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MALE AND FEMALE DIFFERENCES IN RESPONSE TO SOCIAL STRESS: INSIGHTS FROM TRANSCRIPTOMIC AND BEHAVIORAL ANALYSES IN DROSOPHILA MELANOGASTER

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MALE AND FEMALE DIFFERENCES IN RESPONSE TO SOCIAL STRESS: INSIGHTS FROM TRANSCRIPTOMIC AND BEHAVIORAL ANALYSES IN *DROSOPHILA MELANOGASTER*

Elisabeth Alice Tawa

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Abstract

Chronic stress contributes to detrimental health effects, but our understanding of how stress induces sex differences in brain transcriptomics and behavior is incomplete. In this thesis, I use a combination of behavioral testing, RNA sequencing (RNA-seq), and genetic manipulations in *Drosophila melanogaster* to explore how social stress reveals latent sex differences in gene expression and stress-relevant behaviors. I begin by introducing mammalian versus insect stress signaling systems and proceed to review methods for stress induction in *Drosophila*. I also highlight fly behavioral tests for measuring stress-related behaviors, sexual dimorphism in stress responses, and the molecular underpinnings of fly stress behaviors. I then present the effects of social isolation and overcrowding on behavior and survival in *Drosophila melanogaster*. Finally, I identify sex differences in whole head transcriptomics following social isolation and manipulate the expression of *takeout* (*to*) to assess its role in female-specific social isolation-induced increases in sucrose-feeding. Overall, I quantified changes in locomotion, motivated behavior, aggression, and sucrose feeding in flies following social isolation. The most pronounced sex differences were evident in behaviors related to feeding and motivation. RNA profiling revealed isolated female-specific upregulation of over 100 genes, with many of them relating to reproduction and energy metabolism. We manipulated *to* expression and found that downregulating *to* in all *to*-expressing cells decreases sucrose-feeding in isolated females but increases it in isolated males. Upregulating *to* in all *to*-expressing cells also decreases sucrose-feeding in isolated females. Altering *to* expression in neurons did not recapitulate these same effects, suggesting that isolated female-specific *to* overexpression may occur in non-neuronal cell types in the head. Our results suggest that within a chronic stress context, sex-specific effectors in the head may regulate gene expression related to feeding and macronutrient choice to

ensure that females prioritize survival over reproduction. Learning more about this system in flies could provide insight into functionally analogous pathways in humans that may be dysregulated in female-biased stress-related disorders.

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Chapter 1

Introduction

1.1 Defining stress

Stress was first defined in the scientific community by Hans Selye, who defined it as the "nonspecific response of the body to any demand[1]." Selye understood that prolonged exposure to a demand could generate non-specific symptoms of illness. Stress has also been defined as any physical or psychological stimulus (stressor) that brings an organism away from homeostasis and results in a behavioral or physiological change (the stress response)[2]. In humans, some stress can arise from external and systemic factors such as early life adversity, environmental exposures (including from the built environment), poverty, racism, and other social determinants of health[3]. Moreover, stress can be acute or chronic. Acute stress is short-term and results from an immediate challenge. Short-term stress induces the body's fight-or-flight response, in which temporary physiological changes occur to enable the organism to manage a challenge. Chronic stress occurs when a stressor is present over an extended period. This can stimulate prolonged physiological and psychological changes and increase the risk of health problems, ranging from cardiovascular disease to depression, anxiety, and cognitive impairment[2]. Negative stress is referred to as distress, yet stress can also be positive (eustress) and confer health and survival benefits. It is important to study the effects and underlying mechanisms of both eustress and distress to identify targets for optimizing brain and behavioral health.

1.2 Stress-related disorders and sex differences

Stress exposure increases the risk for several mental disorders. Post-traumatic stress disorder (PTSD), acute stress disorder, and adjustment disorders develop following a traumatic event and are marked by dysphoria, anhedonia, external anger and aggression, and dissociation[4]. However, chronic stress can also enhance the risk of developing broader mood, anxiety, and substance use disorders[5]. Sex as a biological variable plays a key role regarding which disorders a person enduring chronic stress is most likely to develop. For example, women are more likely to develop certain forms of PTSD[6,7] and major depression[8,9,10] than men. This may be due to sex differences in molecular and circuit-level mechanisms influencing the neuroendocrine stress response, salience of negative stimuli, and arousal[11]. Sex-specific transcriptional signatures of major depressive disorder have also been identified in males and females, and these can be recapitulated in mice[12]. Conversely, substance use disorders tend to be more prevalent in men than women[13,14]. Including symptoms that are more frequently observed in males, such as aggression, substance abuse, and risk-taking, into the diagnostic criteria for depression erases the sex difference typically observed in prevalence[15]. Taken together, chronic stress or trauma may differentially contribute to the emergence of psychiatric disorders and their symptoms in males and females. Although substantial research has been conducted to elucidate sex differences in the molecular and systems-level mechanisms of mental disorders that present differently between males and females, the field continues to evolve, aided both by studies in humans and in animal models, including invertebrate models (Tawa & Notterman, in review).

1.3 Stress neuroendocrinology across species

In mammals, stress simultaneously activates the faster Sympathetic-Adreno-Medullary (SAM) axis and the slower hypothalamic pituitary adrenal (HPA) axis[2]. The abatement of these

responses returns the body to equilibrium (homeostasis). The activation of the sympathetic nervous system via the SAM axis stimulates the release of catecholamines (epinephrine and norepinephrine) from the adrenal medulla. The metabolic effects of this rapid response comprise increases in heart rate and blood pressure, increased blood flow to muscles, the breakdown of glycogen and synthesis of glucose, the breakdown of fats, elevated oxygen consumption, and increased heat production[2]. Increasing the availability of circulating glucose facilitates uptake of glucose by the brain, which demands energy to execute the stress response.

When stress activates the HPA axis, following the initial SAM response, the hypothalamus releases corticotropin-releasing hormone (CRH), a hormone and neurotransmitter that binds to receptors on the pituitary gland. This action then induces the release of adrenocorticotropic hormone (ACTH) from the pituitary gland to the adrenal glands, stimulating the production of CORT: cortisol in humans and corticosterone in rodents. CORT also acts to mobilize glucose and increase energy, by promoting the synthesis of glucose in the liver, reducing glucose uptake in muscles, and stimulating the lysis of stored fat into free fatty acids in adipose tissue[16,17]. Moreover, CORT breaks down proteins into amino acids, so that they can be more readily accessed for energy production and tissue repair[17]. Circulating glucocorticoids bind to adrenal steroid receptors in the brain to regulate stress responses and, through a well-characterized negative feedback circuit, reduce the further secretion of glucocorticoids[18].

Stress-induced activation of both the SAM and HPA axes generates molecular mediators, which exert their effects through binding to specific receptors throughout the body and brain. In the body, this receptor binding triggers energy mobilization, metabolic changes, immune activation, and digestive and reproductive suppression. In the brain, effects include (but are not limited to) epigenetic modifications and immune reactivity, which can then alter cellular

excitability and neuronal and synaptic plasticity. Taken together, these responses produce changes in physiology and behavior that help the organism adapt to the stressor[19]. However, if chronic stress prolongs the activation of the SAM and HPA axes, cortisol dysregulation or hypercortisolemia can result in detrimental health effects, such as insulin resistance, visceral obesity, and metabolic syndrome[20]. Metabolic syndrome encompasses a cluster of conditions, including hypertension, high blood sugar, and cholesterol abnormalities, which increase the risk of cardiovascular disease, type 2 diabetes, and obesity[20,21]. Extended exposure to high cortisol has also been associated with inflammatory bowel diseases[22] and adverse effects on neurobiology and brain function, such as neurodegeneration, neuroinflammation, exacerbation of mood and anxiety disorders, and cognitive impairment[23].

Although these are the general physiological mechanisms of the stress response, this response becomes highly complex when taking context into account. The pattern and magnitude of an animal's stress response can be influenced by factors such as the duration of stress exposure, the type of stress, context (e.g. time of day), developmental stage, sex, and genetic background[24]. Multiple monoamines, neuropeptides, and steroid hormones act as molecular mediators in the central nervous system to change behavior in a stressor-specific manner. These include noradrenaline, dopamine, serotonin, CRH, urocortins, vasopressin, orexin, dynorphin, corticosteroids, and neurosteroids[24] (Table 1.1). The regulation of the HPA axis is also sensitive to experience and can be altered both during development[25,26,27] and in adulthood[28] as a result of aversive experiences. HPA axis functioning may also change with aging[29,30] and predispose older adults to disorders related to metabolic, immune, and cognitive health.

Table 1.1: Central nervous system molecular mediators responsive to stress

	Source	Regulation	Function
Noradrenaline	Adrenal medulla[31] (adrenal glands), locus coeruleus[32] (brain stem)	Reuptake via the norepinephrine transporter (NET) or extraneuronal uptake (via uptake 2)[33]	Regulates arousal, attention, cognition, and stress reactivity[34]
Dopamine	Substantia nigra, ventral tegmental area, hypothalamus (brain)[35]	Reuptake via the dopamine transporter or plasma membrane monoamine transporter[36]	Primarily involved in reward, movement, motivation, and mood[35,37]
Serotonin	Enterochromaffin cells (intestinal lining)[38], raphe nuclei neurons (brainstem)[39]	Reuptake via serotonin transporter (SERT) protein[40]	Regulates mood, sleep, appetite, digestion, anxiety, blood clotting, and sexual function[39]
Corticotropin-releasing hormone (CRH)	Paraventricular nucleus (PVN) of the hypothalamus (brain)[41]	CRH+ neurons in the PVN receive inputs from the amygdala, hippocampus, and bed nucleus of the stria terminalis[42]; CRH+ neurons are also activated/inhibited by noradrenaline[43] and glucocorticoids[44].	Central regulator of the HPA axis; activates hormonal and neuronal signaling cascades in vertebrates in response to stress[41]
Urocortins	Expressed widely in CNS and PNS, in similar patterns to CRH[45]	Gene encoding urocortin is transcriptionally regulated by CRH, glucocorticoids[45], and inflammatory cytokines[46]	Regulates food intake, anxiety, learning, memory, body temperature, and neuroprotection[47]
Vasopressin	Supraoptic and paraventricular nuclei of the hypothalamus (brain)[48]	Gene expression is regulated by clock genes[49]. Secretion is inhibited by ethanol[50], atrial natriuretic peptide[51], and	Controls and regulates fluid balance, blood pressure, sodium homeostasis, and kidney function[48]

		cortisol[52]. Secretion is stimulated by angiotensin II[51].	
Orexin	Lateral hypothalamic area and perifornical area of hypothalamus (brain)[53]	Primarily regulated by peripheral metabolic factors (ghrelin, leptin, glucose) and neurotransmitters (glutamate, dopamine, serotonin)[54]	Regulates feeding, sleep/wakefulness, and neuroendocrine functions[53]
Dynorphin	CNS (hypothalamus, striatum, hippocampus, spinal cord), adrenal gland, testis, and anterior pituitary[55]	Metabolized by carboxypeptidases and aminopeptidases in the plasma and dynorphin converting enzyme and angiotensin converting enzyme in the brain[56]	Regulates pain, reward and reinforcement, stress response, mood, and cognition[57,58]
Corticosteroids	Produced by the cortex of adrenal glands[59]	Glucocorticoid release is regulated by the HPA axis[59]	Regulate development, homeostasis, metabolism, cognition, and inflammation[59]
Neurosteroids	Produced in hippocampal and cortical neurons and glia. Also synthesized in peripheral tissues (e.g., endocrine glands)[60]	Corticosteroids[61] or gonadal hormones[60] can affect synthesis. Can also be removed by transporter proteins[62]	Regulate epileptic seizure susceptibility, anxiety, and stress[60]

As is the case for all animals, insects must also be able to respond to environmental challenges and return their body systems to homeostasis. Neuropeptides and biogenic amines are produced primarily in the central nervous system and mediate most physiological and behavioral processes[63]. Tachykinin-related peptides and peptides related to the mobilization of reserves for energy, such as adipokinetic hormones, insulin-like peptides, and neuropeptide F, have been

implicated in the general insect stress response, as they contribute to metabolism, circadian rhythms, and feeding[64,65,66]. Octopamine, dopamine, and serotonin are critical biogenic amines that also contribute to the stress response[67,68].

Although there is neither an explicit stress axis nor stress hormones that have been identified in insects, there are pathways which may function similarly to the HPA axis. The *pars intercerebralis* and *pars lateralis* regions of the *Drosophila* larval brain bear some anatomical similarity to the hypothalamus[69], and the Drosophila peptide DH44 is a homolog of CRH[70]. Though the anatomical and functional organizations of the neurosecretory system have not been conserved between insects and mammals, there is some evidence of convergent evolution of hormone function. For instance, mammalian glucagon and *Drosophila* adipokinetic hormone play analogous roles in mobilizing carbohydrates[71,72]. The hormonal axes in flies may be less organized morphologically compared to the HPA axis, but nevertheless, we can identify axes which regulate metabolism, feeding and appetite, reproduction, sleep, activity, water and ion balance, and development[73]. Due to its simplicity relative to that of other animals, the neuroendocrine system of *Drosophila* may present a fruitful model system for understanding the evolutionary conservation of stress hormone signaling pathways and their regulation[74].

1.4 Stress and brain structural plasticity

Stress-induced structural plasticity in the mammalian brain has also been well-characterized. Rewarding experiences that activate the stress system, such as running[75] and sexual experience[76], have been shown to promote adult hippocampal neurogenesis in rodents, while aversive experiences, such as social defeat[77,78] and predator odor exposure[79,80], have been found to hinder it. Chronic stress has also been shown to alter dendritic spine density and complexity and exert its effects in a brain-region specific manner. For example, chronic stress

has been shown to promote growth effects in medium spiny neurons of the nucleus accumbens[81] while inhibiting growth in pyramidal neurons of the prefrontal cortex[82,83,84]. At the outset of my dissertation research, I sought to explore the structural plasticity mechanisms through which eustress, in the form of physical exercise, regulates defensive behaviors in mice (Supplemental Chapter). However, I later decided to transition to *Drosophila melanogaster* as a more tractable model system for studying stress-induced changes in behavior (Chapter 3) and brain transcriptomics (Chapter 4).

1.5 Contribution of dissertation

The goal of this dissertation is to integrate the concepts detailed in the foregoing to establish *Drosophila melanogaster* as a valid non-mammalian animal model for the exploration of sex differences in stress-induced gene expression and behavioral changes. To achieve this, I begin by reviewing the approaches that have been used in the field for inducing stress in flies, with an emphasis on social isolation and crowding as stressors of the social environment (Chapter 2). I then describe behavioral tests that have been used in flies to capture translationally-relevant behaviors, which may reflect internalizing and externalizing symptoms of human stress-related disorders. I highlight sex differences that have been identified in these behaviors following different forms of stress and discuss the molecular and neuromodulatory basis of stress and stress-related behaviors in flies. Next, I present original research on the sex-specific effects of social stress, in the forms of social isolation and overcrowding, on behavior and survival in *Drosophila melanogaster* (Chapter 3). My final chapter addresses sex differences in transcriptomics following social isolation in the *Drosophila* head and brain, and the potential role of the protein takeout (to) in driving social isolation-induced increases in sucrose feeding in females (Chapter 4). Manipulating the expression of *to* in *to*-expressing cells and neurons

produces sex differences in sucrose-feeding. I also explore whether masculinizing neurons through knock down of the gene *transformer (tra*) alters sucrose feeding choice in females. Overall, I hope to contribute to the relatively unexplored domain of sex differences in fly stress signaling pathways and their associated behaviors, which may have some value in better understanding the evolutionary conservation of male and female stress responses.

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Chapter 2

Drosophila melanogaster as a neurobehavioral model for sex differences in stress response

In review at **Frontiers in Behavioral Neuroscience (*Tawa & Notterman, 2025*).**

Sex differences are observed in several neurologic and psychiatric disorders. Many aberrant behavioral symptoms can be characterized clinically as either internalizing or externalizing, which tend to manifest disproportionately in females or males, respectively. Stress may precipitate or amplify these behavioral disturbances, which often start in childhood and adolescence but persist into adulthood. Increased understanding of sex differences in stress-induced behavioral changes and their underlying molecular mechanisms is integral to developing better therapeutics specifically tailored to males and females. Here, we highlight the potential of *Drosophila melanogaster* as a model for the neurobiological study of sex differences in stress-altered behavior. We first review paradigms for stressing *Drosophila*, with an emphasis on social environmental stress, namely social isolation and crowding. We then introduce behavioral tests that can be used to quantify stress-induced behaviors in flies and note behavioral sex differences that emerge in response to stress from the existing literature. Finally, we provide an overview of the known molecular and cellular mechanisms underlying stress-induced behavioral change, with a focus on sex differences and studies incorporating social isolation or crowding.

2.1 Introduction

Across human neuropsychiatric disorders, sex biases manifest through disease prevalence, severity, and development. Late-onset schizophrenia, anorexia, bulimia, post-traumatic stress disorder, anxiety, and depression are more prevalent in females, whereas

attention deficit hyperactivity disorder, autism, dyslexia, stuttering, Tourette's syndrome, and early-onset schizophrenia are more frequent in males[1]. Although it is important to consider the role of sex in the etiology of human mental illness and the biases that influence diagnosis of certain behavioral disorders, establishing relationships between biological sex and aberrations in behavior is the first step to elucidating those behaviors' neurobiological underpinnings.

One lens through which we can consider the contribution of biological sex to the development of maladaptive behavior is that of internalizing and externalizing psychopathology. Psychologists have long established internalizing and externalizing as broad categories of behaviors in humans, particularly in children and adolescents[2]. Internalizing behavior is thought to be self-focused, while externalizing behavior relates to one's interaction with the social environment. Internalizing behaviors include behaviors suggesting anxiety, depression, and emotional dysregulation. Externalizing behaviors encompass episodes of aggression, impulsivity, deviance, and hyperactivity[3]. One recent behavioral genetic study using data from over 3,000 twin families attributed roughly one-third of variance in internalizing and externalizing behavior to genetic influences, leaving the majority of variance to be accounted for by environmental factors, such as stress[3]. The authors suggest that many previous heritability estimates for internalizing and externalizing behaviors may be inflated, reflective of the "missing heritability" problem[4], whereby heritability estimates differ based on whether they were calculated using twin data or DNA-based approaches such as genome-wide association studies (GWAS).

Furthermore, there are prominent sex differences in the prevalence of internalizing versus externalizing disorders in human children and adolescents. Sexual selection theory posits that differences between biological males and females in biomarkers, molecular mechanisms, and development can produce differences in risk and resilience for various forms of

psychopathology[5]. Males may be more predisposed to externalizing risk factors such as disinhibition and sensation-seeking due to higher levels of prenatal testosterone exposure, which result from the male fetus' production of hormones in the testes beginning around week 8 of gestation[6]. This enhanced prenatal testosterone is also hypothesized to make males more susceptible to prenatal stressors with downstream effects on dopaminergic signaling in the brain[5]. Alternately, females may be more at risk for developing internalizing disorders, with the risk markers being negative affect and rumination. These risk factors may arise during puberty from increases in estradiol and its interaction with cortisol and oxytocin, which may act on the serotonergic system to increase females' stress sensitivity[5]. Taken together, in human children and adolescents, psychologists posit that stress exposure in conjunction with differences in sex hormones may trigger different monoaminergic signaling processes in male versus female brains, which manifest as sex-specific stress phenotypes.

While mammalian models have proven useful for gaining mechanistic understanding of how stress differentially impacts male versus female brains and translationally relevant behaviors, *Drosophila melanogaster* offers certain advantages. Beyond the decreased costs and ethical considerations of working with an invertebrate species, flies have a shorter lifespan than rodents, thus facilitating the study of stress and its effects at different developmental timepoints. In *Drosophila*, it is also simple to manipulate the sex determination hierarchy in certain cellular populations without manipulating the sex phenotype of the entire organism or its hormones. This fact, in combination with the recent mapping of the *Drosophila* connectome[7], positions *Drosophila* as a promising potential model for probing sexual dimorphism in neural circuits. While these qualities have made *Caenorhabditis elegans* (*C. elegans*) a good model system for studying the interrelationships among molecular mechanisms, neural circuit function, and

behaviors that differ by sex[8], *Drosophila melanogaster* enables us to study more complex behaviors with excellent availability of genetic tools and greater conservation of genes relevant to human disease[9,10].

The goal of this review is to present *Drosophila melanogaster* as a simple yet elegant model system through which we can probe the molecular mechanisms of sex-specific behavioral changes following stress experience. We begin by highlighting approaches that have been developed for stressing flies in the laboratory, with a focus on social isolation and crowding stress. We next summarize behavioral tests that experimenters have adapted for flies to assess phenotypes such as motivation, locomotion, aggression, activity, and sleep. Then, we consider sex differences in how these behaviors change in response to social or physical stressors. We conclude with an extensive discussion of the molecular mechanisms and neuromodulatory systems underlying *Drosophila* stress-induced behavioral changes, with an emphasis on the effects of social isolation and crowding and how they may differ between males and females.

2.2 *Drosophila melanogaster* as a model system for studying stress

A vast literature exists on stress and its phenotypic and behavioral effects in male and female *Drosophila* (Table 2.1). Numerous paradigms have been developed to subject *Drosophila* to physical or psychosocial stress. Physical stressors include starvation[11], oxidative stress-inducing chemical exposure [11,12], temperature[13], mechanical stress [11,14], sleep deprivation[15], and electric shocks[16]. Social stressors consist of modifications to the fly's social environment, including social isolation and overcrowding. Even the presence of dead conspecifics has been shown to impact fruit flies adversely through changes to the head metabolome and decreases in longevity[17].

Some models of *Drosophila* stress use a chronic unpredictable mild stress approach, in which multiple stressors are used in combination. For example, flies may be exposed to heat, cold, starvation, and sleep deprivation at unpredictable intervals over a period of ten days [18,19]. Chronic unpredictable mild stress in male *Drosophila* has been shown to induce both aggression and depression-like behavior, measured through significantly faster times to immobility in a forced swim test[18]. The same study also found chronic unpredictable mild stress to decrease body weight, decrease preference for sucrose, reduce percentage of time spent in the illuminated compartment of a light-dark box (the preferred side of the apparatus for flies), and diminish whole head levels of serotonin and dopamine[18]. Although taken together these tests imply depressive, anhedonia-like, and defensive behaviors in the flies following chronic unpredictable mild stress, it is important to consider ethological relevance in adapting to flies animal behavior tests traditionally developed for rodents. For instance, interpreting a reduction in time spent in the light portion of a light-dark box as a readout of defensive behavior is plausible in flies, given that flies demonstrate positive phototaxis in contrast to rodents' preference for the dark. Using the forced swim test as an indicator of depression or behavioral despair in *Drosophila* might warrant further scrutiny, as the interpretation of this test has already been debated in the rodent literature[20]. Forced swimming in *Drosophila* also raises the question of whether this is a natural behavior that makes sense to study in the laboratory, or whether scientists' interpretation of the immobility response is over-anthropomorphizing the flies' behavior.

2.2.1 Social isolation stress

In studies of the effects of an individual stressor, social isolation has largely been used in *Drosophila* to examine the behavioral impact of environmental deprivation. Although fruit flies are not considered eusocial insects, they demonstrate behaviors reflective of affective states[21]

and social learning[22]. *Drosophila melanogaster* flies can form social networks, perform collective behaviors[23], and transmit mating preferences across generations[24]. In the absence of social interaction, flies show aberrations in their lifespan, gene expression in the brain, and behaviors. One recent study of social isolation in *Drosophila* established mechanistic connections between social deprivation, activity, and metabolism by finding that chronic social isolation reduces sleep, enhances feeding behavior, and induces transcriptomic changes indicative of a starvation-like brain state[25]. Similarly, another study found the social environment to alter *Drosophila* sleep patterns such that socially isolated flies slept less during the day compared to group-housed controls[26]. Aggression is another behavior that increases in laboratory flies following social isolation. This enhancement in aggression occurs in both sexes, with males fighting for food, territory, and mates[27, 28] and females fighting for food and egg-laying sites[29]. Downregulation of the neuropeptide drosulfakinin, a fly ortholog to mammalian cholecystokinin, has been identified as the potential neural substrate of chronic social isolation-induced aggression[30].

2.2.2 Crowding stress

Crowding, the opposite of social isolation, is another potential source of social environmental stress. Much of the existing literature on overcrowding in flies has focused on the effects of larval crowding[31]. In adult flies, this manipulation has ranged from increasing the density of flies within a given physical space to full restraint, imitating chronic restraint stress in rodent models. One study, which employed full restraint, immobilized male flies between two soft plugs for ten hours a day for up to three days. These flies were then mated in order to examine the epigenetic, transcriptomic, and metabolic effects of paternal "psychological" stress on the offspring. It was found that paternal restraint stress induced epigenetic changes leading to

energy metabolism deficiencies in F1 generation males[32]. Three days of more moderate adult crowding has also been shown to decrease longevity in adult *Drosophila*, though it may be the case that exposure to high density living can build resilience[33].

A study investigating the behavioral effects of physical versus social chronic stress (mechanical stress and adult crowding, respectively) also found that not only did certain phenotypes change following stress exposure, but that these alterations were different by sex and more pronounced following overcrowding compared to mechanical perturbation[34]. Specifically, males demonstrated increased sweet-seeking and activity following adult crowding, whereas females demonstrated reduced sweet-seeking and activity. Females also showed reduced exploratory behavior after chronic crowding. Further research is needed to gain a fuller understanding of the molecular and behavioral impacts of adult crowding in *Drosophila*. However, these preliminary findings support overcrowding as a valid fly stress model for unraveling the cellular and molecular mechanisms that generate stress-related behaviors.

2.3 Models for internalizing behaviors

Human internalizing disorders are frequently marked by symptoms indicative of depression and anxiety. Although we do not believe flies to possess internalizing emotions like humans, they may experience persistent internal states, which generate behaviors with some resemblance to human internalizing symptoms. In male flies, it has been shown that chronic, uncontrollable mechanical stress in the form of vibration is sufficient to produce what is interpreted as learned helplessness and anhedonia-like phenotypes[14]. These are often interpreted in animal research as signs of "depression." Learned helplessness and anhedonia are characterized in *Drosophila* following vibration stress by the failure to climb or the failure of hungry flies to feed on sweet-tasting glycerol, respectively. Upon feeding lithium chloride to

male flies, simulating lithium as a pharmacological treatment for major depressive disorder, researchers observed an alleviation of these depression-like phenotypes, such that both stressed and control flies demonstrated excessive climbing behavior. Serotonergic neurons were found to signal to the fly mushroom bodies to modulate the depression-like state[14]. However, in another study, short-term variable stress consisting of a random sequence of stressors such as social isolation, fasting, heat, and electric shock over a 24 hour period was shown to decrease preference for sucrose in male and female flies. This phenotype was resistant to pharmacological treatment with fluoxetine and diazepam[35]. Although there may be some overlap in the etiologies of human internalizing disorders and similar, stress-induced behaviors in flies, the exact neurobiological mechanisms that produce these behaviors in flies remain ill-defined and are an important area for further research. Some or most of the molecular changes in the stress-exposed fly brain that generate these behaviors may differ from those which current antidepressant and anxiolytic drugs counteract in the human brain.

Drosophila has also been proposed as a possible model for probing the genetic basis of anxiety disorders. The elevated plus maze, open field, and light-dark box tests have been routinely used in rodents to measure approach or avoidance as a readout of defensive behavior[36]. These tests are based on the hypothesis that a more "anxious" animal is thought to avoid light and exposure and prefer darkness and edges, tendencies which are adaptive in the wild for hiding from predators. In humans and other mammals, serotonin has long been the primary neurotransmitter associated with anxiety[37]. Diazepam is a benzodiazepine medication that is used in humans for the management of anxiety disorders. Diazepam exerts its anxiolytic effects via allosteric binding at gamma-aminobutyric acid (GABA)-A receptors, which induces neuronal hyperpolarization within the limbic system[38]. Given that orthologous genes and fundamental

behaviors are frequently conserved across species, researchers who measured avoidance behavior in flies found that upon pharmacologic administration of diazepam, genetic manipulation of the serotonin receptor and serotonin transporter, and environmental stress exposure, flies behaved similarly to mice in the open field test[39]. Thus, fly and mouse wall-following both decrease after consuming a benzodiazepine designed for anxiety reduction or after overexpressing genes encoding the serotonin class 1 receptor and serotonin transporter. Knock-down of these genes yields increases in wall-following in the open field test in both mice and flies. Some scientists characterize the *Drosophila* wall-following behavior as a preference for an arena's boundaries rather than the centrophobism that mice demonstrate in the open field test[40]. However, these authors attribute the arena-boundary preference to the fly's efficient search for escape routes, and they observe a preference for dark corners which may be indicative of shelter-seeking[40]. Regardless of whether the preference for spatial boundaries, attraction to the touch of the wall, or fear of the arena center is driving wall-following, these explanations are all rooted in predatory avoidance. Hence, it is possible that flies could be used in the future as a neurogenetic model for defensive behaviors.

2.4 Models for externalizing behaviors

In addition to studying *Drosophila* as a potential model for stress-induced internalizing-like behaviors, flies have also been used to study behaviors relevant to externalizing disorders. Again, we do not believe that flies have externalizing emotions in the same way as humans, but changes in internal states may stimulate behaviors, such as aggression and hyperactivity, that are analogous to human externalizing symptoms. As previously noted, social isolation has been found to enhance aggression in flies[41]. A study examining genetic variance and gene-environment interactions in aggression in socially isolated and socially experienced individuals

established that significant genetic variation exists in the aggressive response to social isolation[42]. The findings of this study support a polygenic basis of aggression in *Drosophila*, with several of these genes having orthologs previously implicated in mouse aggression and human neurological disorders. The *Drosophila* genes *cac*, *dysf, Fas2, Fas3, ftz-f1, kirre, loco,* and *rst* have orthologs in mice associated with aggression, with *dysf* and *Fas2* also having human orthologs associated with bipolar disorder[43] and suicide attempts in depression and bipolar disorder[44], respectively. Candidate genes identified from the genomic analyses which have been previously implicated in both *Drosophila* aggression and human disorders included *Cad87A, CG42458, Dys, Gug, Ptp99A, Rbfox1, Ten-a,* and *Tl*. The human orthologs of these genes have been associated with bipolar disorder, anxiety, depression, schizophrenia, and Alzheimer's disease. Social isolation has also been linked to hyperactivity in male flies[25], which may be due to histone modifications altering the expression of activity-related transcription factors in dopaminergic neurons[45].

In addition to isolation, short term variable stress, consisting of 24 hours of a random sequence of stressors, has been shown to stimulate hyperactivity in male and female flies which is not abated pharmacologically by fluoxetine or diazepam[35]. Starvation stress also induces hyperactivity in flies, which is thought to facilitate food location and acquisition. This behavior can be attributed to the binding of adipokinetic hormone, the insect analog of glucagon, to its receptor in adipokinetic hormone receptor+ octopaminergic neurons. Silencing these neurons, blocking octopaminergic transmission in these neurons, or binding *Drosophila* insulin-like peptides to the insulin-like receptor either eliminates or suppresses starvation-induced hyperactivity[46].

2.5 Behavioral sex differences in fly models of stress

As noted in the foregoing, sex differences in internalizing and externalizing behaviors may also manifest in *Drosophila* models of stress. It has been previously suggested that *Drosophila* behavioral responses to stress may depend on sex and sexual maturity, and that stress-induced changes in one behavior test may not predict performance in others[47]. One study examining the effects of isolation versus social contact on aging found that lifelong same-sex pairing decreased lifespan more in males than in females[48]. While same-sex pairing in females protected against functional senescence, measured by climbing ability, same-sex pairing produced climbing deficiencies in males. The detrimental effect of same-sex pairing on lifespan doubled in males in combination with wounding. These results suggest that male and female *Drosophila* respond differently to social contact and isolation, and that the adverse effects of social contact on longevity in male-male pairings may be due to the stress of increased competition for mates.

One report that studied sexual dimorphism in *Drosophila* behavioral responses to chronic stress found that stressed females showed more behavioral changes than stressed males, and that the effects on behavior varied based on whether the stress could be categorized as abiotic (mechanical perturbation) or biotic (adult crowding)[34]. Adult crowding produced more sex differences in behavior than mechanical stress in tests of anhedonia, motivation, and locomotion. Following chronic overcrowding, females showed greater anhedonia-like behavior, while males showed less. Females also had a decreased tendency to explore a novel environment, and females' activity declined while males became hyperactive. Different results were obtained when chronic vibration stress was examined. Following chronic vibration stress, both males and females demonstrated greater anhedonic behavior and increased activity. Using this stressor, the

only sex difference was decreased exploratory behavior in the females that was not evident in the males. Hence, flies may respond differently to stress based on whether it is social or non-social in nature, and the multiple behavioral sex differences observed following overcrowding may serve as a model for differentiating female internalizing and male externalizing phenotypes triggered by stress.

Table 2.1. Known stress-induced phenotypes and behavioral effects in *Drosophila melanogaster*.

Form of Stress	Sex	Phenotypes	References
Chronic unpredictable mild stress (heat, cold, starvation, sleep deprivation)	Males	Aggression ↑; depression-like behavior ↑ (forced swim test and light-dark box test); body weight ↓; preference for sucrose ↓	(Araujo et al., 2018)
Social isolation	Males	Sleep ↓; activity ↑; feeding ↑	(Li et al., 2021)
	Males & females	Sleep ↓; daytime activity ↑	(Ganguly-Fitzgerald et al., 2006)
	Males	Aggression ↑	(Wang et al., 2008); (Hoffmann, 1990); (Zwarts et al., 2012)
	Females	Aggression ↑	(Ueda & Kidokoro, 2002)
Adult crowding	Males & females	Longevity ↓	(Joshi & Mueller, 1997)
	Males	Sweet food-seeking ↑; activity ↑	(Lall et al., 2019)
	Females	Sweet food-seeking ↓; activity ↓; exploration ↓	
	Males	Depression-like behavior ↑ (motivation to climb)	(Ries et al., 2017)

Chronic mechanical stress (vibration)	Males	Sweet food-seeking ↓; activity ↑	(Lall et al., 2019)
	Females	Sweet food-seeking ↓; activity ↑; exploration ↓	
Short-term variable stress (social isolation, fasting, heat, electric shock)	Males & females	Preference for sucrose ↓; activity ↑	(Ramos-Hryb et al., 2021)
Starvation	Females	Activity ↑	(Yu et al., 2016)
Lifelong same-sex pairing	Males	Longevity ↓; climbing ability ↓	(Leech et al., 2017)
	Females	Longevity ↓; climbing ability ↑	

2.6 Molecular and neuromodulatory changes in fly models of stress and stress-related behaviors

Well characterized in mammalian systems, neurotransmitters, including the biogenic amines serotonin, dopamine, and octopamine, have been studied extensively as a mechanistic link between stress and behavioral changes in *Drosophila melanogaster*. It has been learned that stress can induce depression-like phenotypes in flies associated with the depletion of serotonin, dopamine, and octopamine, which can be rescued through several approaches with mixed success[14,19,35].

2.6.1 Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), has previously been found to modulate locomotion, sleep and circadian activity, feeding, aggression, anxiety and depressive-like behavior, and learning and memory in *Drosophila*[49]. The literature indicates that treatment with serotonin precursor 5-HTP or activation of 5-HT neurons enhances aggression, while

serotonergic neuronal silencing tends to decrease aggression[50,51,52]. However, the effects of serotonin on aggression may be receptor subtype-specific. Although one report found that activation of the 5-HT1A receptor induces aggression in socially isolated male flies[53], another group found that activating 5-HT1A neurons decreases male aggression[51]. This discrepancy may be reconciled by more recent research, which has identified two distinct 5-HT1A receptor-expressing neuron types with opposing functions in regulating aggression[54]. Activation of the first type, inhibitory GABAergic neurons, results in decreased aggression. Activation of the second type, excitatory cholinergic neurons, increases aggression. It is hypothesized that these two cell types converge in a common sensory integration area, the LC12 optic glomerulus, and that downstream signaling pathways from this brain region may refine the aggression response[54]. The activation of specific receptor subtypes may also produce different forms of aggressive behaviors. For example, activation of 5-HT2 receptors has been associated with lunging and boxing, but manipulation of the 5-HT1A receptor alters wing threats and fencing[53].

2.6.2 Dopamine

Additionally, there is substantial evidence that dopamine signaling in *Drosophila* is associated with behavioral and physiological indicators of stress. Dopaminergic transmission increases heart rate[55], modulates arousal[56], and increases with heat stress[57]. Dopamine has been found to play a role in female sexual receptivity[58] and to modulate the effects of drugs of abuse, including cocaine, nicotine, and ethanol[58,59], in *Drosophila*. Stressors such as starvation, oxidative stress, and mechanical stress have been found to disrupt female sexual receptivity and ovarian development, potentially through alterations in tyrosine hydroxylase activity, which plays a critical role in dopamine biosynthesis[11]. It has also been suggested that social space among flies may be modulated by dopaminergic signaling[60], and flies which have previously

been socially isolated demonstrate reductions in physical distance to other flies[61]. Lastly, differential sensitivity of dopaminergic receptors or differences in receptor subtype expression may account for sexual dimorphism in behaviors, such as locomotion[62].

2.6.3 Octopamine

Octopamine, the structural analog of norepinephrine in insects, has been implicated in *Drosophila* aggression. Silencing of octopaminergic neurons or genetic knockdown approaches have generally yielded decreases in male aggressive behavior[63, 64, 65,] while activation of octopaminergic neurons generates male aggression[64,66]. One research group was able to induce a depression-like state in flies, characterized by reduced climbing motivation, using a battery of chronic stressors, including vibration, overcrowding, and food deprivation. It was found that the depression-like state could be relieved through consumption of 5-HTP, fluoxetine, or sucrose, all of which increase serotonin in the brain[67]. A sugar-induced restoration of climbing motivation was associated with octopaminergic signaling from the ventral unpaired medial neurons in the subesophageal ganglion to dopaminergic protocerebral anterior medial (PAM) neurons. The dopaminergic PAM neurons and serotonergic dorsal paired medial neurons both synapse with cells in the mushroom bodies to modulate and relieve the stress-induced depression state. These findings reiterate the important ways in which monoaminergic signaling systems work together in behavioral regulation, and how disruption of these systems by stress dysregulates behavior.

