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Characterization of a Neuroendocrine Network That Coordinates Sugar and Water Ingestion in Drosophila melanogaster

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Characterization of a Neuroendocrine Network That Coordinates Sugar and Water Ingestion in *Drosophila melanogaster*

By

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Abstract

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Consumption of food and water is tightly regulated by the nervous system to maintain internal nutrient homeostasis. Although generally considered independently, interactions between hunger and thirst drives are important to coordinate competing needs. In chapter 1, I provide a review of hunger regulation and thirst regulation in mammals and *Drosophila melanogaster*. I then discuss the neural and hormonal coordination of hunger and thirst in both mammals and *D. melanogaster*.

In *Drosophila*, four neurons called the Interoceptive Subesophageal zone Neurons (ISNs) respond to intrinsic hunger and thirst signals to oppositely regulate sucrose and water ingestion. In chapter 2, I characterize the neural circuit downstream of the ISNs. This work is presented in the form of a preprint first author manuscript. Using the fly brain connectome, genetic tools, behavioral assays, and functional imaging, I show, together with co-authors Gina Pontes, Nicholas Jourjine and Alexander Del Toro, that the ISNs modulate a peptidergic network of neurons. These include a novel cell type Bilateral T shaped neuron (BiT), insulin producing cells (IPC), crustacean cardioactive peptide (CCAP) neurons, and CCHamide-2 receptor isoform RA (CCHa2R-RA) neurons. These neurons contribute differentially to ingestion of sugar and water, with BiT, IPCs and CCAP neurons oppositely regulating sugar and water ingestion, and CCHa2R-RA neurons modulating only water ingestion. Thus, the decision to consume sugar or water occurs via regulation of a broad peptidergic network that integrates internal signals of nutritional state to generate nutrient-specific ingestion.

In chapter 3, I characterize other neurons that are involved in sugar and/or water ingestion. Using a computational approach to identify neurons in close proximity, genetic manipulation, behavioral assays, and *in vivo* functional imaging, I characterized several neurons involved in sugar and/or water ingestion regulation. Aster, bidirectionally regulates sugar and water ingestion just as the ISNs, Horseshoe, decreases both sugar and water ingestion, and Cowboy likely promotes sugar ingestion. *In vivo* functional connectivity experiments revealed that they were not downstream of the ISNs, however,

Aster, possibly Horseshoe, and another cell type Gallinule are downstream of sensory neurons. Thus, I have identified another cell type that bidirectionally regulates sugar and water ingestion, two cell types that modulate feeding, and a cell type that likely conveys gustatory information to memory centers in the fly brain.

In chapter 4, I describe with co-authors Zoila Alvarez-Aponte, Rachel Brem, Diana Bautista and Denzil Streete, the development and implementation of the Inclusive Excellence in Quals Prep (IEQP) program. This program was designed to provide mentorship, community, and academic support for students from diverse backgrounds as they prepared for their QE. The main components for IEQP program included pairing students with graduate student mentors, academic and wellness workshops, and community building events. This program was first implemented on a pilot cohort of 11 graduate students. After program evaluation, the most significant component of the program was peer mentorship. After program completion, students' perception of their preparedness, QE-related skills, the support received from their advisors, and the agency they felt over their proposed work increased.

In the final part of this dissertation, chapter 5, I summarize the main findings of this dissertation and propose future directions for exploration.

Table of Contents

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Chapter 1: Introduction

Eating is crucial for an animal's survival. Animals are constantly weighing internal nutrient needs against external nutrient availability. More importantly, animals need to distinguish which specific nutrients are in deficit to manage competing needs. Feeding is tightly regulated by the nervous system. Hormonal signals of hunger and thirst, along with circulating nutrients, are detected by the nervous system to promote appropriate ingestion and restore homeostasis. However, how the nervous system coordinates ingestion of sugar and water in response to hunger and thirst signals remains largely unexplored. In this chapter, I briefly review what we know about hunger and thirst regulation in mammals and insects, and recent findings on how these competing needs are coordinated by the nervous system.

Hunger regulation

Hunger regulation in mammals

In mammals, innervation from peripheral nerves as well as detection of circulating factors in the bloodstream serve to signal nutritional status and promote or inhibit food ingestion. The vagus nerve, which carries information from the gut to the brain, senses gut distension and hormones that signal nutritive information (Prescott & Liberles, 2022, Smith et al., 1985). The vagus nerve innervates the brain to control feeding (Prescott & Liberles, 2022, Powley & Phillips, 2004).

Circulating factors released from peripheral organs can also act directly on neurons in the brain to signal hunger and satiety (Schwartz et al., 2000). For example, ghrelin, produced in the gut, and glucagon, produced in the pancreas, increase feeding. Leptin, produced in adipose tissue, and insulin, produced in the pancreas, reduce feeding (Prescott & Liberles, 2022, Schwartz et al., 2000).

Additionally, peptides produced by neurons in the brain also signal nutritive state to alter feeding. Some of the peptides that have been heavily studied include pro-opiomelanocortin (POMC), which inhibits feeding, and agouti-related peptide (AgRP) and neuropeptide Y (NPY), which promote feeding (Aponte et al., 2011, Krashes et al., 2011, Sternson, 2013).

AgRP producing neurons represent a critical node in the feeding pathway (Vohra et al., 2022). These neurons are located in the arcuate nucleus of the hypothalamus and promote food ingestion. Seminal experiments showed that activation of AgRP neurons rapidly promotes feeding in fed mice, while silencing these neurons significantly decreases feeding in starved mice (Aponte et al., 2011, Krashes et al., 2011). Further experiments found that AgRP neurons have an increased firing rate in food-deprived mice (Mandelblat-Cerf et al., 2015). AgRP neurons receive information on nutritive state from circulating factors including insulin, leptin, and glucose (Deem et al., 2022, Zigman and Elmquist, 2003). Additionally, AgRP neurons receive sensory information upon food presentation (Chen et al., 2015, Horio & Liberles, 2021). AgRP neurons in turn secrete AgRP, NPY, and γ-aminobutyric acid (GABA) (Krashes et al., 2013). NPY and GABA are required for fast induction of feeding, while AgRP mediates a delayed and sustained

increase in feeding through its antagonistic action on melanocortin 4 receptors (Krashes et al., 2013, Tao, 2010). AgRP neurons project to multiple brain areas, including the several nuclei of the hypothalamus, preoptic area, suprachiasmatic nucleus, amygdala, periaqueductal gray, and parabrachial nucleus (Deem et al., 2022, Wang et al., 2015, Wu et al., 2009). Despite their innervation of multiple brain areas, AgRP neurons are non-collateralizing, suggesting that there are distinct subtypes of AgRP neurons that could differentially regulate feeding under specific conditions (Betley et al., 2013).

Another important cell type involved in feeding regulation are the POMC producing neurons (Vohra et al., 2022). These neurons are also located in the arcuate nucleus of the hypothalamus and inhibit food ingestion (Sternson, 2013). Deletion of the POMC gene induces an obesity phenotype in mice (Yaswen et al., 1999). POMC mRNA levels in the arcuate nucleus decreased in food-deprived rats (Brady et al., 1990). Furthermore, optogenetic stimulation of POMC neurons reduced food ingestion and body weight in mice (Aponte et al., 2011). POMC neurons can detect circulating ghrelin, glucose, insulin, and leptin secreted from peripheral organs, and receive input from the gut via the vagus nerve to detect nutrient ingestion (Balthasar et al., 2004, Cheung et al., 1997, Cowley et al., 2003, Sternson, 2013). POMC neurons secrete melanocortins, which bind and activate melanocortin receptors, including melanocortin 4 receptors, to suppress feeding (Aponte et al., 2011). POMC neurons project to many brain areas, including several nuclei of the hypothalamus, hippocampus, and brainstem (Cone, 1999, Haskell-Luevano et al., 1999). In addition to reaching several brain regions, POMC neurons co-express different neurotransmitters, suggesting different subtypes of POMC neurons that could potentially regulate feeding distinctly (Hentges et al., 2009; Meister et al., 2006).

AgRP and POMC neurons have opposing effects on feeding: AgRP neurons induce feeding while POMC neurons reduce feeding. Interestingly, both cell types affect melanocortin 4 receptors. Melanocortins secreted from POMC neurons activate melanocortin 4 receptors to decrease feeding, while AgRP secreted from AgRP neurons have an antagonistic effect on melanocortin 4 receptors to increase feeding. Additionally, AgRP and POMC neurons innervate many of the same brain regions. AgRP neurons also directly inhibit POMC neurons (Cowley et al., 2001, Roseberry et al., 2004), providing another mechanism to promote feeding under starvation. Thus, this circuit receives information from circulating factors, the gut and sensory system on the nutritive state of the animal and food availability in order to appropriately regulate feeding.

Hunger regulation in insects

Like mammals, *Drosophila* melanogaster contains neurons that sense nutrient abundance and use many of the same molecules to signal nutrient levels (Pool and Scott, 2014). In starved states, *Drosophila* use adipokinetic hormone (AKH), analogous to mammalian glucagon, and Neuropeptide F (NPF), analogous to mammalian NPY, to signal nutrient release from organ storage and food intake (Brown et al., 1999, Lee et al., 2004). In sated states, *Drosophila* use Unpaired2, a leptin ortholog, and insulin-like

peptides (dILPs) and the insulin receptor to signal nutrient uptake from the hemolymph, the circulatory fluid of insects, and reduce food intake (Hartenstein, 2006, Nässel et al., 2013). Additionally, the concentration of circulating sucrose in the hemolymph also acts as a hunger or satiation signal (Kim & Rulifson, 2004, Kréneisz et al., 2010).

AKH is secreted from the corpora cardiaca, a cluster of endocrine cells adjacent to the brain. During starvation, low levels of circulating glucose induce secretion of AKH (Kim & Rulifson, 2004). AKH binds to the AKH receptor, expressed in the fat body. The fat body, analogous to the human liver and adipocytes, is the main energy storage in flies and responds to signals of nutrient deprivation to mobilize energy storage (Zheng et al., 2016). AKH signals sugar and lipid release from the fat body into the hemolymph, analogous to the function of mammalian glucagon (Lee et al., 2004, Orchard, 1987).

Another hunger signal is NPF, analogous to mammalian NPY. NPF is secreted in the midgut and by neurosecretory cells in the brain. Overexpression of NPF increases food intake, while ablation of NPF expressing neurons reduces food intake (Lee et al., 2004, Wu et al., 2003). NPF neural activity is suppressed by the leptin analog Unpaired2 (Beschel et al., 2017). Interestingly, recent studies of the midgut derived NPF found opposing results, where loss of NPF leads to increase in food intake and decrease in circulating sucrose (Yoshinari et al., 2021). These studies demonstrated that enteroendocrine cells in the midgut secrete NPF in response to dietary sugar which binds to the NPF receptor found in corpora cardiaca cells. NPF signaling suppresses AKH secretion from corpora cardiaca cells (Yoshinari et al., 2021).

In adult flies, insulin signaling has been shown to regulate an array of physiological processes including metabolism and feeding. *Drosophila* has eight insulin-like peptides, dILPs1-8, which have distinct spatiotemporal expression throughout development and adulthood, suggesting they have different functions (Nässel et al., 2013). There is only one insulin receptor (InR), a tyrosine kinase type receptor, which binds dILP1, 2, 3, 5, and 6 (Brogiolo et al., 2001, Clancy et al., 2001, Gronke et al., 2010, Tatar et al., 2001). dILP4 is thought to only be expressed in embryonic stages (Brogiolo et al., 2001). dILP7 is thought to bind to relaxin type receptor Lgr4 (Van Hiel et al., 2015, Veenstra et al., 2012). dILP8 binds to relaxin type receptor Lgr3 (Colombani et al., 2015, Garelli et al., 2015, Vallejo et al., 2015). dILP2, 3, and 5 are co-secreted from insulin producing cells (IPCs) in the brain (Brogiolo et al., 2011). dILP secretion from IPCs depends on the hunger state of the fly (Géminard et al., 2009). Unpaired2, a leptin ortholog secreted by the fat body in fed states, controls insulin release from IPCs. (Hartenstein, 2006; Rajan & Perrimon, 2012). Increased levels of extracellular glucose, as those experienced in fed states, also activate IPCs (Lin et al., 2019, Kréneisz et al., 2010). Additionally, NPF signaling via the NPF receptor promotes dILP secretion from IPCs (Yoshinari et al., 2021).

Thirst regulation

<u>Thirst regulation in mammals</u>

Thirst is the drive to ingest water that results from fluid loss that naturally happens throughout the day (Gizowski & Bourque, 2018, Todini & Fantuz, 2023). Early experiments found that injection of hypertonic solutions into the lamina terminalis (LT) produced a thirst response (Andersson, 1953). The LT is composed of three nuclei: the organum vasculosum lamina terminalis (OVLT), subfornical organ (SFO), and median preoptic nucleus (MnPO) (Zimmerman et al., 2017). The OVLT and SFO are located outside of the blood-brain barrier, meaning that they have direct access to the blood and are poised to sense circulating factors and changes in blood osmolality (McKinley et al., 2004, Zimmerman et al., 2017). