were

allowed to mate freely for 2-3 days. At 3 days post-eclosion, female flies were sorted under light CO_2 and placed into fresh food vials. Contrary to our short-term protocol, experimental flies were chronically exposed to dead animals throughout their life. Lifespan measures were obtained using well-established protocols (Linford, Bilgir et al. 2013). Flies were transferred to fresh food vials every Monday, Wednesday, and Friday, at which time 14 freshly dead flies (~2-3 days old) were added. Unexposed animals were transferred simultaneously, but instead of adding dead flies we removed any flies that had died since the last census time. Flies were maintained at 25°C and 60% humidity under a 12:12 hour light: dark cycle. For experiments in the dark, flies were maintained in a dark incubator at 25°C and 50% humidity. Vials were changed as described above, and dead flies were counted under dim red light.

Drug administration

All drugs were purchased from Sigma-Aldrich. Each drug was initially dissolved in 100% DMSO at 10 mM concentration, aliquoted, and stored at -20°C. Every Monday, Wednesday, and Friday, an aliquot of the drug stock was thawed and diluted 1:500 in water for lifespans (20 µM final concentration). A similar dilution of DMSO alone was made in water as a vehicle control. Then, 100 µl of the diluted drug or vehicle control was added to each vial, coating the top of the food surface. After the liquid evaporated (~2 hours), the vials were ready for use as described above. For the behavior assay, flies were pretreated with 1 mM pirenperone or an equivalent dilution of DMSO for 2 weeks prior to exposure, in the absence of dead flies.

***5-HT2A*$^+$ Neuron Activation Methods**

5-HT2A-GAL4 (*3299-GAL4*, H. Dierick, BCM) flies were crossed to *UAS-dTrpA1* to generate *5-HT2A>dTrpA1* flies. *UAS-dTrpA1* was backcrossed for at least 10 generations to w^{1118} and therefore *5-HT2A-GAL4;+* was used as a genetic control strain. For behavioral assays, progeny from all crosses were maintained at 18°C until they were 10-14 days old, after which they transferred to 29°C for 48 hours prior to mimic exposure to dead individuals. The T-maze assay was established with *5-HT2A>dTrpA1* on one side of the assay and *5-HT2A-GAL4;+* on the other. Canton-S flies were used as naïve choosers. For lifespan experiments, progeny from both crosses were maintained at 18°C throughout development. Following eclosion, females were mated for 3 days, separated by gender, and placed at 29°C to begin lifespan measurement.

Bacterial infection with *P. aeruginosa*

The PA14 *plcs* strain used in this study was obtained from L. Rahme (Harvard Medical School). For each experiment, a glycerol stock was freshly streaked onto an LB/gentamycin plate. After an overnight incubation, a single colony was picked and grown in 1ml of LB/gentamycin until this seed culture reached logarithmic phase. Subsequently, the culture was diluted in 25 ml of LB/gentamycin and grown until the desired A_{600} concentration was reached. Finally, the bacterial culture was centrifuged and the pellet resuspended in LB media to obtain an A_{600} reading of 100. The culture was kept on ice during infection. Needles were directly placed in the concentrated bacterial solution and then poked into the fly abdomen. After infection, flies were transferred to standard food vials and kept in the incubator at 25°C and 60% humidity. Flies were collected 24 hours post infection for behavioral experiments. Infected flies were loaded in one arm of the T-connectors and control flies (not infected with *P. aeruginosa*) were loaded into the opposite arm. Twenty naïve choosing flies were introduced into the central arm of the maze and the number of flies in each arm of the trap was counted at regular intervals.

Quantification and Statistical Analysis

For all preference assays, P-values comparing the Preference Index among treatments was obtained using a randomization procedure and the statistical software R. Briefly, the null distribution of no difference among treatments was obtained by randomizing individual preference indices obtained from groups of 20 flies among all measures (maintaining block structure when appropriate) and 100,000 t-statistics (or F statistics for multiple comparisons). P-values (one-sided or two-sided as appropriate) were determined by computing the fraction of null values that were equal or more extreme to the observed t-statistic (or F-statistic). Mean preference values were plotted and weighted by the number of choosing flies in each trial, with the error bars representing the standard error of the mean. Experiment-wise error rates for experiments comparing three or more treatments were protected by presentation of treatment P-value from non-parametric, randomization ANOVA, which are reported in the Figure Legends when appropriate. For lifespan and starvation assays, we employed survival analysis. Unless otherwise indicated, group- and pairwise-comparisons among survivorship curves (both lifespan and starvation) were performed using the DLife computer software (Linford, Bilgir et al. 2013) and the statistical software R. P-values were obtained using log-rank analysis (select pairwise

comparisons and group comparisons or interaction studies) as noted. Interaction P-values were calculated using Cox-Regression when the survival data satisfied the assumption of proportional hazards. In other cases (as noted in the figure legends), we used ANOVA to calculate P-values for the interaction term for age at death. For all box plots, the box represents Standard Error of the Mean (SEM, centered on the mean), and whiskers represent 10%/90%. For CO_2, TAG, and negative geotaxis measures, P-values were obtained by standard two-sided t-test after verifying normality and equality of variances. Details of the metabolomics analysis are presented above.

Acknowledgments

The authors would like to acknowledge the members of the Pletcher laboratory for their comments on the experimental design and analysis. This research was supported by the US National Institutes of Health, National Institute on Aging (RO1AG030593 and RO1AG023166 to S.D.P); the Glenn Medical Foundation (to S.D.P.), and the NIH Cellular and Molecular Biology and Career Training in the Biology of Aging Training Grants (T32-GM007315 and T32-AG000114 to A.S.M.).

Figures

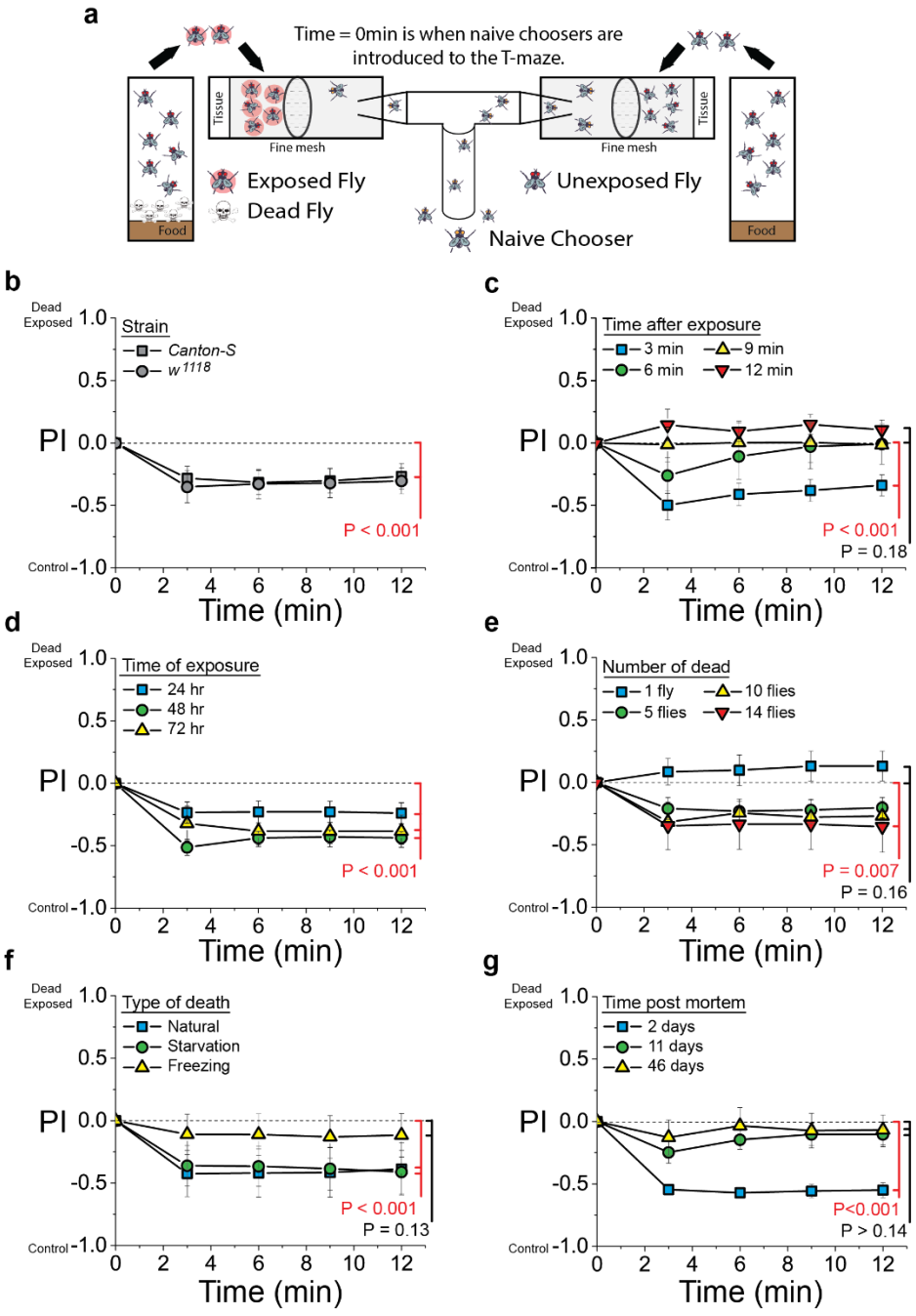

a
Time = 0min is when naive choosers are introduced to the T-maze.
Tissue
Fine mesh
Exposed Fly
Dead Fly
Unexposed Fly
Naive Chooser
Food
b
Strain
Canton-S
w1118
P < 0.001
c
Time after exposure
3 min
6 min
9 min
12 min
P < 0.001
P = 0.18
d
Time of exposure
24 hr
48 hr
72 hr
P < 0.001
e
Number of dead
1 fly
5 flies
10 flies
14 flies
P = 0.007
P = 0.16
f
Type of death
Natural
Starvation
Freezing
P < 0.001
P = 0.13
g
Time post mortem
2 days
11 days
46 days
P<0.001
P > 0.14
Dead
Exposed
Control
PI
Time (min)

Figure 4.1. Flies become aversive after exposure to dead conspecifics. a Cartoon representing the exposure protocol and binary T-maze apparatus used in our choice behavior assays. PI = preference index calculated as (Number of flies in the exposed arm (N_E)-Number of flies in the unexposed arm (N_C))/total (N_C+N_E). **b** Flies of two different laboratory strains (Canton-S and w^{1118}) that were exposed to dead conspecifics for 48 hours were aversive to naïve Canton-S choosing females (N = 9 for Canton-S and N = 7 for w^{1118}, P < 0.001 for Canton-S and P = 0.001 for w^{1118}). **c** Flies exposed to dead conspecifics retained their aversive characteristics to naïve choosing flies for up to 9 min after the dead flies were removed (N = 9 for each treatment, P = 0.01 for 6 min and P = 0.22 for 9 min, group ANOVA P < 0.001). **d, e** When flies were exposed to dead conspecifics, they evoked avoidance behavior in naïve choosing females that was intensified with (**d**) longer periods of exposure (N = 19 for 24 hours, N = 9 for 48 hours, and N = 14 for 72 hours, group ANOVA P = 0.027) and (**e**) the number of dead animals used during the exposure treatment (N = 6 for each treatment, P = 0.043 for 5 flies, P = 0.01 for 10 flies, group ANOVA P < 0.001). **f** Flies exposed to animals that died of natural or starvation-induced death, but not freezing death, evoked avoidance behaviors in naïve flies (N = 8 for natural and starvation-induced death and N = 10 for frozen induced death, group ANOVA P = 0.04). **g** Newly dead flies effectively induced the aversive cues in exposed animals, but long-dead flies did not (N = 6 for each treatment, P = 0.17 for 11 days dead and P = 0.14 for 46 days dead flies, group ANOVA P < 0.001). Except where noted in panel b, all naïve choosing female flies were from the Canton-S strain. Each T-maze sample tests 20 flies. Error bars represent SEM. All P-values were determined by non-parametric randomization (see Methods for details).

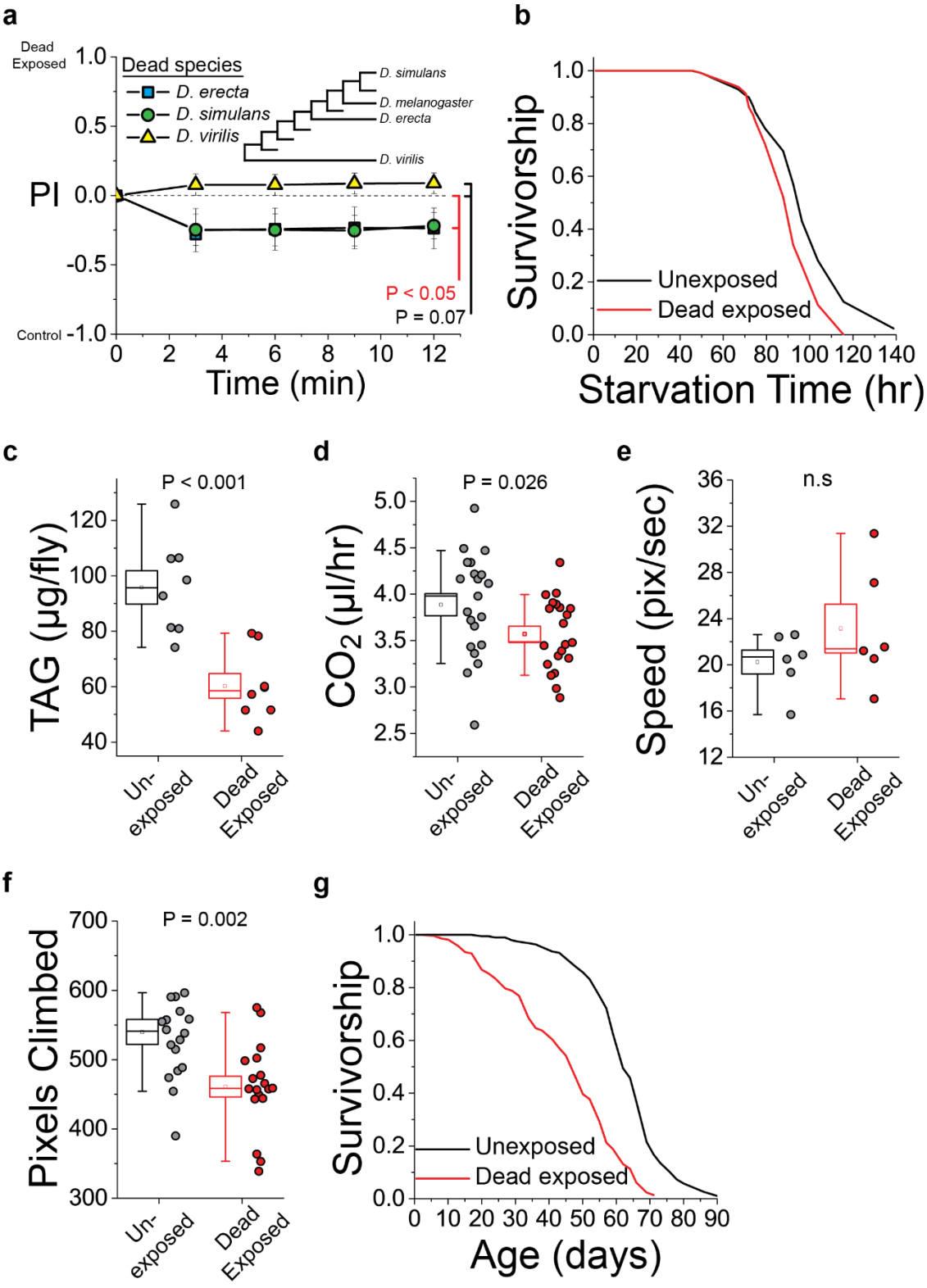


Figure 4.2. Exposure to dead conspecifics alters physiology and lifespan in *D. melanogaster*. **a** When *D. melanogaster* were exposed to dead animals from each of two closely related species (*D. simulans* and *D. erecta*) they presented aversive cues, but exposure to the evolutionarily

more distant *D. virilis* had no effect (N = 10 for *D. erecta*, N = 8 for *D. simulans*, and N = 17 for *D. virilis*, P = 0.046 for *D. erecta* and P = 0.008 for *D. simulans*, group ANOVA P < 0.001). Inset depicts a phylogeny of related *Drosophila* species. **b, c, d** Flies exposed to dead conspecifics exhibited reduced (**b**) starvation survival (N = 100 per treatment, P < 0.001) (**c**) triacylglyceride stores (TAG, N = 8 biological replicates of 10 flies each), and (**d**) metabolic rate as measured by CO_2 production relative to unexposed animals (N = 21 biological replicates of 5 flies/treatment). **e, f** While exposure to dead conspecifics did not affect (**e**) spontaneous movement rates, (N = 6 for each treatment, P = 0.26), (**f**) forced climbing was impaired relative to unexposed animals (N = 18 for each treatment). **g** Chronic exposure to dead animals significantly reduced lifespan flies (N = 190 for unexposed, 212 for exposed, P < 0.001). For panel a, naïve choosing flies were from the Canton-S strain. Each T-maze sample tests 20 flies. Error bars represent SEM. P-values for binary choice were determined by non-parametric randomization. Comparison of survival curves was via log-rank test, and the remaining phenotypes were evaluated for significance by t-test (see Methods for details).

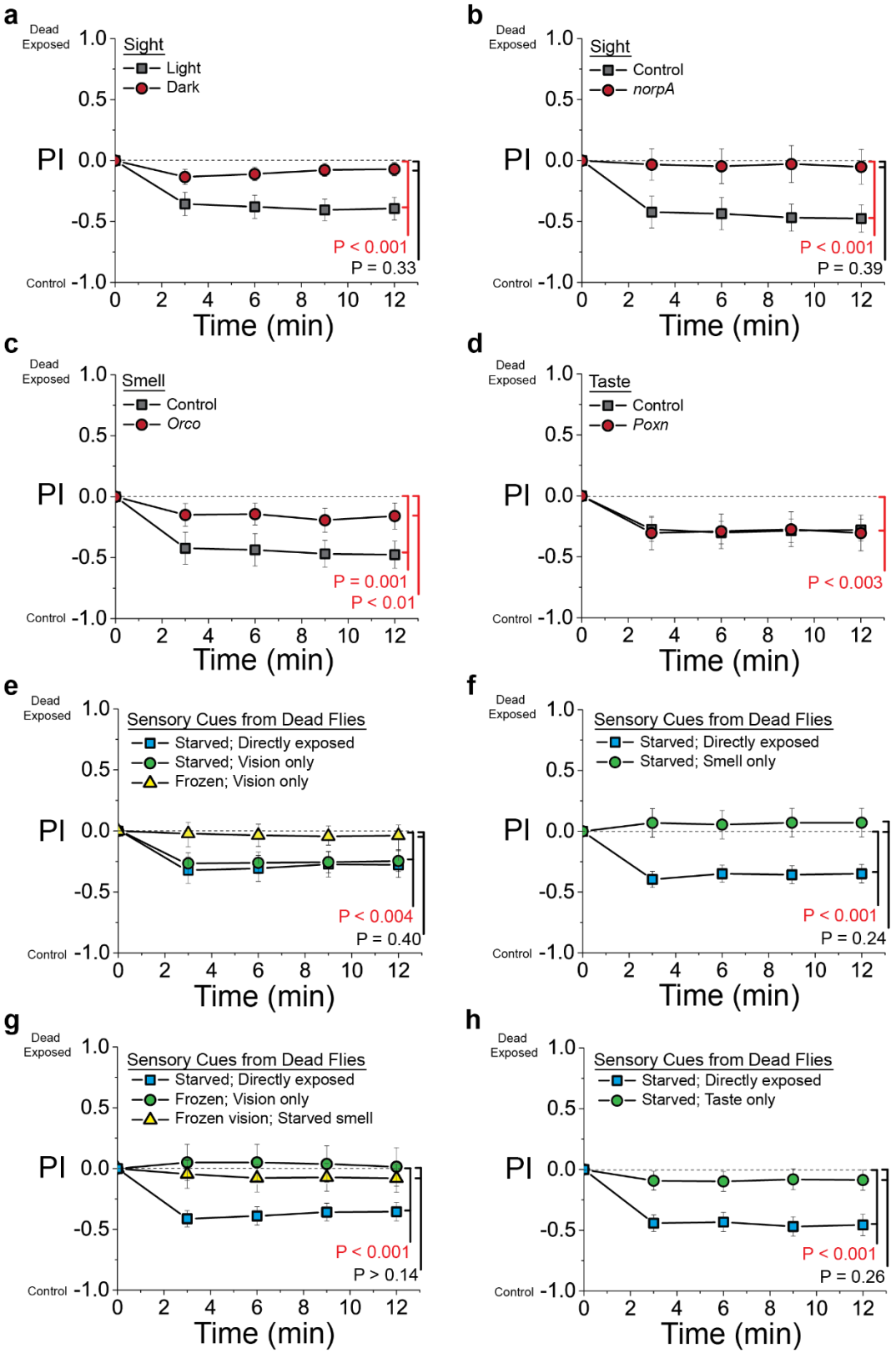


Figure 4.3. Sensory perception is required for the induction of aversive cues following exposure to dead flies. a When flies were exposed to dead flies in the dark, they failed to evoke

avoidance behavior in naïve choosing females (N = 19 for light exposure and N = 20 for dark exposure). **b** Blind, *norpA* mutant flies exposed under lighted conditions also failed to induce aversive cues following exposure to dead flies (N = 8 for each treatment). **c** $Orco^{2}$ mutant flies, which have impaired olfaction, evoked a small, but significant, avoidance behavior in choosing females following death exposure (N = 9 for $Orco^{2}$ and N = 8 for control). **d** Flies carrying the $Poxn^{\Delta M22\text{-}B5\text{-}\Delta XB}$ mutation, which have impaired taste function, exhibited a similar induction of aversive cues in response to death exposure as did control flies (N = 9 for each treatment, P = 0.002 for control and P < 0.001 for *poxn*). **e** The sight of starvation-killed flies was sufficient to induce aversive cues to the same extent as direct exposure, while the sight of flies killed by immersion in liquid nitrogen had no effect (N = 19 for direct exposure, N = 20 for vision only). **f** The smell of starvation-killed flies, which was provided by isolating dead animals behind a fine mesh screen, failed to induce aversive cues (N = 10 for each treatment). **g** The sight of flies killed by freezing in liquid nitrogen failed to induce aversive cues (Frozen; vision only), and this was not affected by simultaneous smell of starvation-killed animals (N = 8 for frozen only, N = 13 for starved and starved +frozen). **h** Homogenized dead flies failed to evoke avoidance behavior in naïve flies (N = 10 for ground up and control treatments). For binary choice assays, all exposed flies and naïve choosing flies were from the Canton-S strain. Each T-maze sample tests 20 flies. Error bars represent SEM. P-values for binary choice were determined by non-parametric randomization.

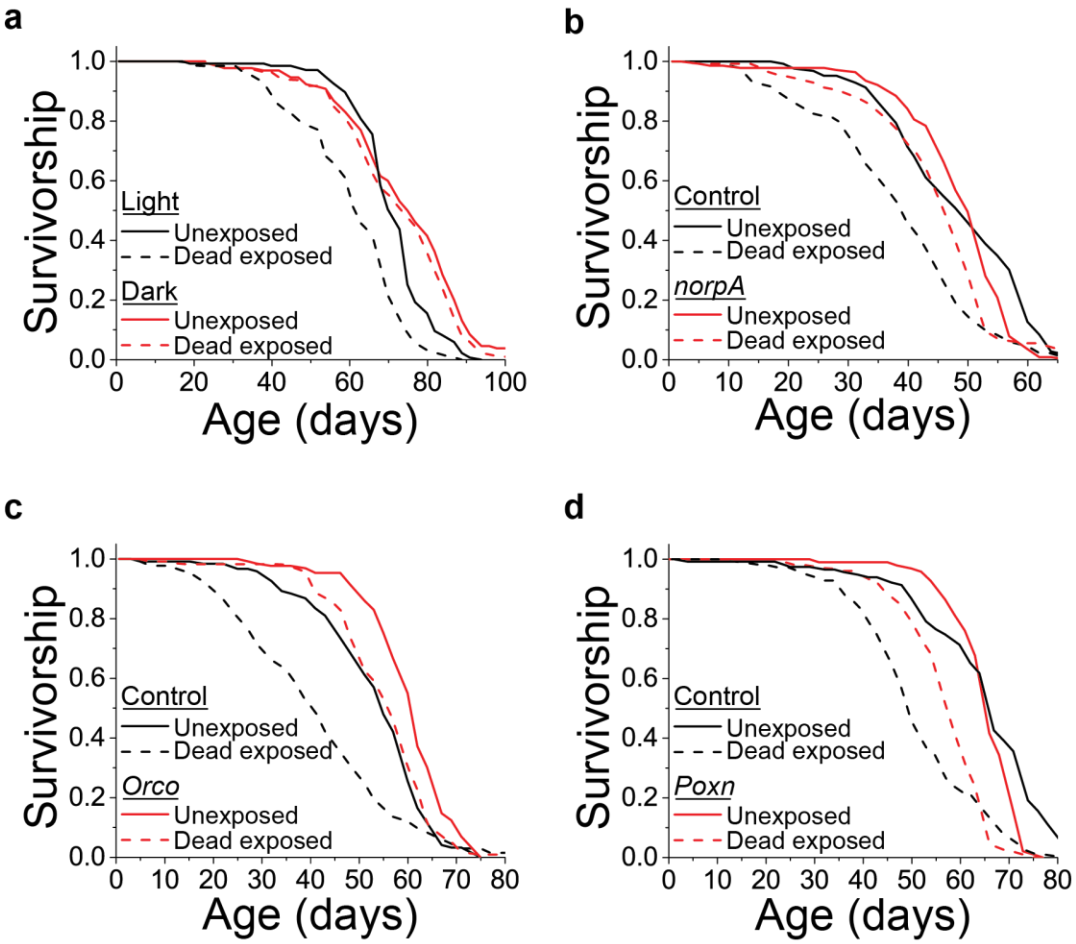


Figure 4.4. Exposure of dead conspecifics causes changes in lifespan that are mediated by sight and smell. **a**, Exposure of live animals to dead in the light, but not the dark, affected lifespan (N = 118 for dark dead exposed, 130 for dark unexposed, 125 for light dead exposed, and 135 for light unexposed, $P < 0.001$ for light and $P = 0.18$ for dark, $P < 0.001$ for the interaction between light and exposure via Cox Regression). **b**, The lifespan effect observed in *norpA* mutant flies following exposure to dead conspecifics was significantly diminished relative to control animals (N = 139 for *norpA* dead exposed, 126 for *norpA* unexposed, 126 for control dead exposed, and 131 for control unexposed, $P < 0.001$ for control and $P = 0.003$ for *norpA*, $P < 0.001$ for the interaction between genotype and exposure via Cox Regression). **c**, Anosmic flies maintained a reduced effect of death exposure on lifespan compared to control flies (N = 115 for $Orco^2$ dead exposed, 129 for $Orco^2$ unexposed, 133 for control dead exposed, and 121 for control unexposed, $P < 0.001$ for control and $Orco^2$, $P = 0.002$ for the interaction between genotype and exposure via ANOVA). **d**, Taste-blind flies showed normal lifespan effects due to dead exposure (N = 101 or *Poxn* dead exposed, 96 for *Poxn* unexposed, 112 for control dead exposed, and 116 for control unexposed, $P < 0.001$ for control and *Poxn*, $P = 0.06$ for the interaction between genotype and exposure via Cox Regression). Survival curve comparison was accomplished using a log-rank test (see Methods for details).

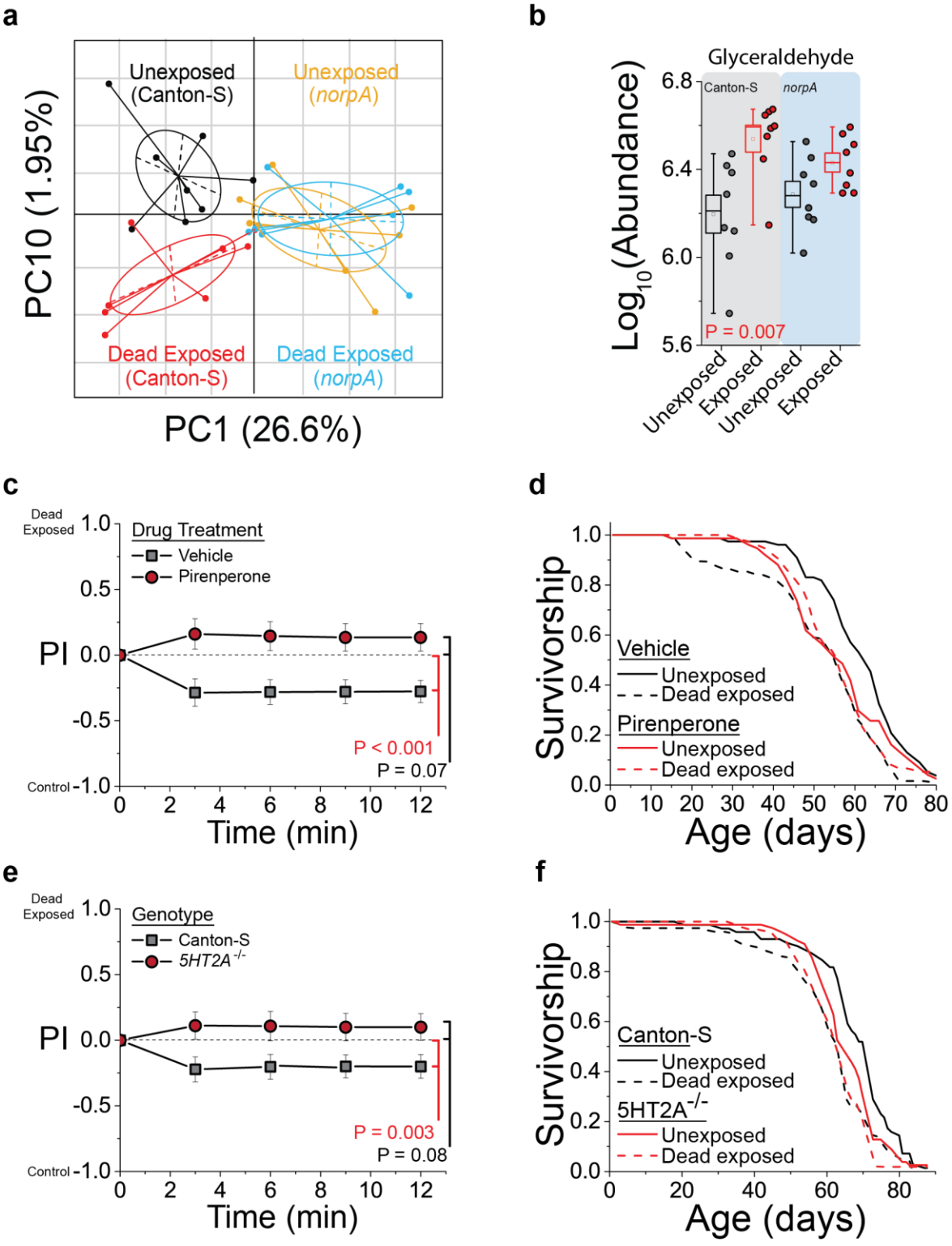


Figure 4.5. Death perception elicits acute changes in the neuro-metabolome, and its effects on health are mitigated by manipulations that attenuate serotonin signaling. a Principal component plot showing the distribution of samples for each treatment. Neuro-metabolites

weighted heavily in PC10 distinguish the effect of death exposure, while those favored in PC1 distinguish genotype. Plots represent mass spectrometry analysis of metabolites identified under positive mode (N = 8 biological replicates, with 40 fly heads per replicate). **b** Glyceraldehyde abundance was significantly increased in flies following death perception, but it was unchanged in blind *norpA* mutant flies similarly treated. **c, d** Pharmacologic treatment of Canton-S females with the serotonin receptor 5HT2-antagonist, pirenperone, during exposure to dead conspecifics effectively protected them from the consequences of death perception on (**c**) aversive cues detected by naïve choosing flies (N = 10 for each treatment) and (**d**) lifespan (N = 75 for pirenperone-fed dead exposed, 76 for pirenperone-fed unexposed, 75 for vehicle-fed dead exposed, and 77 for vehicle-fed unexposed, $P < 0.001$ for vehicle-fed and $P= 0.86$ for pirenperone-fed, $P = 0.007$ for the interaction between drug and exposure via ANOVA). **e, f** Null mutation of serotonin receptor 5-HT2A protected flies from the consequences of death perception on (**e**) aversive cues detected by naïve choosing flies (N = 15 for each treatment) and (**f**) lifespan (N = 63 for *5-HT2A*$^{-/-}$ dead exposed, 78 for *5-HT2A*$^{-/-}$ unexposed, 75 for Canton-S dead exposed, and 72 for Canton-S unexposed, $P < 0.001$ for Canton-S control flies and $P= 0.06$ for *5-HT2A*$^{-/-}$ mutants). A replicate lifespan experiment revealed the same results (see Supplemental Fig. 5c), and $P = 0.05$ for the combined interaction between genotype and exposure via ANOVA. P-values for principal component analysis and for binary choice were determined by non-parametric randomization. Each T-maze sample tests 20 flies. Error bars represent SEM. Comparison of survival curves was via log-rank test, and individual metabolites were evaluated for significance by t-test (see Methods for details).

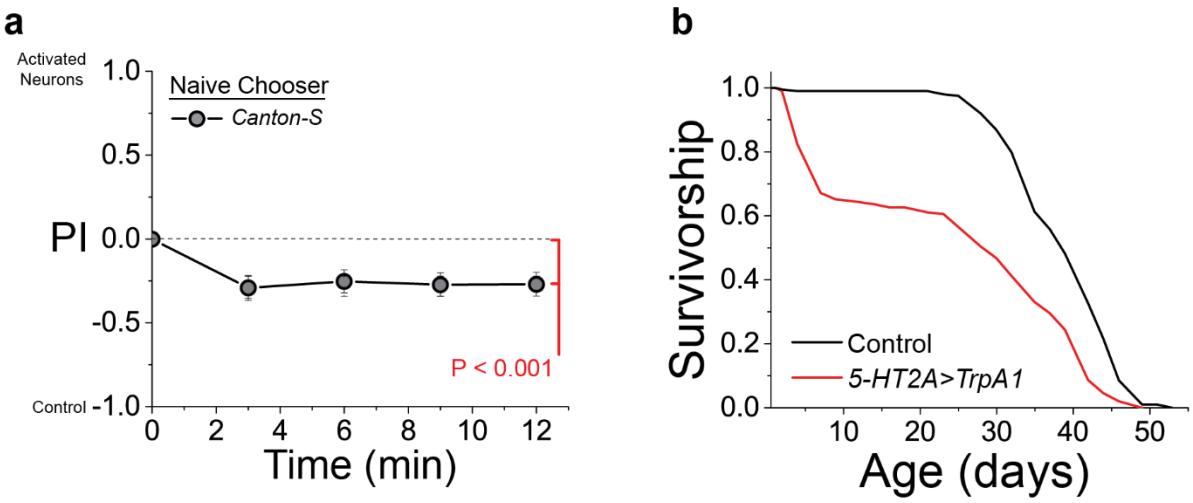
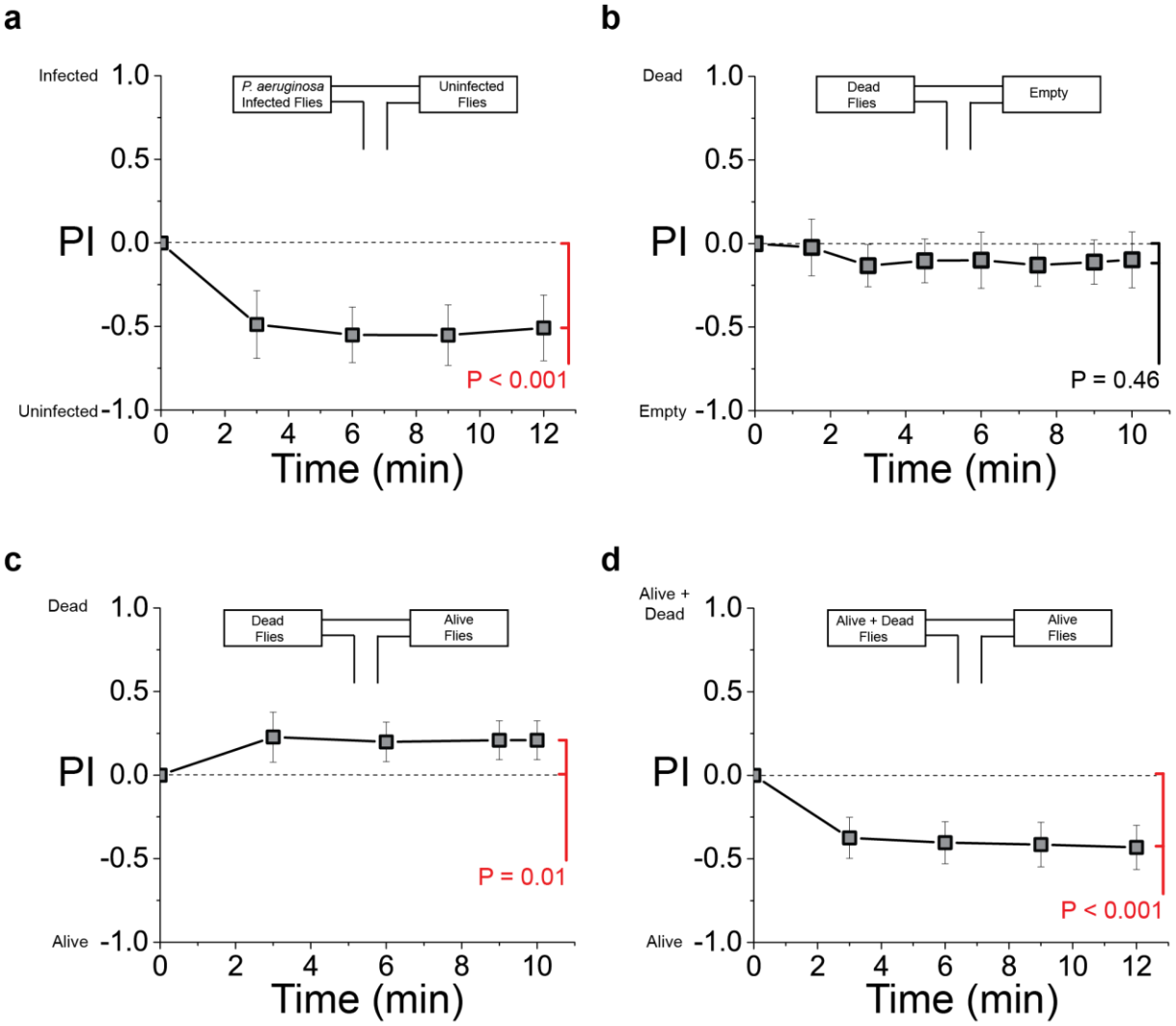
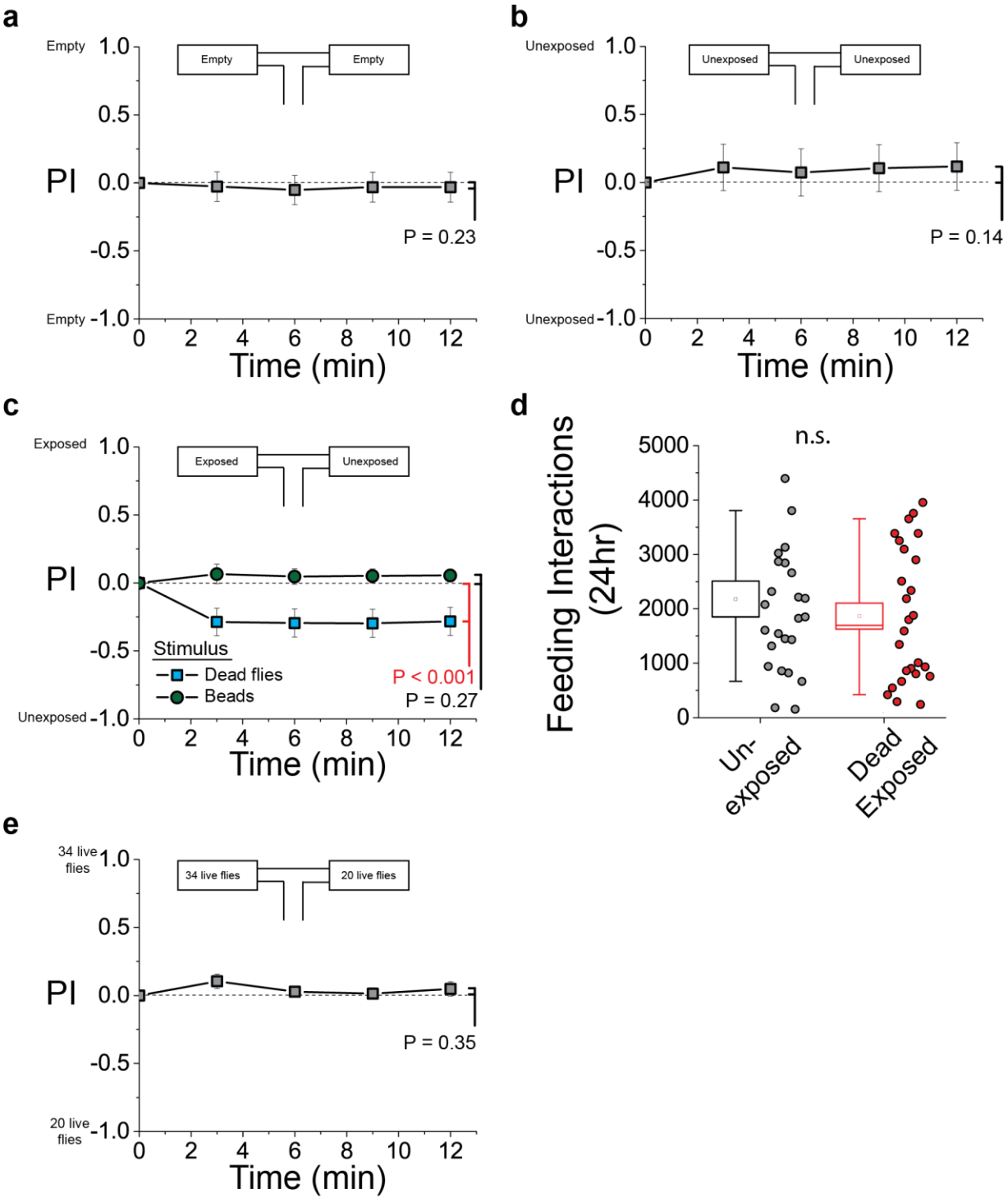


Figure 4.6. Activation of 5-HT2A neurons induces aversiveness and reduces lifespan in *Drosophila*. Constitutive activation of *5-HT2A*$^{+}$ neurons via expression of *UAS-TrpA1* relative to *5-HT2A-GAL4;+* control flies results in **(a)** increased aversiveness (N = 19, P < 0.001) and **(b)** significantly decreased lifespan (N = 194 for *5-HT2A>TrpA1* and N = 195 for *5-HT2A-GAL4;+*, P < 0.001). Each T-maze sample tests 20 flies. Error bars represent SEM. Comparison of survival curves was via log-rank test.

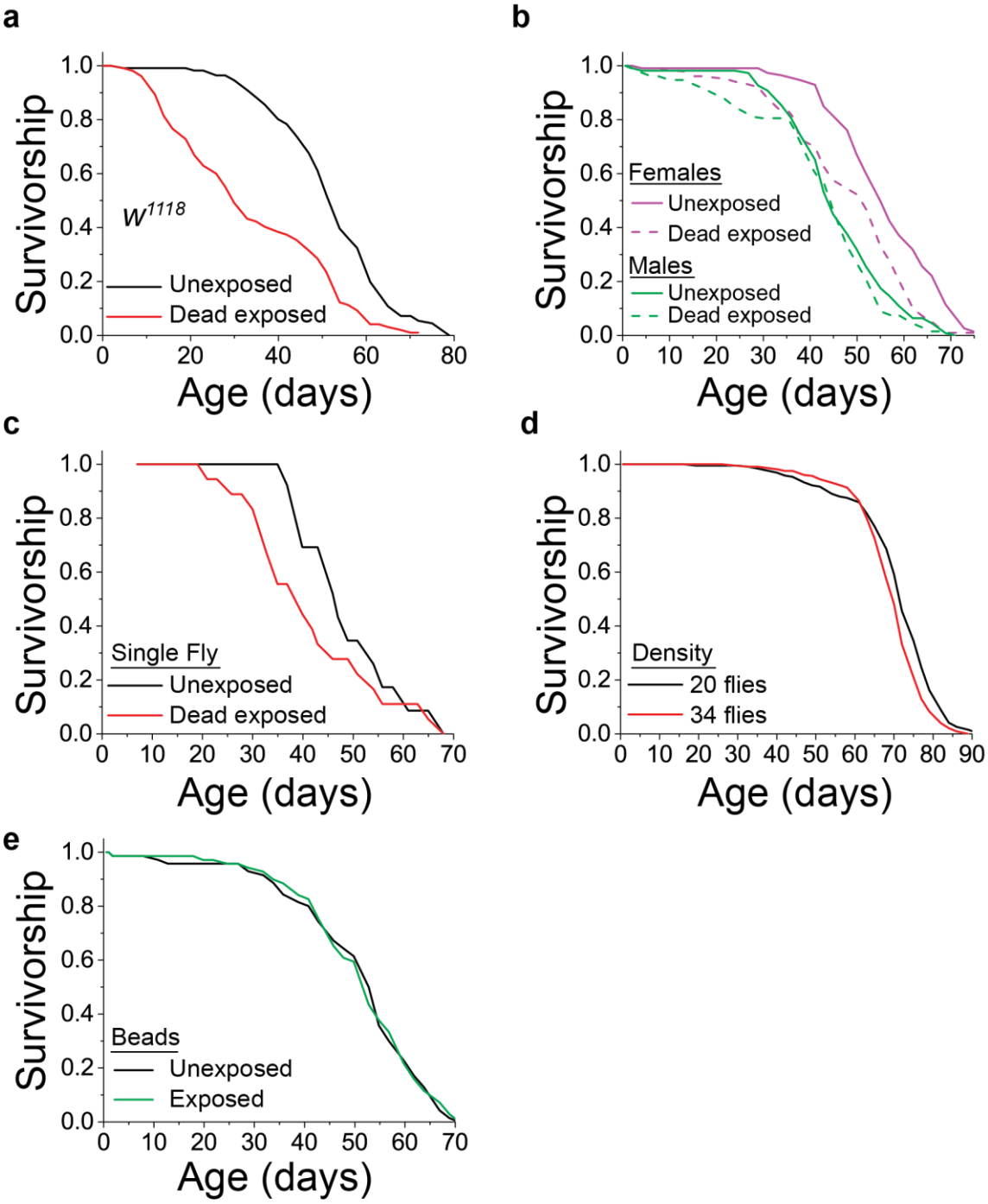


Supplementary Figure 4.1. Initial observations and characterization of the T-maze behavioral assay. a Naïve choosing flies are repelled by infected flies compared to uninfected control flies (N = 6). **b** Naïve choosing flies exhibit no preference when choosing between a chamber containing dead flies or an empty chamber (N = 20). **c** Naïve choosing flies prefer a chamber containing only dead flies over one containing only live flies (N = 4). **d** Naïve choosing flies more often choose a chamber containing only live females to a chamber that contains a mixture of live females and dead conspecifics (N = 10). All naïve choosing flies were females from the Canton-S strain. Each T-maze sample tests 20 flies.



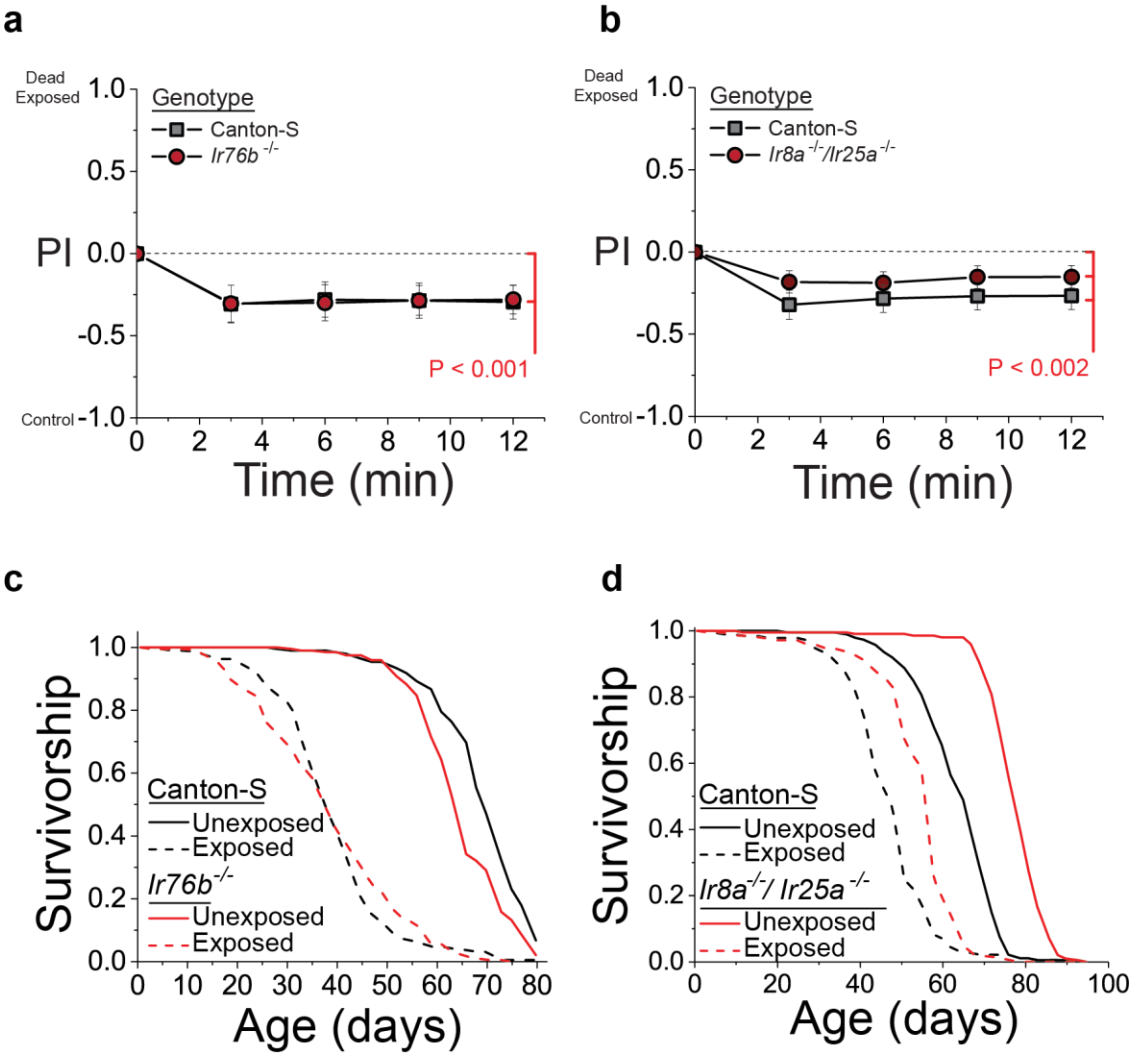
Supplementary Figure 4.2. Further characterization of the T-maze behavioral assay and feeding behavior. a Naïve choosing flies exhibit no behavioral preference when both arms of the T-maze are empty (N = 10). **b** The presence of equal numbers of live female flies in both arms of the T-maze does not result in behavioral preference (N = 8). **c** Naïve choosing flies show no preference between experimental flies that were exposed to mock dead flies (small black

beads) for 48 hours relative to unexposed flies (N = 15). **d** Feeding behavior is not affected by 48 hours exposure to dead conspecifics. Each data point represents the number of feeding interactions with the food for an individual fly (N= 25 for control flies and N = 26 for exposed flies, P = 0.45). **e** Naïve choosing flies exhibit no preference between arms with different densities of live flies (34 female flies vs 20 female flies; N = 10).

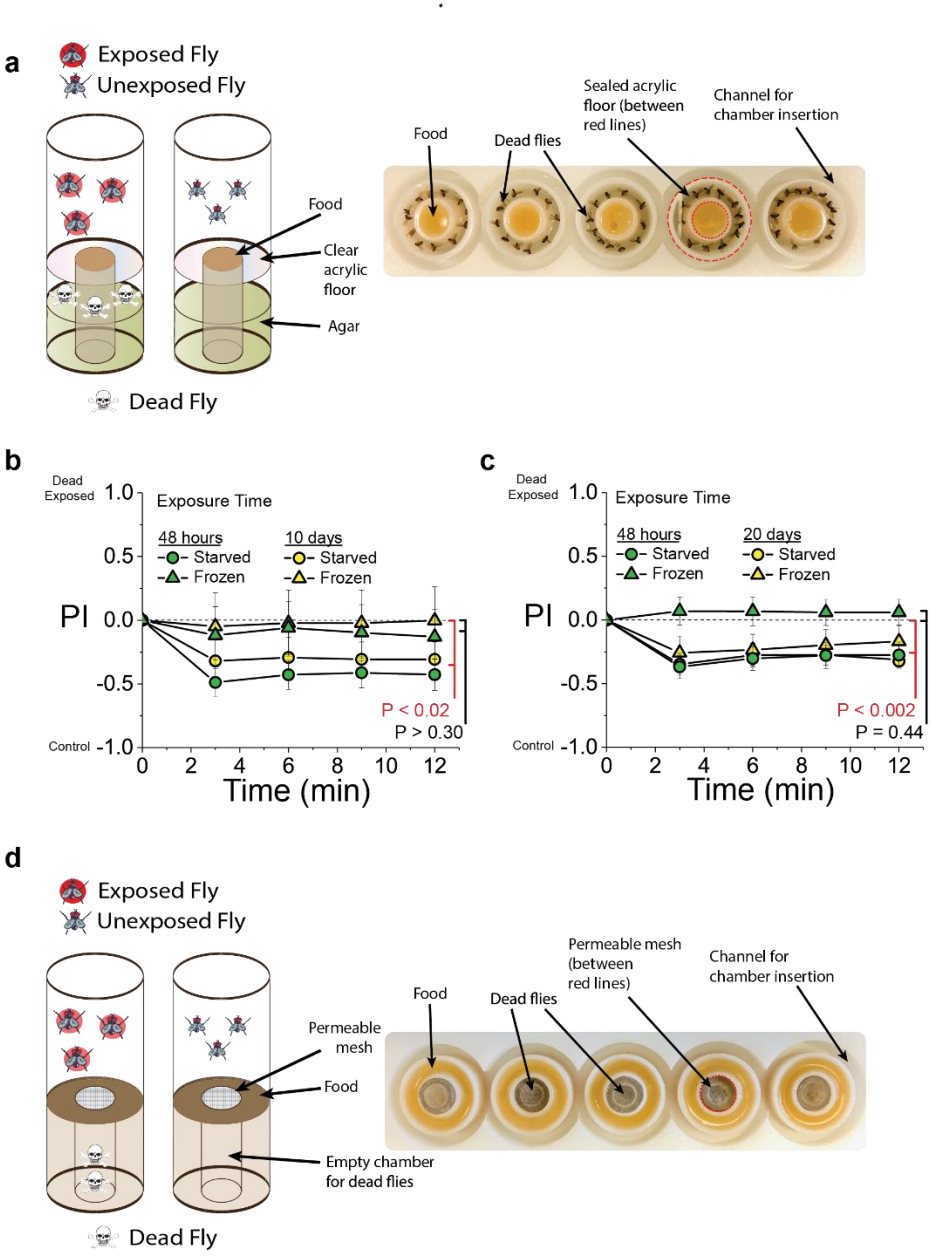


Supplementary Figure 4.3. The effects of death perception on lifespan with regard to genetic background, gender, and density. a Exposure to dead conspecifics shortens lifespan in the w^{1118} genetic background, (N = 111-113 per treatment, P < 0.001). **b** Canton-S female flies exposed to dead flies live significantly shorter compared to control (unexposed) animals, whereas Canton-S male flies exposed to dead females live similarly to controls (N = 85-113 per gender/treatment, P < 0.001 for females and P = 0.33 for males). **c** Single Canton-S female flies exposed to 8 dead female flies repeatedly tend to live shorter, although the small sample size

limits statistical power (N = 16-19 per treatment, P = 0.22). **d** Increased density of live flies has a small effect on lifespan (N = 195 flies for the 20 flies/vial treatment, N = 325 flies for the 34 flies/vial treatment, P < 0.001). **e** Lifespan is unaffected when Canton-S female flies are aged in the presence of mock dead flies (14 fly-sized black beads, N = 71 for control and 70 for the beads exposed, P = 0.90).

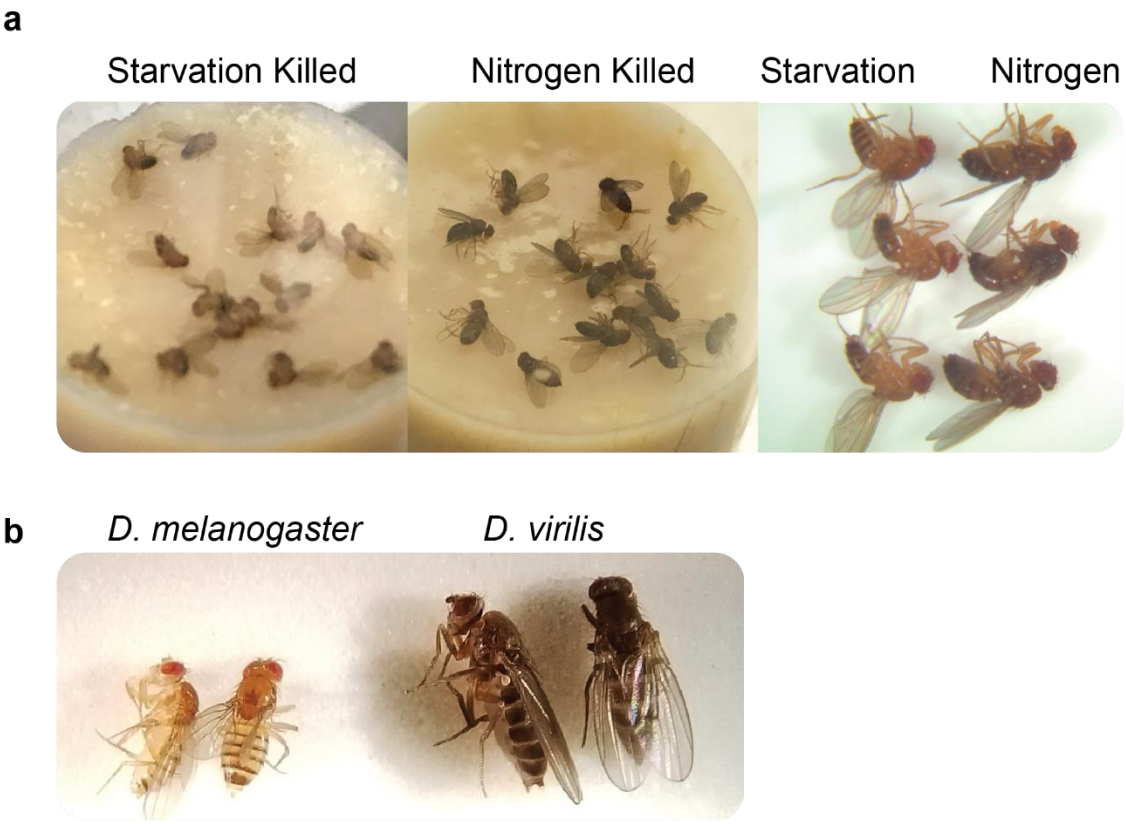


Supplementary Figure 4.4. The effect of sensory manipulations on death perception part 1. **a** *Ir76b*$^{-\backslash-}$ mutant flies retained their aversive characteristics to naïve choosing flies (N = 9 for control and 10 for *Ir76b*$^{-/-}$ mutants). **b** *Ir8a*$^{-/-}$*/Ir25a* $^{-/-}$ mutant flies retained their aversive characteristics to naïve choosing flies (N = 16 for control and 17 for *Ir8a*$^{-/-}$*/Ir25a* $^{-/-}$ mutants). **c** *Ir76b*$^{-/-}$ mutant flies show a significant effect of death exposure on lifespan, comparable to controls (N = 183-197 per genotype/treatment, P < 0.001 for exposed vs. unexposed in both control and *Ir76b* $^{-\backslash-}$ mutants). **d** *Ir8a*$^{-/-}$*/Ir25a* $^{-/-}$ mutant flies show a significant effect of death exposure on lifespan, comparable to controls (N = 184-209 per genotype/treatment, P < 0.001 for exposed vs. unexposed in both control and *Ir8a*$^{-/-}$*/Ir25a* $^{-/-}$ mutants).

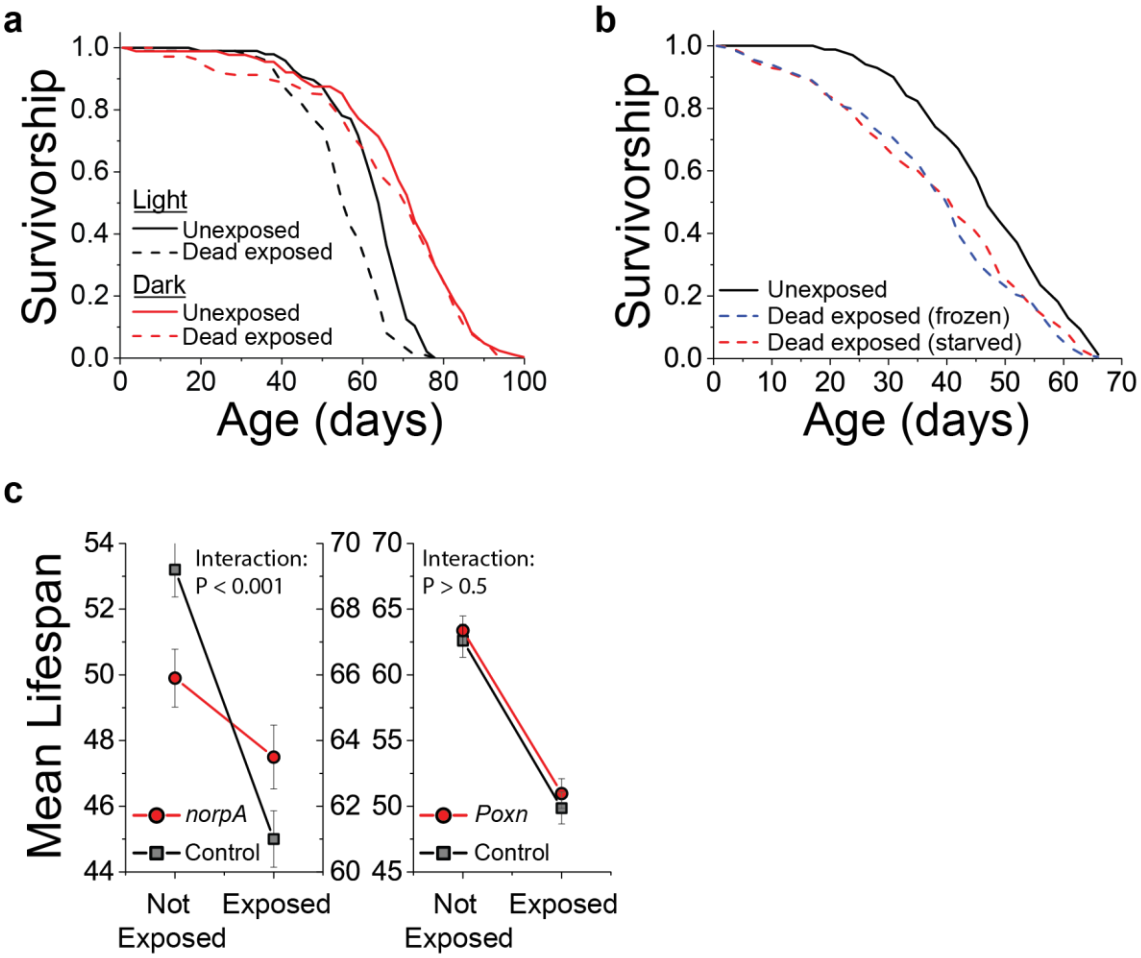


Supplementary Figure 4.5. The effect of sensory manipulations on death perception part 2. **a** Diagram of the visual exposure chamber. Flies have access to the food and can see dead flies, but cannot interact or smell the dead flies. **b** Flies that are quickly killed by immersion in liquid nitrogen do not induce avoidance behaviors when used for short term (48 hours) or medium term (10 day) exposures. Flies that are exposed to dead flies via starvation show aversion at both short and medium term exposures (N = 5 for each treatment, P = 0.30 for 48 hour dead frozen fly exposure, P = 0.0016 for 48 hour dead starved fly exposure, P = 0.42 for 10 day dead frozen fly exposure, and P = 0.016 for 10 day dead starved fly exposure). **c** Flies that are quickly killed by

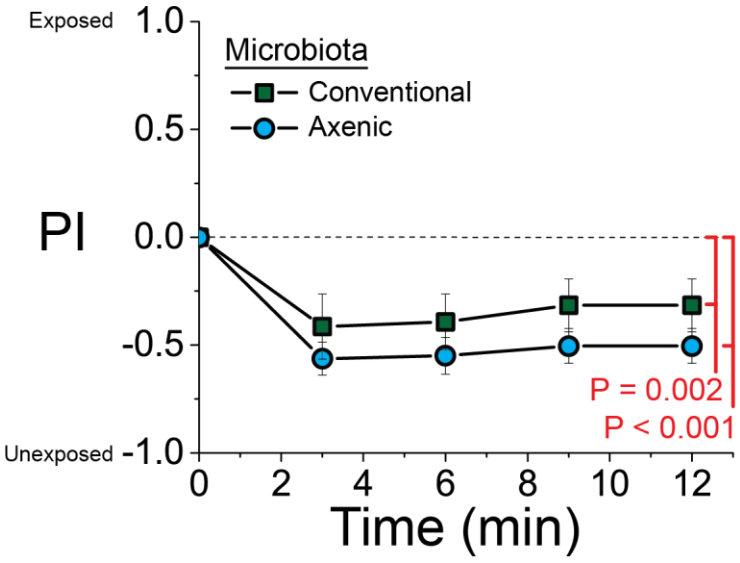
immersion in liquid nitrogen induced avoidance behaviors when used for long term (20 day) exposures (N = 10 for each treatment, P = 0.44 for 48 hour dead frozen fly exposure, P < 0.001 for 48 hour dead starved fly exposure, P = 0.008 for 20 day dead frozen fly exposure, and P = 0.002 for 20 day dead starved fly exposure). **d** Diagram of the odor exposure chamber. Flies have access to the food as well as odors from dead flies, but cannot interact with the dead flies. All of the flies used in these experiments were females from the Canton-S strain. Each T-maze sample tests 20 flies.



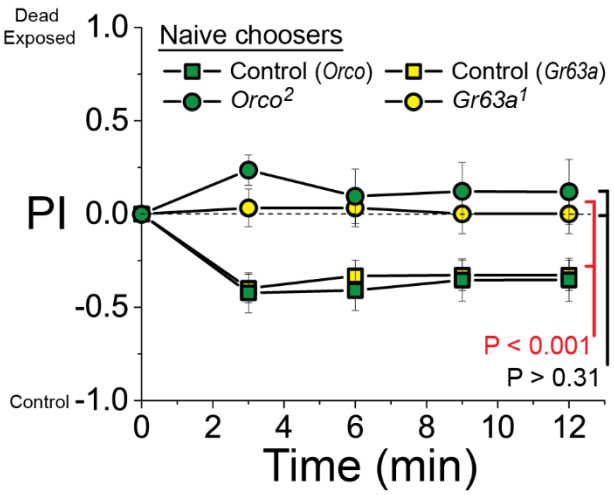
Supplementary Figure 4.6. Images comparing dead flies demonstrate significant visual differences. a *D. melanogaster* that are killed via starvation are visually lighter in color compared to flies killed by immersion in liquid nitrogen. Both groups sat at room temperature for 2 days after dying prior to this picture being taken and were treated in exactly the same manner as those dead flies that were introduced into our experiments. **b** *D. melanogaster* are visually lighter in color and much smaller than *D. virilis*.



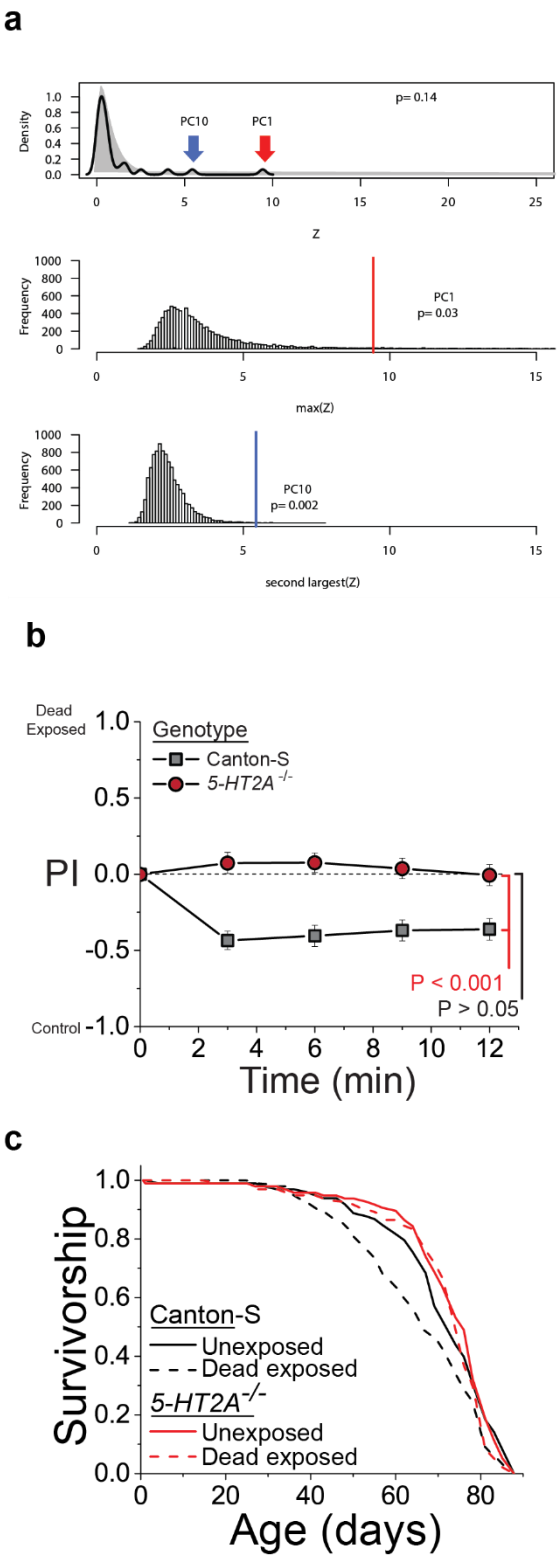
Supplementary Figure 4.7. The effect of sensory manipulations on death perception part 3. **a** This experiment represents a replicate of that presented in Fig. 4a showing that when flies were exposed in the dark, dead animals failed to influence lifespan (N = 82 for dark dead exposed, 88 for dark unexposed, 101 for light dead exposed, and 96 for light unexposed, $P < 0.001$ for light and $P = 0.49$ for dark, $P < 0.001$ for the interaction between light and exposure via Cox Regression) **b** We see similar effects on lifespan when flies are exposed long-term to either dead flies that were killed via starvation or dead flies that were killed via immersion to liquid nitrogen (N = 183 for starvation-dead exposed, 201 for liquid nitrogen-dead exposed, and 200 for unexposed, $P < 0.001$ for each dead exposed treatment compared to the unexposed). **c** The change in mean lifespan when comparing exposed vs. unexposed cohorts of *norpA* flies is significantly decreased when compared to a similar measurement in control flies. There is no significant mean lifespan interaction when comparing exposed vs. unexposed cohorts of control or *Poxn* mutant flies.



Supplementary Figure 4.8. The microbiota is not involved in the effect of exposure to dead conspecifics on aversiveness. Similar levels of aversion were induced when axenic flies were used as both exposed and dead flies as seen when using conventionally reared animals (N = 10 for both treatments). All naïve choosing flies were females from the Canton-S strain.



Supplementary Figure 4.9. Olfaction is required in naïve choosing flies to detect the aversive cues emitted by flies exposed to dead conspecifics. In this experiment, female *Orco*^{2} or *Gr63a*^{1} mutants were used as the naïve choosers, while female Canton S were used as the exposed or unexposed flies in each arm of the T maze (N = 6 for *Orco* control, N = 7 for O*rco*^{2}, N = 10 for *Gr63a* control, and N = 10 for *Gr63a*^{1}). P < 0.001 for both controls and P > 0.31 for both mutants. Each T-maze sample tests 20 flies.



Supplementary Figure 4.10. Details of the principle component analysis and effect of death perception on serotonin *5-HT2A*$^{-/-}$ mutants. a Principle component randomization results. Observed (black) and randomized (grey) distributions of the ability of individual principal

components to effectively distinguish the neurometabolomics signature of flies exposed to conspecific dead. No statistical difference was observed between these two distributions (P = 0.14, Kolmogorov-Smirnov Test). This analysis identified two PCs, PC1 and PC10, that exhibited statistically significant ability to distinguish groups. Shown are the frequency plots and P-values for both PCs. **b** Replicate T-maze behavioral experiment showing that loss of *5-HT2A*$^{-/-}$ protected flies from the consequences of death perception on aversive cues detected by naïve choosing flies (N = 16 for each treatment). **c** Replicate survival experiment showing that *5-HT2A*$^{-/-}$ flies are protected from the consequences of death perception on lifespan (N = 94-98 per genotype/treatment, P = 0.002 for Canton-S control flies and P = 0.22 for *5-HT2A*$^{-/-}$ mutants).

	Vehicle (DMSO)			Drug			
Drug	**Unexposed**	**Exposed**	**% Change**	**Unexposed**	**Exposed**	**% Change**	**P**
Fluvoxamine	55.69 (0.86)	50.24 (1.13)	10%	56.42 (0.78)	51.88 (0.92)	8%	0.35
3-Iodo-L-Tyrosine	61.65 (1.35)	51.93 (1.76)	16%	61.58 (1.36)	49.48 (1.74)	20%	0.35
Thiostrepton	63.59 (1.41)	50.77 (1.29)	20%	67.13 (1.38)	53.26 (1.20)	21%	0.75
Propantheline Bromide	63.59 (1.41)	50.77 (1.29)	20%	67.31 (1.36)	50.47 (1.19)	25%	0.10
Epinastine	63.59 (1.41)	50.77 (1.29)	20%	66.28 (1.24)	50.54 (1.65)	24%	0.21
Ondansetron	63.59 (1.41)	50.77 (1.29)	20%	70.36 (1.01)	49.10 (1.18)	30%	<0.001
3-Iodo-L-Tyrosine	61.65 (1.35)	51.93 (1.76)	16%	61.58 (1.36)	49.48 (1.74)	20%	0.35

Supplementary Table 4.1. Summary of death exposure effects on lifespan of flies fed drugs. Values represent mean lifespan (SEM) in days for each cohort. Interaction P-values represent the results of a randomized ANOVA as described in the Methods. Note that the significant effect of ondansetron reflects an enhancement of the response, not an abrogation.

CHAPTER V

Conclusions and Future Directions

Overview

The overarching goal of this thesis was to understand how different aspects of serotonin signaling influenced health and lifespan in *Drosophila*. Perception of specific environmental stimuli influences lifespan across a wide range of model systems, and conserved neuromodulators, such as serotonin, have been implicated as important mediators of these perceptual events on lifespan; however, very little was known about the ways in which manipulation of specific serotoninergic pathways influences lifespan in the absence of environmental changes.

This section contains a summary of the findings from each chapter, a discussion of how these findings contribute to the current state of science, and promising areas for future studies. Additionally, this chapter includes data relevant to the projects in previous chapters that may form a foundation for future experiments. The techniques used in these particular experiments may also be broadly applicable to future studies examining links between behavior, neuroscience, and lifespan in *Drosophila.*

Summary of Findings and Future Directions

Specific 5-HT Receptors Differentially Modulate Lifespan (Chapter II)

Chapter II addresses the first aim of my dissertation, in which I sought to understand how different aspects of serotonin signaling influenced lifespan in *Drosophila*. Several studies implicated serotonin as an essential mediator of the effects of specific environmental manipulations on lifespan (Ro, Pak et al. 2016, Chakraborty, Gendron et al. 2019, Miller, Huang et al. 2022); however, little was known about the roles of serotonin in aging, independent of changes in the environment. Considering the enormously wide array of behavioral and

physiological processes known to be regulated by serotonin (Berger, Gray et al. 2009), it was likely that this singular molecule may induce vastly different effects on lifespan, depending on the branch of signaling that this manipulated.

Serotonin is capable of such a variety of roles due to its binding to different receptors. Depending on which receptor is activated and in which tissues or neurons, in can alter behaviors like sleep or influence aspects of physiology like heart rate (Berger, Gray et al. 2009). To date, we know of five receptors for serotonin in *Drosophila*: 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, and 5-HT7 (Blenau and Thamm 2011). Null mutants for these receptors displayed a striking level of variability in their effects on lifespan. In both females and males, *5-HT1A*$^{-/-}$, *5-HT1B*$^{-/-}$, and *5-HT2B*$^{-/-}$ mutants were short-lived (Fig. 2.1 and 2.2). Conversely, *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutant females are long-lived; however, these mutations had no effect on male lifespan (Fig. 2.1 and 2.2).

Chapters III and IV are focused on ways in which 5-HT2A influences lifespan; however, nothing is known about the mechanism underlying the *5-HT7*$^{-/-}$ mutant lifespan extension. It is likely that antagonizing the 5-HT7 signaling pathway is beneficial to lifespan, as a pharmacological manipulation recapitulated the mutant lifespan phenotype in a drug screen (Fig. 5.1). This is also a relatively unexplored area, as no data exists on the aging effects of loss of 5-HT7 homologs in other species.

Several key differences in the lifespan phenotypes across receptor mutants could be used as footholds to investigate the mechanisms of these effects. As detailed in Chapter II, potential reasons for similar effects upon loss of 5-HT2A or 5-HT7 may include shared expression patterns in some brain regions or their similar properties upon serotonin binding. Additionally, the sex-specific differences can similarly be utilized to focus on sexually dimorphic neural circuits that co-express 5-HT2A or 5-HT7 as potential candidate populations of neurons underlying the lifespan phenotypes. RNAseq analysis suggests that both 5-HT2A and 5-HT7 co-localize with sex-specific transcription factor *fruitless* (*fru*), and some neurons may even express all three (Janssens, Aibar et al. 2022); however, the precise location of these neurons is unknown. Although antibodies do not exist for 5-HT2A and 5-HT7 in Drosophila, a fruitless

antibody could be used in combination with fluorescent reporters driven by either, or both, receptors. This would allow for a more focused estimate of the neuronal locations of *fru*$^{+}$*/5-HT2A*$^{+}$ or *fru*$^{+}$*/5-HT7*$^{+}$ neurons. Additionally, fru^{+}*/5-HT2A*$^{+}$*/5-HT7*$^{+}$ neurons would be strong candidates for neurons that modulate aging. With the wealth of genetic reagents available in *Drosophila*, *GAL4* drivers for these regions could then be used to ask whether the activity of these neurons is sufficient to alter lifespan. Specifically, split-GAL4 lines that divide the regulatory components of the *GAL4* element between two constructs could be used to selectively target both *fru*$^{+}$*/5-HT2A*$^{+}$ or *fru*$^{+}$*/5-HT7*$^{+}$ neurons and ask whether the "female-ness" of these circuits drives the effects on lifespan. The female-specific transcription factor, *transformer* (*tra*), can be driven by any *GAL4* line, allowing the feminization of neurons in male flies (Ferveur, Stortkuhl et al. 1995).

Another obvious question that follows from the observation that *5-HT2A*$^{-/-}$ and *5-HT7*$^{-/-}$ mutants are both long-lived is whether 5-HT2A and 5-HT7 work in similar or parallel pathways that influence aging. As noted above, there is some evidence to suggest that 5-HT2A and 5-HT7 are co-expressed in at least some populations of neurons (Davie, Janssens et al. 2018). One brain region that expresses both 5-HT2A and 5-HT7 is the ellipsoid body (EB) (Gnerer, Venken et al. 2015). This region was typically thought to be involved in sensory information processing, particularly for visual stimuli (Pfeiffer and Homberg 2014). However, recent work suggests that specific neurons in the EB influence lifespan and that this requires 5-HT2A signaling (Gendron, Chakraborty et al. 2022). The role in lifespan, if any, of 5-HT7 in these neurons is currently unknown. If 5-HT2A and 5-HT7 work in the same neuronal pathway to modulate aging, blocking signaling through both receptors would not further extend lifespan relative to a single manipulation. A simple way to test this quickly would be to measure whether the lifespan of *5-HT2A*$^{-/-}$ mutants is further extended by the selective 5-HT7 antagonist shown to extend lifespan (Fig. 5.1).

If the lifespan phenotype of *5-HT2A*$^{-/-}$ mutants is not further extended by a 5-HT7 antagonist, this could be genetically validated using double mutants, as pharmacological manipulations may have off-target effects. If 5-HT2A and 5-HT7 indeed work in the same pathway, the lifespan of *5-HT2A*$^{-/-}$, *5-HT7*$^{-/-}$ double mutants would be non-additive relative to the single mutants. This is

likely what would be seen if a specific *5-HT2A*$^{+}$*/5-HT7*$^{+}$ neurons' activity modulates lifespan, for instance, certain EB neurons. In that scenario, loss of either receptor would have a similar impact on lifespan, since they influence the activity of the same neuron(s). However, if 5-HT2A and 5-HT7 work in distinct neuronal pathways that influence lifespan, the *5-HT2A*$^{-/-}$, *5-HT7*$^{-/-}$ double mutants would display an even longer lifespan than the single mutants. Given that both receptors induce excitatory responses upon serotonin binding and decreasing global excitatory signaling extends lifespan in *C. elegans* (Zullo, Drake et al. 2019), it is possible that the tuning of excitatory signaling through either 5-HT2A or 5-HT7 influences lifespan. Thus, a prediction might be that the decrease in excitatory signaling through multiple pathways would be even more beneficial to lifespan relative to loss of only one.

It is also worth noting that these long-lived receptor mutants were largely phenotypically normal in a screen evaluating sleep, activity levels, and feeding behaviors (Fig. 2.3, 2.4, 2.5). More targeted experiments may therefore be necessary to better understand the mechanisms underlying these lifespan phenotypes. Although Chapters III and IV focused on 5-HT2A-dependent lifespan phenotypes, relatively little is known about the mechanisms by which 5-HT7 influences lifespan. In *Drosophila*, it is known that 5-HT7 plays important roles in promoting proper courtship behaviors (Becnel, Johnson et al. 2011) and that perception of mates (without the ability to mate) shortens lifespan (Gendron, Kuo et al. 2014). As such, it is attractive to speculate that loss of 5-HT7 may lead to the perceived inability to mate and/or lower mating drive. This reduced reproduction drive may induce a state that promotes metabolic processes that improve the health and longevity of the fly, presumably to allow for reproduction when mates are available. One prediction might be that the *5-HT7*$^{-/-}$ mutant lifespan is not further extended by interventions that reduce mating drive, such as virgin status. Indeed, virgin flies are long-lived relative to mated controls (Service 1989), suggesting that a perceived lack of reproductive demand is beneficial to lifespan, and this may involve 5-HT7 signaling.

New methodology may lead to the identification of previously unknown expression patterns among genes, including the 5-HT receptor mutants. This may generate new hypotheses regarding roles for these receptors in behavioral and/or physiological processes, including lifespan. I generated preliminary data on the expression patterns of each 5-HT receptor in fly heads and

bodies using a recently developed optically clearing protocol (Pende, Becker et al. 2018), which allows for fluorescent imaging of intact flies by depigmentation of the cuticle. This revealed that certain receptors are expressed in regions of the head which might typically be missed with a standard brain dissection. Specifically, all of the 5-HT receptors appear to innervate regions important for feeding, such as the proboscis (Fig. 5.2). The more focused investigations of 5-HT2A in Chapters III and IV also generated curiosity about potential expression of 5-HT2A in other tissues. 5-HT2A appears to be expressed in the salivary glands, as well as potentially expressed in the spiracles (Fig. 5.2), which allow air into the respiratory system in insects. The roles of *5-HT2A*$^{+}$ peripheral neurons in modulating lifespan, if any, is a potential area for further study. This could be addressed by using genetic reagents that allow for knock down of 5-HT2A in all non-neuronal tissues (ex. *Nsyb-Gal80*) and investigating the effects on lifespan.

5-HT2A modulates lifespan in two distinct contexts (Chapters III and IV)

Although loss of either 5-HT2A or 5-HT7 led to a lifespan extension, 5-HT2A became a stronger focus of investigations moving forward as it modulates aging in multiple distinct contexts. 5-HT2A was known to modulate lifespan in a dietary choice environment, in which flies live shorter when they must consume either protein or sucrose as opposed to the standard mixed diet used in aging experiments (Ro, Pak et al. 2016). As 5-HT2A plays a role in evaluating the nutritional environmental in some way, I hypothesized that the magnitude of lifespan extension of *5-HT2A*$^{-/-}$ mutants might be influenced by the ratios of protein to sucrose in the diet. Indeed, Chapter III is centered around the observation that *5-HT2A*$^{-/-}$ mutant females were long-lived specifically on higher protein diets, and their lifespan was more resistant to the detrimental effects of high protein diets (Fig. 3.1a,e).

I then asked how loss of 5-HT2A would influence other behaviors involving protein, such as feeding. When measuring consumption on diets with different levels of protein, I found that control flies consume to a similar mass of protein across all diets (Fig. 3.2b), suggesting flies have the ability to consume to specific nutrient targets. Interestingly, *5-HT2A*$^{-/-}$ mutants consume a similar amount of protein across diets, but their target is lower (Fig. 3.2b). I further found that specific *5-HT2A*$^{+}$ neurons in the supramedial protocerebrum (SMP) are activated upon protein deprivation (Fig. 3.4a) and that optogenetic activation of these neurons can promote protein

feeding behaviors (Fig. 3.5b,d). This suggests that 5-HT2A is recruited to promote protein consumption when protein demand is high. *5-HT2A*$^{-/-}$ mutants lack this ability, and thus, lower their protein set-point as an adaptive response to long-term inability to satisfy the protein demand. This lower protein demand state is likely beneficial to lifespan, as it may promote organismal maintenance, as opposed to growth and reproduction in protein replete conditions.

It is worth noting that the *5-HT2A*$^{-/-}$ mutant lifespan extensions on various diets were restricted to females (Fig. 3.1b and Supplementary Fig. 3.1). This could be due to several factors, which would be worthwhile to explore in future studies. First, perhaps females have more to gain by lowering their protein demand as they tend to pay a higher cost of reproduction; egg production is extremely energetically costly, requiring large amounts of protein (Chippindale, Leroi et al. 1993). Second, perhaps the *5-HT2A*$^{+}$ neurons that influence lifespan lie in female-specific neural circuits. A closer examination of male vs. female 5-HT2A expression in the brain would provide insights into this. If present, *5-HT2A*$^{+}$ neurons specifically expressed in female brains could be targeted to ask whether feminization of those neurons using *UAS-tra* in males is sufficient to extend lifespan, as described above.

In fact, it is to be determined which *5-HT2A*$^{+}$ neurons modulate lifespan under standard conditions. Activation or inhibition of the *5-HT2A*$^{+}$ SMP neurons that promote protein feeding behaviors does not alter lifespan (Fig. 3.5e-f), suggesting that the *5-HT2A*$^{+}$ circuits influencing feeding behaviors and lifespan are at least partially non-overlapping. A potential experiment could be to measure lifespan when *5-HT2A* is knocked-down in different sub-populations of *5-HT2A*$^{+}$ neurons using a *GAL4* screen and identify candidates that recapitulate the mutant phenotype.

This, then, brings us to the second observation in which 5-HT2A signaling was involved in aging. As detailed in Chapter IV, flies live shorter and become aversive to other flies when they are chronically exposed to dead conspecifics, or other dead flies (Fig. 4.1b and Fig. 4.2b). This was not due to environmental artifacts and relied on the sensory perception of dead flies, as primarily vision and, to a lesser extent olfaction, were required for these behavioral and lifespan phenotypes to manifest (Fig. 4.3 and 4.4). A screening of compounds that block these effects

found that one drug, a 5-HT2A antagonist, abrogated the lifespan shortening and aversiveness phenotypes, which was recapitulated by genetic manipulation of 5-HT2A (Fig. 4.5c-f).

It remains to be discovered precisely which *5-HT2A*$^{+}$ neurons are required to mediate the effects of death perception on both behavior and lifespan. Lifespan experiments are time consuming and the behavioral aversiveness assay is tedious and requires hands-on effort for two to three hours. A simpler screening approach may therefore be useful in identifying neuronal populations that block the effects of death perception. An additional phenotype I investigated focused on the behavioral response of naïve flies to dead conspecifics, as measured by video tracking software developed in our lab. In this assay, flies were placed in a dumbbell-shaped behavioral chamber, containing two arenas connected by a small passageway; one arena was empty and the other contained dead flies. Using this assay, I found that flies chose to spend approximately 90% of their time in the arena containing dead flies, and that this attraction was blunted in genetically blind *norpA* mutants (Fig. 5.3), supporting the notion that vision is crucial for the death perception phenotypes (Fig 4.3a-b and 4.4a-b). This may seem counterintuitive, at first, however, this is likely an adaptive response to a perceived threat in the environment. The flies may be investigating the cause of death to assess the risk level to other flies. Many species are known to spend significant periods of time gathered around dead conspecifics (Iglesias, McElreath et al. 2012, van Leeuwen, Mulenga et al. 2016). It may also be a freezing behavior, as flies are known to freeze and slow down subsequent walking when startled (Zacarias, Namiki et al. 2018, Howard, Chen et al. 2019). Interestingly, the release of serotonin is required for this startle response walking behavior; flies deficient in serotonin are not able to slow their walking speed in response to a variety of perceived threats, such as sudden blackout or intense vibration (Howard, Chen et al. 2019). This supports the notion that serotonin is required for the normal response to apparent threats, including death perception. This could be validated by optimizing the video tracking to measure walking speed in addition to arena preference. This may then be a useful tool to screen smaller candidate neuronal populations required in the death perception phenotype, as it could also potentially be coupled to optogenetic manipulations for precise control over neuronal activity.

As mentioned above, it would be very interesting to know whether the *5-HT2A*$^{+}$ neurons that are involved in the death perception phenotype overlap with those required for the lifespan modulation in response to the nutritional environment. In the case that there is significant overlap, this may indicate that any modulation to lifespan requires a limited subset of master longevity regulating *5-HT2A*$^{+}$ neurons. It is also possible that *5-HT2A*$^{+}$ neurons act more upstream to the components of the pathways that alter lifespan. Distinct populations of *5-HT2A*$^{+}$ neurons may receive different sensory inputs which then converge on more downstream neurons or other tissues that are capable of altering lifespan. Regardless of the degree of overlap, this would provide significant insights into the mechanisms by which 5-HT2A signaling modulates aging.

Final Perspectives

Despite clear links between serotonin and aging in specific contexts, very little was known about how a single neuromodulator can impact lifespan through its various signaling pathways. Through a characterization of how each serotonin receptor impacts lifespan and behavior in *Drosophila*, my work provides evidence that different serotonergic signaling pathways modulate aging in profoundly different ways. I identified that loss of either 5-HT2A or 5-HT7 extends lifespan and made substantial progress in understanding the mechanisms by which 5-HT2A influences lifespan in different conditions. I hope that this work will provide a foundation for studies examining the roles of serotonin in aging in humans, with the goal of developing therapeutics for the betterment of human health and lifespan.

Methods

Methods on Fly Husbandry, Survival Assays, and Imaging can be found in Chapters II, III, or IV.

Fly Stocks

Each 5-HT receptor *GAL4* line was generously provided by Herman Dierick (Baylor University). UAS-mCD8::GFP, Canton-S, and *norpA* mutants were obtained from the Bloomington Stock Center.

Drug administration

SB 269970 was purchased from Tocris. SB 269970 was initially dissolved in 100% DMSO at 10 mM concentration, aliquoted, and stored at -20°C. Every Monday, Wednesday, and Friday, an aliquot of the drug stock was thawed and diluted 1:500 in water for lifespans (20 µM final concentration). A similar dilution of DMSO alone was made in water as a vehicle control. Then, 100 µl of the diluted drug or vehicle control was added to each vial, coating the top of the food surface. After the liquid evaporated (~2 hours), the vials were ready for use.

Optical Clearing (Pende, Becker et al. 2018)

To fix the flies prior to depigmentation, flies were placed in a 50mL conical tube and gently shaken (Speed=5, Tilt=15) in 40 mL of 4% PFA, pH=8.5 at 4°C for 1.5 hours then washed 3x with ~40 mL of sterile 1x PBS at room temp. for 20 min/wash. The conical was covered for all remaining steps to prevent photobleaching of fluorescent proteins. To depigment the flies, the PBS from the final wash was removed and replaced with 30 mL of Solution 1 was added to the conical (8% THEED, 5% Triton-X, 25% Urea in MilliQ water) and gently shake (Speed=5, Tilt=15) at 37°C for 3-5 days (eye pigmentation was checked at 3 days). To prepare the samples for imaging/storage, flies were washed with ~40 mL of sterile 1x PBS at room temp for 20 min and washed a second time with ~40 mL of sterile 1x PBS at room temp overnight. Next the PBS solution was removed, leaving just enough to cover the flies. Flies were transferred to a 1.5mL Eppendorf tube with a wide-bore or cut pipette tip, and any remaining PBS was removed with a thin pipette tip. Flies were then submerged in VectaShield (tubes were covered with tinfoil) and

imaged within 2-3 days, following standard mounting and imaging procedures (see Methods in Chapter II).

Video Tracking and Analysis

One-week old, mated Canton-S female flies were starved for 4 hours in a vial with moist tissue paper prior to the experiment to encourage movement within the chambers. Each chamber contained two dumbbell-shaped arenas comprised of two circles (Internal diameter = 1.0”) separated by a narrow corridor connecting them. A thin 2% layer of agar served as the floor of the chambers. Dead flies were lightly pressed into the agar of 1 arena to secure them. The positions of these stimuli were randomized within each experiment. Five minutes prior to recording, single flies were loaded into the chambers by aspiration. Movement in each arena was recorded for 2 hours in a 25°C incubator under white light. Recordings were analyzed using the DTrack Software, developed in the Pletcher laboratory (Linford, Kuo et al. 2011, Kuo, Fedina et al. 2012). From the tracking data, we calculated the amount of time each fly spent in each side of the arena, which was then used to calculate the relevant Preference Index (PI). PI = (Time spent in stimulus arena (T_S)-Time spent in empty arena (T_E))/(T_S+T_E).

Figures

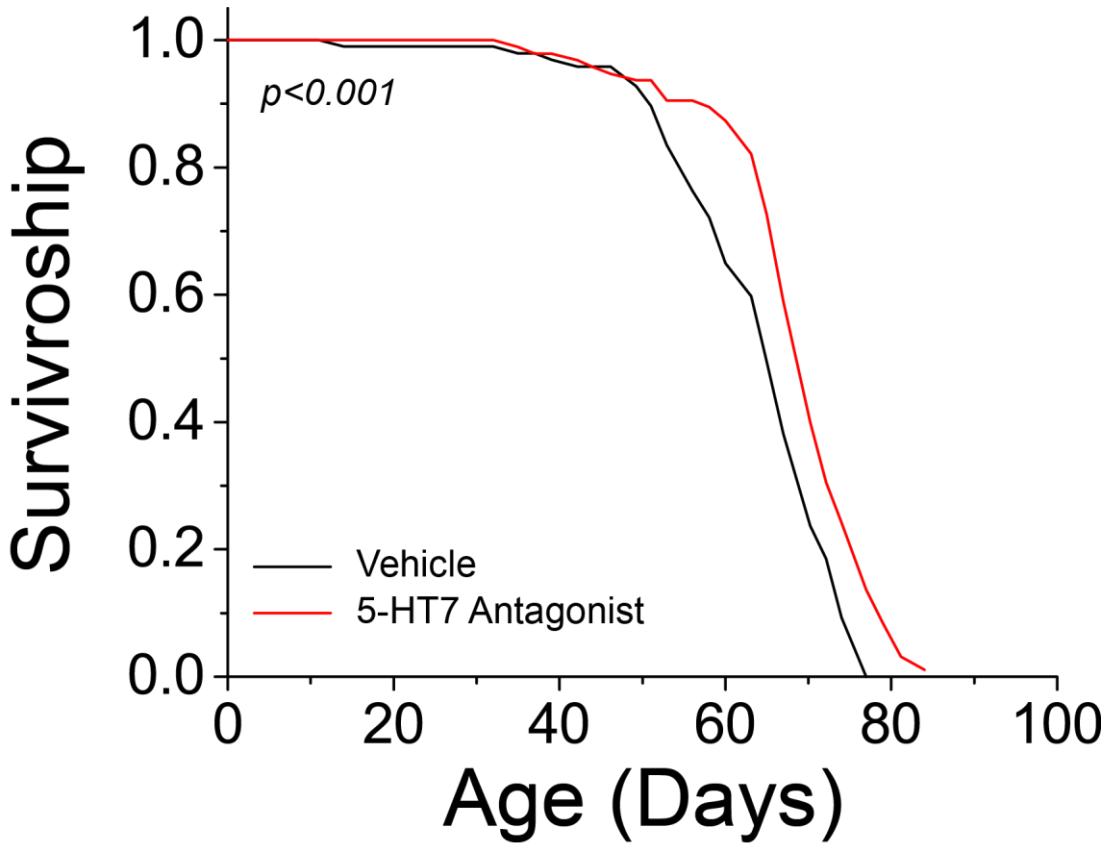


Figure 5.1. Administration of a 5-HT7 antagonist extends lifespan. Female flies fed the selective 5-HT7 antagonist, SB 269970, are long-lived relative to DMSO vehicle controls (N = 95 and 97, log-rank analysis $P < 0.001$).

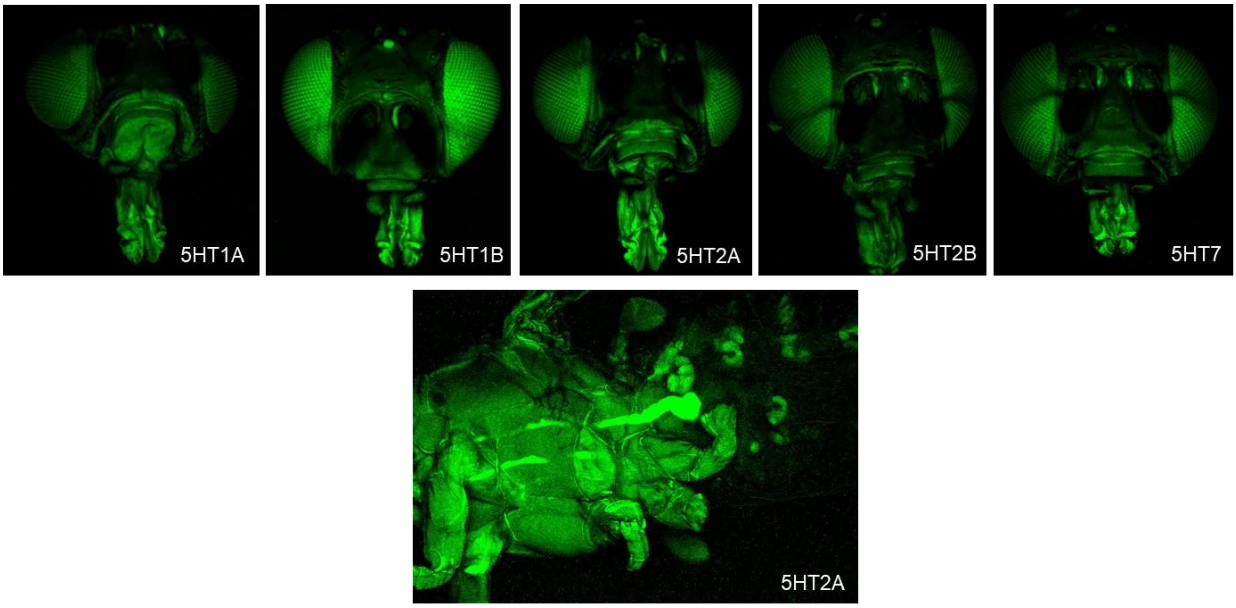


Figure 5.2. Expression of 5-HT receptors in intact flies. Upper panels: Expression of each 5-HT receptor in the head, visualized by driving expression of GFP using receptor specific *GAL4* lines. Lower panel: Peripheral expression of 5-HT2A, ventral view.

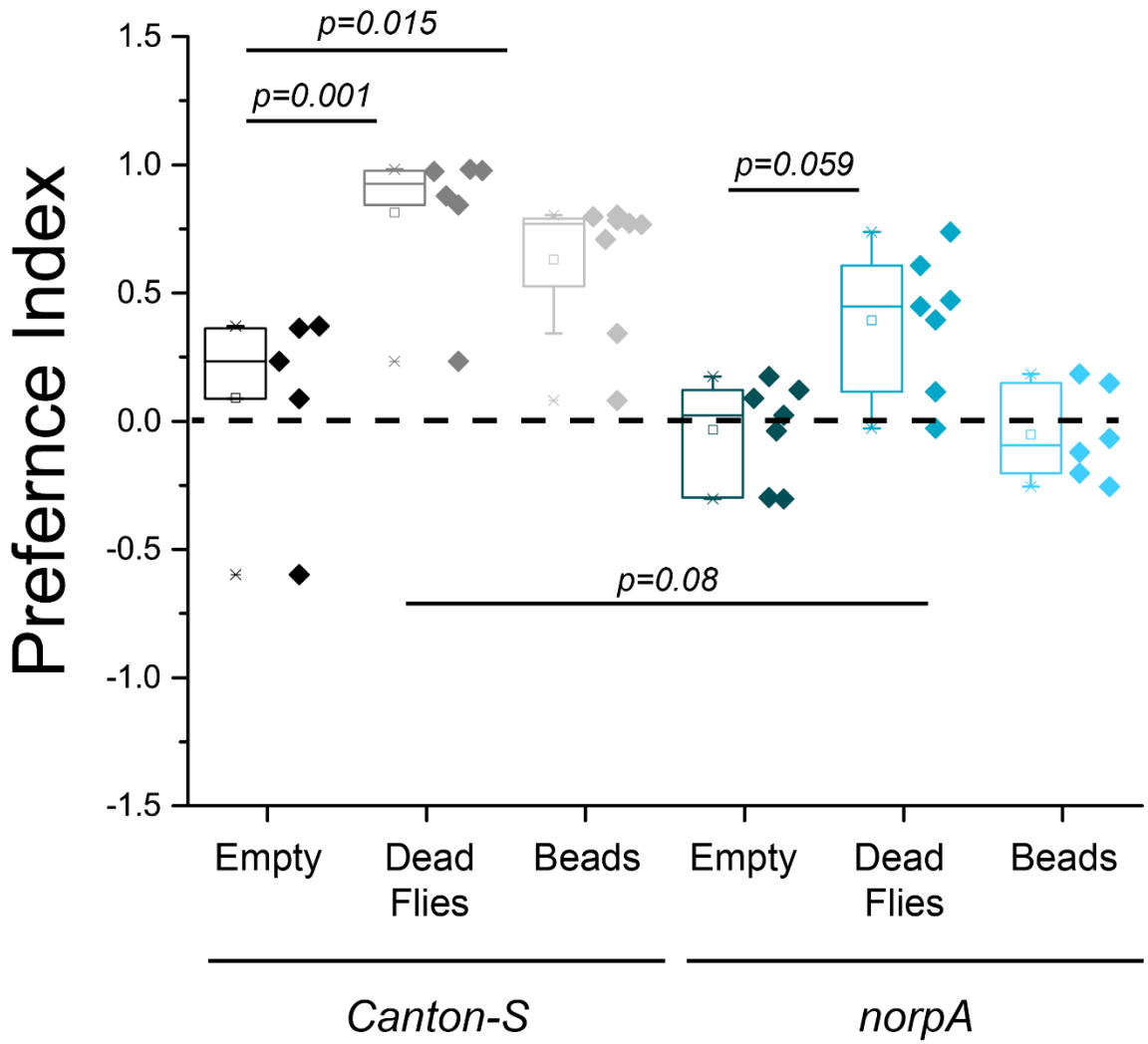


Figure 5.3. Flies are visually attracted to dead flies. Preference index represents time spent between two arenas, and all comparisons are made against an empty arena. A preference index of 1 indicates 100% of time spent in the arena with the stimulus. Canton-S flies spend significantly more time in arenas occupied with visual stimuli (N = 5-8, Two-way ANOVA w/ Tukey Post-hoc Test: dead flies P = 0.001 and beads P = 0.015). *norpA* flies do not spend significantly more time in an arena with beads and trend towards less time in arena with dead flies relative to Canton-S controls (N = 5-7, Two-way ANOVA w/ Tukey Post-hoc Test P = 0.08).

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