2.6.4 Drosulfakinin (Dsk)

Neuropeptides are another subset of neuromodulators that have been found to play a role in behavior change driven by social stress. Cholecystokinin (CCK) is a mammalian gut hormone that also functions as a neurotransmitter. CCK+ neurons regulate aggression, anxiety, and social defeat responses in rodents[68, 69, 70, 71], and CCK-4 has been shown to induce panic attacks in

humans[72, 73]. Drosulfakinin (Dsk), the fly ortholog of CCK, has been implicated in regulating gut function, feeding and satiety, hyperactivity, aggression, locomotor behavior, and synaptic plasticity during the development of the neuromuscular junction[74]. The *Drosophila* genome encodes two distinct receptors to which Dsk binds: CCKLR1 (CCKLR-17D1) and CCKLR2 (CCKLR-17D3). Both *Dsk* and *CCKLR-17D1* mutants show deficits in larval stress-induced escape behavior[75], and knockdown of these genes in the *pars intercerebralis* in Dsk-GAL4 or Dilp2-GAL4 adults increases aggression in socially isolated males[30]. Generally, social isolation tends to increase aggression in *Drosophila* towards conspecifics, and another group has found that chronic social isolation decreases *Dsk* expression, while decreasing sleep and increasing feeding behavior[25]. Although there is abundant evidence that the Dsk signaling pathway may play a role in stress-related phenotypes in *Drosophila*, little is known about whether chronic stress induces sexual dimorphism at the transcriptomic level in this pathway, and if that generates differences in behaviors such as activity, feeding, or aggression.

2.6.5 Neuropeptide F (NPF)

Neuropeptide F (NPF), the fly homolog of mammalian neuropeptide Y (NPY), has also been linked to essential behaviors which are impacted by stress. In mammals, NPY is involved in the regulation of diverse biological functions, including energy balance, circadian rhythms, feeding, reproduction, anxiety, learning and memory, and alcohol dependence[76]. In *Drosophila*, NPF regulates feeding, wakefulness, metabolism, and reproduction[77, 78]. A related class, short NPFs, which belong to a separate invertebrate neuropeptide family from NPFs but bind to NPY-like receptors, have been associated with feeding and growth, stress response, movement, olfaction, hormone release, and learning and memory[77]. One study has found that inhibitory signaling from NPF+ neurons to dopaminergic neurons confers resilience to chronic stress-

induced learning deficits through the maintenance of normal autophagic flux[79]. Silencing NPF+ cells has also been shown to increase aggression in socially isolated males[50] and decrease courtship behavior in group-housed controls[80].

2.6.6 Corazonin (Crz)

Finally, corazonin (Crz) is a lesser studied but highly conserved neuropeptide in insects that may contribute to sexually dimorphic neural circuitry and behavioral responses to stress. *Drosophila* cells expressing *Crz* have receptors for two diuretic hormones, DH44 and DH31[81], which are related to the mammalian stress hormones corticotropin releasing factor and calcitonin gene-related peptide[82, 83]. It has been suggested that Crz plays important functions in development and survival by regulating growth, feeding, mating behavior, and ethanol sensitivity[84]. Although Crz is expressed in dorsomedial, dorsolateral, and ventral nerve cord neurons in the fly nervous system, there is a small population of four neurons in the abdominal ganglion which are present only in males and are involved in ejaculation and copulation duration[85]. A study examining the role of *Crz*-expressing neurons in stress sensitivity and stress-related behavior identified sex differences in lifespan following starvation, osmotic, or oxidative stress. Activation of Crz+ neurons using NaChBac channel expression decreased lifespan only in males, even though neural silencing with open rectifier K+ channel expression improved lifespan in *both* sexes under conditions of starvation or oxidative stress[86]. This group also found male-specific effects of Crz+ neuron activation and silencing on motor activity, with silencing resulting in male hyperactivity and activation resulting in male hypoactivity relative to wild type flies. Starvation and osmotic stress decreased transcript expression of *Crz* more in males compared to females, and males with silenced Crz+ neurons showed elevations in dopamine in the hemolymph relative to parental genotypes. Activation and silencing of corozonin neurons did

not alter dopamine levels compared to parental genotypes in females[86]. Taken together, the results of these experiments suggest that corozonin neurons may receive sex-specific inputs or may directly or indirectly signal to sex-specific downstream targets to produce sexual dimorphisms in stress phenotypes. To date, no studies have explored the effects of social isolation on corozonin neural signaling and behavior.

2.7 Isolation and molecular and cellular sex differences

Overall, the studies outlined below offer many potential genetic targets for exploring male- versus female-specific cell types and circuits underlying stress-induced and social context-dependent sex differences in behaviors (Table 2.2). In the context of social isolation, a recent review[87] summarized extensive research on the genes, neuromodulators, cell types, and behaviors that are differentially altered by single versus group housing conditions, with courtship, sleep, and aggression being the most widely studied behaviors. In addition to *Dsk* downregulation[30], social isolation-induced aggression has also been linked to upregulation of *Obp69a* and downregulation of *Cyp6a20*[88]. Interestingly, *Obp69a*, a gene encoding an odorant binding protein, and *Cyp6a20*, a gene encoding a cytochrome P450, are expressed in a sexually dimorphic manner. *Obp69a* is naturally overexpressed in males relative to females and increases only in males following single housing. In females, expression of *Obp69a* does not seem to be affected by single or group housing with same-sex conspecifics, but it increases when they are group-housed with males. Expression of *Cyp6a20* does not vary based on social environment in females but is significantly reduced in males following single housing[88]. The authors believe that the inverse regulation of *Obp69a* by social environment in males versus females may promote mating, by decreasing aggression and increasing aggregation in groups for males and enhancing sexual receptivity for females.

NPF, which is altered by stress and associated with stress-induced behavior change, is regulated in both a sex-specific and sex-nonspecific manner[76]. Male-specific *NPF* expression is regulated by fruitless (fru), a transcription factor which plays a critical role in the sex determination hierarchy in the fly central nervous system and is required for normal male courtship. *NPF* is also regulated by clock factors in a subset of male dorsolateral neurons, suggesting that this gene may play a role in sexually dimorphic courtship behavior which is also circadian[76]. Since NPF+ neuronal silencing increases aggression in socially isolated males and decreases courtship in group-housed males, it would be interesting to explore whether social isolation impacts *NPF* gene expression in a sex-specific way (e.g. decreases *NPF* expression in males), and, if so, whether *NPF* expression changes in this particular population of neurons. P2 neurons expressing *NPF* have also been identified as the cells through which chronic social isolation reduces sleep and increases feeding, but this study only tested male flies[25].

In addition to NPF, tachykinin (Tk) is a neuropeptide which may play sex-specific roles in stress-related behaviors. Substance P, a member of the Tk family, has been linked to the initiation of aggressive behavior in mammals[89] and may perform a similar function in insects. A class of neurons in the male fly brain that express the male-specific form of *fru* and release Drosophila Tk have been found to generate male-specific aggression[90]. A recent report has also found that in male flies, social enrichment (relative to social isolation) upregulates *Diuretic Hormone 44* (*DH44*) and downregulates *Tk* to decrease spontaneous locomotor activity[91]. These authors also uncovered a neural circuit in which male-specific P1 neurons stimulate movement through positive feedback with interneurons expressing both *doublesex* (*dsx*) and *Tk* and inhibit movement with negative feedback from interneurons co-expressing *dsx* and *DH44*[91]. This neural circuit could be integral to understanding how changes to the social environment produce

sexually dimorphic changes in innate behaviors. Taken together, the results of these studies allude to male-specific neural substrates in flies for two externalizing behaviors, aggression and hyperactivity.

Interestingly, the feminization of cholinergic neurons in male flies via expression of *transformer* (*tra*) also increases aggression[92]. *Tra* is an important gene in the sex determination hierarchy that is only functional in females. It has been found that a small number of cholinergic cells in female fly brains, pC1 dsx+ neurons, are required for female hyperaggression[93]. Although females are typically less aggressive than males at baseline[94], they will become more aggressive, similarly to males, following social isolation[95]. Further research is needed to determine if social isolation induces female-specific aggression by altering gene expression in such a way that makes pC1 dsx+ or other cholinergic neurons more active.

Table 2.2. Genes implicated in social experience-dependent and stress-induced phenotypic changes.

Gene	Manipulation	Sex	Social Experience	Phenotypes	References
Dsk	Genetic knockdown	Males	Social Isolation	Aggression ↑	(Agrawal et al., 2020)
	Chronic social isolation ↓ expression	Males	Social Isolation	Sleep ↓, feeding ↑	(Li et al., 2021)
Obp69a	Social isolation ↑ expression	Males	Social Isolation	Aggression ↑	(Bentzur et al., 2018)
	Group housing with males, but not females, ↑ expression	Females	Group Housing	Sexual receptivity ↑	
Cyp6a20	Social enrichment ↑ expression	Males	Group Housing	May contribute to aggression ↓ in combination with	(Bentzur et al., 2018)

	(relative to isolation)			*Obp69a* expression ↓	
NPF	Neural silencing	Males	Social Isolation	Aggression ↑	(Dierick & Greenspan, 2007)
	Neural silencing	Males	Group Housing	Courtship ↓	(Lee et al., 2006)
Tk	Social enrichment ↓ expression (relative to isolation)	Males	Group Housing	Spontaneous locomotion ↓	(Zhao et al., 2024)
DH44	Social enrichment ↑ expression (relative to isolation)	Males	Group Housing	Spontaneous locomotion ↓	(Zhao et al., 2024)
Crz	Neural activation	Males	Group Housing	Lifespan ↓ under starvation, osmotic, or oxidative stress; activity ↓; no change in dopamine in hemolymph	(Zhao et al., 2010)
		Females	Group Housing	Lifespan ↑ under starvation or oxidative stress; no change in activity; no change in dopamine in hemolymph	
	Neural silencing	Males	Group Housing	Lifespan ↑ under starvation, osmotic, or oxidative stress; activity ↑; dopamine in hemolymph ↑	
		Females	Group Housing	Lifespan ↑ under starvation or oxidative stress; no change in activity; no change in	

				dopamine in hemolymph	

2.8 Crowding and molecular and cellular changes

In *Drosophila melanogaster*, there has been more research conducted on larval than adult crowding. Larval crowding has been associated with transcriptomic changes in adulthood, including the upregulation of genes relating to sugar, steroid, and amino acid metabolism and DNA replication and downregulation of genes involved in immune signaling. Downregulation was also observed in gene classes relating to excretion and recycling pathways, namely taurine metabolism and lysosome and ABC transporters[96]. Overall, high density living early in life may remodel metabolic pathways in flies to cope with a limited-resource environment and build tolerance to toxic compounds.

However, one study has suggested that the stress resistance built in adulthood by larval crowding may not be the same in both sexes. During adult heat stress, it was found that after multiple generations of larval crowding, males but not females improved their heat shock tolerance[97]. Crowding increases expression of the heat shock protein *Hsp70* in larvae, but *Hsp70* expression in adulthood did not account for the observed sexual dimorphism in heat shock tolerance, as there were no *Hsp70* expression differences in the adult populations. Another study of larval crowding in the fruit fly *Bactrocera tryoni* found that high density early living decreased emergence, body weight, energetic reserves, flight ability, and fertility and increased feeding in adulthood[98]. Although flies of both sexes from the larval crowding treatment increased their total food consumption (relative to body weight), males ate more yeast, while females ate more sucrose compared to their respective uncrowded controls. Further research is needed to

uncover the molecular mechanisms by which larval crowding induces sex differences in stress sensitivity and nutrient preference in adulthood.

In adult flies, one study discussed previously in this review[79] used adult crowding stress in combination with mechanical shock to examine the molecular and neuronal mechanisms underlying chronic stress-induced learning deficits, a common behavioral symptom of depression in humans and other mammals. The researchers found that 4 days of crowding and mechanical shock treatment induced learning deficits associated with disruption of autophagic degradation in neurons. RNA-seq differential expression analysis from fly heads collected after 96 hours of stress treatment revealed changes in gene expression in the autophagy-lysosome pathway. This is an evolutionarily conserved stress pathway[99] that functions to maintain cellular homeostasis through the degradation of abnormal intracellular macromolecules and has been implicated in both neurodegenerative diseases[100] and depression[101, 102]. Activation of NPF+ cells during chronic stress protects against learning deficits by inhibiting dopaminergic neurons and thus maintaining autophagic flux[79]. Another paper studying adult restraint stress, which bears some similarity to overcrowding in that the flies were restricted in physical space in a group, found that restraint of fly fathers induced gene expression changes in the offspring[32]. It was found that paternal restraint disrupted heterochromatin, upregulated the expression of genes involved in one-carbon metabolism, and downregulated the expression of genes involved in glycolysis and the tricarboxylic acid (TCA) cycle in F1 males. These authors identified a novel pathway by which paternal restraint stress reduces energy metabolism activity and produces epigenetic changes in male offspring to transmit stress information from somatic to germline cells. Overall, adult crowding or restraint stress seems to alter gene expression in *Drosophila* in a manner that is behaviorally maladaptive and detrimental to survival. However, the extent to

which adult crowding or restraint stress produces behavioral sex differences in *Drosophila* remains fairly unknown, as well as the underlying molecular mechanisms if such sexual dimorphism exists.

2.9 Conclusion

Drosophila melanogaster has long served as a useful model system for identifying conserved molecular pathways underlying fundamental behaviors and diseases. The genetic toolkit combined with established methods for stressing flies and quantifying internalizing-like and externalizing-like behaviors make *Drosophila* an attractive organism for exploring the molecular basis of sex differences in stress-induced behavior change. It is possible that evolutionarily conserved molecular mechanisms could play a role in human psychiatric and behavioral disorders that are precipitated by stress and manifest differently in males and females. Combined with knowledge from rodent models, increased understanding of these relationships could inform novel therapeutic interventions for stress-induced mental disorders and emphasize the need for equal representation of both sexes in preclinical research.

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Chapter 3

Behavioral sex differences in the fly response to social stress

This work was presented as a poster at the* Princeton University Department of Molecular Biology annual retreat (2021, 2022, and 2023), *as well as at* Society for Neuroscience (2023). *It was presented as a talk at the* Princeton University Department of Molecular Biology annual retreat (2024). *Katherine Cho and Daniel Notterman contributed to work described in this chapter.

Drosophila melanogaster provides a model system to elucidate how environmental stress interacts with sex to induce changes in behavior and gene expression. Previous research suggests that social isolation induces changes in gene expression that encode a starvation-like brain state and reduce sleep[1]. However, the extent to which behaviors are differently altered by isolation and crowding in male and female flies is unclear. The overall goal of this chapter was to explore sex differences in behavior that arise in response to social stress in *Drosophila*. Male and female adult flies were exposed to chronic stress through either social isolation or crowding, and multiple behavioral sex differences were identified through tests of activity, motivation, aggression, and sugar consumption. Our findings suggest that social isolation is sufficiently stressful to produce behavioral changes and sex differences, despite having no impact on survival, whereas overcrowding decreases survival in both sexes but induces fewer alterations in behavior and behavioral sex differences.

3.1 Introduction

Social isolation has emerged in recent decades as a worldwide public health issue. The World Health Organization estimates that one in four older adults and five to fifteen percent of

adolescents experience social isolation and loneliness globally[2], while the Centers for Disease Control and Prevention estimates that one in three adults experience these problems in the United States[3]. In humans, isolation increases the risk of early death and is associated with several diseases and psychiatric conditions, including cardiovascular disease, stroke, type 2 diabetes, depression, anxiety, suicidality, and dementia[2,3]. Social isolation has also been found to enhance aggression, depression, and anxiety in humans undergoing solitary confinement[4,5].

Common social stressors across animal species include maternal separation, social deprivation, reduced parental care, and aggression from caregivers early in life[6]. In adulthood, social stressors manifest as competition for mates and territory, within-group competition for resources and social rank, and between-group conflict[6]. Individuals within a social group may benefit the most in terms of fitness by living in a group of intermediate size. This provides safety in numbers and allows for engagement in cooperative behaviors. If the social group is too large or dense, overcrowding occurs, resulting in increased competition for mates and resources, aggression, parasite transmission, and sometimes cannibalism[7]. If the individual is isolated, it poses a challenge to finding mates and resources and induces a chronic stress response[7]. Using animal models, the molecular, cellular, and behavioral effects of social isolation can be studied by separating animals of social species from their social group. This has been done using rodents[8,9], bees[10], ducklings[11], zebrafish[12], and fruit flies[13,14, 15–18]. Although *Drosophila* are not eusocial insects, they tend to aggregate and can adapt their behavior and physiology to other flies to function as part of a group[7]. In *Drosophila melanogaster*, chronic social isolation has been shown to reduce sleep, increase feeding, and contribute to a transcriptomic profile indicative of a starvation-like brain state[1]. However, the cited study only examined the effects of chronic social isolation on activity and feeding in males.

In this chapter, our goal was to develop a battery of behavioral tests and other phenotypes that could be used to assess sex differences in the fly response to social environmental stress. The flies underwent social isolation or overcrowding, representing opposite poles along the spectrum of social environment. Overall, our aim was to establish *Drosophila* as a valid non-mammalian animal model for studying the neuro-molecular mechanisms of social stress-induced changes in behavior. If we can identify multiple translationally relevant behaviors in flies that change in response to isolation or crowding, and change differentially between males and females, we can then harness them as a model system to explore the molecular and physiological bases of sex differences in stress-related neurodegenerative and psychiatric disorders.

3.2 Results

3.2.1 Locomotion increases to a greater extent in males compared to females following social isolation or overcrowding

To characterize social isolation and overcrowding as valid fly stress models, we began by performing a series of behavioral tests that could capture changes of translational relevance. We first recorded fly walking behavior in a 55mm diameter arena and used EthoVision tracking software (Noldus Information Technology Inc., Wageningen, the Netherlands) to quantify distance traveled in a 10-minute period to see if the social stress paradigms induced hypo- or hyperactivity, and if the behavioral effect differed by sex. We found that social isolation increased distance traveled in both males (Figure 3.1A, $p < 0.0001$) and females (Figure 3.1A, $p < 0.0001$), but that the magnitude of the change was significantly greater in males (Figure 3.1B, $p < 0.0001$). Overcrowding only increased distance traveled in males (Figure 3.1C, $p < 0.0001$), and the difference in distance traveled between crowded flies and their respective controls was significantly greater in males compared to females (Figure 3.1D, $p < 0.0001$).

3.2.2 Social isolation impacts motivation to seek food (yeast) differentially in males versus females

Anhedonia, defined as a reduced ability to experience pleasure, has been previously conceptualized as an endophenotype of depression indicative of abnormal reward processing[19]. To determine if social isolation or crowding stress induces an anhedonia-like phenotype in flies, we used two different stimuli, yeast paste and apple cider vinegar, to measure flies' interest and motivation to explore a physical space containing an attractive food. Yeast volatiles, either alone or as byproducts of fermenting fruit, attract *Drosophila*[20–23]. Apple cider vinegar, through its high acetic acid content, mimics the odor of rotting fruit, which attracts flies as a source of food and mates. We developed a novel behavior test, inspired by a sucrose preference test[24], to assess whether social stress alters the motivation of flies to investigate an attractive food or food odorant, and if so, whether sex differences are evident.

In our set up (Figure 3.2A), we placed briefly anesthetized flies along the midline of a petri dish that was filled with agarose and topped with a piece of parafilm and filter paper, to limit the flies' space and encourage exploration. We recorded the flies for 60 minutes and calculated a preference index (Figure 3.2B) at 5-minute intervals, to get a sense of the flies' "preference" over time between a side that had yeast or fermenting fruit odor (apple cider vinegar) and a side that contained nothing.

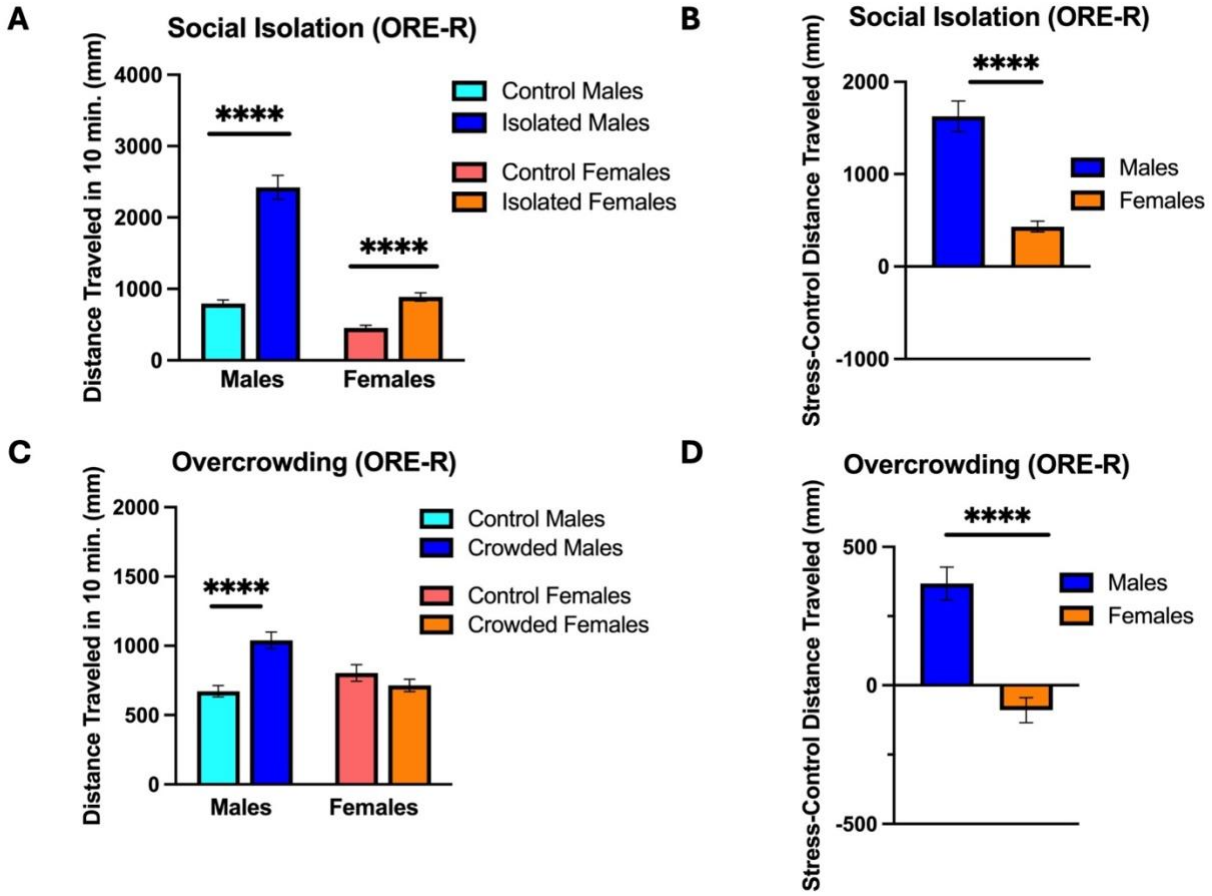

Figure 3.1. Motor activity increases following social isolation and crowding stress to a greater extent in males compared to females. A) Flies that experienced social isolation (n = 99 individuals) traveled greater distances in a 10-minute period than controls (n = 100 individuals) for both males ($U = 1729$, $p < 0.0001$) and females ($U = 2808$, $p < 0.0001$). **B)** Taking differences in distance traveled between socially isolated flies and the control average for each sex revealed that the increase in locomotion in isolated males relative to control males was significantly greater (n = 99, $U = 2850$, $p < 0.0001$) than that observed between isolated females and control females (n = 99). **C)** Distance traveled in 10 minutes only increased significantly in males after crowding stress (n = 100 crowded males, n = 102 control males, $U = 3224$, $p < 0.0001$). No difference was observed between control (n = 100) and crowded (n = 100) females. **D**) Taking differences in distance traveled between crowded flies and the control average for each sex further corroborated that crowding altered the motor behavior of males (n = 100), relative to their controls (n = 100), more than that of females (n = 100 in each group, $U = 2825$, $p < 0.0001$). Error bars are ± SEM. ****$p < 0.0001$.

The preference index (PI) was calculated such that a value of 0 indicated no discrimination between the food side versus non-food side. Using a repeated measures two-way ANOVA, we found that social isolation reduced females' preference for yeast (Figure 3.2C, F(1,

19) = 8.65, p = 0.0084) such that it eliminated the difference between males and females observed in the control groups, wherein females' preference had exceeded that of males (Figure 3.2C, F(1, 18) = 18.43, p = 0.0004). Upon subtracting the control group average from individual PI values of socially isolated males and females at each time point, we found a significant difference between males and females in how each stress group deviated from its control group (Figure 3.2D, F(1, 22) = 46.08, p < 0.0001). That is, following social isolation, females demonstrated a reduced preference for the yeast side of the arena relative to control females, while isolated males showed an increased preference for the yeast side compared to control males. Using apple cider vinegar as an attractive odorant, we observed a significant difference in preference index between control males and control females (Figure 3.2E, F(1, 14) = 14.55, p = 0.0019) but did not see an effect of social isolation in either sex. For apple cider vinegar, both sexes were more attracted to the odorant side of the arena following social isolation relative to controls, yielding no sex difference (Figure 3.2F).

For the overcrowding paradigm, we found that PI for yeast paste was heightened in control females relative to control males (Figure 3.3A, F(1, 18) = 6.19, p = 0.02), and that this effect of sex was maintained with crowding experience (F(1, 18) = 13.55, p = 0.002); thus, there was no effect of crowding on PI in either sex. There was also no difference between males and females in how crowded flies deviated from controls (Figure 3.3B). Using apple cider vinegar, we did not see any significant effects of crowding or sex on preference (Figure 3.3C), nor was there a difference in how crowded males' and females' preferences for apple cider vinegar changed relative to controls' (Figure 3.3D). However, despite being non-significant (F(1, 16) = 3.83, p = 0.07), crowding seemed to decrease females' interest in the apple cider vinegar side, while crowding had little to no effect on apple cider vinegar PI in males.

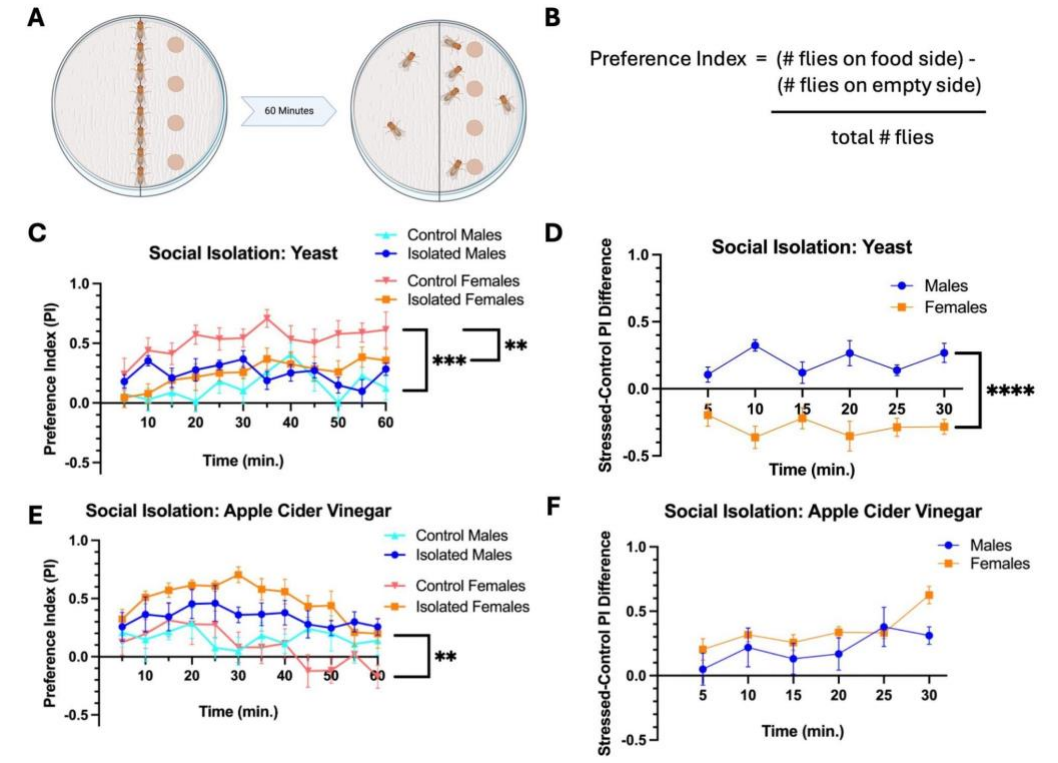

Figure 3.2. Social isolation differentially impacts males' and females' motivation to explore an area containing an attractive food stimulus. A) Set up: flies were briefly anesthetized, positioned along the midline of the arena, and recorded during 60 minutes of exploration time. One half of the arena contained four dots of yeast paste or apple cider vinegar, and the other half had no food. Created with BioRender.com. **B)** A preference index (PI) was calculated at five-minute intervals, where PI = (the number of flies on the food side of the arena) - (the number of flies on the empty side of arena) / the total number of flies. A value of 0 can be interpreted as at chance, due to equal numbers of flies being on each side. **C)** Isolated females (n = 12 groups) are less attracted to yeast paste relative to control females (n = 12 groups, $F(1, 19) = 8.65$, $p = 0.0084$). The baseline difference between control males (n=12 groups) and control females ($F(1, 18) = 18.43$, $p = 0.0004$) is abrogated upon social isolation. **D)** When comparing the differences in preferences between isolated and control flies, isolated male flies (n = 13 groups) are relatively more attracted to yeast, whereas isolated females are relatively less attracted (n = 11 groups, $F(1, 22) = 46.08$, $p < 0.0001$). **E)** Isolated females (n = 12 groups) are more attracted to apple cider vinegar relative to control females (n = 12 groups, $F(1, 14) = 14.55$, $p = 0.0019$). **F)** However, trends do not differ significantly between males (n = 8 groups) and females (n = 8 groups) when examining differences in apple cider vinegar preference between isolated and control flies. Error bars are ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

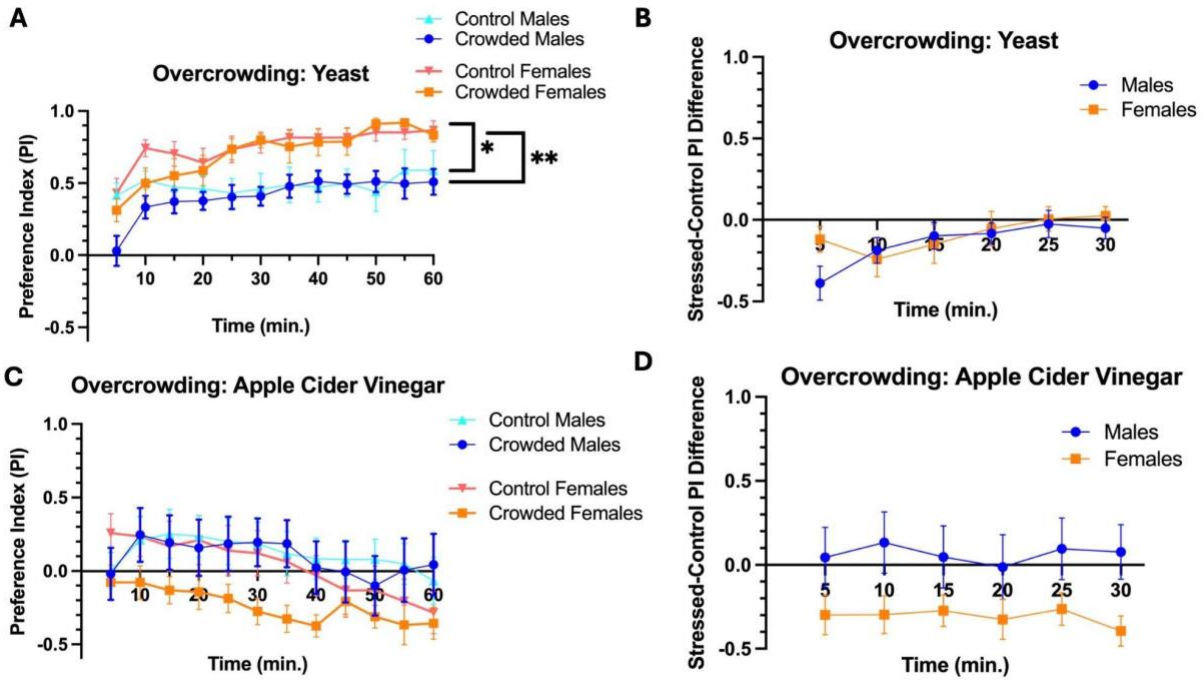

Figure 3.3. Flies' attraction to yeast paste is greater at baseline for females relative to males and does not change with crowding stress. A) For both control (n = 12 groups, $F(1, 18) = 6.19$, $p = 0.02$) and crowded flies (n = 12 groups, $F(1, 18) = 13.55$, $p = 0.002$), females demonstrated a heightened preference for yeast relative to males. **B)** The differences in yeast paste preference between crowded and control flies for males (n = 10 groups) versus females (n = 10 groups) were non-significant. **C)** No meaningful differences in preference for apple cider vinegar were detected between experimental groups (n = 12 groups per condition). **D)** While crowded females seemed overall less attracted to apple cider vinegar relative to controls, the differences in preference between crowded and control flies for males (n = 9 groups) versus females (n = 9 groups) were non-significant ($F(1, 16) = 3.83$, $p = 0.07$). Error bars are ± SEM. *$p < 0.05$, **$p < 0.01$.

3.2.3 Social isolation increases aggression in males and females across different measures

After testing flies for hyperactivity and anhedonia-like behavior, we next decided to assess aggression, a behavior that has been previously associated with social isolation in fly models[13,18,25]. We used a set up (Figure 3.4A) similar to one used by other research groups studying *Drosophila* aggression[26]: microtube caps were filled with standard fly food, topped with yeast paste, and placed in a 12-well plate, such that each well contained a single cap. This was

used to create an arena, where flies would encounter each other and begin fighting for territory and food. We defined aggressive "hits" in male flies as the lunge, a behavior in which the aggressor rears up on his hind legs and quickly snaps down on his opponent[26]. Aggressive "hits" in females were defined as the head butt, a movement in which the aggressor produces an abdominal thrusting-type motion and strikes the opponent with her head[27]. Same-sex pairs of flies were aspirated into a well and recorded for 60 minutes. We noted the time at which the flies first encountered each other on the food cup to measure *latency to first hit* from that initial encounter. A shorter latency to first hit indicates a higher level of aggression[28]. Once the first hit occurred, we counted the number of hits and any social interactions that did not result in a hit for the next 10 minutes. We found that social isolation increased the number of hits in males ($p < 0.05$) but not females ($p = 0.87$) (Figure 3.4B). Social isolation also decreased latency to first hit in males ($p < 0.03$) but not females (0.57), relative to the respective control groups (Figure 3.4C). Interestingly, females at baseline may have a lower latency to first hit (i.e., "greater aggression") compared to males. This is reflected in the significant difference in latency to first hit between male controls and female controls ($p = 0.002$). It is possible that we did not observe a difference between socially isolated females and control females in latency to first hit because the latency in control females is so short to begin with, creating a floor effect. Isolated males also showed a greater number of total social interactions (both aggressive and non-aggressive encounters) compared to controls ($p = 0.002$) which was not evident in females (Figure 3.4D). Finally, when examining aggressive hits as a proportion of all social interactions (Figure 3.4E), there is a difference between isolated females and their controls, such that hits make up a significantly greater proportion of social behaviors in isolated females ($p = 0.002$). This difference is not seen between isolated males and control males. Both control males ($p = 0.0006$) and isolated males (p

< 0.0001) showed a higher hit proportion compared to control females, but isolated females' increase in hit proportion rose to the extent that there was no difference between isolated females and isolated males. Thus, the increase in number of hits observed in isolated males may be a product of the fact that isolated males are also interacting more overall; there does not seem to be an increase in the frequency of hitting relative to other social behaviors. Isolated females' increased proportion of hits out of total social interactions in combination with females' overall short latency to hit may be indicative of enhanced female territoriality.

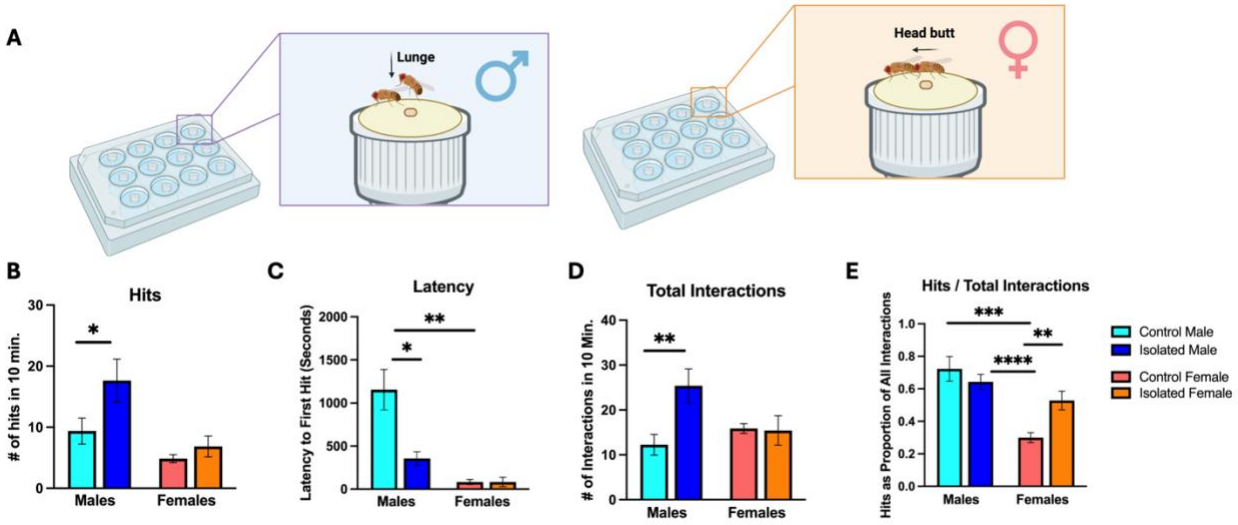

Figure 3.4. Males and females show increases in different measures of aggression following social isolation. A) Set up: caps filled with standard fly food and topped with yeast paste were placed in a 12-well plate. Same-sex pairs of flies from the same experimental condition were aspirated into each well and recorded for 60 minutes. Male "hits" were defined as lunges and female "hits" were defined as head butts. Created with BioRender.com. **B)** Socially isolated males (n = 16 pairs) hit more compared to group housed controls (n = 16 pairs, U = 76, p < 0.05). There was no difference in the number of hits between isolated (n = 15 pairs) and control females (n = 15 pairs, U = 101, p = 0.87). **C)** The latency between the flies' first encounter on the food cup and the first hit was lower in socially isolated males relative to controls (U = 71, p < 0.03). Female controls had a significantly shorter latency to first hit compared to male controls (U = 40, p = 0.002) that did not differ from that of isolated females (U = 79, p = 0.57). **D)** Isolated males had a greater number of social interactions, aggressive or non-aggressive, than control males (U = 49.5, p = 0.002) in the 10 minutes following the first hit. **E)** Measuring the proportion of all social interactions that were hits, both control (U = 37, p = 0.0006) and isolated males (U = 14, p < 0.0001) had hits as a greater proportion of all social interactions relative to control females. However, social isolation significantly raised this proportion in females (U = 39.5, p = 0.002) to the levels of males. Error bars are ± SEM. **P* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

Following crowding stress, male flies (n = 14 pairs) did not demonstrate changes in any measures relative to male controls (n = 15 pairs), but females showed decreases in number of hits (Figure 3.5A, n = 15 pairs per group, p = 0.0006) and number of social interactions (Figure 3.5C, p = 0.001). Neither males nor females showed changes in latency to first hit (Figure 3.5B) or hits as a proportion of all social interactions (Figure 3.5D) following overcrowding. These data suggest that females may hit fewer times following overcrowding because they are also having fewer social interactions with each other in general.

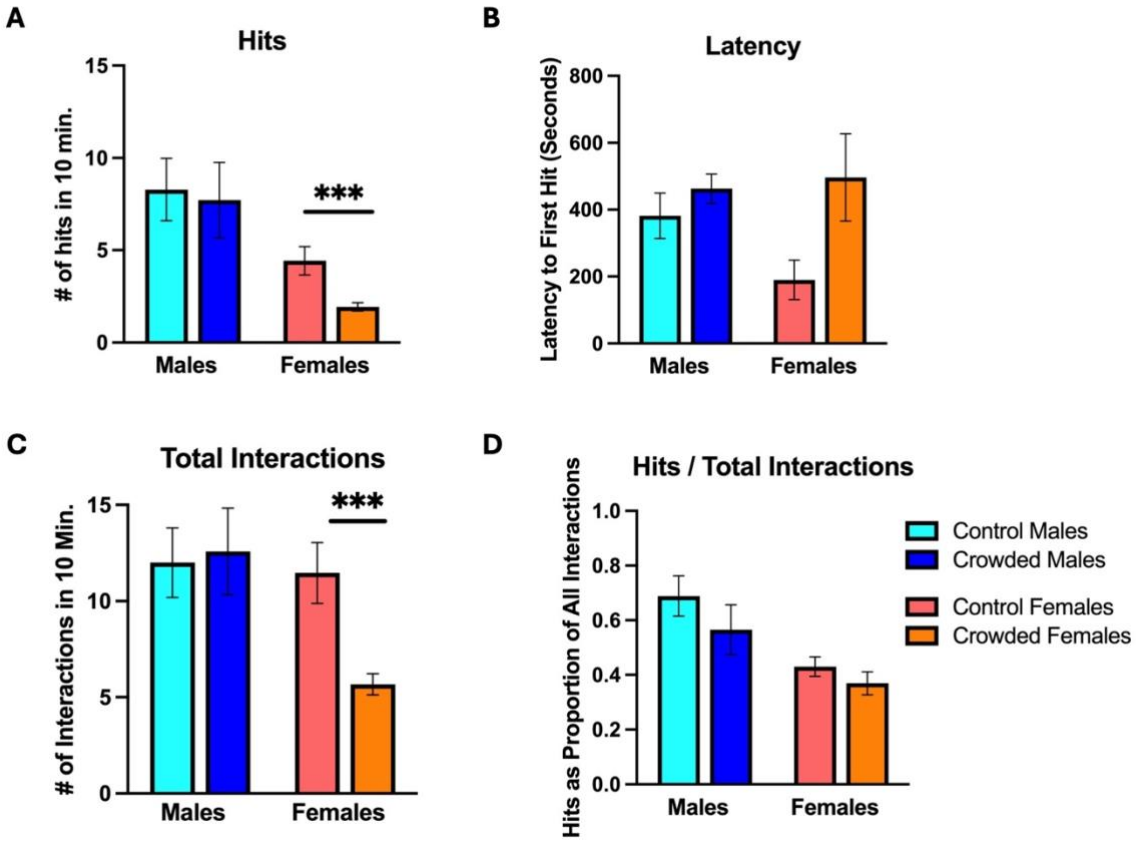

Figure 3.5. Overcrowding decreases the number of hits and interactions between females. **A)** Females that experienced crowding stress (n = 15 pairs) hit fewer times compared to controls (n = 15 pairs, U = 31.5, p = 0.0006) in the first 10 minutes following the first strike. **B)** Crowding did not affect the latency to the first hit from the first encounter on the food cup in either sex. **C)** Crowded females, relative to controls, had fewer overall social interactions in the first 10 minutes following the first strike (U = 36.5, p = 0.001). **D)** Crowding did not change the proportion of total social interactions that were hits in either sex. Error bars are ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

3.2.4 Social isolation enhances sucrose feeding in females

The Value-Based Feeding Decision (VBFD) test was the final behavioral test that we employed to assess whether our social isolation and overcrowding paradigms were sufficiently stressful and repeatable in flies to generate behavioral changes. We followed an existing protocol[29], in which flies were placed in an arena for two hours to feed freely on either a blue solution of sucrose, a more nutritious sugar, or a red solution of arabinose, a less nutritious sugar (Figure 3.6A). The developers of this behavioral test argue that the results of the VBFD indicate the flies' cognitive status, as feeding on the more nutritious food source necessitates learning and decision-making, and this discrimination ability declines with age and in models of neurodegenerative disease[29]. We decided to interpret the results of our test at face value, as a readout of sugar consumption, to complement the previously described tests of motivated behavior. We used the color (blue, red, purple, or none) of the crop, the fly equivalent of the mammalian stomach, to sort the flies into categories of which sugar they primarily consumed (Figure 3.6B). We performed additional testing to confirm that the flies did not have a color preference between blue and red.

Following social isolation, female flies significantly increased in the proportion of flies consuming sucrose (Figure 3.6C, $t(15) = 3.14$, $p = 0.007$). No differences were observed in feeding between control males and isolated males. Focusing on sucrose, the nutritious sugar used in this test, we observed that both control males (Figure 3.6D, $t(22) = 2.85$, $p = 0.009$) and isolated males (Figure 3.6D, $t(19) = 2.67$, $p = 0.02$) had a greater proportion of flies consuming sucrose relative to control females, but that these differences were erased when comparing males to socially isolated females. Thus, isolation makes females more closely resemble males regarding sucrose feeding.

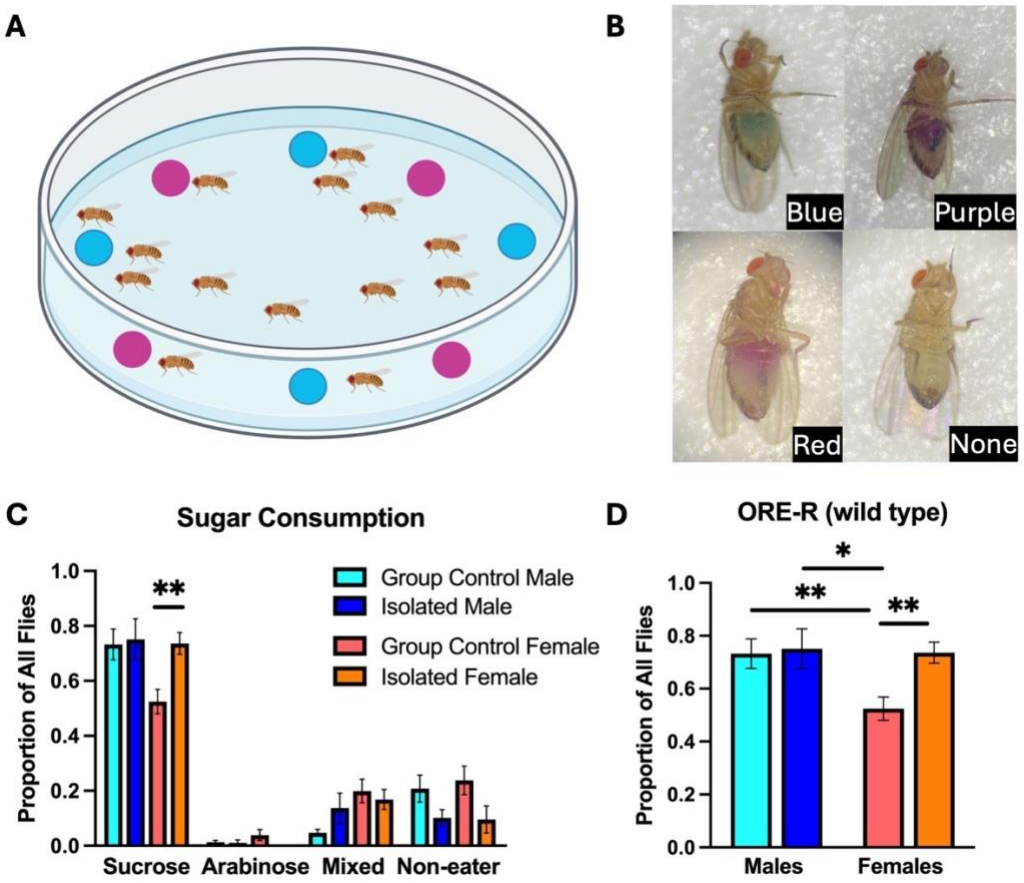

Figure 3.6. Social isolation increases sucrose consumption in ORE-R females. A) Set up: flies were briefly anesthetized and allowed to explore an agarose plate with 2 different sugar solutions for 2 hours. Sucrose (150mM) was dyed blue, and arabinose (150mM) was dyed red. Created with BioRender.com. **B)** Flies were identified as either blue, red, purple, or none and counted at the end of each 2-hour period. **C)** Social isolation increased sucrose feeding in females ($t(15) = 3.14$, $p = 0.007$). **D)** Control (n = 13 groups, $t(22) = 2.85$, $p = 0.009$) and isolated (n = 10 groups, $t(19) = 2.67$, $p = 0.02$) males both consume more sucrose than control females (n = 11 groups), but there are no significant differences between these groups and isolated females. Error bars are ± SEM. *$p < 0.05$, **$p < 0.01$.

3.2.5 Both social isolation and overcrowding yield sex differences across behaviors

The outcomes of all behavioral tests are summarized and compared by sex in Table 3.1. Overall, both social isolation and overcrowding produced alterations in tests of activity, motivation, and aggression. For the VBFD test, we only tested flies that had undergone the social isolation manipulation. We had begun concentrating on social isolation at this stage of the

project, based on preliminary RNA-seq results (see Chapter 4) and the outcomes of the other behavioral tests.

Table 3.1: Summary of behavioral changes and sex differences following social isolation or overcrowding.

SOCIAL ISOLATION

	Activity	Motivation		Aggression				Feeding Decision
	Walking	Yeast Pref.	ACV Pref.	Hits #	Latency	Social Interactions #	Hits %	VBFD % sucrose
Male								
Female								

OVERCROWDING

	Activity	Motivation		Aggression			
	Walking	Yeast Pref.	ACV Pref.	Hits #	Latency	Social Interactions #	Hits %
Male							
Female							

= UP
= NO CHANGE
= DOWN

Following isolation, males *increased* their activity, their motivation to explore a space containing yeast paste or apple cider vinegar odorant, their number of aggressive hits (lunges), and overall social interactions in the aggression assay compared to male controls. Males' latency to strike an opponent *decreased*, indicating heightened aggressive behavior. Females also *increased* their activity, motivation to explore the apple cider vinegar odorant, their percentage of overall social interactions that were aggressive hits (head butts), and their sucrose consumption relative to arabinose and non-feeding. Isolated females *decreased* their exploration of the yeast paste side of the arena relative to control females.

Following crowding stress, overcrowded males increased their activity but otherwise did not deviate from control males. Crowded females demonstrated decreased motivation to explore the apple cider vinegar odorant, number of aggressive hits (head butts), and overall social interactions in the aggression test compared to control females.

3.2.6 Social isolation does not impact survival, but crowding decreases lifespan

To further explore the stress effects of social isolation and crowding, we wanted to expand our experiments beyond behavioral change and examine whether isolation or crowding impacted survival or nucleic acid biomarkers. We performed survival analyses in which newly-eclosed flies were set up in groups of 50. The "stressed" groups experienced either social isolation or crowding for the duration that was used prior to behavioral testing, and then they returned to group-housed standard conditions for the remainder of the experiment. Flies were checked every 24 hours for new deaths until no flies remained alive.

We found no differences in survival between socially isolated and control flies for males (Figure 3.7A, $\chi^2 = 2.71$, df = 1, p = 0.10) or females (Figure 3.7A, $\chi^2 = 0.25$, df = 1, p = 0.62). Additional statistical tests, beyond the log-rank test, are detailed in Table 3.2. However, for flies that experienced crowding, we found that crowding significantly decreased lifespan relative to controls in both males (Figure 3.7B, $\chi^2 = 8.60$, df = 1, p = 0.003) and females (Figure 3.7B, $\chi^2 = 10.10$, df = 1, p = 0.002). Additional statistical tests are provided in Table 3.3.

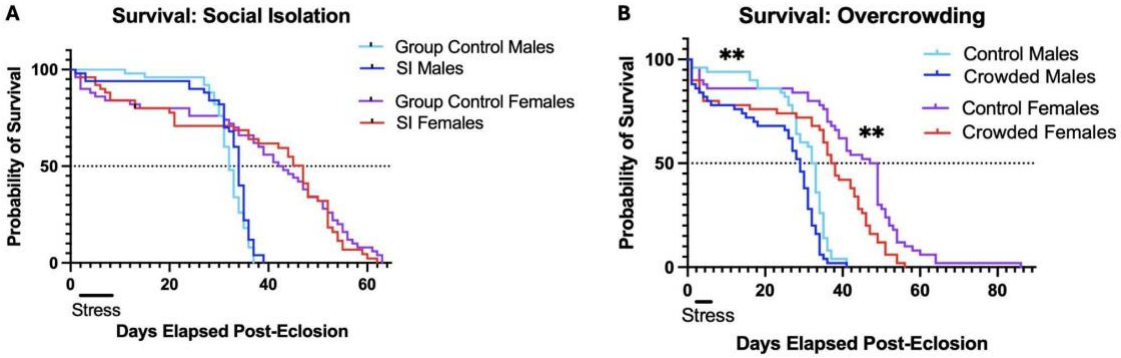

Figure 3.7. Overcrowding stress, but not social isolation (SI), decreases lifespan in male and female flies. A) Log-rank tests showed no significant differences in survival between isolated males (n = 50) and group-housed control males (n = 50, $\chi^2 = 2.71$, df = 1, p = 0.10) or isolated females (n = 50) and group-housed control females (n = 50, $\chi^2 = 0.25$, df = 1, p = 0.62). **B)** Log-rank tests indicated significant differences in survival following overcrowding, with overcrowding decreasing survival in both males (n = 50 in each group, $\chi^2 = 8.60$, df = 1, p = 0.003) and females (n = 50 in each group, $\chi^2 = 10.10$, df = 1, p = 0.002) relative to their respective controls.

Table 3.2: Statistical analysis of survival for social isolation

MALES	Chi square	df	P value
Log-rank test	2.711	1	0.0997
Gehan-Breslow-Wilcoxon test	2.448	1	0.1177
Hazard ratio (Mantel-Haenszel): 0.6770 (0.4255, 1.077)			
Hazard ratio (logrank): 0.7560 (0.5089, 1.123)			

FEMALES	Chi square	df	P value
Log-rank test	0.2481	1	0.6184
Gehan-Breslow-Wilcoxon test	0.006334	1	0.9366
Hazard ratio (Mantel-Haenszel): 1.114 (0.7276, 1.707)			
Hazard ratio (logrank): 1.102 (0.7355, 1.651)			

Table 3.3: Statistical analysis of survival for overcrowding

MALES	Chi square	df	P value
Log-rank test	8.602	1	**0.0034**
Gehan-Breslow-Wilcoxon test	8.676	1	**0.0032**
Hazard ratio (Mantel-Haenszel): 1.966 (1.251, 3.088)			
Hazard ratio (logrank): 1.683 (1.122, 2.524)			

FEMALES	Chi square	df	P value
Log-rank test	10.10	1	**0.0015**
Gehan-Breslow-Wilcoxon test	7.205	1	**0.0073**
Hazard ratio (Mantel-Haenszel): 2.060 (1.319, 3.215)			
Hazard ratio (logrank): 1.777 (1.181, 2.672)			

3.2.7 Mitochondrial DNA copy number does not change after social isolation or crowding

Mitochondrial DNA copy number (mtDNA-CN) has been identified as a biomarker of mitochondrial function that correlates with energy reserves and oxidative stress[30]. The increased production of reactive oxygen species in response to oxidative stress, environmental stressors, infection, or medications may induce mitochondrial dysfunction and reductions in mtDNA-CN. It is hypothesized that these alterations in mtDNA-cn then lead to broader gene expression changes in energy, immune, and cell signaling pathways that ultimately result in chronic diseases in humans. These diseases include cardiovascular diseases, chronic kidney disease, liver disease, neurodegenerative diseases, and cancers[31]. Thus, mtDNA-CN has emerged as a potential biomarker of stress, with a lower mtDNA-CN indicating stress-induced mitochondrial dysfunction.

One recent study examined the effects of sex and age on stress resistance and mtDNA-CN in *Drosophila melanogaster*[32] and concluded that females may be more tolerant to environmental stress with age than males. Hence, we decided to see if social isolation or crowding stress impacted mtDNA-CN, as a read out of stress susceptibility or physiological robustness. We piloted three samples of DNA extracted from 50 fly heads for each stress paradigm, sex, and experimental condition but did not find any stress/control differences that achieved statistical significance (Figure 3.8). One deviation from the original protocol[32] was that we used *GAPDH* as a housekeeping gene instead of *rosy*, which could have impacted our findings. However, the preliminary results did not encourage us to pursue mtDNA-CN further as a stress phenotype.

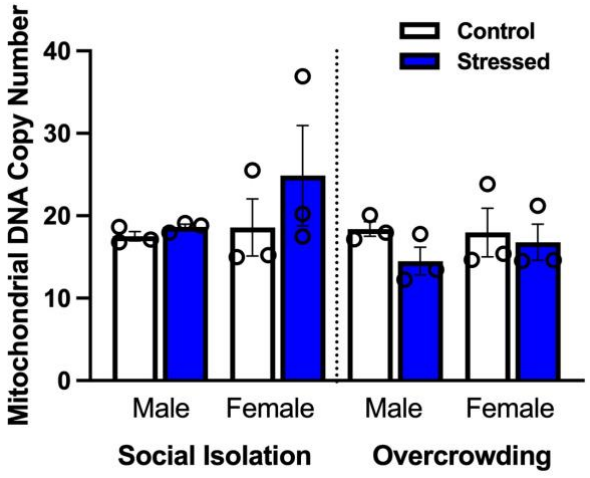

Figure 3.8. Pilot data do not suggest an effect of social stress on mitochondrial DNA copy number. No statistically significant differences were detected between stressed and control samples in either sex for social isolation or overcrowding. Circles represent individual DNA samples that were extracted from groups of 50 whole heads. Error bars are ± SEM.

3.3 Discussion

Our first goal for this project was to establish social isolation and crowding as valid stress paradigms in flies, using behavior, survival, or biomarkers to capture stress-induced phenotypic changes. Overall, we found changes in males and females across behavioral tests of locomotion,

food motivation, and aggression following both social isolation and overcrowding. Crowding stress was found to decrease survival in both male and female flies. There were no significant effects of social isolation on lifespan in either males or females. We also did not see any significant differences in mtDNA-CN between stressed and control experimental groups of either sex following social isolation or overcrowding.

Most of our behavioral findings relating to social isolation were consistent with those from the literature: that social isolation in *Drosophila* increases activity[1,16], feeding[1], and aggression[15,18,33,34]. While we did not quantify food consumption, which is frequently measured with capillary feeding[35], we used a novel combination of tests to measure sex differences in motivated behavior and different subcomponents of aggression. These tests reflect general increases in feeding or food motivation and aggressive behaviors in isolated flies of both sexes but provide more granular detail of how the behaviors are changing.

The study of overcrowding in *Drosophila* adults is limited. However, the existing literature suggests that adult crowding decreases longevity in both males and females[36] and alters sweet food-seeking and activity in opposite directions between males and females, such that these behaviors increase in males and decrease in females[37]. Our findings of the effects of early adult overcrowding on long-term survival are consistent with those of the previous report. Similarly, we found that males increased their activity following overcrowding, while in females there was no change. We did not study overcrowded flies in the VBFD test, but we also saw that crowded females' motivation to explore apple cider vinegar odor decreased relative to controls, while there was no difference in males. The apple cider vinegar is intended to signal the presence of rotting fruit, which would be a sweet food source for the flies.

Although we did not see reductions in mtDNA-CN that suggest oxidative stress, there is

evidence of both crowding[38,39,40,41] and social isolation[42,43,44,45] inducing oxidative stress across the animal kingdom. Increased oxidative stress may contribute to shortened lifespan. Despite declines in survival in male and female flies following overcrowding, we did not see any survival differences between socially isolated flies and group-housed controls of either sex. One possible explanation for this outcome is that the social isolation we employed was sufficiently stressful to alter behavior but insufficiently stressful to decrease lifespan. We isolated the flies for seven days and then returned them to group housing for the remainder of their lives. It is possible that the isolation experience fostered resilience in the flies once they were returned to standard conditions, and that we may have observed declines in lifespan had we employed lifelong social isolation. Another explanation could be that all of our experimental groups were same sex. Long term same-sex pairing has been found to decrease lifespan in males and females[46], so it is possible that our group-housed controls were not completely unstressed controls, making it more difficult to detect stressed versus control differences. Although the way in which we set up our experiments accounts for the confounding effect of same-sex living on survival, it may also be worthwhile in the future to compare the survival of isolated males and females to that of a mixed sex group-housed control group.

Finally, it should be noted that there is some evidence of a common molecular signaling mechanism between feeding and aggression in *Drosophila*. *Drosulfakinin* (*Dsk*) is a neuropeptide that has been implicated in both satiety and feeding[47–49] and aggression[13,50,51]. Some of the sex-specific increases in different subcomponents of aggression and changes in food motivation may be related to distinct male and female neural circuitry downstream of Dsk signaling. We will explore this concept further in the next chapter.

In our behavioral data, we observed a potential female shift in macronutrient preference

from protein to sugar following social isolation, implied by isolated females' decreased interest in yeast paste and increased interest in apple cider vinegar and preference for sucrose. Isolated males were less discerning, showing increased interest in both yeast and apple cider vinegar and no change in sucrose consumption compared to controls. One potential explanation could be that while under the environmental stress of isolation, females need energy for survival over reproduction and thus switch their food choice from protein to sugar. Although yeast is composed of sugar, protein, and salt, flies rely on yeast as their primary protein source, and both males and females increase their consumption of yeast prior to mating to prepare for reproduction[52]. Mated females also increase their yeast consumption for the benefit of their offspring to the detriment of their own stress resistance and lifespan, suggesting a fitness-fecundity trade off[52]. While social isolation may increase general feeding in males, based on the literature, the male macronutrient preference may be less clear, given that males do not have the reproductive demand of egg-laying. Additionally, the different manifestations of isolation-induced aggression between males and females may reflect different ways in which males versus females display territoriality over a food source.

For the remainder of this project, we decided to focus on the sex-specific effects of social isolation on gene expression, and the potential stress-signaling pathways in which these genes participate. The social isolation paradigm is experimentally simple and robust in generating a greater number of behavioral alterations and sex differences compared to overcrowding.

3.4 Methods

3.4.1 Fly stocks and rearing

All fly stocks were raised on standard medium (cornmeal/yeast/molasses/agar) at 25°C and 40% relative humidity on a fixed 12-h light/dark cycle. Male and female *Oregon-R* flies

(wild type) were used for the experiments detailed above and were a gift from Dr. Jodi Schottenfeld-Roames, Princeton University. Behavioral experiments were conducted within eight hours after lights on.

3.4.2 Experimental setups and design

Social isolation

For the social isolation stress model, we used an experimental procedure for chronic social isolation already characterized in the literature[1]. Newly-eclosed male and female flies were housed together for three-five days to acquire social experience. Flies were then housed either singly or in same sex groups of 25 for seven days. Within 24 hours of the final day of isolation or group housing, flies were either frozen on dry ice for RNA extraction, tested for behavior, or moved to/kept in same sex group housing for survival analysis.

Overcrowding

For the crowding model, one-day-old flies underwent three sets of restraint stress, consisting of 10 hours of limited physical space in a same sex group of 25 without food, and then 14 hours of rest (free movement and access to food). The control group consisted of freely moving flies that underwent three iterations of no food for 10 hours and then 14 hours of rest. Within 24 hours of the final day of crowding or free movement, flies were either frozen on dry ice for RNA extraction, tested for behavior, or moved to standard conditions in their same groups for survival analysis.

Survival

To measure survival, flies (n=50 per experimental group) underwent either social isolation, overcrowding, or the respective control conditions of the prior stress paradigms. At the end of the stress period, all flies were moved into group housing in standard food vials. The flies

were counted once per day, and any deaths were recorded, along with the date of death, until all flies were deceased.

3.4.3 Behavior tests

Locomotion assay

Flies were transferred to the testing chamber via aspiration or brief anesthesia with CO_2. Upon analysis, we found that the use of CO_2 did not alter activity when compared to aspiration. Each chamber consisted of a 55mm diameter petri dish filled with 1% agarose gel, to provide the flies with enough room to walk but not fly. The flies were put into same sex groups of 10, recorded for 10 minutes, and frozen on dry ice. Videos were initially analyzed using the Iowa Fly Locomotion and Interaction Tracker (IowaFLI Tracker)[53], a MATLAB based video analysis system, and later validated using EthoVision XT video tracking software (Noldus Information Technology, VA).

Motivated behavior tests

Flies were tested in the arena previously described in the locomotion assay to evaluate anhedonia-like behavior or discrimination of an attractive food source. Flies were briefly anesthetized using CO_2 and lined up along the midline of a filter paper evenly divided into 2 halves: one with yeast paste and one with nothing. Behavior was recorded for 60 minutes, and a preference index (PI) was calculated at five minute intervals as PI = [(# of flies on the yeast side) – (# of flies on the empty side)] / (total). This test was repeated using apple cider vinegar in place of yeast paste, as an additional attractive stimulus.

Aggression assay

To test for aggression, we modified an experimental protocol previously described by the Kravitz lab[26]. Testing occurred within 24 hours following the end of social isolation or crowding

stress. Twelve-well plates were used as behavior chambers for testing same sex, same experimental condition pairs of flies. For group housed controls, flies in a fighting pair were taken from different vials to account for social novelty. To create an arena/territory, white plastic microtube caps were filled with standard fly food, topped with a dot of yeast paste, and placed in the wells. A transparent plastic covering with drilled holes was placed on top of the well plate so that flies could be gently aspirated into the wells at the same time. The holes were then plugged with short screws. Fights were recorded for one hour using a Sony Digital Handycam camcorder (Sony Group Corporation, Tokyo, Japan).

Only behavior that took place in the arena was scored. Flies that did not encounter each other on the food cup within 45 minutes of recording were excluded from analysis. A goal sample size of n=15 fighting pairs was decided prior to the start of experiments. For the fighting pairs, the times of the first encounter on the food cup and the first hit were recorded to calculate latency to initiate fighting. Fights were then scored for 10 minutes following the first hit (lunge for males or head butt for females). The male lunge was defined as a fly rearing up on its hind legs and snapping downwards onto its opponent. The female head butt was defined as a fly thrusting forward horizontally and then recoiling from the opponent. Number of hits, latency, number of overall encounters, and hits as a percentage of all encounters were used as measures to quantify and interpret aggressive behavior.

Value-Based Feeding Decision test

A previously described behavior test, the value-based feeding decision test[29], was used as a two-choice assay to examine feeding decision making. Flies were briefly anesthetized with CO_2 and placed in the center of the arena used for the locomotion and motivated behavior tests, a 55 mm diameter petri dish filled with 1% agarose gel and topped with a layer of parafilm. Sugars

were dissolved in plain water to make two solutions: a 150 mM sucrose solution and a 150 mM arabinose solution. Each solution was labeled with a blue dye (0.01% erioglaucine disodium salt, Acros Organics, Cat# 229730250) or red dye (0.1% Food Red No. 106, TCI, Cat# F0143). Alternating droplets (20 µL volume) of each liquid food were placed evenly around the perimeter of the dish, such that there were eight droplets total, four of each solution. Flies were allowed to explore the arena and feed in the dark for two hours. At the end of this period, flies were collected and counted under a microscope. They were scored by color: blue (ingested primarily blue food), red (ingested primarily red food), purple (ingested both foods), or non-eater (no color accumulation in the abdomen).

3.4.4 DNA extraction

For the mitochondrial DNA copy number analysis, DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). For each experimental group, DNA was extracted from three samples of 50 whole fly heads each. The mitochondrial NADH dehydrogenase 4 L gene (*ND4L*) and *GAPDH* were targeted. The reactions were performed in a total volume of 10 µL using SYBR Green PCR Master Mix (Qiagen), 5 µM of each of the primers and 2 ng of DNA. We used the following PCR conditions: 95 °C for 20 s, 40 cycles of 0.3 s at 95 °C, 15 s at 52 °C, and 30 s at 72 °C. The formula $2^{(\text{ctGAPDH median}-\text{ctND4L median})}$ was used to calculate a value for mtDNA copy number, as previously reported[32].

3.4.5 Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.4.0 software (GraphPad Software, Inc., La Jolla, CA). The Shapiro-Wilk test of normality (passed at $p > 0.05$) was used to determine whether data followed a parametric distribution. Unpaired t-tests were used for parametric data assuming equal variances, and Welch's t-tests were used if the samples had

unequal variances based on the result of the F-test. If the data did not pass the test for normality distribution, nonparametric testing was used. All nonparametric data were analyzed with the Mann–Whitney *U* test for between-group comparisons. Effects were deemed statistically significant at $p < 0.05$. The Grubbs' test was applied to identify and exclude outliers in the data. Log-rank tests were used to determine differences in survival curves, with $p < 0.05$ deemed statistically significant. A repeated measures 2-way ANOVA was used to compare differences between stressed and control flies' preferences for yeast or apple cider vinegar between males and females over time.

3.5 Acknowledgments

We would like to acknowledge Gillian Hilscher, Sarah Brown, and Ella DePaolo for their contributions to the development and execution of the behavioral tests. We thank Caroline Palavicino-Maggio (McLean Hospital/Harvard Medical School) for guidance regarding the aggression testing.

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Chapter 4

Social isolation-induced sex differences in Drosophila transcriptomics and the role of *takeout* in female isolation-induced sucrose feeding

This work was presented as a poster at the* Princeton University Department of Molecular Biology annual retreat (2021, 2022, and 2023), *as well as at* Society for Neuroscience (2023). *It was presented as a talk at the* Princeton University Department of Molecular Biology annual retreat (2024). *Lisa Schneper, Katherine Cho, Mindy Sim, Eve Schoeffler, and Daniel Notterman contributed to work described in this chapter.

We can attribute differences between the male and female stress response to sex-specific brain changes in molecules and circuits. However, our understanding of these mechanisms is far from complete. Here, we use *Drosophila melanogaster* to explore sex differences in chronic social isolation-induced transcriptomic changes and focus on *takeout* (*to*) as a gene that is upregulated solely in females following social isolation experience. We performed RNA-seq to identify candidate genes that may be mechanistically linked to sex differences in social stress-induced behavioral changes. *To* and sucrose consumption are upregulated exclusively in females following social isolation. Consistent with our hypothesis that *to* expression changes and sucrose consumption are causally linked, sucrose consumption decreases in socially isolated females, but increases in males, following both *to* knockdown and overexpression in *to*-expressing cells. Contrary to our expectations, these changes do not hold when *to* is knocked down or overexpressed only in neurons. We also do not observe differences in sucrose feeding choice

between isolated and control females when neurons are masculinized via *transformer* (*tra*) knock down. Our findings suggest that manipulating *to* expression influences sucrose feeding in opposite directions between females and males following social isolation, and that isolation-induced *to* overexpression in non-neuronal cells in the brain or head may play a role in communicating information about females' nutritional status to the brain. Additional roles for *to* in stress relevant behavioral sex differences should be explored, as well as whether it functions in a signaling pathway that may be conserved in human disorders.

4.1 Introduction

In the previous chapter, we identified a set of behaviors that changed in a sexually dimorphic manner following seven days of social isolation. The sex differences in these phenotypes, coupled with preliminary data from quantitative polymerase chain reaction (qPCR) experiments (Figure 4.1) prompted us to perform RNA sequencing (RNA-seq) on male and female samples of socially isolated and group-housed control flies as an unbiased approach for target identification. From here, we could probe any emerging genetic candidates as potential players in the generation of sex-specific behavioral responses to social isolation stress.

The transcriptome is the entirety of RNA transcripts that the genome produces. It can be measured using high-throughput approaches. By comparing transcriptomes, we can identify changes in gene expression and networks in response to different experimental treatments. RNA-seq is one such high-throughput sequencing method that allows for mapping and quantifying transcriptomes. In RNA-seq, RNA samples are converted to a library of cDNA fragments with adaptors attached to the ends. Each cDNA molecule is sequenced to generate short sequence reads, which are later aligned to a reference genome. This alignment provides a genome-scale transcriptional map containing information about RNA expression level for each gene in the

sample[1].

Drosophila produces many different types of RNAs. Out of total RNA, only messenger RNA (mRNA) codes for the production of proteins. The remainder of the RNAs, which comprise the vast majority, are non-coding RNAs (ncRNAs), and include transfer RNA (tRNA), ribosomal RNA (rRNA), long ncRNA (lncRNA), circular RNA, and short ncRNAs. Short ncRNAs include smaller ncRNAs such as microRNAs, PIWI-interacting RNAs, and small interfering RNAs. tRNA and rRNA are involved in translation, while the other ncRNAs perform diverse regulatory functions[2]. RNA-seq is useful for gaining a comprehensive understanding of all transcripts present in the sample, regardless of abundance. Although RNA-seq can be used to measure both coding and non-coding RNAs, and novel techniques have been developed for selectively sequencing mRNA, small RNA, and specific transcripts[3], we chose to focus on mRNA expression to examine stress-induced changes in genes which code for proteins.

RNA-seq data can provide information about transcriptional regulation that occurs in response to a particular condition, and whether gene expression is changing within certain molecular pathways. Methods such as mass spectrometry are useful for understanding whether gene expression changes ultimately result in changes to protein presence and quantity, but this approach captures changes downstream of gene expression and can miss low-abundance proteins. Thus, we decided that RNA-seq would be more appropriate for exploratory purposes.

In the following set of experiments, we began by using RNA-seq to identify sex differences in gene expression in fly heads following social isolation. We then manipulated the expression of one of the emerging targets, *takeout (to*), and the sex determination gene *transformer* (*tra*) to evaluate mechanistic relationships between isolation, sex, gene expression changes, and behavior (sucrose feeding in the value-based feeding decision test) that may be

relevant to sex differences in the human response to chronic stress. We decided to focus on the value-based feeding decision (VBFD) test because we observed the clearest sex differences in motivated behavior and feeding choice in our behavioral data from Chapter 3, *to* has been previously associated with feeding in the literature, and the simplicity of the VBFD test provides ease of analyzing feeding behavior for large numbers of flies in a short amount of time.

4.2 Results

4.2.1 Preliminary qPCR tests suggest sex differences in the expression of candidate stress genes following social isolation

To explore the effects of social environmental stress on transcriptional regulation, we began by selecting five candidate genes and using qPCR to test for expression differences between isolated and control flies of each sex. In their study of transcriptomic changes in male *Drosophila melanogaster* following chronic (seven day) and acute (one day) social isolation, Li et al. (2021) found alterations in gene expression that they interpreted as indicators of a "starvation-like brain state[4]." These included upregulations in *Limostatin* (*Lst*) and *target of brain insulin* (*tobi*) and downregulation in *drosulfakinin* (*Dsk*) in the heads of flies that experienced seven days of social isolation, in comparison to flies that were isolated for only one day or group-housed. Whole heads are often used in place of brains in behavioral and molecular studies of *Drosophila* to facilitate experimental tractability and gain a more comprehensive understanding of how the brain integrates with sensory organs in the head. In the present project, we first performed RNA-seq using whole heads and later validated the expression of our primary gene of interest in both whole heads and brains using qPCR.

Dsk is a satiety-related cholecystokinin-like peptide that is secreted when flies are fed and reduced upon starvation[5]. Li et al. (2021) found a 2.03-fold decrease in *Dsk* in adult males

following chronic social isolation[4]. *Lst*, a decretin hormone that is induced by fasting and suppresses insulin secretion[6], was upregulated 1.67-fold, and *tobi* expression increased 1.76-fold[4]. *Tobi* has been found to encode an evolutionarily conserved alpha-glucosidase that is regulated by fly analogs of insulin and glucagon[7]. This RNA profiling then allowed Li et al. (2021) to identify a population of neurons responsible for chronic social isolation-induced decreases in sleep and increases in feeding[4]. We decided to include *Dsk* and *tobi* in our initial analysis, to see if our social isolation paradigm replicated their results or reflected sex differences in gene expression.

We also selected 5-hydroxytryptamine (serotonin) receptor 2B (*5-HT2B*), cholecystokinin-like receptor at 17D1 (*CCKLR-17D1*), and cholecystokinin-like receptor at 17D3 (*CCKLR-17D3*) based on a paper that identified these genes as "fly anxiety factors[8]." This research group found strong correlations between rodent and fly thigmotaxis in the open field test in response to common genetic, pharmacologic, and stress manipulations. The three genes cited above are orthologs of candidate mouse anxiety-related genes, and knockdown of these genes yielded significant decreases in fly wall-following[8]. *5-HT2B* encodes a G protein-coupled receptor that binds serotonin and has been associated with the regulation of sleep[9] and social behaviors[10]. *CCKLR-17D1* and *CCKLR-17D3* both encode neuropeptide receptors that bind Dsk. *CCKLR-17D1* has been implicated in locomotion[11], neuromuscular junction growth[12], and aggression[13]. *CCKLR-17D3* has been associated with feeding[14], as well as female sexual receptivity[15] and suppression of male sexual behavior[16]. *CCKLR-17D3* is the ortholog of the gene encoding the mammalian cholecystokinin type B receptor, *CCKBR*, which plays a role in regulating anxiety, arousal, feeding, locomotion, neuroleptic activity, and opiate-induced analgesia[17,18,19]. Taken together, the five genes for which we quantified expression (*Dsk, tobi, 5-*

HT2B, CCKLR-17D1, and *CCKLR-17D3*) have been previously associated with the stress-related behaviors we tested in the previous chapter (locomotion, feeding or food motivation, and aggression), and some of them participate in a common signaling pathway (Dsk as the ligand that binds to the CCKLRs).

We found sex differences in the expression of *5-HT2B* and *CCKLR-17D1* following seven days of social isolation. Expression of *5-HT2B* generally increased in socially isolated males relative to control males, whereas it decreased in isolated females relative to control females, resulting in a sex difference in relative gene expression fold change (Figure 4.1A, $t(10) = 3.00$, $p = 0.01$). Expression of *CCKLR-17D1* increased in socially isolated males relative to control males, despite little to no difference in expression between isolated and control females. This resulted in a sex difference in relative gene expression fold change that bordered on statistical significance (Figure 4.1A, $t(10) = 2.08$, $p = 0.06$). In both males and females, 7 days of social isolation decreased expression of *Dsk* and increased expression of *tobi* (Figure 4.1A), consistent with the literature[4]. Following three days of overcrowding stress, we did not observe any sex differences that achieved statistical significance between stressed and control flies (Figure 4.1B). However, similar to social isolation, overcrowding seemed to upregulate expression of *tobi* in both sexes. These initial qPCR findings motivated us to collect additional samples for RNA-seq. Because we observed more sex differences in both our preliminary gene expression data and the behavioral data from the previous chapter in socially isolated flies, compared to the overcrowding model, we decided to focus on social isolation only for the remainder of the project.

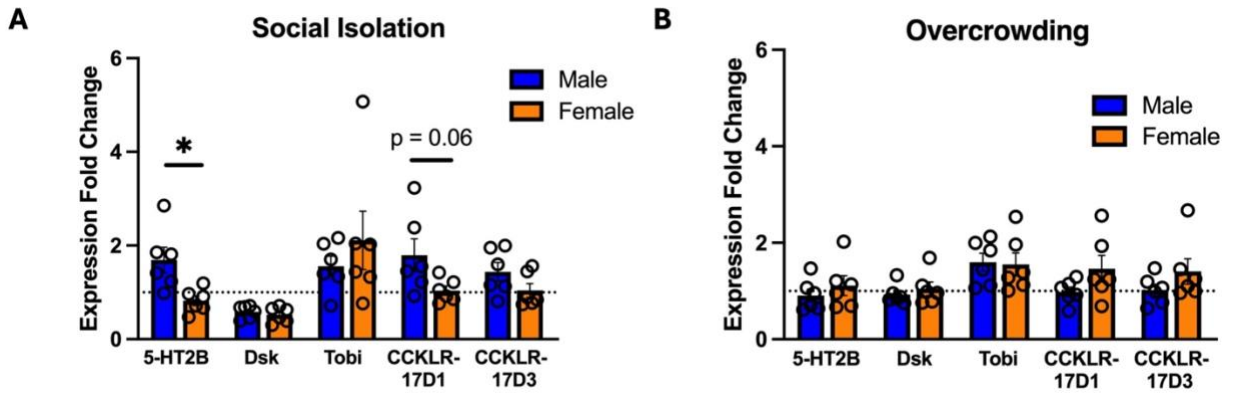

Figure 4.1. Socially isolated males, but not females, demonstrate increases in expression of *5-HT2B* relative to controls. A) Expression of *5-HT2B* ($t(10) = 3.00$, $p = 0.01$) in isolated males relative to control males is significantly greater than that observed in isolated females relative to control females. **B)** The overcrowding paradigm did not yield significant differences in relative gene expression fold change fold change between the sexes for the five candidate genes tested. Circles represent individual cDNA samples synthesized from stressed fly RNA relative to a control average expression value. Error bars are ± SEM. *$p < 0.05$.

4.2.2 Differential expression analysis indicates female-specific overexpression of fat body-synthesized proteins following social isolation

RNA samples were extracted in triplicate from whole fly heads for each experimental group: social isolation (SI) male, control male, SI female, and control female. The samples were then submitted to the Princeton Genomics Core Facility for cDNA library preparation and sequencing (see section 4.4.6, "Sequencing"). Upon receiving the FASTQ files, we processed these raw files through a bioinformatics pipeline to identify differentially expressed genes (see section 4.4.6, "Analysis Pipeline").

We began by generating a Multidimensional Scaling (MDS) Plot as a dimensionality reduction approach to discern the overall similarity and dissimilarity across our samples (Figure 4.2A). Of our triplicates, we removed one sample from each of the female groups that failed this quality test (see Figure S4.2 for original plot) for more accurate target discovery. The resulting

MDS plot shows a separation between the samples, along the top two dimensions that account for the greatest percentage of variance in log fold change, by sex and condition (Figure 4.2A).

Next, we prepared the data for linear modeling with *limma* using *voom*. Our design matrix modeled the relationship between gene expression and condition (isolated female, control female, isolated male, and control male) adjusted for batch. Specifically, we focused on identifying differences in gene expression between the sexes under social isolation, relative to their respective controls. Differentially expressed genes were filtered based on an FDR-adjusted p-value of $p_{adj} < 0.05$ to determine significant genes with positive or negative changes when comparing socially isolated males to control males, socially isolated females to control females, and the interaction between social isolation and sex. The heatmap in Figure 4.2B depicts the genes that met these criteria when comparing the interaction between social isolation and sex. We observed clusters of genes that were upregulated in isolated females compared to control females and males (Figure 4.2B). Upon running these significant genes through Metascape[20], a gene annotation and pathway analysis resource, we identified changes in groups of genes related to cell division (e.g. mitotic cell cycle process, asymmetric cell division) and metabolism (e.g. cellular catabolic process, fatty acid metabolic process) (Figure 4.2C).

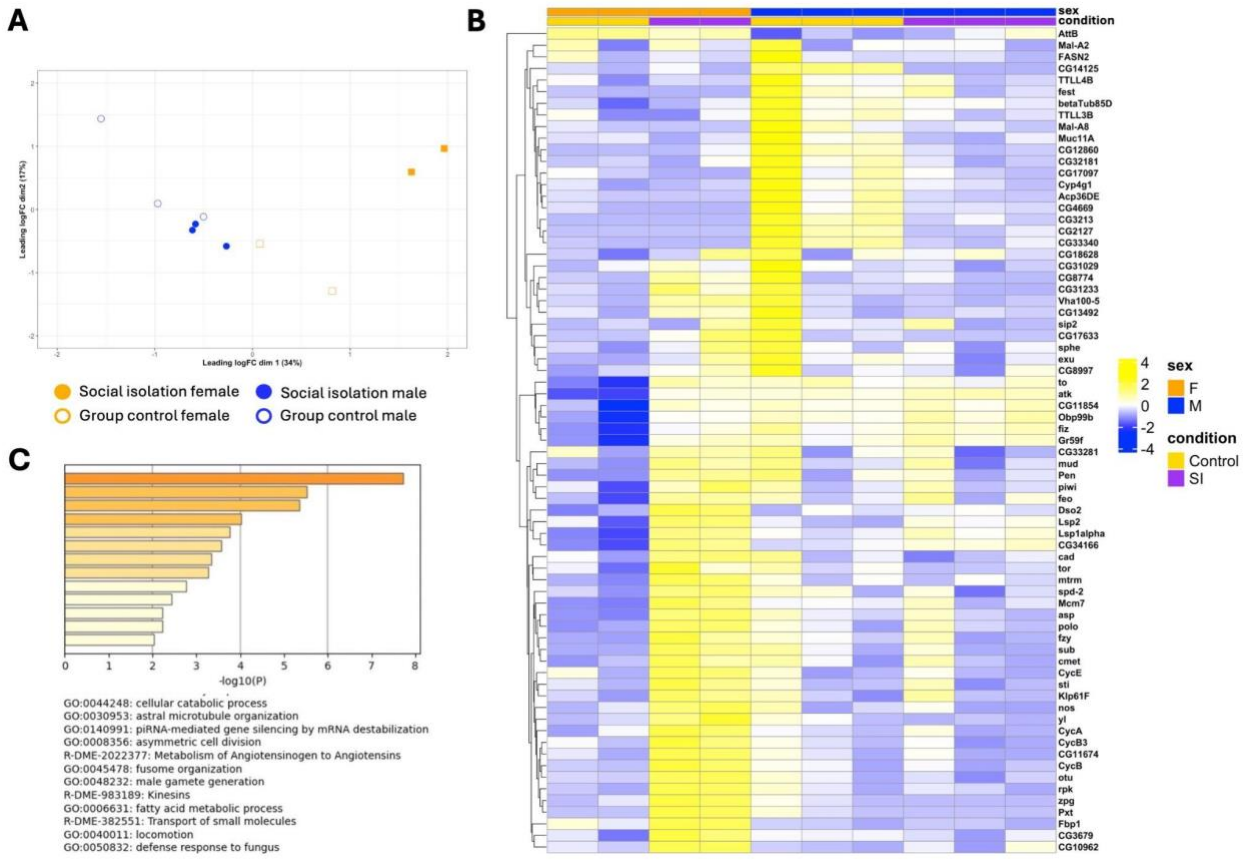

Figure 4.2. Socially isolated females demonstrate upregulation of multiple genes related to metabolic processes relative to control females and males. A) Multidimensional Scaling reflects similarity and dissimilarity within and between experimental groups. Key: open = control, solid = social isolation; blue = male, orange = female; square = batch 1, circle = batch 2. **B)** Heat map depicting the scaled (z-score transformed) log2 counts per million of genes differentially expressed (absolute value of log2-fold change > 2 and adjusted p-value < 0.05) when comparing the difference between SI female and control female with the difference between SI males and control males. **C)** Gene ontology (Metascape) of the significantly differentially expressed genes captured in the heat map reflects changes in broad categories of cell division and metabolism.

Additionally, entering the significantly differentially expressed genes (comparing the interaction of social isolation and sex) into the STRING database[21] returned a network map of potential functional protein-protein interactions among them (Figure 4.3A). Applying the Markov Cluster (MCL) Algorithm[22] to our data then parsed our genes into 21 potential functional clusters, many of which coincided with the Metascape gene ontology results. Clusters with the strongest functional enrichment included genes related to mitotic cell cycle (Figure

4.3B), female reproduction (Figure 4.3C), and hemocyanins/hexamerins and secreted storage proteins (Figure 4.3D). STRING calculates measures of enrichment strength, signal, and statistical significance. *Strength* captures the size of the enrichment effect by calculating $\log_{10}$(observed/expected), where the (observed/expected) ratio represents the ratio between the number of genes in my network annotated with a term and the number that we would expect to see annotated with this term in a random network of the same size. *Signal* measures a weighted harmonic mean between the (observed/expected) ratio and -log(false discovery rate). *False discovery rate* (FDR) describes the significance of the enrichment, with p-values corrected for multiple testing with the Benjamini-Hochberg procedure[23].

We also used STRING to visualize our top functional enrichments, based on gene count, signal, and FDR, categorized by biological process, local network clustering, and *Drosophila* phenotype (Figure 4.4). Reiterating the findings from our clustering, the top biological process terms related to mitotic cell cycle processes and cell division (Figure 4.4A). Local networking clustering showed enrichment in egg activation, piRNA metabolic process, and hemocyanins/hexamerins (Figure 4.4B). Finally, our significant genes were implicated in *Drosophila* phenotypes of abnormal meiosis and female sterility (Figure 4.4C).

Taken together, these enrichments suggest that gene expression may be altered in socially isolated females compared to isolated males, relative to their respective controls, in a manner that disrupts reproduction. Hemocyanin and hexamerin proteins, which are also upregulated in the isolated females, have been implicated across numerous functions in invertebrates. Hemocyanins largely play a role in oxygen transport but have also been associated with innate immunity, wound healing, hormone transport, and ecdysis (moulting)[24]. Hexamerins act as storage proteins in the hemolymph of insects that provide the organism with amino acids and energy during non-

feeding periods. However, hexamerins may also play roles in cuticle formation, hormone transport, and humoral immunity[25].

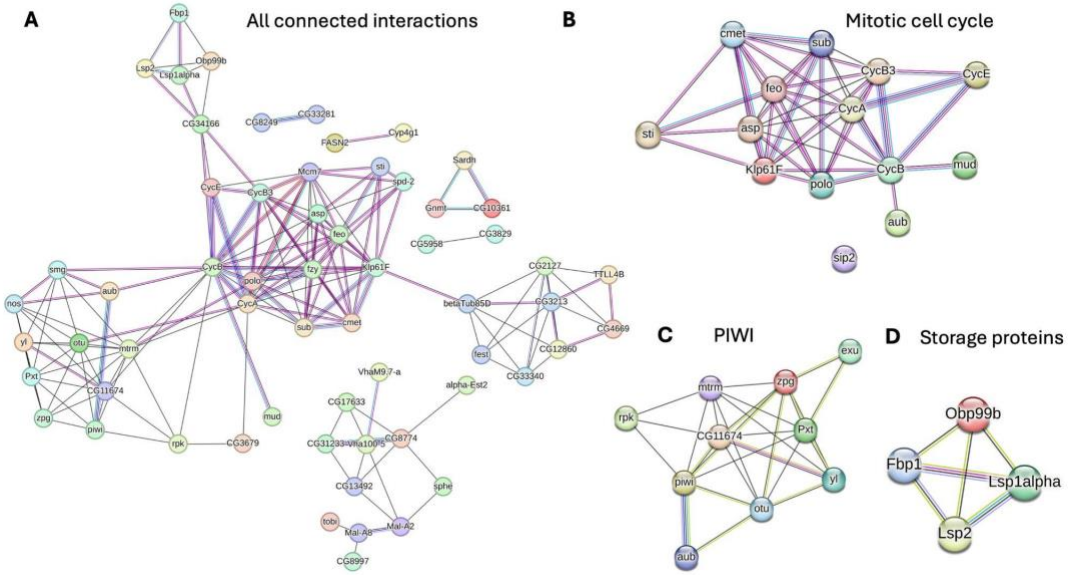

Figure 4.3. Significant differentially expressed genes, when comparing the interaction between social isolation and sex, are connected in broader networks of protein interactions. **A)** All connected interactions are depicted, with non-connected genes removed. We specified a setting of "medium interaction strength" in STRING. The thickness of the connecting line between genes roughly corresponds to the strength of data support for gene interaction. **B)** One of our clusters that showed the strongest functional enrichment included genes implicated in the mitotic cell cycle process (strength = 0.82, signal = 0.78, FDR: p = 0.0003). **C)** Another top cluster consisted of genes connected to piwi, with functional enrichments in cellular process involved in reproduction in multicellular organism (strength = 0.49, signal = 0.45, FDR: p = 0.006), piRNA metabolic process (strength = 1.31, signal = 1.72, FDR: p = 6.97e-08), and egg activation and regulation of oskar mRNA translation (strength = 1.60, signal = 1.62, FDR: p = 9.30e-07). **D)** The third cluster of interest contains three genes encoding secreted (strength = 0.76, signal = 0.83, FDR: p = 8.01e-05) storage proteins (strength = 1.89, signal = 0.70, FDR: p = 0.004): Lsp2, Lsp1alpha, and Obp99b. All four genes are categorized as hemocyanin/hexamerin (strength = 1.72, signal = 0.92, FDR: p = 0.0006).

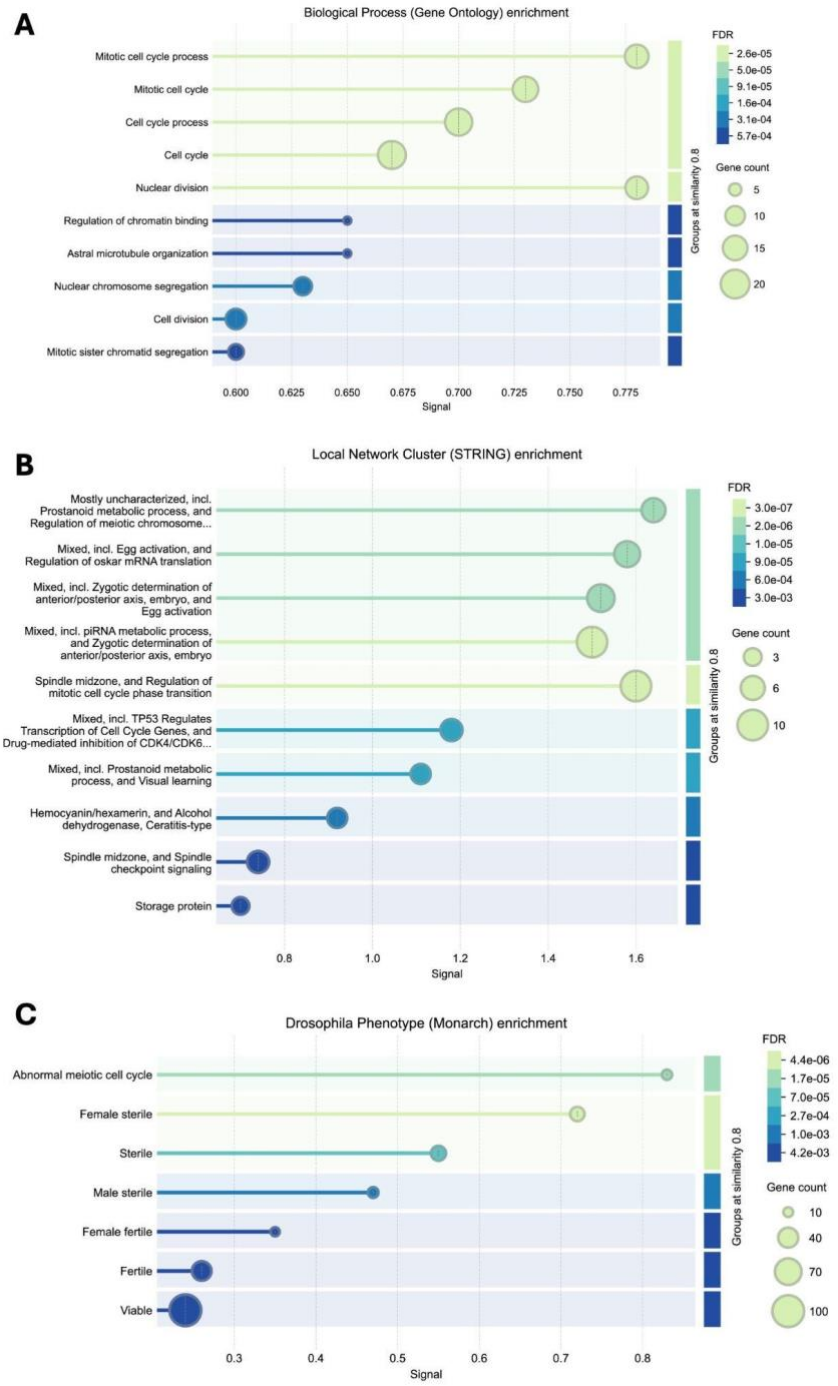

Figure 4.4. Significant differentially expressed genes, when comparing the interaction between social isolation and sex, are enriched across biological process, local network clustering, and *Drosophila phenotype* terms relating to cell division, reproduction, and hemocyanin/hexamerin proteins. A) Top biological process terms include mitotic cell cycle process and nuclear cell division. **B)** Top local network cluster terms include egg activation, piRNA metabolic process, and regulation of mitotic cell cycle phase transition. **C)** Top *Drosophila* phenotypes include abnormal meiotic cell cycle and female sterility.

Finally, we used our significance cut-off of adjusted p-value < 0.05 to visualize our differentially expressed genes in a volcano plot (Figure 4.5). Only three significant genes were downregulated when comparing the interaction of social isolation and sex, while 105 were upregulated. The most significantly differentially expressed genes, by adjusted p-value, are listed in Table 4.1, along with their log fold change values. These genes were all upregulated with respect to the interaction term, suggesting that expression in isolated females exceeds that of isolated males when taking into account the differences between the isolated groups and their respective controls.

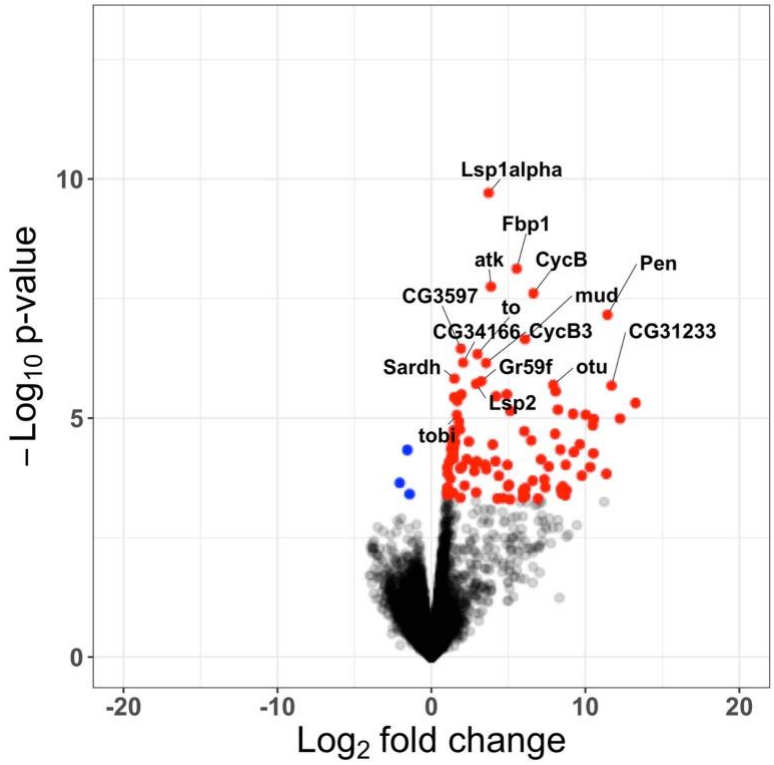

Figure 4.5. The top differentially expressed genes (absolute value of log2-fold change > 1 and adjusted p-value < 0.05), when comparing the interaction between social isolation and sex, reflect upregulation in isolated females. The red points represent the genes upregulated in socially isolated females (relative to female controls) compared with socially isolated males (relative to male controls). In contrast, blue points represent the genes (*CG11211*, *VhaM9.7-a*, and *AttB*) upregulated in socially isolated males compared to the socially isolated females, relative to their respective controls.

Table 4.1: Top differentially expressed genes (interaction of social isolation and sex)

Gene Symbol	Log Fold Change	P-value	Adj. P-value
Lsp1alpha	3.72	1.96E-10	2.04E-06
Fbp1	5.54	7.54E-09	3.93E-05
atk	3.87	1.79E-08	6.21E-05
CycB	6.63	2.47E-08	6.43E-05
Pen	11.44	6.94E-08	0.000145
mud	6.08	2.25E-07	0.000391
CG3597	1.92	3.54E-07	0.000527
to	2.99	4.59E-07	0.000598
CycB3	3.55	7.05E-07	0.000734
CG34166	2.07	6.90E-07	0.000734
Sardh	1.50	1.49E-06	0.00141
Lsp2	2.90	1.92E-06	0.00146
otu	7.92	2.03E-06	0.00146
Gr59f	3.25	1.70E-06	0.00146
CG31233	11.70	2.10E-06	0.00146

An analysis of expression quantity data (transcripts per million, TPM), of some of the top genes identified by our model, confirmed female specific overexpression of RNA transcripts for *larval serum protein-2* (*Lsp-2*), *larval serum protein-1a* (*Lsp-1a*), *fat body protein 1* (*Fbp1*), and *takeout* (*to*). *Lsp-2, Lsp-1a, Fbp1*, and *to* are all synthesized in fat body cells, with the first three genes functioning as amino acid storage proteins and *to* being implicated in feeding behavior. *To* expression is higher in control (Figure 4.6A, $t(4) = 5.07$, $p = 0.007$) and isolated males (Figure 4.6A, $t(4) = 5.79$, $p = 0.004$) relative to control females, but this male-female difference is abolished when females have undergone social isolation. *To* expression is significantly higher in isolated females compared to control females (Figure 4.6A, $t(4) = 8.56$, $p = 0.001$). Similarly, social isolation increases *Lsp-2* (Figure 4.6B, $t(4) = 7.06$, $p = 0.002$), *Lsp-1a* (Figure 4.6C, $t(4) = 4.14$, $p = 0.01$), and *Fbp1* (Figure 4.6D, $t(2.06) = 8.64$, $p = 0.01$) expression relative to control levels in females but not in males.

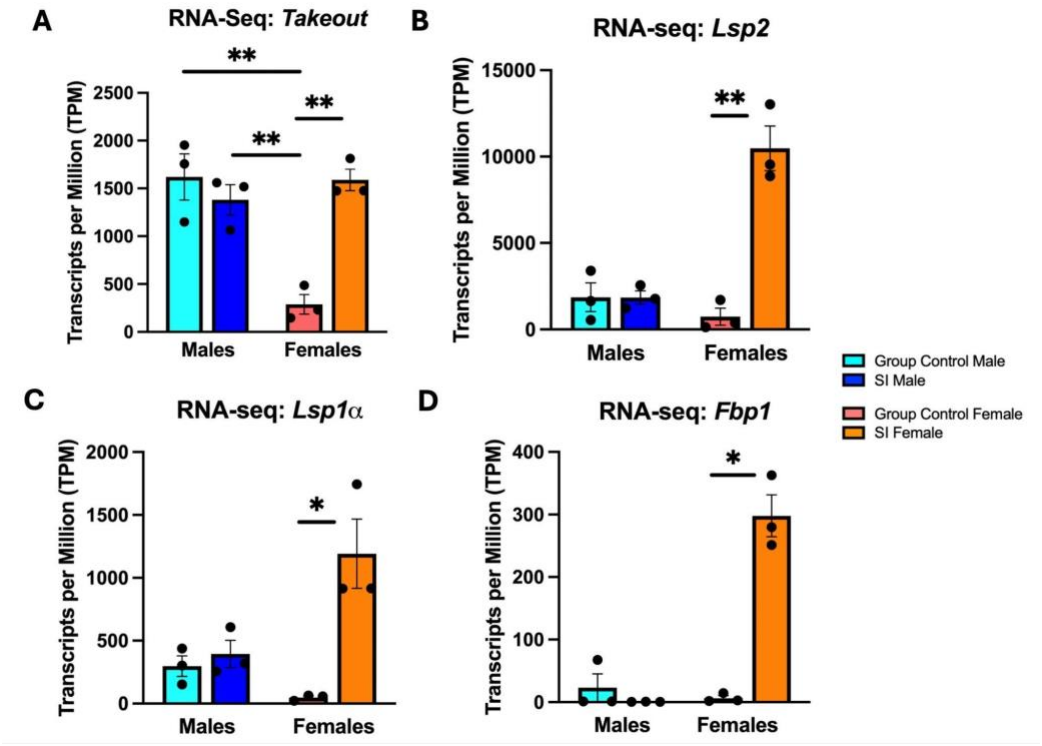

Figure 4.6. Gene expression, quantified in transcripts per million (TPM) from RNA-seq, indicates elevation in *takeout, Lsp2, Lsp1α,* and *Fbp1* in isolated females relative to control females that is not seen in males. A) At baseline, control females had lower TPMs compared to control males ($t(4) = 5.07$, $p = 0.007$) or isolated males ($t(4) = 5.79$, $p = 0.004$). However, socially isolated females had significantly higher TPMs compared to control females ($t(4) = 8.56$, $p = 0.001$) which are comparable to those of males. **B)** TPMs were also elevated following social isolation in females for *Lsp2* ($t(4) = 7.06$, $p = 0.002$), **C)** *Lsp1α* ($t(4) = 4.14$, $p = 0.01$), and **D)** *Fbp1* ($t(2.06) = 8.64$, $p = 0.01$, with Welch's correction). Closed circles represent values derived from individual RNA samples, which were extracted from 200 fly whole heads. Error bars are ± SEM. $*p < 0.05$, $**p < 0.01$.

Insect larval serum proteins, or hexamerins, are generated in the fat body, the functional analog of both the liver and fat tissue in vertebrates. The fat body plays a role in storing excess energy in the form of triglycerides and glycogen[26]. It also generates hexamerin storage proteins that are accumulated during the insect's larval stage[25,27] and secreted from the fat body into the hemolymph. Hexamerins are then circulated as reservoirs of amino acids and metal ions that can be accessed during periods of high metabolic need, such as metamorphosis and reproduction[27]. This is beneficial for insects such as flies, which undergo a period in their life cycle when they

cannot access food (in the pupal stage), to be able to store nutrients when food is available. Amino acid storage consequently fluctuates over developmental time periods to ensure proper development and adult fitness[28]. The fat body also plays a crucial role in nutrient sensing by communicating the nutritional status of the organism to the brain via multiple molecular signaling pathways. These signals activate insulin-producing cells (IPCs), which form and release *Drosophila* insulin-like peptides (DILPs) to regulate levels of glucose circulating in hemolymph[29].

Lsp2 encodes a major hexamerin that serves as a nutrient store in the late larval and pupal stages but also continues accumulation in adulthood[30]. Recently, Fbp1 has also been characterized to play a role in the reuptake of LSPs and FBPs from the hemolymph back into fat body cells[28]. Based on the results from our STRING functional enrichment, which suggested that female social isolation may be disrupting reproduction, these genes could be upregulated due to the fact that the female will not be reproducing. If amino acid stores are accessed during reproduction, in response to high metabolic demand, it follows that the stores may be built back up during periods when reproduction is not occurring.

To was originally characterized as a gene encoding a secretory protein that is mainly expressed in the fly head[31] and mediates circadian feeding behavior[32]. It is also expressed in organs related to feeding and olfaction and is upregulated in these structures in response to starvation, with *to* mutants dying shortly following the onset of starvation[31]. *To* expression may also be regulated by sex-specific factors. In the pericerebral fat body surrounding the brain, the male-specific forms of Doublesex (Dsx^M) and Fruitless (Fru^M) proteins activate *to* expression, but the female-specific form of Doublesex (Dsx^F) protein suppresses it[33]. This is consistent with our *to* TPM data from the male and female controls (Figure 4.6A). Moreover, *to* has been

associated with male courtship behavior, which significantly decreases upon feminization of fat body cells via expression of the female Transformer protein (Tra^F) in males[33,34]. To the best of our knowledge, the effect of social isolation on *to* expression has not been studied.

To confirm our RNA-seq analysis, we next used qPCR as a secondary method to validate the expression of *Lsp-2, Lsp-1a, Fbp1*, and *to* in male and female fly heads. Using remaining RNA from the same samples that we submitted for sequencing, we analyzed expression of *to* in SI males and SI females relative to their respective controls. The qPCR data largely confirmed the RNA-seq results. Overexpression of *to* following social isolation was specific to females and differed significantly from the gene expression change between isolated and control males (Figure 4.7A, $t(4) = 6.14$, $p = 0.004$). Although the sex differences in isolation/control relative expression did not reach statistical significance in *Lsp-2* and *Lsp-1a* (*Lsp-2*: Figure 4.7B, $t(4) = 2.56$, $p = 0.06$; *Lsp-1a:* Figure 4.7C, $t(4) = 2.21$, $p = 0.09$), this is likely due to small sample sizes ($n = 3$ samples/group) and large variances in the expression fold change in females. We also did not find a statistically significant sex difference between isolation-control expression for *Fbp1*, likely due to our small sample sizes and large variances in male and female expression fold change values (Figure 4.7D, $t(4) = 1.71$, $p = 0.16$). However, there may still be biologically meaningful differences such that *Lsp-2, Lsp-1a,* and *Fbp1* expression is elevated in socially isolated females relative to female controls more so than in socially isolated males versus male controls.

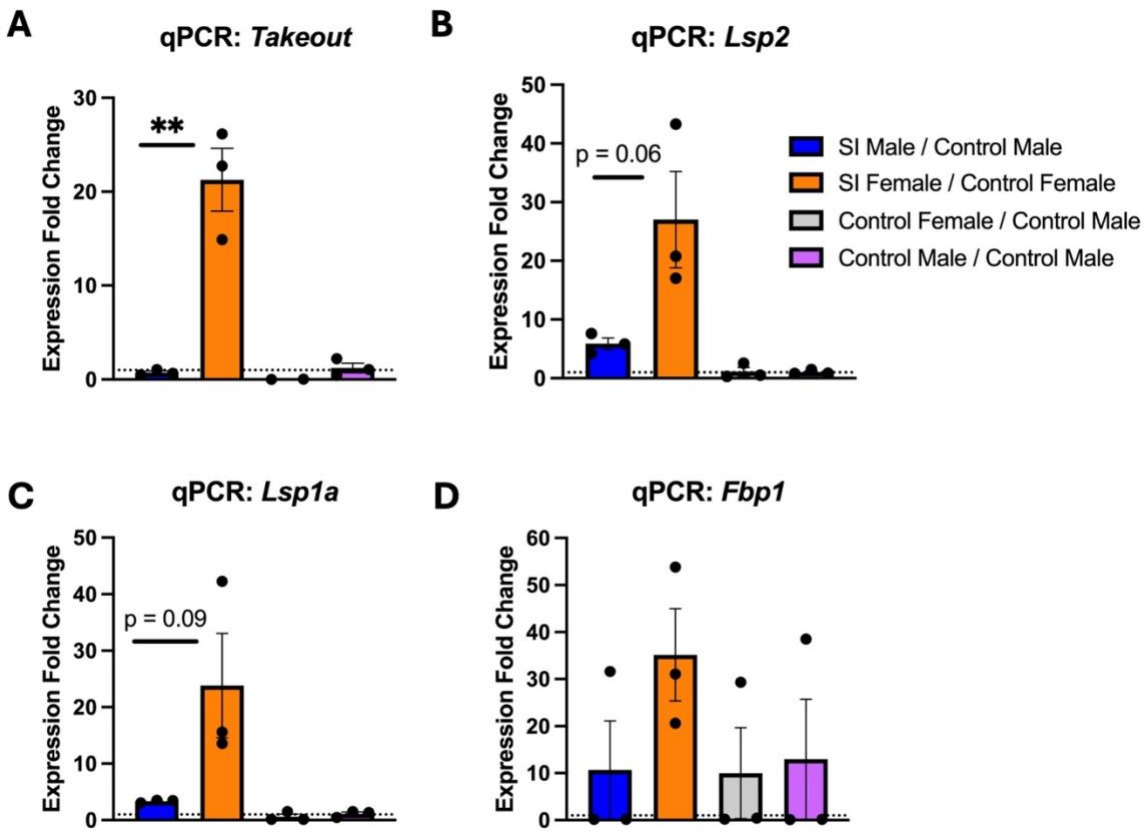

Figure 4.7. Validation of the RNA-seq-derived target genes with qPCR indicates enhanced expression of *takeout* in socially isolated females relative to isolated males. A) A comparison of relative expression fold change values reveals that *takeout* is overexpressed in females but not males following social isolation. The difference in expression between isolated and control flies is significantly greater in females versus males ($t(4) = 6.14$, $p = 0.004$). **B)** Social isolation enhances *Lsp-2* expression in males and females ($t(4) = 2.56$, $p = 0.06$). **C)** Social isolation enhances *Lsp-1α* expression in males and females ($t(4) = 2.21$, $p = 0.09$). **D)** We did not find a statistically significant male-female difference in expression of *Fbp1* following social isolation ($t(4) = 1.71$, $p = 0.16$). Data points represent individual cDNA samples for a particular condition relative to a control average. Error bars are ± SEM. $^{**}p < 0.01$.

4.2.3 qPCR of ORE-R brains suggests that social isolation increases expression of *takeout* in females but not males

Following RNA-seq profiling and validation, we identified *to* as a gene demonstrating female-specific overexpression in the head following social isolation. However, this finding raised the question of whether *to* expression was changing in the brain or elsewhere in the head. For instance, the sex difference in isolation-induced *to* expression could stem from

transcriptomic change in the pericerebral fat body, where *to* has already been established to be transcriptionally regulated in a sexually dimorphic manner, or in the antennae or esophagus. To gain a better understanding of whether *to* expression is changing within or outside of the fly brain, I dissected and extracted RNA from the brains of additional male and female flies that had experienced either social isolation or group housing (control conditions). The subsequent qPCR experiment again reflected female-specific overexpression of *to* following isolation (Figure 4.8, $t(10) = 2.28$, $p < 0.05$).



Figure 4.8. *Takeout* expression is elevated in the brains of females but not males following social isolation. Using relative expression fold change as a measure of the magnitude of change in *takeout* (*to*) expression between the social isolation and control groups, we observed that the values for males are randomly distributed around no change (1), while the female values are largely positive and significantly higher compared to males ($t(10) = 2.28$, $p < 0.05$). Data points represent individual cDNA samples for a particular condition relative to a control average. Error bars are ± SEM. *$p < 0.05$.

4.2.4 Adult *Drosophila* express *to* primarily in olfactory receptor neurons, epithelial cells of the gastrointestinal tract, and antennal nerve glia

In addition to validating our bulk RNA-seq results with qPCR, we also compared our findings with existing data from *Drosophila melanogaster* RNA-seq atlases. RNA-seq data from the modENCODE project[35] indicate that adult male *Drosophila* have higher whole organism-wide expression of *to* compared to adult females, which is consistent with our data comparing

males to control females. Based on data from the Fly Cell Atlas project[36], *to* was most highly expressed in (sensory) neurons, sensory organ cells, glial cells, and epithelial cells out of all adult cell types in the *Drosophila* body (Figure 4.9A). The prominence of *to* across many olfactory receptor neuron (ORN) subtypes (Figure 4.9B) suggests a potential function for *to* in regulating feeding through sensitivity to food odor. ORNs express chemoreceptors that bind odor molecules, and their axons synapse with projection neuron dendrites in antennal lobe glomeruli. Olfactory information is then transmitted through the circuit to higher order brain regions, the lateral horn and mushroom bodies[37]. *To* was also shown to be expressed in multiple epithelial cell types throughout the digestive system (Figure 4.9C), including the cells of the crop (the fly equivalent of the stomach), midgut, esophagus, hindgut, and enteroendocrine cells, which secrete hormones to regulate digestion, metabolic homeostasis, and feeding behavior[38]. Finally, in glial cells, *to* showed the most widespread expression in adult antennal nerve glia (Figure 4.9D). Antennal glial cells ensheath ORNs and may modulate the neuronal response to olfactory stimuli[39]. It is plausible that sex differences in *to* expression in any of these cell types could contribute to the differential response in sucrose consumption and food motivation between males and females following isolation stress. However, the replication of our whole head RNA-seq finding in isolated brains narrowed our focus to neurons and glia. Because glial cells only constitute approximately 10% of cells in the *Drosophila* nervous system[40], we decided to manipulate *to* expression using GAL4 driver lines specific to *to*-expressing cells and postmitotic neurons.

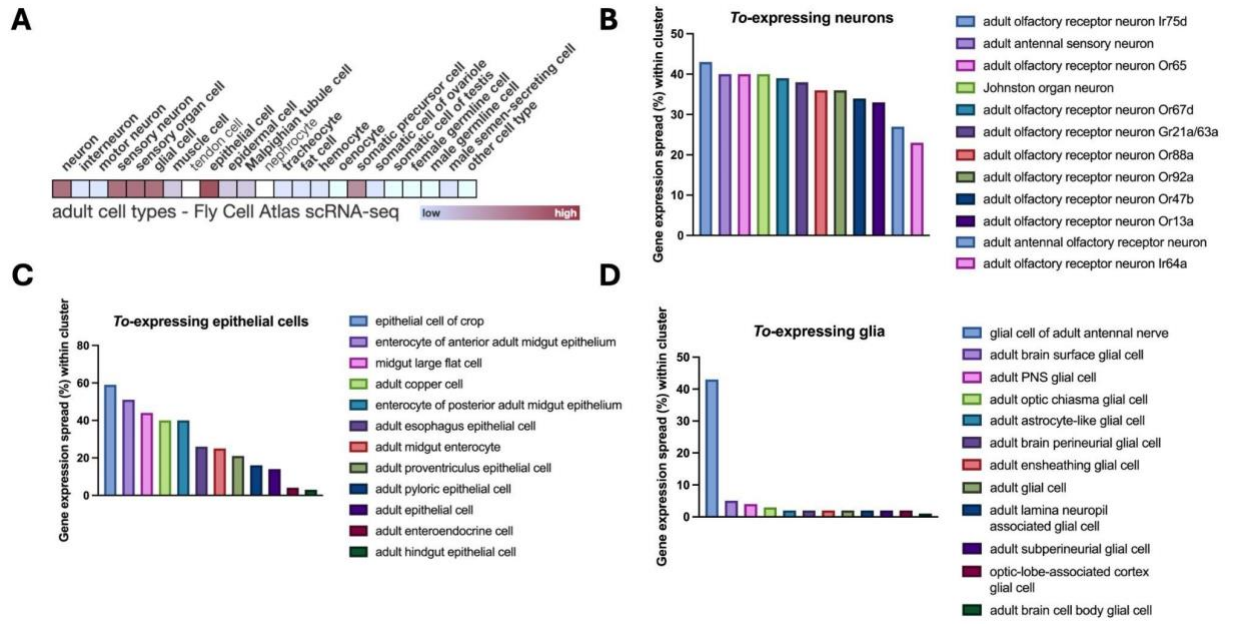

Figure 4.9. Single cell RNA-seq data from the Fly Cell Atlas identify *takeout* (*to*) expression in neuronal, glial, and epithelial cell types. A) Colors in the ribbon indicate that *to* expression was identified in that cell type through single nucleus RNA sequencing (snRNA-seq) performed by the Fly Cell Atlas project[36]. A redder color on the blue-red gradient indicates that a greater proportion of cells of that particular cell type express *to*. White means that there is no available snRNA-seq data available for that cell type. **B)** For neurons, *to* was expressed primarily in adult olfactory receptor neurons. **C)** For epithelial cells, *to* was expressed across numerous cell types of the gastrointestinal tract (crop, midgut, esophagus, and hindgut). **D)** For glial cells, *to* expression was most widespread within the glia of the adult antennal nerve.

4.2.5 Knocking down *to* in *to*-expressing cells decreases sucrose-feeding choice in socially isolated females and increases it in socially isolated males

In order to manipulate the expression of *to* in *to*-expressing cells, we mated virgin female flies with GAL4 expressed under the control of a *to*-specific promoter (*to*-Gal4) to males with a UAS enhancer 1) inducing the generation of RNA interference (RNAi) specifically targeted to *to* mRNA (UAS-to^{RNAi}), or 2) driving expression of *to* (UAS-*to*). We performed qPCR tests to validate that *to* expression was downregulated in *to*-Gal4xUAS-to^{RNAi} flies relative to ORE-Rs and upregulated in *to*-Gal4xUAS-*to* flies relative to ORE-Rs across experimental groups (Figure S4.1A). The offspring of the *to*-Gal4xUAS-to^{RNAi} mating were tested in the Value-Based

Feeding Decision (VBFD) test described in the previous chapter, to assess the effect of *to* knockdown in *to*-expressing cells on the proportion of flies that chose to feed on sucrose, a more nutritious sugar, over arabinose, a less nutritious sugar. The "proportion of all flies" dependent measure in the figures below represents the proportion of all flies in a test group that fed exclusively or almost exclusively on sucrose, compared to all other combinations of feeding choice (a sucrose-arabinose mixture, arabinose only, or neither). Sucrose is the optimal choice for nutritional value.

Control males, isolated males, control females, and isolated females of *to*-Gal4xUAS-to^{RNAi} and each parent genotype were tested. Unlike the sucrose feeding difference observed between control female and isolated female ORE-R flies (Figure 4.10A, $t(15) = 3.14$, $p = 0.007$), we did not see a difference in sucrose consumption between control and isolated females of the *to*-Gal4 genotype (Figure 4.10B, $t(14) = 0.57$, $p = 0.58$). However, in the other parent genotype, UAS-to^{RNAi}, there was a female-specific elevation in sucrose feeding in isolated females relative to controls (Figure 4.10C, $t(21) = 3.41$, $p = 0.003$) that resembled the pattern observed in wild type females. For the *to*-Gal4xUAS-to^{RNAi} flies, we observed an increase in sucrose feeding in isolated males compared to control males (Figure 4.10D, $t(14) = 2.31$, $p = 0.04$), but a decrease in sucrose feeding in isolated females relative to female controls (Figure 4.10D, $t(15) = 5.35$, $p < 0.0001$). This male isolation-induced increase in sucrose choice is specific to the knockdown manipulation and is not evident across either of the parent genotypes or the wild type. Moreover, knocking down *to* reverses the trend observed in isolated females relative to control females that is observed in the UAS-to^{RNAi} and ORE-R flies.

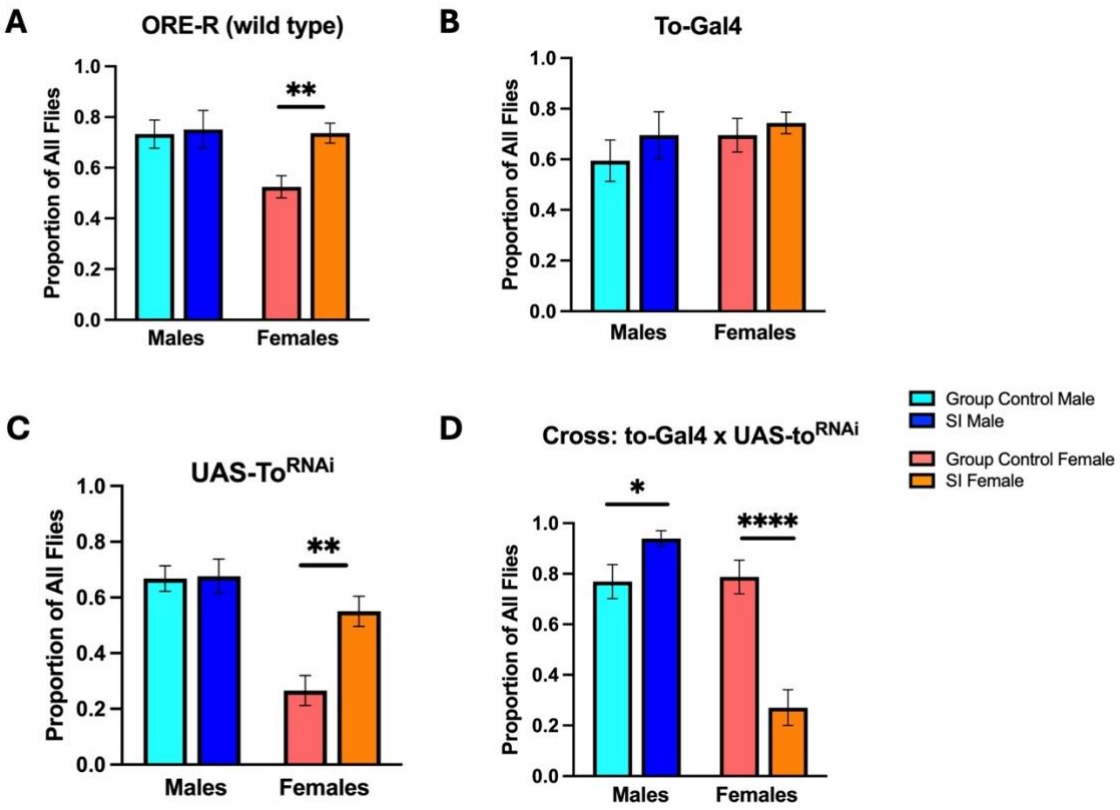

Figure 4.10. Knock down of *to* via RNA interference (RNAi) in *to*-expressing cells significantly decreases sucrose feeding choice in isolated females and increases sucrose feeding in isolated males relative to their respective controls. A) In ORE-R flies, our preliminary results indicated a female-specific increase in sucrose feeding following social isolation ($t(15) = 3.14$, $p = 0.007$). **B)** Flies expressing GAL4 under the control of a *to*-specific promoter do not show differences in sucrose feeding following social isolation ($t(14) = 0.57$, $p = 0.58$). **C)** For flies with a UAS enhancer sequence that induces RNAi targeted to *to* mRNA when activated, isolated females increase sucrose consumption relative to control females ($t(21) = 3.41$, $p = 0.003$). **D)** When RNAi targeted to *to* mRNA is induced in *to*-expressing cells, social isolation increases sucrose feeding in males ($t(14) = 2.31$, $p = 0.04$) yet decreases it in females ($t(15) = 5.35$, $p < 0.0001$) relative to respective controls. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

Upon examining the effect of genotype on sucrose consumption, within a specific experimental group, we found that *to*-Gal4xUAS-*to*^{RNAi} control females had a greater proportion of flies eating sucrose relative to ORE-R control females (Figure 4.11A, $t(18) = 3.41$, $p = 0.003$) and UAS-*to*^{RNAi} control females (Figure 4.11A, $t(22) = 6.07$, $p < 0.0001$). However, since the behavior only differed in the cross from one parent genotype and did not differ from the inert RNAi group, it was difficult to interpret an effect of *to* knockdown in control females. In isolated

females, the effect of the *to* knockdown was much clearer. *To*-Gal4xUAS-*to*^{RNAi} isolated females had a significantly *lower* proportion of sucrose-feeding flies compared to all other genotypes: ORE-R (Figure 4.11B, $t(12) = 5.24$, $p = 0.0002$), *to*-Gal4 (Figure 4.11B, $t(13) = 5.53$, $p < 0.0001$), UAS-*to*^{RNAi} (Figure 4.11B, $t(14) = 3.15$, $p = 0.007$), and *to*-Gal4 x UAS-mCherry^{RNAi} (Figure 4.11B, $t(12) = 3.84$, $p = 0.002$).

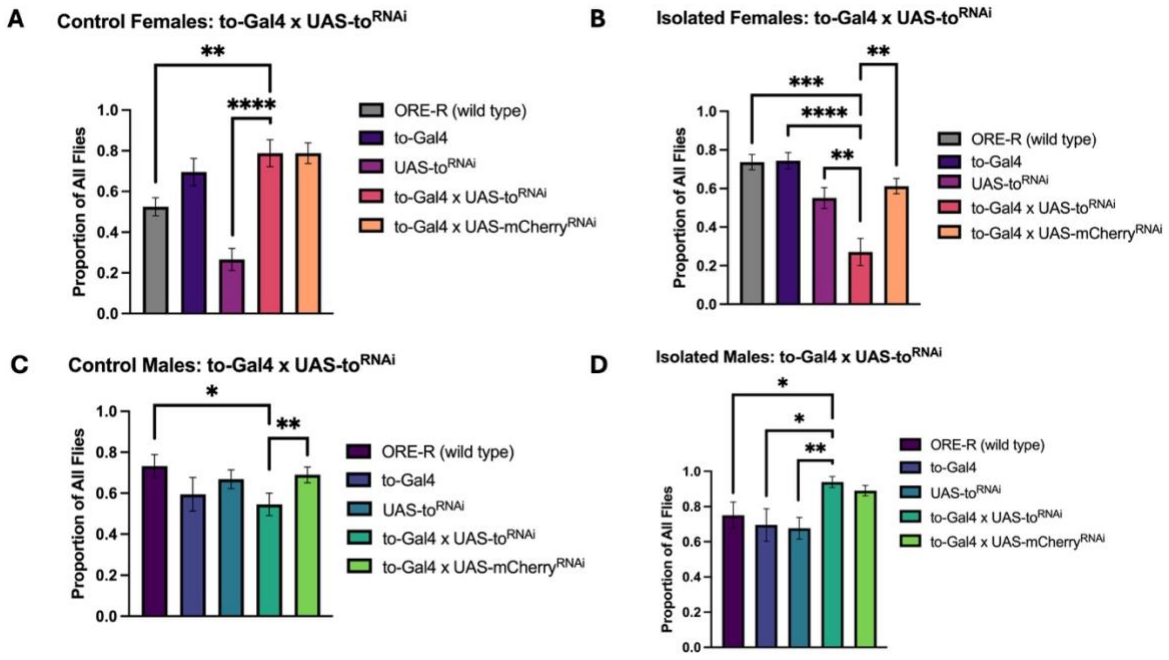

Figure 4.11. The proportion of all flies that primarily consumed sucrose changes in opposite directions, relative to other genotypes, between isolated females and isolated males upon knock down of *to* in *to*-expressing cells. A) Sucrose feeding is elevated in control females of the to-gal4 x UAS-to^{RNAi} cross relative to ORE-R ($t(18) = 3.41$, $p = 0.003$) and UAS-to^{RNAi} ($t(22) = 6.07$, $p < 0.0001$) control females but does not differ from to-Gal4 or to-Gal4 x UAS-mCherry^{RNAi} control females. **B)** Sucrose feeding is reduced in isolated females of the to-gal4 x UAS-to^{RNAi} cross relative to isolated females of all other genotypes: ORE-R ($t(12) = 5.24$, $p = 0.0002$), to-Gal4 ($t(13) = 5.53$, $p < 0.0001$), UAS-to^{RNAi} ($t(14) = 3.15$, $p = 0.007$), and to-Gal4 x UAS-mCherry^{RNAi} ($t(12) = 3.84$, $p = 0.002$). **C)** Sucrose feeding is reduced in control males of the to-gal4 x UAS-to^{RNAi} cross relative to ORE-R control males ($t(20) = 2.31$, $p = 0.03$) and to-Gal4 x UAS-mCherry^{RNAi} control males ($t(14) = 3.89$, $p = 0.002$) but does not differ from parent genotype control males. **D)** Sucrose feeding increases in isolated males of the to-gal4 x UAS-to^{RNAi} cross relative to wild type and parent genotype isolated males: ORE-R ($t(16) = 2.14$, $p < 0.05$), to-Gal4 ($t(15) = 2.39$, $p = 0.03$), and UAS-to^{RNAi} ($t(14) = 3.84$, $p = 0.002$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Regarding control males, *to* knockdown decreased sucrose feeding choice in *to*-gal4 x UAS-*to*^{RNAi} flies compared to ORE-Rs (Figure 4.11C, $t(20) = 2.31$, $p = 0.03$) and *to*-Gal4 x

UAS-mCherry^{RNAi} flies (Figure 4.11C, t(14) = 3.89, p = 0.002). However, the knockdown manipulation in control males did not produce a change in behavior relative to either parent genotype. In isolated males, the *to*-gal4 x UAS-*to*^{RNAi} flies increased their proportion of sucrose feeding relative to both parent genotypes (*to*-Gal4: Figure 4.11D, t(15) = 2.39, p = 0.03; UAS-*to*^{RNAi}: Figure 4.11D, t(14) = 3.84, p = 0.002) and the ORE-Rs (Figure 4.11D, t(16) = 2.14, p < 0.05).

4.2.6 Overexpressing *to* in *to*-expressing cells decreases sucrose feeding in isolated females, but not males, relative to the ORE-R and parent genotypes

As alluded to earlier, we also tested the offspring of *to*-Gal4 flies crossed with UAS-*to*, in order to drive *to* expression. We found that, similar to the result observed in the ORE-Rs (Figure 4.12A, t(15) = 3.14, p = 0.007) but different from that of the *to*-Gal4 flies (Figure 4.12B, t(14) = 0.57, p = 0.58), a greater proportion of UAS-*to* females ate sucrose following social isolation (Figure 4.12C, t(14) = 7.86, p < 0.0001). Within the *to*-Gal4 x UAS-*to* genotype, isolation did not seem to affect sucrose choice in either sex (males: Figure 4.12D, t(14) = 0.30, p = 0.77; females: Figure 4.12D, t(15) = 1.59, p = 0.13).

When comparing across genotypes, sucrose choice was only elevated in control female *to*-Gal4 x UAS-*to* relative to UAS-*to* control females (Figure 4.13A, t(16) = 4.50, p = 0.0004). In isolated females, sucrose feeding choice was dampened in *to*-Gal4 x UAS-*to* compared to ORE-Rs (Figure 4.13B, t(11) = 5.54, p = 0.0002) and both parent genotypes (*to*-Gal4: Figure 4.13B, t(12) = 5.65, p = 0.0001; UAS-*to*: Figure 4.13B, t(13) = 2.74, p = 0.02). In both control (Figure 4.13C, t(18) = 3.91, p = 0.001) and isolated males (Figure 4.13D, t(14) = 3.31, p = 0.005), sucrose feeding choice was increased in *to*-Gal4 x UAS-*to* flies compared to UAS-*to* males but did not differ from *to*-Gal4 or ORE-R flies of the same experimental condition.

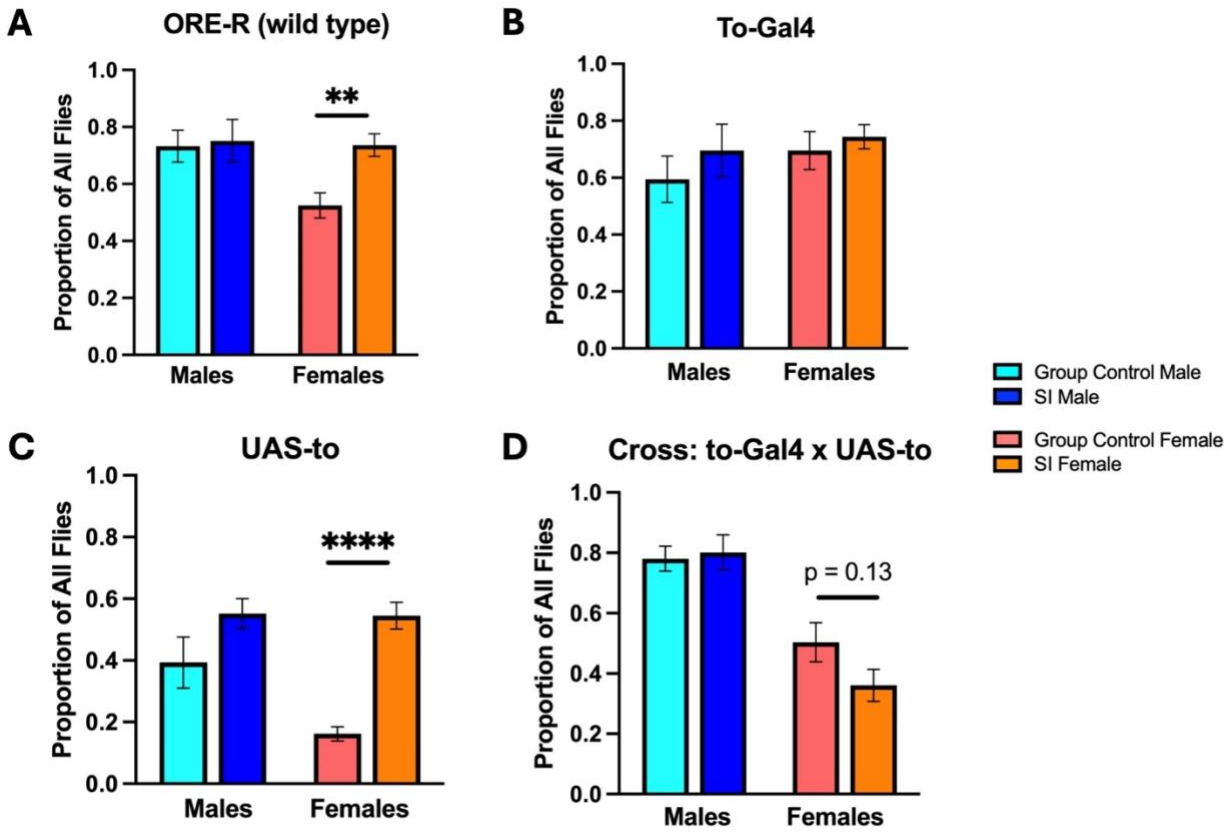

Figure 4.12. Overexpression of *to* in *to*-expressing cells results in no differences in sucrose feeding choice between isolated flies and their respective controls. A) In ORE-R flies, our preliminary results indicated a female-specific increase in sucrose feeding following social isolation ($t(15) = 3.14$, $p = 0.007$). **B)** Flies expressing GAL4 under the control of a *to*-specific promoter do not show differences in sucrose feeding following social isolation. **C)** For flies with a UAS enhancer sequence that induces *to* expression when activated, isolated females increase sucrose consumption relative to control females ($t(14) = 7.86$, $p < 0.0001$). **D)** When *to* expression is induced in *to*-expressing cells, social isolation does not alter sucrose feeding in males ($t(14) = 0.30$, $p = 0.77$) or in females ($t(15) = 1.59$, $p = 0.13$) relative to respective controls. $^{**}p < 0.01$, $^{****}p < 0.0001$.

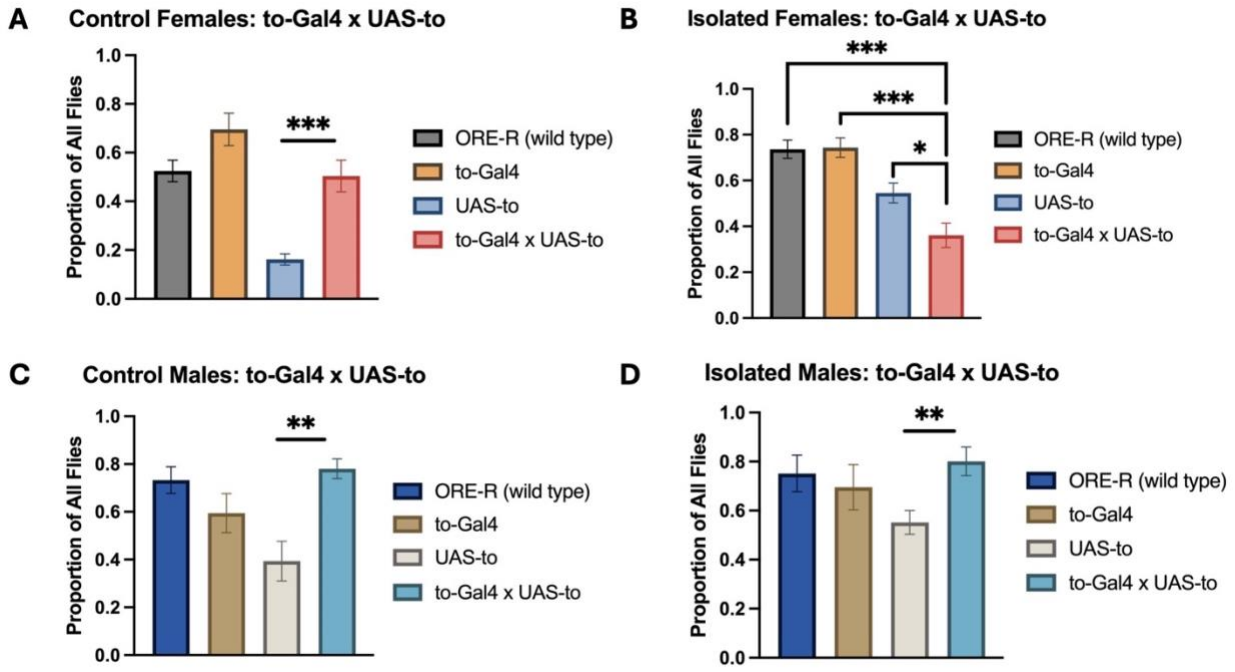

Figure 4.13. The proportion of all flies that primarily consumed sucrose decreases relative to other genotypes in isolated females upon overexpression of *to* in *to*-expressing cells. A) Sucrose feeding is elevated in control females of the to-gal4 x UAS-to cross relative to UAS-to control females ($t(16) = 4.50$, $p = 0.0004$) but does not differ from ORE-R or to-Gal4 control females. **B)** Sucrose feeding is reduced in isolated females of the to-gal4 x UAS-to cross relative to isolated females of the wild type and parent genotypes: ORE-R ($t(11) = 5.54$, $p = 0.0002$), to-Gal4 ($t(12) = 5.65$, $p = 0.0001$), and UAS-to ($t(13) = 2.74$, $p = 0.02$). **C)** Sucrose feeding is enhanced in control males of the to-gal4 x UAS-to cross relative to UAS-to control males ($t(18) = 3.91$, $p = 0.001$) but does not differ from wild type or to-Gal4 control males. **D)** Sucrose feeding is elevated in isolated males of the to-gal4 x UAS-to cross relative to UAS-to isolated males ($t(14) = 3.31$, $p = 0.005$) but does not differ from wild type or to-Gal4 control males. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.2.7 Driving *to* expression in neurons increases sucrose-feeding in males

After observing sex differences in sucrose feeding upon *to* manipulation in all *to*-expressing cells, we also wanted to down- and upregulate expression of *to* in neurons. Given that our qPCR results from extracted brains corroborated female-specific upregulation of *to* following chronic social isolation, and the single cell RNA-seq atlas data indicated *to* present across several olfactory receptor neurons, we thought that neurons would be a logical target to identify whether changes in *to* expression in the brain itself are generating sexual dimorphism in feeding behavior.

We began by mating *elav*-Gal4 virgin female flies with UAS-*to*^{RNAi} males to generate flies expressing RNAi targeted to *to* in postmitotic neurons. The elav-Gal4 line is commonly used in *Drosophila* research to drive the expression of GAL4 under control of the *elav* gene, which is active in postmitotic neurons throughout development and is expressed throughout most cells of the fly nervous system[41,42]. Via qPCR, we validated that *to* expression was downregulated in *elav*-Gal4xUAS-*to*^{RNAi} flies relative to ORE-Rs across experimental groups (Figure S4.1B).

Although isolated females of the ORE-R (Figure 4.14A, $t(15) = 3.14$, $p = 0.007$) or UAS-*to*^{RNAi} (Figure 4.14C, $t(21) = 3.41$, $p = 0.003$) genotypes demonstrate a higher proportion of flies choosing sucrose compared to control females, social isolation does not affect sucrose feeding choice in *elav*-Gal4 females or males (Figure 4.14B). We also did not observe any differences in sucrose-feeding following social isolation in male or female offspring of the elav-Gal4 x UAS-*to*^{RNAi} cross (Figure 4.14D).

An examination of within-experimental group effects of genotype on sucrose feeding choice also failed to show an effect of *to* knock down in neurons on behavior. Although elav-Gal4 x UAS-*to*^{RNAi} control females increased sucrose feeding compared to ORE-R (Figure 4.15A, $t(16) = 2.25$, $p = 0.04$) and UAS-*to*^{RNAi} (Figure 4.15A, $t(20) = 4.72$, $p = 0.0001$) control females, there was no difference relative to the other parent genotype, *elav*-Gal4. Across isolated females (Figure 4.15B), control males (Figure 4.15C), and isolated males (Figure 4.15D), the elav-Gal4 x UAS-*to*^{RNA} flies did not differ in their feeding behavior from any other experimental control group (the wild type and parent genotypes) in a statistically significant manner.

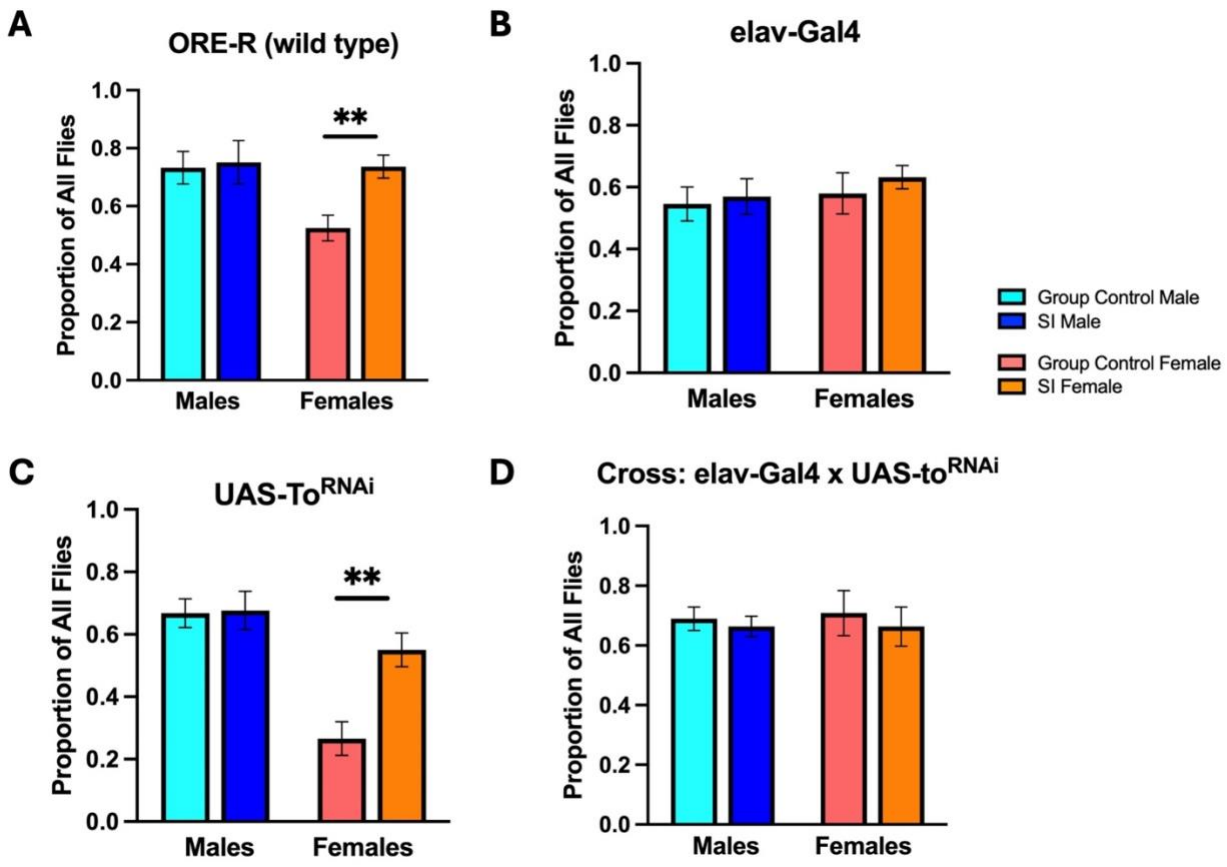

Figure 4.14. Knock down of *to* in neurons does not alter sucrose feeding across experimental groups. A) In ORE-R flies, females increased their sucrose feeding following social isolation ($t(15) = 3.14$, $p = 0.007$). **B)** Flies expressing GAL4 under the control of an *elav*-specific promoter do not show differences in sucrose feeding following social isolation in either sex. **C)** For flies with a UAS enhancer sequence that induces RNAi targeted to *to* mRNA when activated, isolated females increase sucrose consumption relative to control females ($t(21) = 3.41$, $p = 0.003$). **D)** When RNAi targeted to *to* mRNA is induced in neurons, there is no difference in sucrose feeding choice between socially isolated and control flies of either sex. $^{**}p < 0.01$.

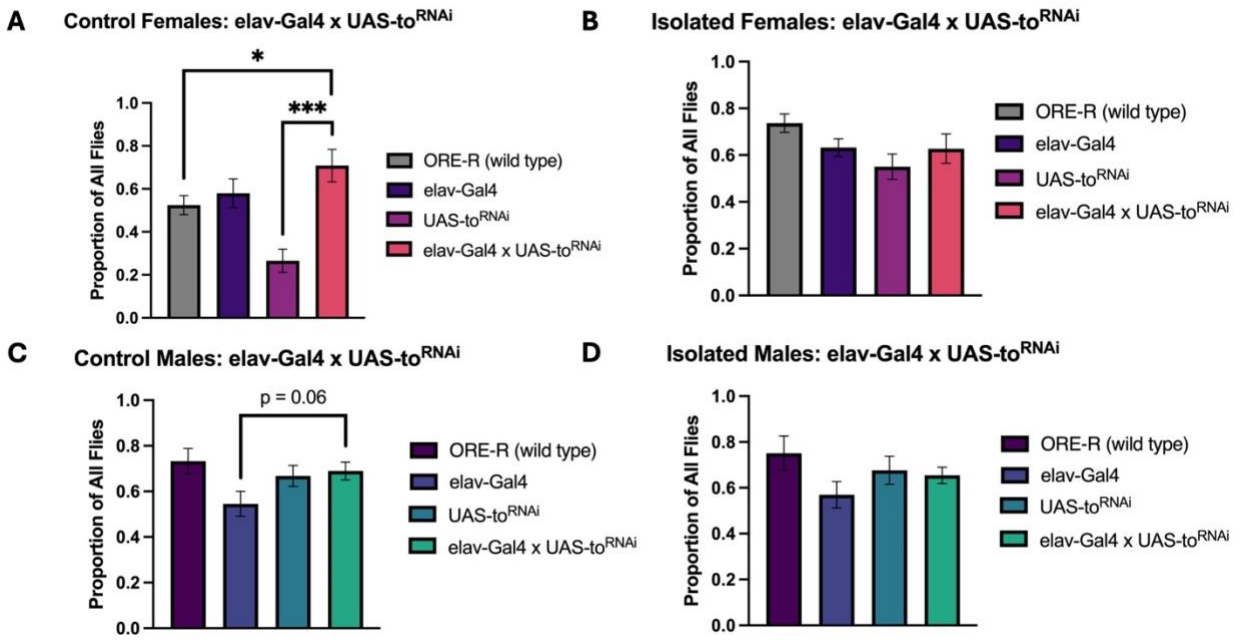

Figure 4.15. The proportion of all flies that primarily consumed sucrose does not change in any experimental groups upon knock down of *to* in neurons. A) Sucrose feeding is elevated in control females of the elav-Gal4 x UAS-to^{RNAi} cross relative to ORE-R ($t(16) = 2.25$, $p = 0.04$) and UAS-to^{RNAi} ($t(20) = 4.72$, $p = 0.0001$) control females but does not differ from elav-Gal4 control females. **B)** Sucrose feeding does not differ between elav-Gal4 x UAS-to^{RNAi}, wild type, and parent genotype isolated females. **C)** Sucrose feeding does not differ between elav-Gal4 x UAS-to^{RNAi}, wild type, and parent genotype control males. **D)** Sucrose feeding does not differ between elav-Gal4 x UAS-to^{RNAi}, wild type, and parent genotype isolated males. $^{**}p < 0.01$, $^{***}p < 0.001$.

Next, we set up elav-Gal4 x UAS-to matings to assess behavioral effects of *to* overexpression in neurons. In UAS-to flies, there was a female-specific increase in sucrose-feeding in isolated females relative to control females (Figure 4.16C, $t(14) = 7.86$, $p < 0.0001$) that resembled the pattern seen in the wild type isolated and control females (Figure 4.16A, $t(15) = 3.14$, $p = 0.007$). When we tested the offspring of the elav-Gal4 x UAS-to mating, we no longer saw an effect of isolation on sucrose choice in females (Figure 4.16D, $t(9) = 0.87$, $p = 0.41$), similar to the pattern noted in the elav-Gal4 females (Figure 4.16B, $t(18) = 0.69$, $p = 0.50$).

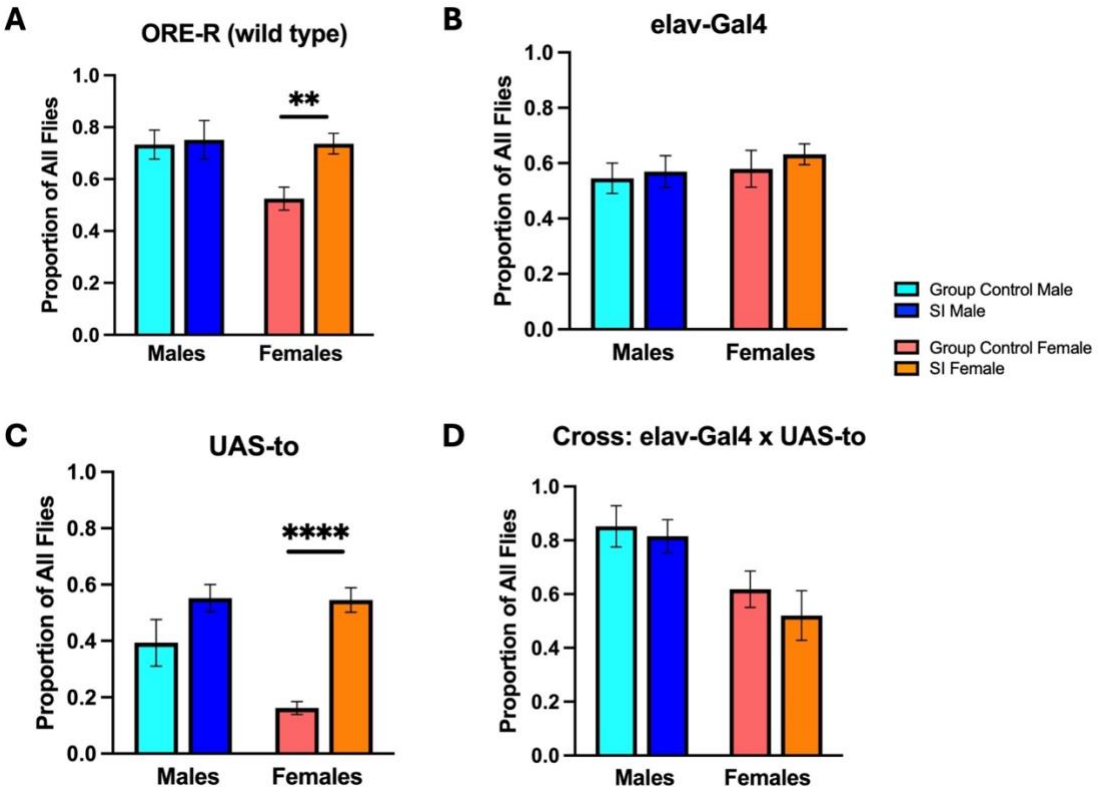

Figure 4.16. Overexpression of *to* in neurons does not yield sucrose feeding differences between isolated and control flies of either sex. A) In ORE-R flies, females increased their sucrose feeding following social isolation ($t(15) = 3.14$, $p = 0.007$). **B)** Flies expressing GAL4 under the control of an *elav*-specific promoter do not show differences in sucrose feeding following social isolation in either sex (males: $t(16) = 0.30$, $p = 0.77$; females: $t(18) = 0.69$, $p = 0.50$). **C)** For flies with a UAS enhancer sequence that induces *to* expression when activated, isolated females increase sucrose consumption relative to control females ($t(14) = 7.86$, $p < 0.0001$). **D)** When *to* expression is driven in neurons, there is no difference in sucrose feeding choice between socially isolated and control flies of either sex ($t(9) = 0.87$, $p = 0.41$). $^{**}p < 0.01$, $^{****}p < 0.0001$.

However, for within-experimental group effects of genotype on sucrose feeding choice, we noted a behavioral sexual dimorphism independent of social housing conditions. In control females, elav-Gal4 x UAS-to flies had an enhanced sucrose choice relative to UAS-to control females (Figure 4.17A, $t(12) = 7.15$, $p < 0.0001$), yet there was no difference between elav-Gal4 x UAS-to and ORE-R (Figure 4.17A, $t(15) = 1.20$, $p = 0.25$) or elav-Gal4 flies (Figure 4.17A, $t(14) = 0.38$, $p = 0.71$). In isolated females, sucrose-feeding was only depressed in elav-Gal4 x UAS-to flies compared to ORE-R flies (Figure 4.17B, $t(9) = 2.30$, $p < 0.05$). There were no

behavioral differences between isolated female elav-Gal4 x UAS-to flies and isolated females of the parent genotypes (*elav*-Gal4: t(13) =1.35, p = 0.20; UAS-*to*: t(11) = 0.28, p = 0.79). Yet, in males, both control flies (Figure 4.17C) and socially isolated flies (Figure 4.17D) increased sucrose-feeding relative to both parent genotypes. Thus, overexpressing *to* in postmitotic neurons seems to increase sucrose feeding choice in males, but not females, irrespective of social housing or isolation.

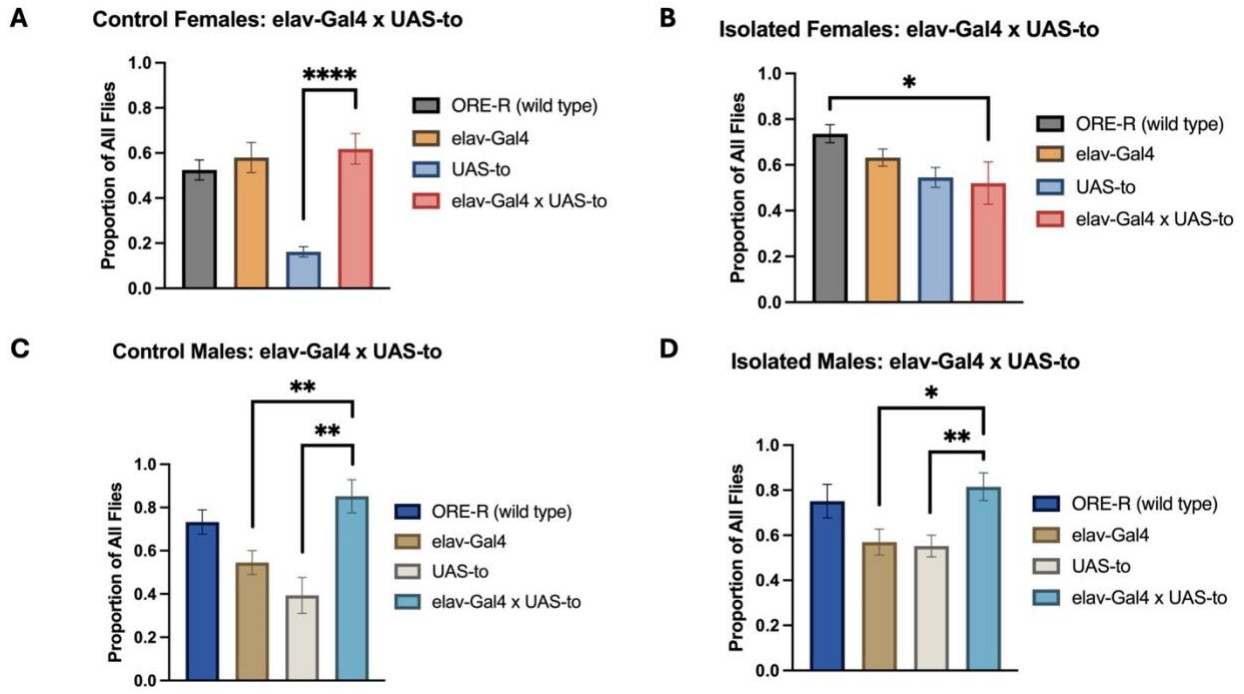

Figure 4.17. The proportion of all flies that primarily consumed sucrose increases relative to parent genotypes in control males and isolated males upon overexpression of *to* in neurons. A) Sucrose feeding is elevated in control females of the elav-gal4 x UAS-to cross relative to UAS-to control females (t(12) = 7.15, p < 0.0001). **B)** Sucrose feeding is reduced in isolated females of the elav-gal4 x UAS-to cross relative to ORE-R isolated females (t(9) = 2.30, p < 0.05). **C)** Sucrose feeding is enhanced in control males of the elav-gal4 x UAS-to cross relative to UAS-to control males (t(15) = 3.63, p = 0.003) and elav-Gal4 control males (t(13) = 3.33, p = 0.005). **D)** Sucrose feeding is also elevated in isolated males of the elav-gal4 x UAS-to cross relative to UAS-to isolated males (t(12) = 3.30, p = 0.006) and elav-Gal4 isolated males (t(12) = 2.71, p = 0.02). *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

4.2.8 Masculinizing female neurons via RNAi targeted to *transformer (tra)* does not alter sucrose feeding choice

Finally, we wanted to test whether altering the sexual identity of neurons to make female neurons more male-like affected sucrose feeding choice. To do this, we crossed elav-Gal4 virgin females with UAS-transformer (tra)^{RNAi} males. This genetic manipulation has been used previously as a tool to masculinize neurons in female flies[43]. The UAS-tra^{RNAi} line uses double-stranded RNA to knock down *transformer* (*tra*), a gene in the *Drosophila* sex determination pathway that is crucial for female somatic sexual differentiation[44]. In female flies only, the gene *Sex-lethal* (*Sxl*) produces a functional protein, which then splices *tra* as a downstream target. This yields a functional Tra protein in females but not males, which then performs sex-specific alternative mRNA splicing on *doublesex* and *fruitless*[45,46,47,48,49,50]. Sex-specific forms of *doublesex* and male *fruitless* then act as transcription factors to influence sex-specific development of somatic and neural tissues and behavior[51,52,53]. Hence, we were interested in knocking down *tra* in neurons, to assess whether this manipulation made sucrose feeding choice more "male-like" in females. We used qPCR to validate that *tra* expression was downregulated in *elav*-Gal4xUAS-*tra*^{RNAi} flies relative to ORE-Rs across experimental groups (Figure S4.1C).

Overall, we observed increases in sucrose-feeding in isolated males and isolated females relative to their respective controls in the UAS-tra^{RNAi} flies (Figure 4.18C, males: $t(14) = 3.91$, $p = 0.002$; females: $t(14) = 2.79$, $p = 0.01$) but not in the elav-Gal4 x UAS-tra^{RNAi} offspring (Figure 4.18D, males: $t(17) = 1.42$, $p = 0.17$; females: $t(17) = 0.91$, $p = 0.38$).

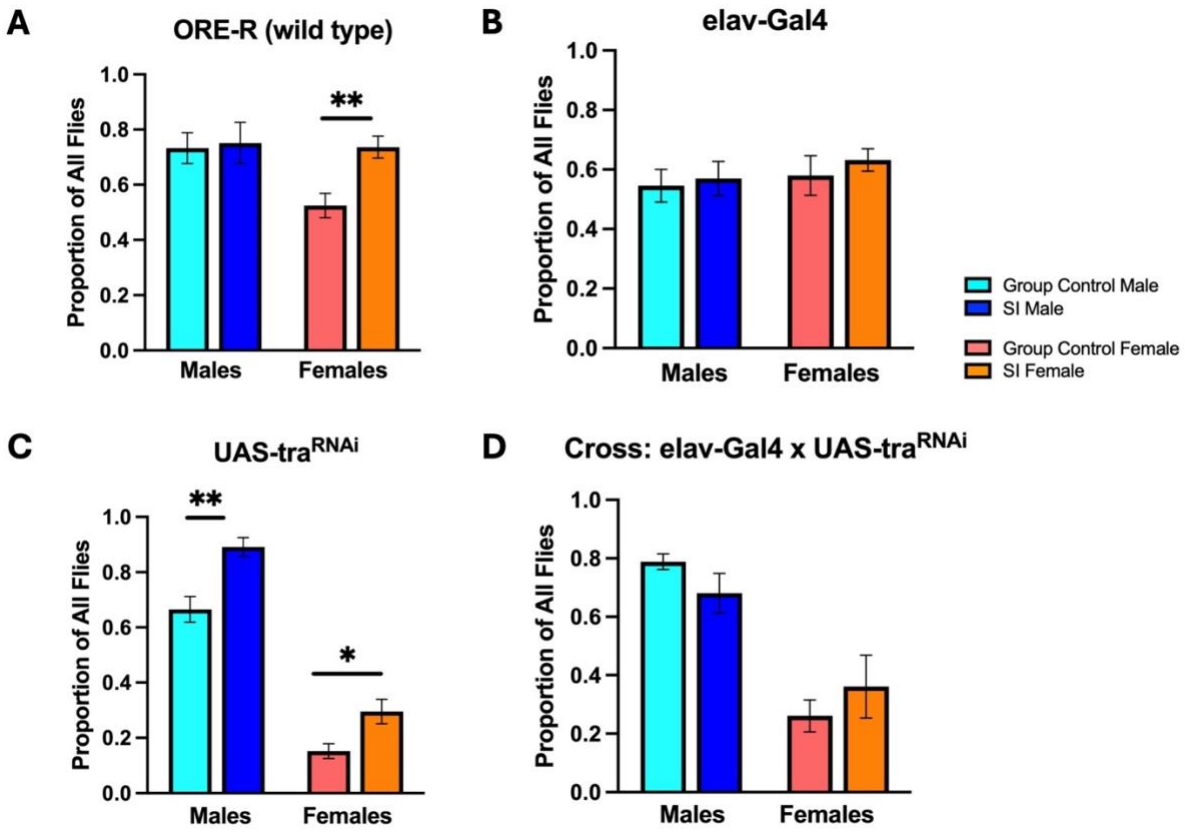

Figure 4.18. Isolated males and isolated females show no differences in sucrose-feeding relative to controls following knock down of *tra* in neurons. A) In ORE-R flies, sucrose feeding is higher in isolated females compared to control females ($t(15) = 3.14$, $p = 0.007$). **B)** Flies expressing GAL4 under the control of an *elav*-specific promoter do not show differences in sucrose feeding following social isolation in either sex. **C)** For flies with a UAS enhancer sequence that induces RNAi targeted to *tra* mRNA when activated, isolated females ($t(14) = 2.79$, $p = 0.01$) and isolated males ($t(14) = 3.91$, $p = 0.002$) increase sucrose consumption relative to their respective controls. **D)** When RNAi targeted to *tra* is induced in neurons, there is no difference in sucrose feeding choice between socially isolated and control flies of either sex (males: $t(17) = 1.42$, $p = 0.17$; females: $t(17) = 0.91$, $p = 0.38$). *$p < 0.05$, **$p < 0.01$.

Within-condition comparisons across genotypes also did not reflect any clear differences in sucrose feeding choice upon *tra* knock down in neurons. We did not see any differences across all genotypes in control females (Figure 4.19A) or isolated males (Figure 4.19D). In isolated females, sucrose-feeding decreased in elav-Gal4 x UAS-tra^{RNAi} flies relative to ORE-R (Figure 4.19B, $t(10) = 2.54$, $p = 0.03$) and elav-Gal4 flies (Figure 4.19B, $t(14) = 2.19$, $p < 0.05$) but did not change when compared to the other parent genotype. In control males, there was only an elevation in sucrose-feeding in the elav-Gal4 x UAS-tra^{RNAi} flies relative to the elav-Gal4

group (Figure 4.19C, $t(13) = 2.61$, $p = 0.02$). Interestingly, qPCR validation revealed that knockdown of *tra* resulted in decreased expression of *to* across all experimental groups relative to ORE-R levels (Figure S4.1D). However, since knocking down *to* expression in neurons through the elav-Gal4 x UAS-to^{RNAi} manipulation did not yield any changes in sucrose-feeding, it is consistent that decreasing *to* expression through neuron masculinization similarly did not produce clear changes in behavior.

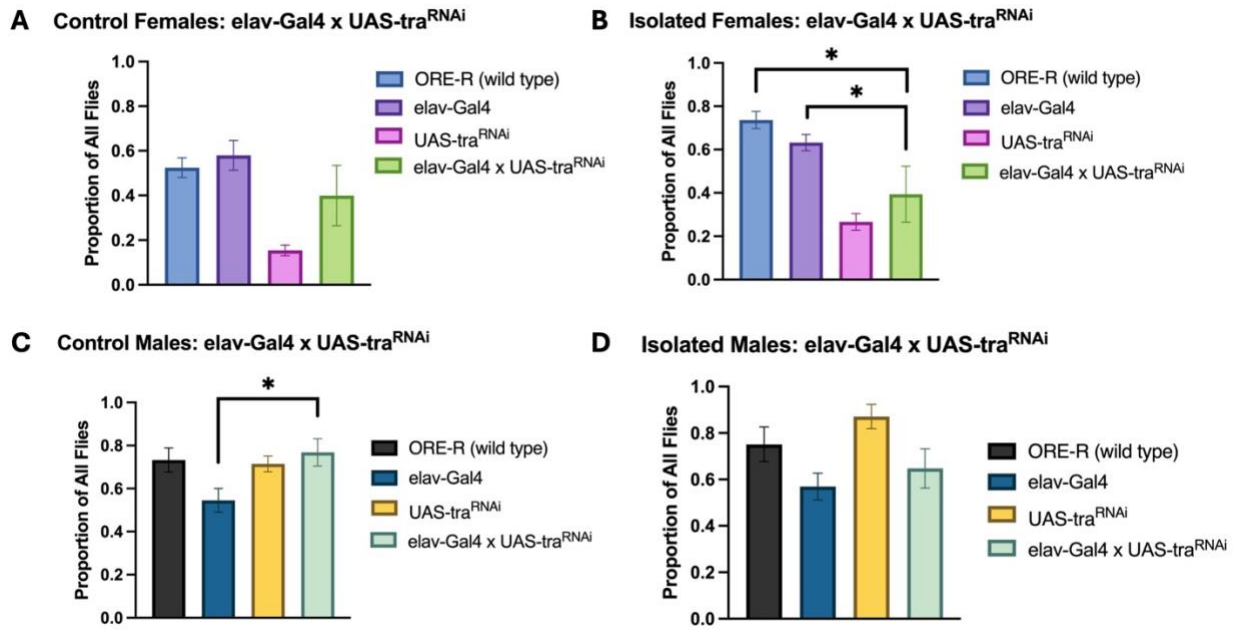

Figure 4.19. The proportion of all flies that primarily consumed sucrose does not differ between elav-Gal4 x UAS-tra^{RNAi} flies and other genotypes, across experimental groups. A) No significant differences were detected in sucrose feeding between elav-gal4 x UAS-tra^{RNAi} control females and control females of the wild type and parent genotypes. **B)** Sucrose feeding is reduced in isolated females of the elav-gal4 x UAS-tra^{RNAi} cross relative to ORE-R ($t(10) = 2.54$, $p = 0.03$) and elav-Gal4 isolated females ($t(14) = 2.19$, $p < 0.05$). **C)** Sucrose feeding is elevated in elav-gal4 x UAS-tra^{RNAi} control males relative to elav-Gal4 control males ($t(13) = 2.61$, $p = 0.02$). **D)** No significant differences were detected in sucrose feeding between elav-gal4 x UAS-tra^{RNAi} isolated males and isolated males of the wild type and parent genotypes. *$p < 0.05$.

Hence, through these genetic manipulations, we found that either up- or downregulating *to* in *to*-expressing cells decreases sucrose feeding choice in socially isolated females. However, these same manipulations produce either increases or no changes in sucrose-feeding in socially isolated males. Altering gene expression of *to* in neurons does not seem to generate changes in

sucrose-feeding in females, but *to* overexpression in neurons may increase sucrose-feeding in males, regardless of social condition. Masculinizing neurons did not produce changes in sucrose feeding behavior across experimental groups, but it did abrogate the feeding difference that we see between ORE-R isolated females and control females. A complete summary of our manipulations and behavioral findings is provided below (Table 4.2).

Table 4.2: Summary of behavioral results in VBFD test across genetic manipulations and experimental groups

Effect of manipulation on sucrose feeding (yellow = up, grey = no change, blue = down)				
	Control Males	Social Isolation Males	Control Females	Social Isolation Females
to^{RNAi} in *to*-expressing cells (to-Gal4 x UAS-to^{RNAi})				
to overexpression in *to*-expressing cells (to-Gal4 x UAS-to)				
to^{RNAi} in neurons (elav-Gal4 x UAS-to^{RNAi})				
to overexpression in neurons (elav-Gal4 x UAS-to)				
tra^{RNAi} in neurons (elav-Gal4 x UAS-tra^{RNAi})				

4.3 Discussion

4.3.1 Summary

In this set of experiments, we used RNA-seq as an unbiased approach for target identification. We sought to understand how social isolation impacts gene expression, and subsequently behavior, differentially in male versus female flies. Our differential expression analysis revealed that *to* is upregulated in females but not males following social isolation

experience. We were able to validate our RNA-seq result with qPCR of both the original samples and new samples derived from extracted brains.

Upon manipulating *to* expression with various GAL4-UAS combinations, we found that knocking down *to* in *to*-expressing cells decreases sucrose feeding in isolated females relative to control females of the same genotype but increases sucrose feeding in isolated males relative to same-genotype control males. Within-condition comparisons supported these findings, in that there were effects of genotype on sucrose feeding. For isolated females, offspring of the *to*-Gal4 x UAS-to^{RNAi} parents showed *decreases* in sucrose consumption compared to ORE-R (wild type) flies, flies of each parent genotype, and the offspring of *to*-Gal4 x UAS-$mCherry^{RNAi}$ parents. This group was used as an inert RNAi control. For isolated males, offspring of the *to*-Gal4 x UAS-to^{RNAi} parents showed *increases* in sucrose consumption compared to ORE-Rs and flies of each parent genotype.

In our model that aimed to overexpress *to* in *to*-expressing cells, we did not see an effect of condition (social isolation) on sucrose choice in either sex within the *to*-Gal4 x UAS-*to* offspring. However, when broken down by experimental condition and sex, there was an effect of genotype in isolated females such that *to*-Gal4 x UAS-*to* isolated females consumed less sucrose relative to ORE-R and parent genotype isolated females.

Taken together, these results suggest that takeout may function like a neuromodulator in isolated females; when either downregulated or upregulated in *to*-expressing cells, in the context of social isolation, sucrose feeding choice is suppressed. Interestingly, we see the opposite behavioral response in males. Isolated males increase sucrose consumption when *to* is knocked down and at least maintain their level of consumption when it is overexpressed in *to*-expressing cells.

When we manipulated the expression of *to* in neurons, we observed no effects of *to* knock down in any experimental groups, but *to* overexpression increased sucrose-feeding exclusively in males regardless of isolation or control status. Validation via qPCR indicated that the overexpression model may not have worked (Figure S4.1B); compared to ORE-R expression of *to*, the elav-Gal4 x UAS-to flies showed expression that was either the same or slightly decreased. The failure of this experiment could be due to technical error or an actual neurobiological phenomenon, such as *to* overexpression in neurons killing the cells. The cause is presently unclear and necessitates the experiment being repeated. *To* expression *was* enhanced in elav-Gal4 x UAS-to flies relative to both parent genotypes in males only (Figure S4.1E and Figure S4.1F), which may be consistent with sucrose-feeding increasing in males, but not females, in elav-Gal4 x UAS-to flies relative to parent genotypes (but not ORE-R).

Masculinizing neurons in females via *tra* knock down did not produce differences in feeding behavior in either control or isolated flies relative to other genotypes, but we no longer observed the female-specific elevation in sucrose-feeding following social isolation in masculinized females that we see in ORE-R females.

In the future, it would be worthwhile to try masculinizing the fat body instead of neurons, if we believe the pericerebral fat body is where the sex difference in isolation-induced *to* expression may be influencing feeding behavior. *Tra* has been found to control male-female differences in fat storage in *Drosophila* by regulating adipokinetic hormone producing cells and the pathways in which they participate in a sex-specific manner[54]. Another study established that feminizing the fat body in adult male *Drosophila* decreases both courtship behavior and the levels of Takeout protein in the head[34]. As many as 46 sex-biased genes have been identified in the fly head, with some being localized to the fat cells[55]. These sex-specific transcripts in the

head fat body may encode protein products, which are secreted and act on the brain to generate sex differences in behavior. Moreover, manipulations of *tra* and its cofactor *tra-2* in the context of starvation or oxidative stress have been shown to reverse the expected male versus female stress response (measured by heart rate and locomotor patterns)[56]. These results suggest that effectors produced in the fat body may influence sexually dimorphic actions in the brain outside of its own transcriptome.

A recent paper identified a population of *to*-expressing gustatory receptor neurons (GRNs) which also express *CCKLR-17D3*, one of my initial fly "anxiety" candidate genes, and *Gr64f*, which encodes a gustatory receptor for sugar[14]. These researchers proposed a model in which food intake triggers the release of Dsk from MP1/MP3 neurons, which then binds to CCKLRs on GRNs to increase expression of *to* and decrease the expression of *Gr64f*. This functions to decrease GRN responsivity to sweet sensation in order to decrease food ingestion. Interestingly, these MP1/MP3 neurons have reciprocal connections with P1 neurons. It has been found that MP1/MP3 cells can synapse with P1 neurons to dampen male sexual behavior, and that MP1/MP3 neurons may regulate mating behavior by integrating external, sex-related sensory cues with factors such as age, feeding, and housing conditions[16]. P1 neurons project back to MP1/MP3 cells to modulate male aggression[13]. MP1/MP3 and P1 both express Fru^{M}, the male-specific form of fruitless, and P1 expresses *Dsx*. Although this model is somewhat consistent with the data from my isolated males upon *to* knockdown in *to*-expressing cells, it does not explain why *to* upregulation in socially isolated females would produce *increases* in sucrose feeding in wild type flies. It is plausible that information about the social environment is processed differently in males and females to generate different behavioral responses. MP1/MP3 and P1-induced increases in *to* expression and reductions in sugar consumption that apply to both

sexes under normal conditions may become male-biased upon chronic social isolation.

It is more likely that the *to* overexpression in isolated females driving sucrose feeding is expressed elsewhere in the head or brain. It is possible that our qPCR of extracted brains detected *to* upregulation in non-neuronal cell types, or that some fat body cells remained on the brains when they were extracted. *To* was originally characterized as a pericerebral fat body gene that is upregulated in response to starvation[31]. This would be consistent with social isolation inducing a starvation-like brain state, thus upregulating *to* expression. The single-cell RNA-seq atlas data indicate that *to* is also widely expressed in ORNs, epithelial cells, and glia. ORNs play an important role in feeding behavior by enabling odor-detection during foraging. Although it does not seem like *to* expression in neurons is the source or key driver of female isolation-induced sucrose preference, *to* could also be expressed in glia that support the function of these neurons.

Alternatively, *to* could influence behavior through the binding of juvenile hormone, a fly hormone that canonically regulates developmental transitions. The protein encoded by *to* is highly structurally similar to juvenile hormone binding protein (JHBP), and it contains a putative binding domain for juvenile hormone[31,32]. The secretion of takeout into the hemolymph from the pericerebral fat body and its binding of juvenile hormone could either hinder juvenile hormone signaling, by reducing levels of the hormone actively circulating, or facilitate juvenile hormone acting on its targets by functioning as a carrier protein.

In humans, adipose tissue has been established to function as a neuroendocrine organ that supports essential bodily functions. Adipose tissue can communicate with the brain through adipokine release and afferent outputs, and the brain communicates back via systemic hormones and efferent inputs[57]. Dysregulation of the brain-fat axis may contribute to obesity and its

associated conditions. Takeout may participate in a functionally analogous system in flies, in which a combination of *to* expression in neurons, glia, and fat body cells communicates nutritional status to the brain and stimulates or represses feeding behavior to return the organism to homeostasis. The fact that *to* is expressed both in the head and throughout the gastrointestinal tract also suggests that it may be involved in a gut-brain axis to maintain energy and metabolic homeostasis.

The question remains that if social isolation induces a starvation-like brain state, according to Li et al. (2021)[4], and my preliminary qPCR results showed similar indicators of this brain state in both sexes via *Dsk* downregulation and *tobi* upregulation, why are the changes in *to* expression and sucrose feeding female-specific? There could be a ceiling effect in males, given that wild type control and socially isolated males both consume more sucrose than control females at baseline. However, the sex differences in *to* expression and feeding behavior could be connected to changes in sexual behavior and reproduction in response to chronic stress. In Chapter 3, I found evidence of a sex difference in motivated behavior in the yeast preference test in addition to the VBFD test. Females were less interested in the yeast side of the arena relative to controls, whereas males were more interested, following social isolation. If isolated females are less interested in yeast and more motivated to seek out and consume sugar, it is possible that they are adapting their macronutrient needs to prioritize survival over reproduction.

Yeast is the primary food source through which flies obtain protein, and mating has been shown to increase female flies' preference for protein-rich food to support egg development[58,59,60]. Sex peptide transferred to females through male seminal fluid has been found to act on neural circuitry that increases preference for salt and protein and appetite for yeast[58]. However, protein consumption involves a tradeoff between reproduction and lifespan across

animal species[61]. In insects and mammals, excessive protein consumption promotes fertility, yet low-protein diets are associated with improved longevity[62]. A recent study in *Nature Communications* found that prior to mating, male and female *Drosophila melanogaster* both consume more protein-rich food to prepare for reproduction. After mating, only male flies decrease protein feeding. When their role in reproduction ends, males may change their diet to recover lost fitness[63], possibly to be able to mate again. Female flies increase yeast consumption, relative to an all-sugar food source, after mating to support the development of their offspring, even if it comes to the detriment of their own survival[63]. The anterior leucokinin neurons in the brain were identified as key mediators of sexually dimorphic macronutrient choice based on mating status[63]. Interestingly, leucokinin also limits female receptivity when early adult flies transition from an asexual phase to full sexual maturity[64]. Hence, leucokinin-expressing neurons may play a dual function in regulating sexual behavior and macronutrient feeding choice in a sexually dimorphic manner.

In our research, it could be that in an environmentally stressful context, in which the fly is perceiving social isolation similarly to hunger, there could be a macronutrient shift from protein to sugar in females, as the females attempt to bolster their own stress resistance and lifespan. Females may have a lower stress threshold than males to start changing feeding and mating behavior, as males do not have to support and lay eggs. We may only see increases in *to* expression and sucrose feeding in females if males do not perceive isolation-induced "starvation" to be as threatening to survival as actual starvation. Food-deprived male flies do tend to prioritize feeding over courtship, and this preference can be reversed on the time scale of minutes by consuming protein-rich food[65]. This male behavioral switch from feeding to courtship occurs when ingested amino acids trigger the release of Diuretic hormone 31 (Dh31), a neuropeptide

hormone, from the enteroendocrine cells in the gut into the hemolymph. Dh31 then binds to its receptors in the brain in two distinct neuronal populations: one that inhibits feeding, and one that stimulates courtship[65]. It would be interesting to add courtship and sexual receptivity to our battery of behavioral tests to test the hypothesis further that social isolation-induced *to* upregulation in females functions to divert nutritional resources away from reproduction and towards survival. It might follow that females may be less sexually receptive following social isolation if a stressful environment is not conducive to reproduction.

There is some initial evidence that takeout may play a role in the tradeoff-switch between fertility and fitness, and that it may exert its effects by binding juvenile hormone, thus restricting juvenile hormone's signaling activity. Overexpression of *to*, specifically in the fat body, has been found to increase fly longevity and decrease male courtship behavior. These effects of *to* overexpression are nullified when flies are treated with methoprene, a juvenile hormone analog[66]. If *to* overexpression improves lifespan, this could also be a reason why I did not observe a survival difference between isolated females and group-housed control females in my experiment from Chapter 3; female isolation-induced increases in *to* expression could have improved lifespan, counteracting any negative stress effects of social isolation that shorten lifespan. Finally, in addition to overexpression, downregulation of *to* via a loss-of-function mutation also decreases male courtship behavior[33]. These researchers hypothesized that takeout may play a redundant role with other Fru^M-regulated factors that influence mating in males. Since there is evidence of both upregulation and downregulation of *to* reducing male courtship, this strengthens the argument that takeout may act similarly to other neuromodulators.

4.3.2 Limitations and future directions

Our study was limited with respect to certain aspects of our behavioral testing and RNA

sequencing. In the VBFD test, we quantified sucrose feeding choice by categorizing the flies by crop color as primarily consuming sucrose, arabinose, both sugars (in roughly equal quantity), or nothing, and then calculating a proportion of sucrose-consuming flies out of all animals. We did not quantify the exact amount of food consumed, nor more closely examine how flies were making their choice. For example, for flies that consumed both sugars, we did not distinguish between flies that began eating arabinose and then switched to sucrose midway through the feeding period, or flies that were indiscriminately feeding between sucrose and arabinose during the entirety of the experiment. We could use an approach such as the CApillary FEeder (CAFE) assay[67], which directly measures food intake in *Drosophila* via capillary tube liquid feeding, to better quantify actual food consumption in the future.

Our RNA-seq analyses were based on a low number of replicates, which necessitated validation using qPCR tests. We also used bulk RNA-seq, whereas single-cell RNA sequencing (scRNA-seq) would have been more informative in determining if female-specific *to* overexpression is driven by a specific cell type. If we do not want to recollect samples and perform scRNA-seq, we could try projecting our current data onto a single cell atlas[68].

A final, major limitation in this work was that our manipulations of *to* expression were highly non-specific and only focused on spatial control of gene expression. In our *to*–Gal4 x UAS-to^{RNAi} flies, we knocked down *to* in all *to*-expressing cells of the body, not just the ones in the head that were detected in our RNA profiling. For future experiments, it would be helpful to perform immunohistochemistry or fluorescence in situ hybridization to determine where *to* is being overexpressed in the isolated female head or brain, and then target manipulations to a more specific cell or tissue type. We could also expand our genetic tools to allow for temporal control to see if activation or inactivation of to+ cells during the behavior experiment impacts feeding

choice. This would only be relevant to sex differences in *to* expression in a specific population of neurons, but optogenetic manipulations could extend our studies to elucidate the role of *to*-expressing neurons and their potential targets in the neural circuitry underlying sexually dimorphic food choice within a chronic stress context.

4.4 Methods

4.4.1 Fly stocks and rearing

All fly stocks were raised on standard medium (cornmeal/yeast/molasses/agar) at 25°C and 40% relative humidity on a fixed 12-h light/dark cycle. Male and female *Oregon-R* flies (wild type) and lines from the Bloomington *Drosophila* Stock Center (BDSC) were used for the majority of these experiments. Behavioral experiments were conducted within 8 hours after lights on. The following fly lines were obtained from the BDSC: *to-Gal4* (#80938), *elav-Gal4* (#8765), *UAS-to* (#81000), and *UAS-to*^{RNAi} (#55982). *Oregon-R* and *UAS-mCherry*^{RNAi} were gifts from Dr. Jodi Schottenfeld-Roames, Princeton University. *Canton-S* and *UAS-tra*^{RNAi} (Vienna *Drosophila* Resource Center #2560, also known as traIR) stocks were kindly provided by Dr. Rachel Monyak, Stonehill College.

4.4.2 Experimental setup

Social isolation

For the social isolation stress model, we used an experimental procedure for chronic social isolation already characterized in the literature[4]. Newly-eclosed male and female flies were housed together for 3-5 days to acquire social experience. Flies were then housed either singly or in same sex groups of 25 for 7 days. Within 24 hours of the final day of isolation or group housing, flies were either frozen on dry ice for RNA extraction, tested for behavior, or moved to/kept in same sex group housing for survival analysis.

4.4.3 Behavioral testing

Value-Based Feeding Decision test

A previously described behavior test, the value-based feeding decision test[69], was used as a two-choice assay to examine feeding decision making. Flies were briefly anesthetized with CO_2 and placed in the center of the arena used for the locomotion and motivated behavior tests, a 55 mm diameter petri dish filled with 1% agarose gel and topped with a layer of parafilm. Sugars were dissolved in plain water to make 2 solutions: a 150 mM sucrose solution and a 150 mM arabinose solution. Each solution was labeled with a blue dye (0.01% erioglaucine disodium salt, Acros Organics, Cat# 229730250) or red dye (0.1% Food Red No. 106, TCI, Cat# F0143). Alternating droplets (20 µL volume) of each liquid food were placed evenly around the perimeter of the dish, such that there were 8 droplets total, 4 of each solution. Flies were allowed to explore the arena and feed in the dark for 2 hours. At the end of this period, flies were collected and counted under a microscope. They were scored by color: blue (ingested primarily blue food), red (ingested primarily red food), purple (ingested both foods), or non-eater (no color accumulation in the abdomen).

Table 4.3: Average group size and number of groups tested in the VBFD test for all experimental groups across all genotypes

	Control Male	Social Isolation Male	Control Female	Social Isolation Female
ORE-R	n = 13 groups, avg. = 22 flies/group	n = 10 groups, avg. = 21 flies/group	n = 11 groups, avg. = 23 flies/group	n = 6 groups, avg. = 20 flies/group
to-Gal4	n = 8 groups, avg. = 20 flies/group	n = 9 groups, avg. = 16 flies/group	n = 9 groups, avg. = 16 flies/group	n = 7 groups, avg. = 16 flies/group
UAS-to^{RNAi}	n = 13 groups, avg. = 18 flies/group	n = 8 groups, avg. = 19 flies/group	n = 15 groups, avg. = 20 flies/group	n = 8 groups, avg. = 21 flies/group
UAS-to	n = 11 groups, avg. = 15 flies/group	n = 9 groups, avg. = 15 flies/group	n = 8 groups, avg. = 18 flies/group	n = 8 groups, avg. = 19 flies/group
to-Gal4 x UAS-to^{RNAi}	n = 8 groups, avg. = 15 flies/group	n = 8 groups, avg. = 13 flies/group	n = 9 groups, avg. = 16 flies/group	n = 8 groups, avg. = 14 flies/group
to-Gal4 x UAS-to	n = 9 groups, avg. = 14 flies/group	n = 7 groups, avg. = 18 flies/group	n = 10 groups, avg. = 16 flies/group	n = 7 groups, avg. = 15 flies/group
to-Gal4 x UAS-mCherry^{RNAi}	n = 7 groups, avg. = 17 flies/group	n = 9 groups, avg. = 15 flies/group	n = 8 groups, avg. = 15 flies/group	n = 9 groups, avg. = 14 flies/group
elav-Gal4	n = 9 groups, avg. = 23 flies/group	n = 9 groups, avg. = 16 flies/group	n = 10 groups, avg. = 24 flies/group	n = 10 groups, avg. = 15 flies/group
elav-Gal4 x UAS-to^{RNAi}	n = 7 groups, avg. = 14 flies/group	n = 8 groups, avg. = 15 flies/group	n = 7 groups, avg. = 14 flies/group	n = 8 groups, avg. = 14 flies/group
elav-Gal4 x UAS-to	n = 6 groups, avg. = 11 flies/group	n = 5 groups, avg. = 10 flies/group	n = 6 groups, avg. = 12 flies/group	n = 5 groups, avg. = 12 flies/group
UAS-tra^{RNAi}	n = 8 groups, avg. = 22 flies/group	n = 8 groups, avg. = 20 flies/group	n = 8 groups, avg. = 22 flies/group	n = 8 groups, avg. = 18 flies/group
elav-Gal4 x UAS-tra^{RNAi}	n = 9 groups, avg. = 16 flies/group	n = 10 groups, avg. = 14 flies/group	n = 11 groups, avg. = 17 flies/group	n = 8 groups, avg. = 14 flies/group

4.4.4 RNA extraction

For each sample, flies were decapitated on dry ice. RNA was extracted and purified from homogenized whole heads using a phenol chloroform organic extraction. If guanidine isothiocyanate contamination was detected via NanoDrop (Thermo Fisher Scientific, MA), all samples were treated with 1-butanol[70]. Samples were also treated using either the RQ1 RNase-Free DNase kit (Promega Corporation) or the TURBO DNA-*free*™ Kit (Invitrogen) to address genomic DNA contamination. Total RNA samples were sequenced by the Princeton Genomics Core or used for downstream applications. The samples submitted for RNA-seq were extracted from 200 whole heads. To obtain brains, 20-40 *Drosophila* heads were dissected one at a time in cold Schneider's medium. Brains were transferred to a microtube on ice containing 200 µL of

Schneider's medium, briefly spun down on a benchtop centrifuge, and then frozen at -80°C. Prior to homogenization in TRIzol (Invitrogen), samples were spun down, and the Schneider's medium covering the brains was removed.

4.4.5 Quantitative Polymerase Chain Reaction (qPCR)

RNA was reverse transcribed to complementary DNA (cDNA) using the SuperScript™ III First-Strand Synthesis System (Invitrogen). Random primers and a 200 ng total RNA template were used in a 20 µL reaction, following the instructions.

For real-time qPCRs, samples used SYBR Green PCR Master Mix (Qiagen or Applied Biosystems, Thermo Fisher Scientific). The PCR was performed in a 10 µL reaction including 3 µL of cDNA and 7 µL of master mix. The master mix for each gene consisted of 0.5 µL of each primer (10 µM), 5 µL SYBR Green, and 1 µL RNase-free water. We used the following PCR conditions: initial incubation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification was performed using the $2^{-\triangle\triangle CT}$ method[71].

Table 4.4: List of PCR primers used for experiments

Target mRNA	Forward primer sequence	Reverse primer sequence
GAPDH	CCACTGCCGAGGAGGTCAACTAC	ATGCTCAGGGTGATTGCGTATGC
5-HT2B	CCAACTACTTCCTTATGTCG	GATAAATATCTGTCCACGGA
Dsk	CCGATCCCAGCGCAGACGAC	TGGCACTCTGCGACCGAAGC
tobi	CCACCAAGCGAGACATTTACC	GAGCGGCGTAGTCCATCAC
CCKLR-17D1	GCTACGTGAGCGACAATGAA	AGCCCACGTACTCGTACACC
CCKLR-17D3	ACGCGTACCCTGTACGTAGG	GGTCTCGTTGTCAAGGTGGT

Lsp2	CTTCCAGCACGTCGTCTACTG	CCCTGCATATCATCACGGAACA
Fbp1	ATCGTGGCGGCATTGATAAGG	CGAAGGGTGTCAAAGTCCTG
Lsp1α	ACATCAAGGTCGCTGACAAGG	TTCCCATCGCAATGTACTCTTC
To	ATCGCATTCGCAGTGGTTTTG	CGCCATCACCATACTTACAAGGT
Tra	CCGATGAAAATGGATGCCG	TGCTCTCTCTGATGGACGACTGTG

4.4.6 Transcriptome analysis by RNA Sequencing (RNA-Seq)

Sequencing

RNA-seq was performed by the Princeton University Genomics Core Facility. The integrity of total RNA samples was assessed on the 2100 Bioanalyzer system using the RNA 6000 Pico Chip (Agilent Technologies, CA). The poly-A containing RNA transcripts were enriched from one microgram of total RNA for each sample using oligo-dT beads, further fragmented, and converted to cDNA and Illumina sequencing libraries using the PrepX RNA-seq library preparation protocol on the Apollo 324^{TM} NGS Library Prep System (Takara Bio, CA). Different DNA barcodes were attached to each library. The RNA-seq libraries were examined on Agilent Bioanalyzer DNA High Sensitivity chips for size distribution, quantified by Qubit fluorometer (Invitrogen, CA), and pooled at equal molar amounts. Each library pool was denatured and sequenced on one Illumina NovaSeq 6000 S Prime lane using the 100 cycle v1.5 kit. Raw sequencing reads were filtered by Illumina NovaSeq Control software, and only the Pass-Filter (PF) reads were used for further analysis.

Analysis Pipeline

Sequences were aligned to the *Drosophila melanogaster* genome (BDGP.46)[72,73] using STAR[74] (v. 2.7.10b). Duplicates were removed using the Picard Toolkit[75] (v. 2.27.5) MarkDuplicates function. The number of reads mapping to each gene was determined using

htseq-count (HTSeq v. 2.0.5[76]). Differential gene expression analysis was performed in R[77] (v. 4.4.0). All read counts from rRNA and pseudo-rRNA genes were removed. Normalization was performed using edgeR[78] (v. 4.4.1). Multidimensional scaling (MDS) plots were used to identify outlier samples, which were then removed from the analysis. Data was prepared for linear modeling with limma[79] (v. 3.60.6) using voom[80]. The design matrix was constructed to model the relationship between gene expression and condition (Female-SI, Female-Control, Male-SI, Male-Control) adjusted for batch. Contrasts were used to compare gene expression differences between the following groups: 1. SI and control females, 2. SI and control males, 3. SI vs controls ((SI Female + SI Male)/(Control Female + Control Male). A fourth contrast was included to compare the difference between SI Female and Control Female with the difference between SI males and control males. Differentially expressed genes were filtered based on an FDR-adjusted p-value of < 0.05 to determine significant genes with positive or negative values of log2FoldChange when comparing socially isolated males to control males, socially isolated females to control females, and the interaction between social isolation and sex. Heatmaps and additional plots were created using the R packages ComplexHeatmap[81] (v. 2.21.2) and ggplot2[82] (v. 3.5.1).

4.4.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.4.0 software. The Shapiro-Wilk test of normality (passed at $p > 0.05$) was used to determine whether data followed a parametric distribution. Unpaired t-tests were used for parametric data assuming equal variances, and Welch's t-tests were used if the samples had unequal variances based on the result of the F-test. If the data did not pass the test for normality distribution, nonparametric testing was used. All nonparametric data were analyzed with the Mann–Whitney *U* test for between-group

comparisons. Effects were deemed statistically significant at $p < 0.05$. The Grubbs' test was applied to identify and exclude outliers in the data. All statistics for two-way (Table S4.1) and one-way (Table S4.2) ANOVAs are provided in "Supplementary Figures and Tables" below.

4.5 Supplementary Figures and Tables

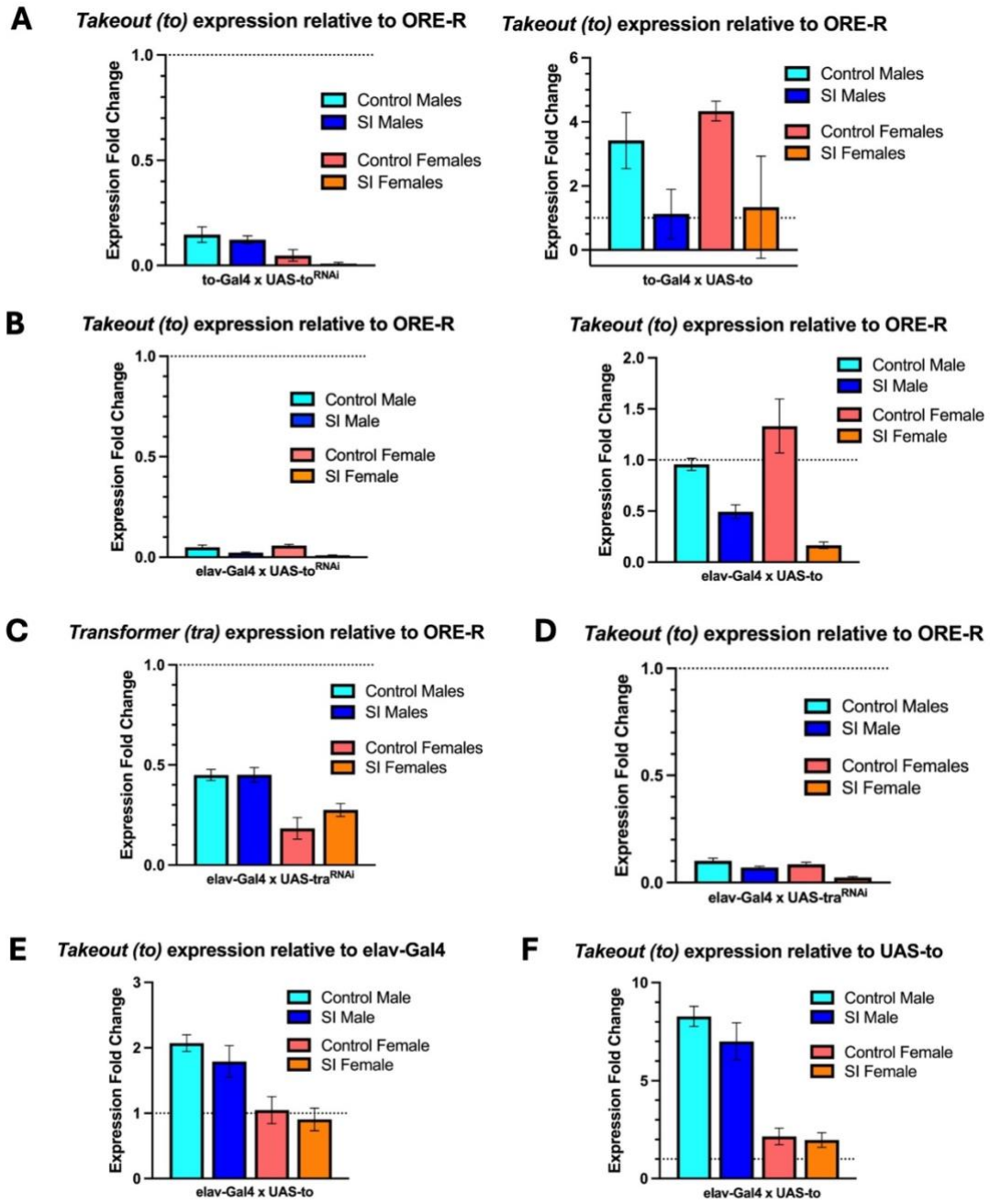

Figure S4.1. Downregulation and overexpression of *to* and downregulation of *tra* align with expectations across experimental groups. A) We performed qPCR to confirm genetic knockdown of *to* in the to-Gal4 x UAS-to^{RNAi} flies and overexpression of *to* in to-Gal4 x UAS-to flies. *To* overexpression was clearer in control males and control females, which could be due to ORE-R isolated males and isolated females already having relatively high expression of *to*. **B)** Although the elav-Gal4 x UAS-to flies showed no change or negative expression of *to* relative to ORE-Rs, the elav-Gal4 x UAS-to^{RNAi} flies showed downregulation of *to* across experimental groups. **C)** Genetic knockdown of *tra* in elav-Gal4 x UAS-tra^{RNAi} flies decreased *tra* expression relative to ORE-Rs. **D)** Genetic knockdown of *tra* in elav-Gal4 x UAS-tra^{RNAi} flies also decreased expression of *to* across groups. **E)** In elav-Gal4 x UAS-to flies, *to* expression was elevated relative to elav-Gal4 flies in control males and isolated males but not in females. **F)** In elav-Gal4 x UAS-to flies, *to* expression was elevated relative to UAS-to flies across all experimental groups. After completing the VBFD test, flies were frozen on dry ice and decapitated. RNA was extracted from whole fly heads and reverse transcribed into cDNA. All groups represent n = 3 samples/group. Error bars are ± SEM.

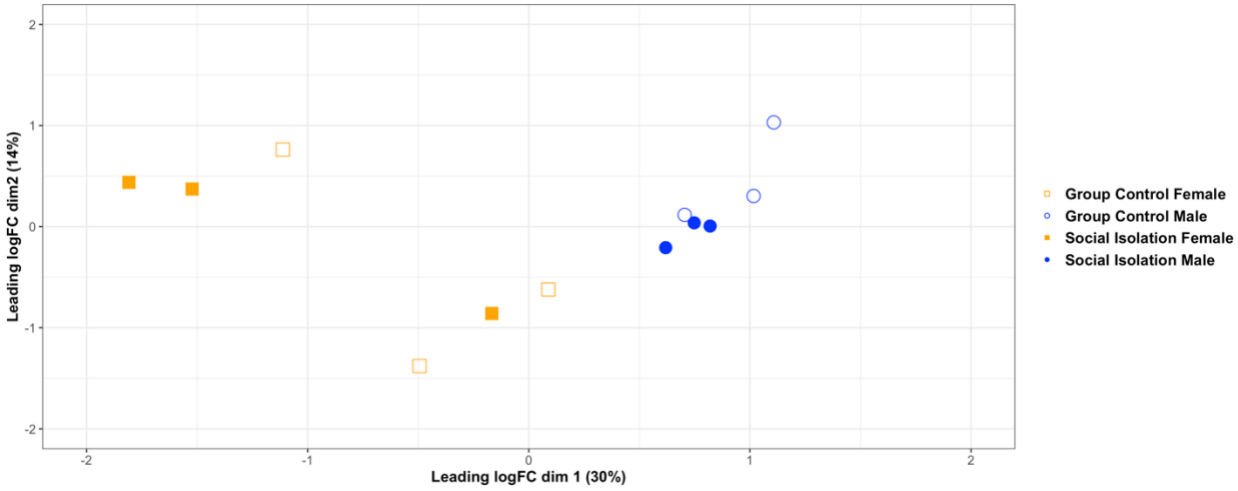

Figure S4.2. Original MDS plot shows triplicate samples for each experimental group. One sample (that was most dissimilar from the other two) was removed from each of the female groups to improve target discovery.

Table S4.1: Statistics for all 2x2 ANOVAs

Figure	Panel	Factors	F Statistic	P-value
3.1	A	Interaction	$F(1, 394) = 41.39$	$p < 0.0001$
		Sex	$F(1, 394) = 102.00$	$p < 0.0001$
		Stress	$F(1, 394) = 123.10$	$p < 0.0001$
	C	Interaction	$F(1, 398) = 19.40$	$p < 0.0001$
		Sex	$F(1, 398) = 3.47$	$p = 0.06$
		Stress	$F(1, 398) = 7.16$	$p = 0.0078$
3.4	A	Interaction	$F(1, 57) = 1.81$	$p = 0.18$

		Sex	F (1, 57) = 10.78	p = 0.002
		Stress	F (1, 57) = 4.84	p = 0.03
	B	Interaction	F (1, 55) = 8.37	p = 0.0054
		Sex	F (1, 55) = 23.64	p < 0.0001
		Stress	F (1, 55) = 8.37	p = 0.0055
	C	Interaction	F (1, 57) = 5.76	p = 0.02
		Sex	F (1, 57) = 1.26	p = 0.27
		Stress	F (1, 57) = 5.04	p = 0.03
	D	Interaction	F (1, 58) = 7.67	p = 0.01
		Sex	F (1, 58) = 23.42	p < 0.0001
		Stress	F (1, 58) = 1.78	p = 0.19
3.5	A	Interaction	F (1, 53) = 0.50	p = 0.48
		Sex	F (1, 53) = 12.53	p = 0.0008
		Stress	F (1, 53) = 1.27	p = 0.27
	B	Interaction	F (1, 50) = 0.63	p = 0.43
		Sex	F (1, 50) = 0.20	p = 0.66
		Stress	F (1, 50) = 2.65	p = 0.11
	C	Interaction	F (1, 51) = 4.01	p = 0.05
		Sex	F (1, 51) = 5.46	p = 0.02
		Stress	F (1, 51) = 2.68	p = 0.11
	D	Interaction	F (1, 55) = 0.24	p = 0.63
		Sex	F (1, 55) = 12.72	p = 0.0008
		Stress	F (1, 55) = 2.10	p = 0.15
3.6	D	Interaction	F (1, 36) = 2.48	p = 0.12
		Sex	F (1, 36) = 3.31	p = 0.08
		Stress	F (1, 36) = 3.53	p = 0.07
4.6	A	Interaction	F (1, 8) = 22.16	p = 0.002
		Sex	F (1, 8) = 11.82	p = 0.009
		Stress	F (1, 8) = 10.55	p = 0.01
	B	Interaction	F (1, 8) = 34.63	p = 0.0004
		Sex	F (1, 8) = 20.51	p = 0.002
		Stress	F (1, 8) = 34.45	p = 0.0004
	C	Interaction	F (1, 8) = 11.54	p = 0.009
		Sex	F (1, 8) = 3.16	p = 0.11
		Stress	F (1, 8) = 16.19	p = 0.004
	D	Interaction	F (1, 8) = 60.72	p < 0.0001

		Sex	F (1, 8) = 48.38	p = 0.0001
		Stress	F (1, 8) = 44.48	p = 0.0002
4.10	A	Interaction	F (1, 36) = 2.48	p = 0.12
		Sex	F (1, 36) = 3.31	p = 0.08
		Stress	F (1, 36) = 3.53	p = 0.07
	B	Interaction	F (1, 29) = 0.12	p = 0.73
		Sex	F (1, 29) = 0.96	p = 0.34
		Stress	F (1, 29) = 0.95	p = 0.34
	C	Interaction	F (1, 40) = 5.96	p = 0.02
		Sex	F (1, 40) = 21.85	p < 0.0001
		Stress	F (1, 40) = 6.72	p = 0.01
	D	Interaction	F (1, 29) = 31.40	p < 0.0001
		Sex	F (1, 29) = 28.10	p < 0.0001
		Stress	F (1, 29) = 7.97	p = 0.009
4.12	A	Interaction	F (1, 36) = 2.48	p = 0.12
		Sex	F (1, 36) = 3.31	p = 0.08
		Stress	F (1, 36) = 3.53	p = 0.07
	B	Interaction	F (1, 29) = 0.12	p = 0.73
		Sex	F (1, 29) = 0.96	p = 0.34
		Stress	F (1, 29) = 0.95	p = 0.34
	C	Interaction	F (1, 32) = 3.40	p = 0.07
		Sex	F (1, 32) = 3.80	p = 0.06
		Stress	F (1, 32) = 19.69	p = 0.0001
	D	Interaction	F (1, 29) = 2.05	p = 0.16
		Sex	F (1, 29) = 39.41	p < 0.0001
		Stress	F (1, 29) = 1.14	p = 0.30
4.14	A	Interaction	F (1, 36) = 2.48	p = 0.12
		Sex	F (1, 36) = 3.31	p = 0.08
		Stress	F (1, 36) = 3.53	p = 0.07
	B	Interaction	F (1, 34) = 0.07	p = 0.80
		Sex	F (1, 34) = 0.76	p = 0.39
		Stress	F (1, 34) = 0.48	p = 0.49
	C	Interaction	F (1, 40) = 5.96	p = 0.02
		Sex	F (1, 40) = 21.85	p < 0.0001
		Stress	F (1, 40) = 6.72	p = 0.01
	D	Interaction	F (1, 26) = 0.03	p = 0.87

		Sex	F (1, 26) = 0.03	p = 0.87
		Stress	F (1, 26) = 0.40	p = 0.53
4.16	A	Interaction	F (1, 36) = 2.48	p = 0.12
		Sex	F (1, 36) = 3.31	p = 0.08
		Stress	F (1, 36) = 3.53	p = 0.07
	B	Interaction	F (1, 34) = 0.07	p = 0.80
		Sex	F (1, 34) = 0.76	p = 0.39
		Stress	F (1, 34) = 0.48	p = 0.49
	C	Interaction	F (1, 32) = 3.40	p = 0.07
		Sex	F (1, 32) = 3.80	p = 0.06
		Stress	F (1, 32) = 19.69	p = 0.0001
	D	Interaction	F (1, 18) = 0.16	p = 0.69
		Sex	F (1, 18) = 12.25	p = 0.003
		Stress	F (1, 18) = 0.80	p = 0.38
4.18	A	Interaction	F (1, 36) = 2.48	p = 0.12
		Sex	F (1, 36) = 3.31	p = 0.08
		Stress	F (1, 36) = 3.53	p = 0.07
	B	Interaction	F (1, 34) = 0.07	p = 0.80
		Sex	F (1, 34) = 0.76	p = 0.39
		Stress	F (1, 34) = 0.48	p = 0.49
	C	Interaction	F (1, 28) = 1.13	p = 0.30
		Sex	F (1, 28) = 205.10	p < 0.0001
		Stress	F (1, 28) = 22.79	p < 0.0001
	D	Interaction	F (1, 34) = 2.42	p = 0.13
		Sex	F (1, 34) = 39.85	p < 0.0001
		Stress	F (1, 34) = 0.002	p = 0.96

Table S4.2: Statistics for all one-way ANOVAs

Figure	Panel	F Statistic	P-value
4.11	A	F (4, 33) = 14.55	p < 0.0001
	B	F (4, 23) = 12.31	p < 0.0001
	C	F (4, 33) = 3.51	p = 0.02
	D	F (4, 27) = 2.99	p = 0.04
4.13	A	F (3, 24) = 20.82	p < 0.0001
	B	F (3, 17) = 22.27	p < 0.0001
	C	F (3, 25) = 7.25	p = 0.001

	D	F (3, 22) = 2.25	p = 0.11
4.15	A	F (3, 25) = 7.42	p = 0.001
	B	F (3, 18) = 4.63	p = 0.01
	C	F (3, 26) = 3.01	p < 0.05
	D	F (3, 21) = 2.16	p = 0.12
4.17	A	F (3, 21) = 12.23	p < 0.0001
	B	F (3, 16) = 4.92	p = 0.01
	C	F (3, 23) = 8.70	p = 0.0005
	D	F (3, 20) = 4.22	p = 0.02
4.19	A	F (3, 18) = 5.32	p = 0.008
	B	F (3, 16) = 8.72	p = 0.001
	C	F (3, 18) = 5.88	p = 0.006
	D	F (3, 19) = 3.48	p = 0.04

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Chapter 5

Concluding remarks

5.1 General discussion

This dissertation was motivated by the goal of gaining a better understanding of sex-specific molecular and behavioral responses to social stress. Human males and females experience and respond to stress differently, which can be attributed to a combination of hormonal, genetic, and environmental factors. Biological and psychosocial factors in tandem can trigger the emergence of stress-related disorders, which are often characterized by internalizing symptomatology in females and externalizing symptoms in males. Women show increased susceptibility to mood, anxiety, and post-traumatic stress disorders, whereas men are more likely to develop substance use disorders and demonstrate antisocial behavior, aggression, and impulsivity in response to stress[1,2,3]. Women and men may also have different cortisol responses to stress. Previous research suggests that males generally show greater cortisol reactivity relative to females in response to stressors[4,5,6], but that females may be more sensitive to social stress[7]. Sex hormones have also been implicated in differential stress responses between human males and females. For instance, estrogen may be a protective factor against stress-related brain damage in women[8,9], but it also fluctuates across phases of the menstrual cycle. When estrogen is relatively lower, female susceptibility to stress increases[10]. Testosterone, which is higher in men, may protect against anxiety-related behaviors in the face of stress[11] but may also exacerbate aggression[12]. Animal models allow us to perform manipulations to gain a better understanding of the cellular, molecular, and circuit-level bases of sex-specific stress responses. This knowledge is

valuable for informing interventions and treatments for stress-related conditions that may manifest differently between males and females.

In this thesis, I began by reviewing definitions of stress, sex differences in human stress-related disorders, and comparative stress neuroendocrinology between mammals and insects. In Chapter 2, I presented *Drosophila* as a potential neurobehavioral model for studying sex differences in the stress response, with a focus on social isolation and crowding stressors. I outlined known stress-induced phenotypes and behavioral effects in flies, as well as cellular, molecular, and neuromodulatory mechanisms that are impacted by stress. In Chapter 3, I found that both social isolation and overcrowding alter locomotion, motivated behavior, and aggression in flies, but that social isolation yields a greater number of sex differences. Social isolation also produces female-specific increases in preference for sucrose over a non-nutritious sugar, arabinose. In Chapter 4, I used RNA sequencing (RNA-seq) complemented with qPCR to identify female-specific, social isolation-induced transcriptomic changes in the fly head across several genes related to feeding, metabolism, and reproduction. When I manipulated the expression of *takeout (to)*, one of the top candidate genes from our RNA-seq analysis that has also been implicated in feeding and male courtship behavior, the same alteration in gene expression produced differences in sucrose feeding choice between socially isolated males and females. Downregulation of *to* in all *to*-expressing cells decreased sucrose-feeding in isolated females but increased it in isolated males. Upregulation of *to* in all *to*-expressing cells also decreased sucrose-feeding in isolated females but had no effect in isolated males. Overexpressing *to* in neurons only increased sucrose-feeding in males, with no effect in females. Masculinizing neurons via *transformer* knockdown did not have an effect on sucrose-feeding in males or females of either social condition (isolation or group-housed control).

Overall, the findings from Chapter 4 suggest that, like in mammals, states of satiety and starvation affect behavior and metabolism. Chronic social isolation has been found to induce a starvation-like brain state in *Drosophila*, and my preliminary qPCR results replicated two changes in gene expression (*Dsk* and *tobi*) that formed the basis of this previous study[13]. Certain behavioral effects that are influenced by hunger and fullness, such as the decision to feed and macronutrient choice, are sexually dimorphic and may relate to nutritional needs for survival versus reproduction. The insect fat body is functionally analogous to the adipose tissue and liver in mammals, and it plays a key role in controlling the overall metabolic response to satiety and starvation. The fat body may also communicate with the brain to regulate the appropriate behavior to mediate those states. By changing the expression of storage proteins and proteins such as takeout in response to stress and hunger, flies can overcome perturbations in food abundance and adjust their behavior to achieve nutritional balance. Chylomicrons, low-density lipoproteins, and amino acid binding proteins in humans bear some similarity to these molecules, in that they are involved in the response to feeding and nutritional depletion[14].

Collectively, the findings of this dissertation provide a start to exploring female-specific molecular mechanisms related to stress-induced changes in feeding, metabolism, and possibly fertility. In the future, it would be interesting to explore other potential sexually dimorphic functions of *to*, as a gene that has been found to be activated downstream of Dsk[15], a protein which plays a dual role in feeding and aggression. Although often viewed as less translationally valid compared to mammalian models, *Drosophila melanogaster* can be an ethical, cost-effective, and useful model system for elucidating stress-related sex differences in gene expression, circuit function, and behavior.

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Supplemental Chapter

Neural mechanisms underlying murine physical exercise-induced anxiety regulation

This work was done during my time in Elizabeth Gould's lab* and was presented at* Society for Neuroscience (2019). ****This chapter has not been reviewed by Professor Gould and may not reflect her views.

In humans, aerobic exercise has been shown to enhance brain development, bolster cognition and academic performance[1], and decrease anxiety in both non-clinical and clinical populations[2]. Because the rodent ventral hippocampus (vHIP) is required for anxiety regulation[3], this brain region is a likely candidate for mediating the beneficial effects of physical activity. Previous studies have shown that running stimulates widespread structural plasticity throughout the hippocampus, including the formation of large numbers of new neurons[4], a process referred to as neurogenesis. Still, it is unclear whether running-induced structural plasticity in the vHIP contributes to the anxiolytic effects of exercise. To investigate the potential roles of hippocampal new neurons and perineuronal nets (PNNs) in the anxiety-reducing effects of physical activity in mice, we used a combination of behavioral and histological analyses. Initial results revealed a decrease in defensive behaviors accompanied by increases in neurogenesis, decreases in PNNs, and increases in inhibitory neural activity in the dentate gyrus in runners compared to sedentary controls. This project was stopped due to significant replicability issues, but it could provide a foundation for future research exploring the cellular, molecular, and systems-level bases of how exercise alters hippocampal inhibitory activity and decreases anxiety.

6.1 Introduction

On a global scale, obesity, mental illness, and non-communicable diseases associated with inactivity have risen, necessitating public health intervention. Individuals who participate in regular exercise typically demonstrate reduced obesity, less anxiety and stress, and improved feelings of self-esteem and well-being[5]. However, physical activity is either hindered or not possible for many due to aging, disabilities, restricted access to exercise, and lifestyle changes associated with contemporary industrialized societies[6]. This project aimed to identify targets for manipulating brain plasticity to mimic the anxiety-reducing effects of aerobic exercise. The manipulation of these targets pharmacologically or through deep brain stimulation could perhaps be refined as a future therapy for cognitive, mood, and behavioral dysfunction.

Several studies have shown that long-term aerobic exercise reduces anxiety and defensive behavior in humans and rodents[7,8,9,10,11], respectively. In comparison with the human posterior and rodent dorsal hippocampi, which primarily support cognitive functions, the human anterior hippocampus and its rodent homolog, the ventral hippocampus (vHIP), have been implicated in anxiety regulation[3]. Running has been found to drive adult neurogenesis, or the formation of new neurons from neural stem and progenitor cells, throughout the hippocampus in rodents[12], and these results likely extend to humans[13]. Despite previous findings that running regulates anxiety and stimulates hippocampal structural plasticity, it remains unresolved whether widespread running-induced structural plasticity in the vHIP plays a causal role in anxiety reduction.

The hippocampus contains a large number of parvalbumin-expressing (PV+) cells ensheathed in extracellular matrix (ECM) structures called perineuronal nets (PNNs). PNNs are known to regulate synaptic and structural plasticity by encapsulating the cell body and proximal dendrites of neurons to form a physical and chemical barrier and limit the motility of receptors at

synapses[14]. PNN formation can be influenced by experience, including early life trauma[15], environmental enrichment[16,17,18], and physical exercise[19]. Although adult-born neurons are known to form connections with PV+ inhibitory interneurons[20,21], and exercise can alter PNN formation[19], no studies to date have investigated whether the new neurons generated during running send projections to PNN+ PV+ cells, and whether such connections help reduce anxiety.

Additionally, during different behavioral states, large populations of hippocampal neurons fire synchronously. Synchronized activity among PV+ cells gives rise to neuronal oscillations in the hippocampus, where PV+ cells are a major target of postnatally-generated granule cells that have been found to desynchronize neural activity in their postsynaptic recipients[22]. Anxious states have been linked to neuronal oscillations in the theta frequency range (4-12 Hz) in the vHIP of mice[23] and the analogous anterior hippocampus of humans[24]. However, no studies have examined how long-term running alters neuronal oscillations, whether any such changes decrease anxiety, or whether running-induced changes in PNN expression together with enhanced adult neurogenesis may causally contribute to reduced theta rhythm synchrony.

This project was designed to explore the mechanistic relationships between new neurons, PNNs, and defensive behaviors in the context of physical activity. We hypothesized that running would induce changes in vHIP microcircuitry as a result of reduced PNNs and increased connections between excitatory new neurons and inhibitory PV+ cells. We predicted that these changes in the vHIP would desynchronize neuronal oscillations and, consequently, decrease anxiety-like behaviors (Figure 6.1). Although we decided to stop the project due to replicability issues and my departure from the lab, the preliminary results detailed below begin to shed light on how physical exercise may sculpt vHIP circuits to reduce anxiety-like behavior.

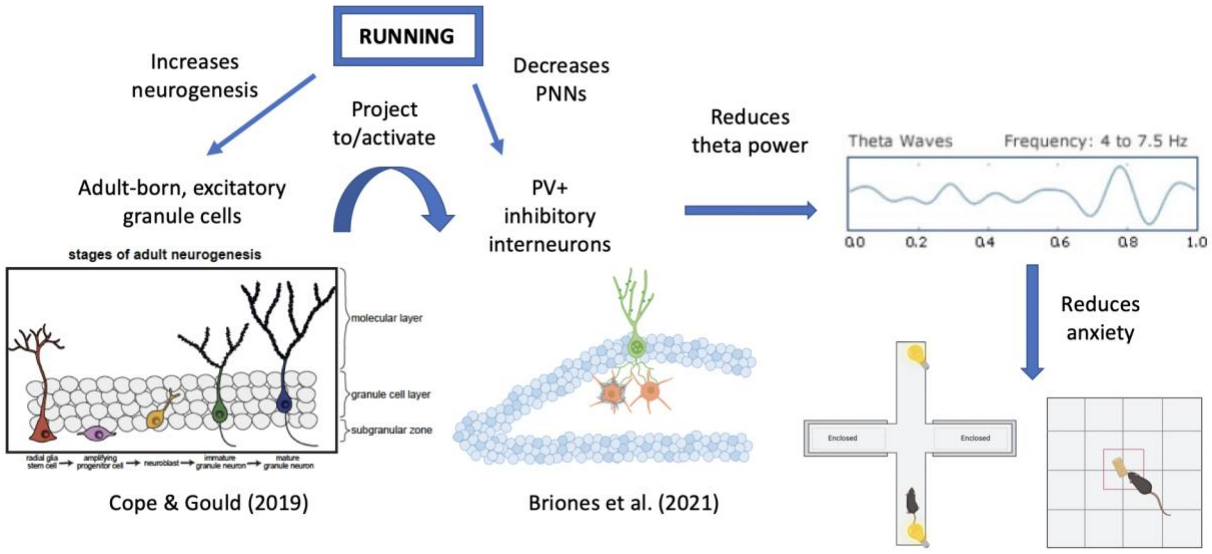

Figure 6.1. A model connecting the potential relationships between exercise, hippocampal structural plasticity, oscillatory activity, and behavior. We predicted that running would decrease anxiety-like behavior and the synchrony of theta oscillations in the vHIP by enhancing adult neurogenesis, diminishing PNN expression, and promoting connectivity between vHIP new neurons and PV+ inhibitory interneurons.

6.2 Results

6.2.1 Running reduces anxiety-like behavior

Rodent models have shown that running reduces anxiety-like behavior in canonical approach-avoidance paradigms[11]. We used the Elevated Plus Maze (EPM) and Novelty Suppressed Feeding (NSF) behavioral paradigms to quantify defensive behaviors in mice[25]. The dependent measures which we interpreted as anxiety reduction in the EPM test were higher percentages of entries and time spent in the open arms of the apparatus. The dependent measure in the NSF test corresponding to anxiety reduction was a shorter latency to approach a food pellet in the center of a brightly-lit novel environment. Our preliminary data indicate that after 6 weeks of running, adult male runner C57BL/6 mice demonstrate significantly lower anxiety-like behaviors compared to controls on both the EPM (Figure 6.2A) and NSF tests (Figure 6.2B). Runner and control mice did not differ in their general activity or hunger levels, which were assessed by the number of open ($p = 0.34$)/closed ($p = 0.43$) arm entries and the amount of food

pellet consumed in the home cage ($p > 0.99$). These data replicate previous findings that aerobic exercise reduces anxiety-like behavior in rodents[11,26].

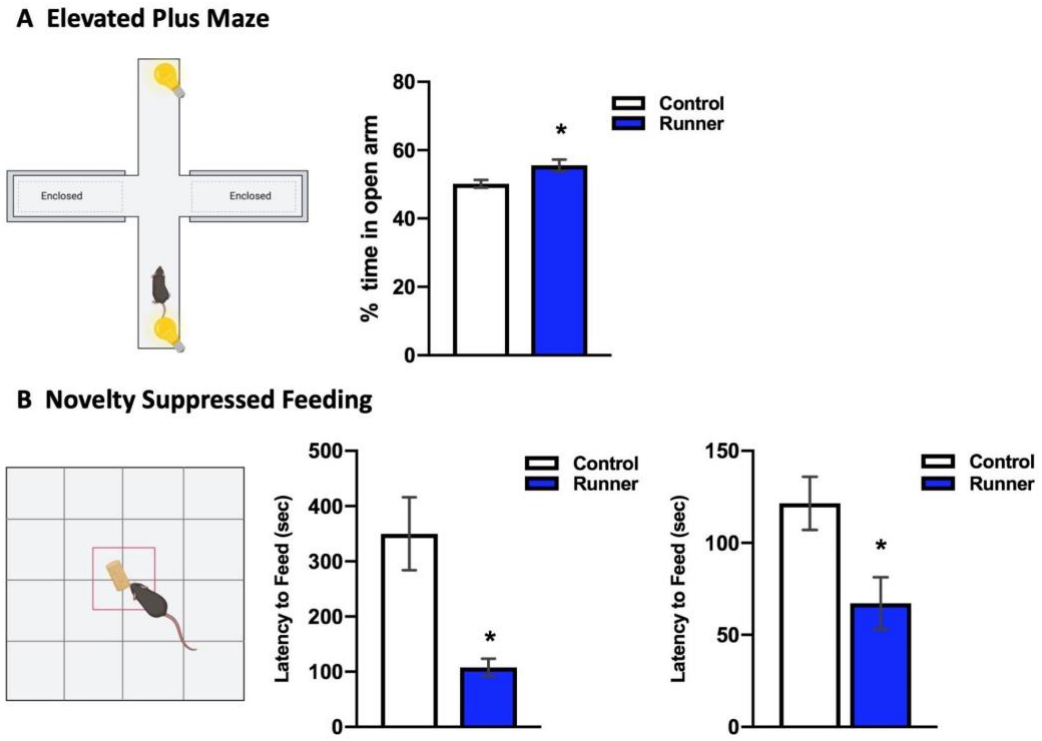

Figure 6.2. Adult male mice demonstrate less anxious behavior after 6 weeks of voluntary wheel running compared to non-runner controls. A) Male runners (n = 6) spent a significantly greater percentage of time in the open arms ($p = 0.02$) of the elevated plus maze compared to controls (n = 6). **B)** Left: male runners (n = 6) had a significantly reduced latency to feed in the novelty-suppressed feeding test ($p = 0.005$) compared to controls (n = 6). Right: this effect was replicated ($p = 0.02$) in a second cohort (n = 10 per group). Error bars are ± SEM. *$p < 0.05$.

6.2.2 Running alters adult neurogenesis, PNN+ cells, and inhibitory plasticity in the dentate gyrus

Previous findings have established that voluntary wheel running enhances both the proliferation of progenitor cells and the survival of new neurons in the dentate gyrus (DG) of the rodent hippocampus[12,27,28,29]. Because running has been shown to stimulate adult neurogenesis throughout the entirety of the DG[30], we expected and found similar increases in new neuron densities in runner mice in both ventral DG (vDG) and dorsal DG (dDG), using PSA-NCAM as

a marker of immature neurons (Figure 6.3A). These adult brains came from mice that began running in adolescence.

Additionally, PNNs and ECM molecules that comprise or interact with PNNs are altered in an array of neuropsychiatric disorders, suggesting that synaptic dysfunction across psychopathologies may be attributed to aberrations in the ECM[31]. It has also been shown that drug or environmental stimuli have the capacity to change PNN expression[14], and that exercise training reduces the expression of PNNs in the rat hippocampus[19]. Considering that PNNs ensheath inhibitory interneurons, and excitatory granule cells both form connections with inhibitory interneurons and desynchronize activity in their postsynaptic targets[22], it is possible that running might decrease PNNs surrounding DG PV+ cells to enhance synaptic plasticity between the PV+ and newborn granule cells. With increased innervation from new neurons, vDG PV+ cells could be activated asynchronously, reducing theta power as well as anxiety. Our preliminary data suggest that running may decrease PNN expression in the DG. Upon analyzing confocal images for the density of PNN+ cells, we found lower cell densities of PNN+ neurons in runner mice in the dDG ($p = 0.01$). Cell densities of PNN+ cells were not reduced in runners relative to controls in the vDG based on statistical significance ($p = 0.16$), but the analysis used a small sample size ($n = 6$ brains) and displayed a trend towards decreased densities of PNN+ cells in runner vDG. The adult brains in this analysis came from the cohort that began running in adolescence. These findings suggest that running may exert its positive effects on behavior through decreases in DG PNNs (Figure 6.3B). This finding is consistent with prior data from our lab showing increases in anxiety-like behavior correlated with increased PNN optical intensity in vDG[32]. Together, these results suggest that PNNs may modulate DG synaptic plasticity in a way that is important for anxiety regulation.

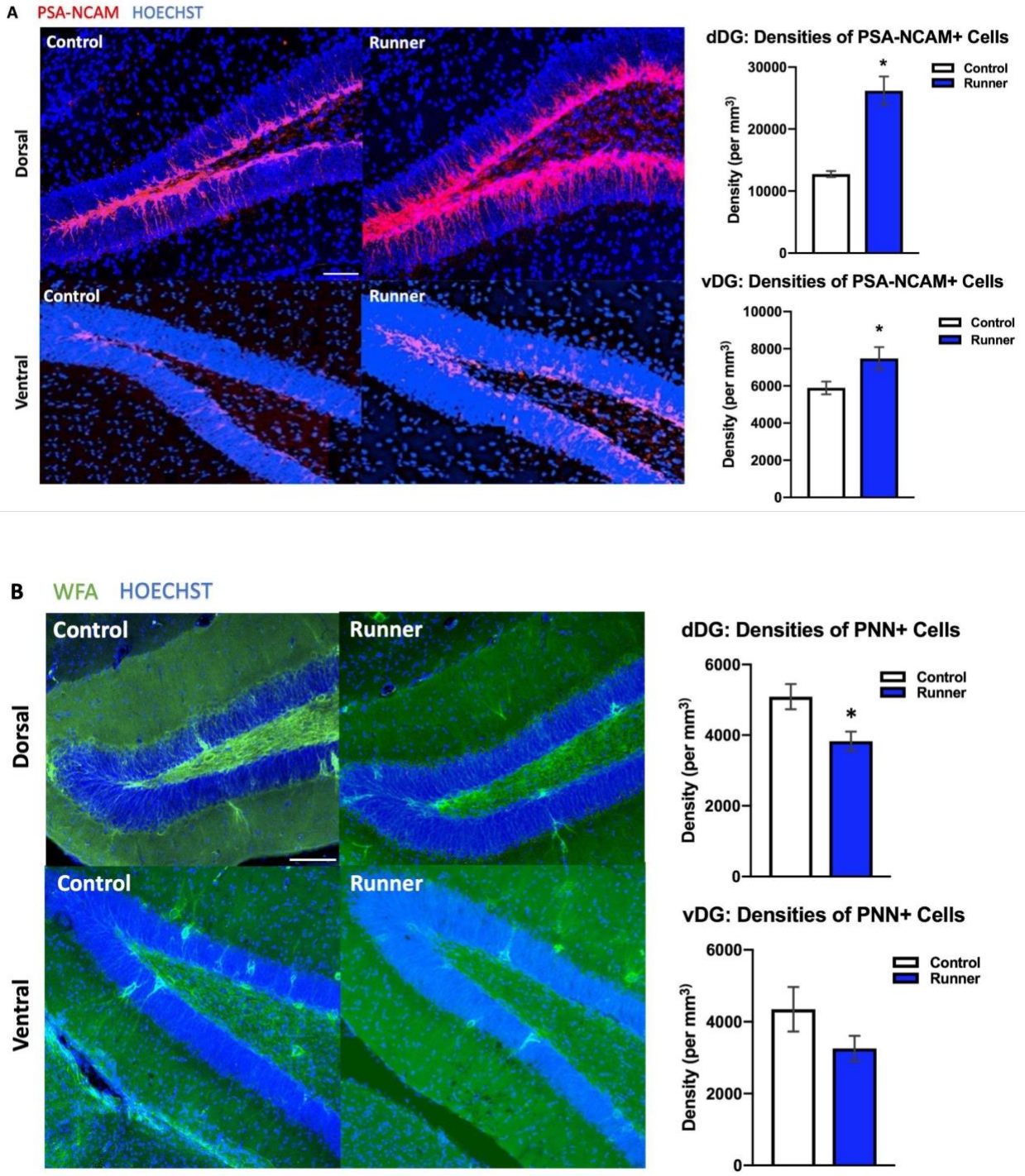

Figure 6.3. Running is associated with higher densities of new neurons and lower densities of PNNs in the hippocampal dentate gyrus. A) Male runners (n =7) show higher densities of PSA-NCAM+ immature neurons (red) in the dDG ($p = 0.0001$) and vDG ($p = 0.04$) after 6 weeks of running compared to non-runner controls (n = 7). **B)** Male runners (n = 6) demonstrate lower PNN+ cell densities in the dDG after 6 weeks of voluntary wheel running ($p = 0.01$) compared to non-runner controls (n = 6). Histochemical staining labels *Wisteria floribunda agglutinin* (green) and Hoechst (blue). Error bars are ± SEM. *$p < 0.05$. Scale bars = 200 μm.

To assess vDG neural activity after long-term running, we analyzed the expression of the immediate early gene (IEG) c-Fos, a proxy for neuronal activity[33], in hippocampal cells. Studies have shown that IEG expression occurs approximately 1 hour after activation in excitatory neurons and approximately 2 hours after activation in inhibitory neurons[34]. Mice were challenged at the end of behavioral testing with 15 minutes of bright light exposure on a wet, blocked off open arm of the EPM and perfused 2 hours later, to target inhibitory neural activity. We observed higher densities of c-Fos+ cells in runners (Figure 6.4A) in both the dDG and vDG. Across all animals, independent of experimental condition, we also found a negative correlation, albeit not statistically significant ($r = -0.42$, $p = 0.08$), between latency to feed on the NSF test and c-Fos+ cell density in the vDG (Figure 6.4B), suggesting an association between inhibitory neural activity in the vDG and reductions in anxiety-like behavior. This result is consistent with previous findings showing that runner mice demonstrate decreased anxiety-like behavior and increased GABAergic measures in the vHIP, including greater percentages of vDG inhibitory interneurons co-labeled with c-Fos, in response to a stressor[35]. The mice used for these experiments began running in adulthood.

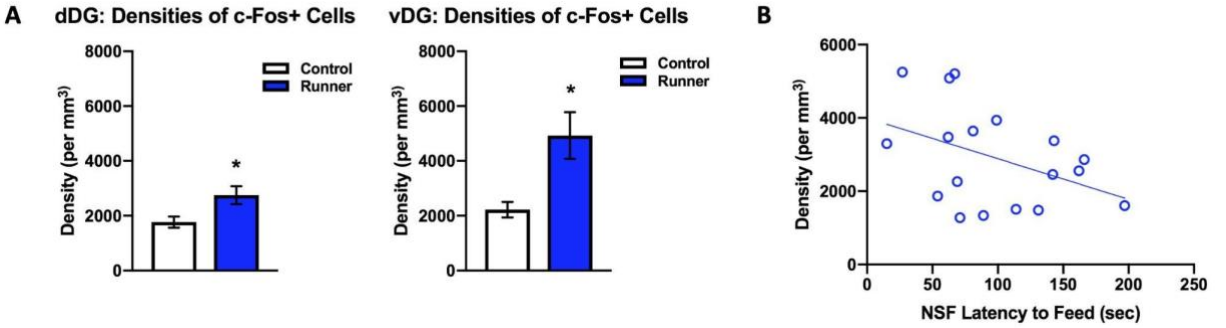

Figure 6.4. Runner mice show increased densities of active inhibitory cells in the hippocampal dentate gyrus in response to an environmental challenge. A) Adult male runner mice (n = 10) show higher densities of c-Fos+ cells compared to controls (n = 10) in both the dDG ($p = 0.02$) and vDG ($p = 0.007$) 2 hours following bright, wet EPM open arm exposure. **B)** A marginal negative correlation exists ($r = -0.42$, $p = 0.08$) between latency to feed (seconds) on the NSF test and c-Fos+ cell density in the vDG, suggesting a potential association between enhanced vDG inhibition under stressful environmental conditions and decreased anxiety. Error bars are ± SEM. *$p < 0.05$.

6.2.3 Running increases PNN and c-Fos expression in ventral CA1

Although our discussion of the relationship between the vHIP and anxiety[36] has thus far focused on the vDG, as newborn granule cells are located in the DG, the ventral CA1 (vCA1) hippocampal subregion has also been implicated in anxiety-like behavior. In vivo calcium imaging has shown that the vCA1, but not the dorsal CA1 (dCA1), is rich in anxiety cells, and optogenetic inactivation of these cells produces anxiolytic effects by reducing avoidance behavior[37]. Basolateral amygdala (BLA)-vCA1 synapses have been found to bidirectionally modulate anxiety-related behaviors[38], and populations of neurons in the vCA1 which "double-project" to both the medial prefrontal cortex (mPFC) and BLA play a crucial role in contextual fear conditioning[39]. Upon cell counting and analysis, we found higher cell densities of PNN+ neurons in the vCA1 of runner mice (Figure 6.5A, $n = 10$ per group, $p = 0.02$) compared to sedentary controls, as well as higher densities of c-Fos+ cells (Figure 6.5B, $p = 0.0002$), which negatively, although non-significantly, correlated with anxiety-like behavior on the NSF test (Figure 6.5C, $r = -0.45$, $p = 0.08$). The mice used for these experiments began running in adulthood.

In contrast, there was no difference in density of PNN+ cells (Figure 6.5A, $p = 0.58$) between runners and controls and a significant reduction in density of c-Fos+ cells (Figure 6.5B, $p = 0.03$) in runners in the dCA1. Although these findings contrast with the running-induced decrease in PNN expression observed in the DG and previous results showing decreased PNN expression in the CA1[19], they suggest the possibility of running-induced, ventral-specific molecular plasticity and inhibitory neural activity in hippocampal CA1. To the best of our knowledge, no direct vDG-vCA1 projections have been established. Thus, it may also be

worthwhile to investigate and target the vCA1 while exploring the relationship between PNN+ neuron density and anxiety-related phenotypes[40].

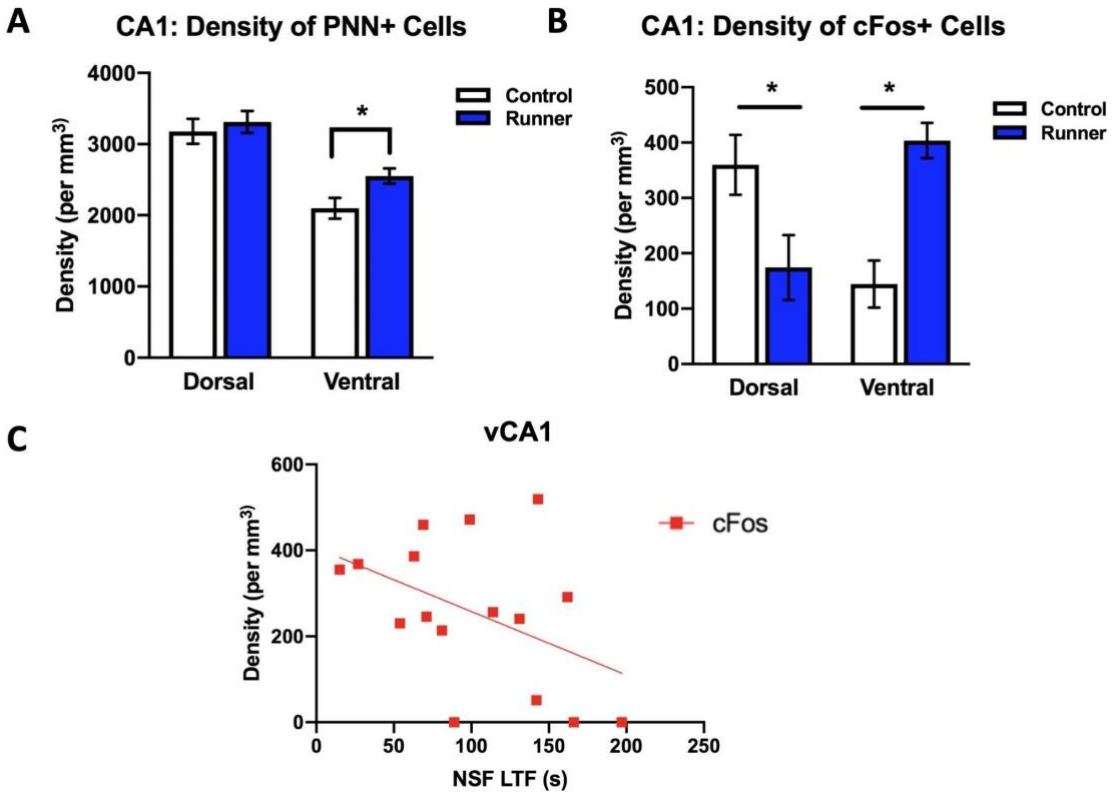

Figure 6.5. Runner mice show increases in PNN+ and cFos+ cell densities in the vCA1. A) Male runner mice (n = 10) show higher densities of PNN+ cells compared to controls (n = 10) in the vCA1 ($p = 0.02$) but not dCA1 ($p = 0.58$). **B)** Male runner mice (n = 10 per group) show higher densities of c-Fos+ cells in the vCA1 ($p = 0.0002$) but lower densities of c-Fos+ cells in the dCA1 ($p = 0.03$) 2 hours following bright, wet EPM open arm exposure. **C)** A marginal negative correlation exists ($r = -0.45$, $p = 0.08$) between latency to feed (seconds) on the NSF test and c-Fos+ cell density in the vCA1. Error bars are ± SEM. *$p < 0.05$.

6.2.4 Exercise-induced changes in PNN+ cell density observed in the hippocampus do not extend to the broader corticolimbic circuitry

Beyond the vHIP, we also wanted to consider whether long-term running induces molecular plasticity in other brain regions within the corticolimbic neural circuitry underlying rodent anxiety-like behaviors. Hence, we examined non-cell type-specific PNN expression in the BLA, mPFC, and anterior cingulate cortex (ACC) (Figure 6.6), as these brain regions are

involved in threat detection and appraisal, and the subsequent generation of defensive and anxiety-like behaviors[40]. Between runner and control brains of the mice that began running in adulthood, we found no significant differences in PNN+ cell densities in the BLA ($p = 0.86$), mPFC ($p = 0.23$), or ACC ($p = 0.95$), reinforcing our focus on the hippocampus, specifically the DG and vCA1 subregions, in studying the effects of exercise on molecular plasticity.

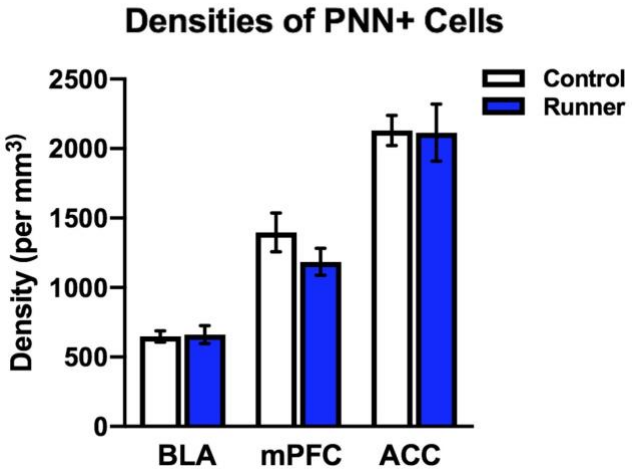

Figure 6.6. Physical exercise does not alter the overall density of PNN+ cells in corticolimbic brain regions implicated in rodent defensive behaviors. No changes were observed in PNN+ cell densities in the BLA ($p = 0.86$), mPFC ($p = 0.23$), or ACC ($p = 0.95$) between runners ($n = 10$ brains) and controls ($n = 10$ brains).

6.3 Discussion

6.3.1 Summary

The experiments detailed above were designed to test the potential roles of hippocampal new neurons and PNNs in the anxiety-reducing effects of physical activity in mice. After 6-8 weeks of running, adult male runner C57BL/6 male mice demonstrated significantly lower anxiety-like behaviors compared to controls on both the EPM and NSF assays. Consistent with the literature and previous work in the lab, our preliminary results showed increases in vDG and dDG new neuron densities in runner mice, using PSA-NCAM as a marker of immature neurons. Analysis of PNN+ cell densities also suggested that running may exert its positive effects on

behavior through decreases in PNNs. This is consistent with previous work in the lab, done by Sahana Murthy, in which degrading PNNs via chondroitinase ABC (chABC) infusion in the sedentary mouse vDG significantly reduced anxiety-like behavior in the EPM test, mimicking the behavioral effects of running. We also found evidence of increased inhibitory activity in the DG and increases in PNN+ neurons and c-Fos+ cells in the vCA1 in runners relative to controls following an environmental challenge. The lack of differences between runners and control in PNN+ cell densities in other brain regions previously implicated in rodent defensive behaviors focuses our attention to probing molecular plasticity further within subregions of the hippocampus. However, it remains possible that within these other brain regions, there could be meaningful PNN density differences in specific cell types, which cancel each other out and were simply not captured by this analysis.

Overall, preliminary results reflected a decrease in defensive behaviors accompanied by increases in neurogenesis, decreases in PNNs, and increases in inhibitory neural activity in the mouse DG following physical exercise. Subsequent experiments could extend these findings by examining colocalization, or lack thereof, of PV and PNNs and connections between new neurons and PV+ cells in the vDG versus the dDG. Next steps would include recording and analyzing local field potentials from runner and control mice during behavior, to determine whether running alters neuronal oscillations in association with anxiety regulation. Final experiments would entail manipulating new neurons and PNNs with alternative strategies, to evaluate whether these changes are sufficient to recapitulate the effects of running on oscillatory activity and behavior.

Since conducting this work, new research has emerged showing that voluntary wheel running can decrease brainstem sensory nuclei PNNs and restore lumbar PNNs following spinal

cord injury[41], correlating with improvements in multiple locomotor parameters. More relevant to my work, it has also been found that removing PNNs in hippocampal CA1 with chABC disrupts the retention of long-term fear memory while reducing the firing rate of PV+ neurons and inhibitory synaptic transmission in both PV+ and excitatory cells in CA1[42]. These findings are potentially consistent with my own, as these authors saw a decrease in inhibitory neural activity upon a decrease in PNN density, and I observed an increase in inhibitory activity with an increase in PNN density in vCA1. Although loss of fear memory may be in line with anxiety regulation, these authors did not distinguish between subregions of the CA1. It is possible that their electrophysiological and behavioral findings are more reflective of dCA1, where I also found suggestive evidence of decreased inhibitory activity in runners. Another research group has found that voluntary wheel running stimulates neurogenesis and decreases fear memory retention while decreasing expression of PNNs and c-Fos in CA1[43]. Discrepancies in our findings could again be due to the failure to distinguish between dorsal and ventral CA1 or be attributed to sex differences in the mechanisms of exercised-induced anxiety regulation. The aforementioned study used only female mice, whereas I used only males.

Despite the initial promise of some of my findings, we ultimately decided to stop the project due to replicability issues. Although the preliminary behavioral and immunohistochemical results shown here, from the adolescent cohort that ran for 6-8 weeks from 3 weeks of age into adulthood, were consistent with our hypotheses and the existing literature, I could not replicate the behavioral differences once we expanded our numbers. The initial reductions in anxiety-like behavior after running were also specific to males. Our behavioral pilot tests revealed that anxiety decreased in male runners in the EPM and NSF tests but not in females. Female runners only showed decreased anxiety-like behavior in the open field test[44],

displaying a greater amount of time in the center of the brightly-lit arena and less time in the periphery, which was not observed in males. Although these behavioral differences could have provided an opportunity to explore sex differences, we were initially focused on replicating the anxiety-reducing effects of running from the literature. Studying males seemed more promising for gaining a better mechanistic understanding of this phenomenon, as we could establish a phenotype that was consistent across 2 different anxiety tests. One of the greatest challenges of working with animal models of anxiety in behavioral neuroscience remains that the results of different anxiety-like behavior tests frequently do not correlate with one another[45–48]. Thus, multiple tests must be performed to gain an accurate understanding of whether and how an experimental manipulation may be impacting anxiety.

To address the inconsistencies between our expanded behavioral data and the literature, we decided to test a new cohort that ran for 4 weeks beginning at 12 weeks of age, as opposed to adolescence. Although we were able to replicate the result from the original NSF test, the EPM result was the opposite; male runners spent a decreased percentage of time in the open arms of the plus maze, indicating heightened anxiety. We also failed to establish a significant decrease in PNN+ cells in the vDG in this cohort, which was a core tenet of the project. Without being able to establish a highly reliable phenotype (anxiety reduction) and a clear molecular plasticity change (PNN degradation), it did not seem prudent to proceed with further experiments. However, if the hypothesized phenotypes and structural plasticity changes could be replicated, I have several ideas for how this project could be executed.

6.3.2 Future Directions

If the foundational principles of running increasing adult hippocampal neurogenesis, decreasing PNN density, and reducing anxiety-like behavior could be established with greater

confidence, this project could be advanced by 1) determining whether running enhances connectivity between new neurons and PV+ cells in the adult vDG, and if these connections play a causal role in regulating anxiety. It would then be important to 2) investigate the effects of long-term running on vDG molecular plasticity and anxiety-like behavior, and determine the role of PNNs in regulating anxiety, if any.

Assessing neuronal connectivity and anxiety regulation

Previous literature and findings from the Gould laboratory suggest that running promotes the production and survival of new hippocampal neurons[27–29], regulates anxiety[12], and enhances GABAergic signaling in the vHIP[35,49]. However, it remains unresolved how exactly abGCs are integrated into vHIP circuitry together with inhibitory interneurons to produce these effects. A combination of behavioral analyses, retroviral labeling of new neurons, histology, optogenetics, and *in vivo* electrophysiology in awake behaving mice could be used to determine whether new hippocampal granule cells reduce anxiety through their direct connections to PV+ cells. Rene Hen's lab has developed strategies to target and optically stimulate or silence newborn neurons[21,50]. Although one prior study concluded that new neurons were not necessary for running induced anxiety reduction, using the GFAP-TK (thymidine kinase) transgenic mouse model to ablate adult neurogenesis[51], these experimenters blocked adult neurogenesis globally and for several weeks prior to the start of wheel running[26]. This design could have allowed for reorganization of the DG and engagement of compensatory mechanisms which enabled post running anxiety reduction. It would be important to reassess the role of new neurons in exercise-induced anxiety reduction if the cells are allowed to develop normally in response to sedentary living or running and are functionally activated or silenced *during* behavioral testing.

Ideally, we would see that running increases the density of new neurons in the vDG and

the connections of these new neurons with PV+ inhibitory interneurons, which in turn disrupts theta oscillations and decreases anxiety. If running does not produce the expected anxiety reductions in our behavioral tasks, we could reexamine the results of individual animals on the EPM and NSF tests to determine if they could be split into low/high anxiety subpopulations, as is often done in stress studies[52,53,54]. We could also pilot additional defensive behavioral tests or use automated home cage monitoring to assess behavioral differences between runner and control mice in a more ethologically relevant context.

To further explore the relationship between exercise-induced new neurons and anxiety regulation, the transgenic mouse line iBax^{nestin} [55] could be used to enhance hippocampal neurogenesis in sedentary animals, as an alternative approach to the optogenetic activation of new neurons. The iBax^{nestin} line harnesses a genetic gain of function strategy to eliminate expression of *Bax*, a gene that regulates programmed cell death, selectively in neural stem cells in the adult brain[55]. If enhancing adult neurogenesis through the iBax^{nestin} model does not reduce anxiety, it would also be important to stimulate cell proliferation to determine if targeting that particular stage of adult neurogenesis is necessary for reductions in anxiety-like behavior. The iBax^{nestin} model promotes neurogenesis through the survival of abGCs, whereas running is known to stimulate both cell proliferation and survival[27]. Drugs such as ISX-9[56] and P7C3[57] have been found to be "neurogenic," and are one means by which we could assess the necessity of cell proliferation in adult hippocampal neurogenesis for anxiety reduction. In place of optogenetically silencing new neurons in runners, we could use the aforementioned GFAP-TK mouse line to ablate hippocampal neurogenesis. In this mouse line, herpes simplex virus thymidine kinase is activated in dividing GFAP+ cells to selectively eliminate neural progenitor cells upon treatment with the antiviral drug valganciclovir.

Investigating molecular plasticity and anxiety regulation

Previous data from the Gould lab suggest that running enhances local inhibitory mechanisms in the vHIP of mice[35], and it has been found in rats that physical exercise may differentially alter PNNs in the brain and spinal cord, such that running significantly decreases PNN expression in the hippocampus[19]. Taken together, it is plausible that running could reduce the number of PV+ cells surrounded by PNNs in the vDG and subsequently expose PV-expressing inhibitory interneurons to greater synaptic input from adult-generated granule cells. Presynaptic excitatory inputs from abGCs cells may promote overall inhibition of vHIP circuitry in anxiety-provoking contexts in part by disrupting synchronous firing in the theta range of PV+ interneurons. If larger sample sizes could be used to demonstrate that PNN density decreases in the vDG following running, these hypotheses could be tested further using viral injections and transcription factor infusions. Specifically, viral injections inducing Cre-lox recombination in aggrecan-loxP$^{+/+}$ mice could be used to decrease PNNs in the vHIP. Local infusions of the transcription factor OTX2 could be used to increase PNNs in the vHIP. These methods have been used previously to decrease and increase PNNs in other brain regions[58,59].

The cre-dependent viral manipulation in an aggrecan knockout mouse model[58] could be used to degrade PNNs in the vDG before running and behavioral testing. Aggrecan is a primary constituent of PNNs[60], and PNNs are ablated upon aggrecan removal[58]. The Acan loxP$^{+/+}$ mouse line conditionally knocks out aggrecan by removing exon 4 of the *Acan* gene by Cre-lox recombination[58]. Bilateral OTX2 infusion could be used to increase PNNs surrounding PV+ cells in the vDG prior to running and behavioral testing. OTX2 is a transcription factor that stimulates PNN assembly and has previously been used to enhance PNN expression and maturation in PV+ cells upon direct injection to the visual cortex[59].

If running could be shown to decrease the densities and possibly intensities of vDG PNNs ensheathing PV+ inhibitory interneurons, with the effects of disrupting theta oscillations and decreasing anxiety, and if PNN decreases are sufficient to reproduce these effects of running, we would expect sedentary AAV-injected aggrecan-loxP$^{+/+}$ mice to resemble running AAV-injected aggrecan-loxP$^{+/-}$ mice. If PNN reductions are necessary for running-dependent improvements in anxiety regulation and reductions in vDG theta power, we would expect runner mice receiving a control vehicle to show behavioral improvements and reduced neuronal oscillations, but not runner mice receiving OTX2. If OTX2 runners show reduced anxiety-like behavior on the EPM and NSF tests compared to vehicle controls, running may influence hippocampal microcircuitry and behavior independent of PNNs.

Given the conflicting findings between the Gould lab and a previous study[19] regarding the effects of physical exercise on PNN expression in hippocampal CA1, it would be worthwhile to explore if manipulating PNNs, using these same methods, in the vCA1 alters behavior. Because we found a ventral-specific PNN density increase in runners, we predict that increasing vCA1 PNN expression in sedentary mice would decrease anxiety-like behavior and decreasing vCA1 PNN expression in runners would increase anxiety-like behavior. However, if we repeat the initial histology with a larger n size and find a decrease in vCA1 PNN expression in C57BL/6 runners, consistent with the previous published report, we would expect the same outcomes as those observed in the vDG.

If knocking down aggrecan expression did not fully degrade PNNs in the vDG, an alternative strategy would be to infuse chABC directly into the brain region. ChABC is a bacterial enzyme that is widely accepted as a method of digesting PNNs in experimental animals[61,62,63] by dissolving the chondroitin sulfate proteoglycans that PNNs are composed of[61]. One drawback of

chABC injection is that it non-specifically digests the entire ECM. Thus, it is imperative to pilot the time frame and dose of the enzyme so that the resulting PNN reduction in sedentary mice roughly matches that of running. However, this technique might provide greater confidence that the PNNs are fully removed. Preliminary work from the Gould lab has shown that sedentary mice bilaterally injected with chABC in the vDG (n = 11) demonstrate less anxiety-like behavior on the EPM test at 10 dpi compared to penicillinase (control enzyme)-injected mice (n = 5), spending a higher percentage of overall time in the open arms of the apparatus ($p = 0.0002$). These findings would need to be repeated and extended, but preliminary work suggests that decreases in PNNs in the vDG mimic the behavioral effects of running.

Overall, it is possible that either stimulating adult neurogenesis or degrading PNNs in these experiments could dissociate the dependent measures such that we could observe reductions in anxiety-like behavior but not decreased synchrony of theta rhythm, or vice versa. If our manipulations would yield anxiety reduction, but no change in theta oscillatory activity, it could be the case that other cell types beyond PV+ cells influence the vDG excitatory/inhibitory network. While overall inhibition through the activation of PV+ inhibitory interneurons may promote anxiety reduction, other running-induced changes could make it so that neural firing is not less synchronous in the theta range. For example, enhanced theta power due to increased locomotion could cancel out the diminished theta power typically associated with anxiety reduction for a net zero change in synchronous activity. Furthermore, running has been demonstrated to spur hippocampal structural alterations beyond adult neurogenesis, including increased dendritic spine density and dendritic tree complexity on CA1 pyramidal cells[64]. It is possible that running could promote changes in CA1 pyramidal cells which could also influence theta rhythm independent of anxiety like behavior.

6.4 Methods

6.4.1 Setup

Environmental optimization to isolate the effects of running on behavior

We conducted extensive piloting to determine the ideal running environment for mice. It is widely accepted that stress generates anxiety-related behaviors. Subsequently, we aimed to 1) eliminate any unanticipated stressors that could negatively impact adult neurogenesis and 2) isolate the effects of running from the project's outset. The literature suggests that housing a group of male mice with only one wheel in the home cage increases aggression, and that this aggression can be diminished by lowering the ratio of mice to wheels[65]. Hence, we piloted different home cage set-ups, with pair versus group-housing (5 mice per cage), more versus less living space, and 2 different types of running wheels: vertical and disc. Overcrowding from reduced cage space, competition for a limited desired resource, and the development of "wheel tail" malformations are potential negative repercussions which could make running an aversive rather than a rewarding experience, reducing neurogenesis throughout the hippocampus. Our initial observations of group-housed mice on a disc running wheel showed that the mice were competing for the wheel and developing tail deformities. Additionally, when mice are housed in larger enclosures, they tend to demonstrate less anxious behavior on two commonly used behavioral tests of anxiety, the Elevated Plus Maze (EPM) and Novelty-Suppressed Feeding (NSF) tasks, even in the sedentary condition. Thus, we believe there could be an anxiolytic impact of living in a larger enclosure alone. To study the effects of running and not environmental enrichment in general, we decided to use a pair-housed mouse cage arrangement.

Animals

Male C57BL/6 mice (The Jackson Laboratory) were pair-housed, provided food and water *ad libitum*, and maintained on a 12 h light/dark cycle. Mice were randomly assigned to two groups: runner and control. Runners had *ad libitum* access to a wireless running wheel (Med Associates Inc.), and control mice received a locked wireless running wheel inside the home cage. Within 3 weeks of receiving the wheel, each cage of mice ran approximately 20 kilometers per day or greater. The mice either ran or lived sedentarily prior to testing on the Elevated Plus Maze (EPM) and Novelty Suppressed Feeding (NSF) tasks as assays of anxiety-like behavior. Two cohorts of mice were studied: one adolescent cohort, in which the mice were bred in-house and began running at 3 weeks of age for 6-8 weeks, and one adult cohort, in which the mice arrived and began running at 12 weeks of age for 4 weeks.

Following behavioral testing, the mice were deeply anesthetized with Euthasol (120 mg/kg, Virbac) and then transcardially perfused with 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS). All experiments were approved by the Princeton University Institutional Animal Care and Use Committee and compliant with the NRC Guide for the Care and Use of Laboratory Animals.

6.4.2 Behavioral assays

The Elevated Plus Maze (EPM) and Novelty Suppressed Feeding (NSF) behavioral paradigms were used to quantify anxiety-like behavior in mice. The dependent measures to be interpreted as anxiety reduction in the EPM and NSF tests, respectively, are higher percentages of entries and time spent in the open arms of the apparatus, and shorter latencies to approach a food pellet in the center of a brightly-lit novel environment. Analyses of movement and home cage feeding behavior were performed as control tests to evaluate general activity and hunger

levels, which were assessed respectively through number of open and closed arm entries and amount of food pellet consumed in the home cage.

Elevated Plus Maze (EPM)

Mice were placed in the center region of the elevated plus maze (44 × 44 × 20 inches). Two of the arms had high walls (13 inches), and the other two arms and central intersection were open. All arms were 20 inches in length, with the open arms illuminated to 330-350 lux. Exploratory behavior was recorded for 5 minutes and analyzed. The videos were hand scored by an experimenter measuring the number of entries and time spent in each of the open/closed arms for each individual animal. More time spent in the open arms is interpreted as a measure of reduced anxiety, due to the open arms having greater exposure to light and open air.

Novelty-Suppressed Feeding (NSF)

The NSF test was performed as previously characterized in the literature[66]. Mice were food deprived in the home cage for 24 hours prior to testing. A single pellet of rodent chow (LabDiet) was placed on white filter paper in the center of a square acrylic open field (17.5 inches x 17.5 inches), with opaque walls and center illumination of ~700 lux. A camera was suspended above the middle of the arena, and each mouse was video recorded until it ate or reached the time limit. Each mouse was placed in the corner of the open field, and the latency to approach and consume the food pellet was observed. If the mouse did not feed within 600 seconds, the test was ended, and a latency of 600 seconds was assigned to that animal. Mice were returned to their home cages immediately after testing.

6.4.3 Histology, imaging, and analysis

Immunohistochemistry

Because IEG expression has been found to be maximal approximately 2 hours following the initial activation of inhibitory neurons[34], mouse perfusion was timed to occur 2 hours following our defensive behavior tests. Upon transcardial perfusion, brains were extracted from the mice and post-fixed for 48 hours in 4% PFA. Brains were then cryoprotected via sucrose immersion for 48 hours. Coronal sections (40μm-thick) were cut from half brains using the cryostat (Leica Biosystems) and refrigerated until processing.

We used c-Fos as a marker for neuronal activation[67] and polysialylated neuronal cell adhesion molecule (PSA-NCAM) as a marker for immature neurons. For c-Fos labeling, sections were washed with PBS and pre-blocked with 0.3% Triton X-100 and 3% normal donkey serum in PBS for 1.5 hours at room temperature. Sections were then incubated in preblock solution containing biotinylated *Wisteria floribunda* agglutinin (WFA), a plant-based lectin that labels PNNs via binding to the N-acetylgalactosamine component of the nets, and rabbit anti-c-Fos for 24 hours at 4°C. Washed sections were then incubated for 1.5 hours at room temperature with anti-streptavidin Alexa Fluor 488 for WFA and donkey anti-rabbit Alexa Fluor 568 for c-Fos. Sections were then counterstained with Hoechst 33342, mounted onto slides, and coverslipped over glycerol in PBS (3:1).

The procedure for PSA-NCAM immunostaining was nearly identical to the one described above. For this stain, free-floating sections were rinsed in PBS and incubated in a pre-block solution containing rat anti-PSA-NCAM for 24 h at 4°C. They were then incubated for 1.5 hours at room temperature with donkey anti-rat Alexa Fluor 568, counterstained with Hoechst 33342, mounted, and coverslipped.

Table 6.1: Reagent Resource Table

Name	Species	Dilution	Type	Company
Polysialylated neuronal cell adhesion molecule (PSA-NCAM)	rat	1:400	Primary	BD Pharmingen (San Diego, CA)
c-Fos	rabbit	1:500	Primary	Cell Signaling Technology
Wisteria floribunda agglutinin (WFA) lectin	N/A	1:500	Primary	Sigma-Aldrich
Alexa Fluor anti-rat 568	donkey	1:250	Secondary	Invitrogen
Alexa Fluor anti-rabbit 568	donkey	1:500	Secondary	Invitrogen
Alexa Fluor anti-streptavidin 488	N/A	1:500	Secondary	Molecular Probes

Confocal imaging and cell density quantification

Images were acquired using a confocal microscope (Zeiss-LSM 700) at 20x objective. All slides were coded to blind the experimenter to group (runner or control). Whole cell counts of PSA-NCAM were made in the granule cell layer of the dorsal and ventral dentate gyrus. Whole cell counts of WFA and c-Fos were made in the granule cell layer of the dentate gyrus, throughout the BLA, and throughout all cortical layers of the mPFC and ACC. Counts were taken using Stereo Investigator software (MBF Bioscience, Williston, VT) or images were analyzed with ImageJ. The perimeter of each subregion was outlined based on the Allen Mouse Brain Atlas, and cells within the delineated region were labeled and counted. Cell densities were determined by dividing the total number of positive cells by the volume of the region measured.

Statistics

For behavioral and cell density analyses, differences between groups were determined using unpaired two sample t-tests. Correlations were calculated based on the Pearson's

correlation coefficient. All figures depict mean ± SEM, with "*" indicating statistical significance at $p < 0.05$. GraphPad Prism 8.0 (GraphPad Software) and RStudio 3.0.1 were used for statistical analyses and data visualization.

6.5 Acknowledgments

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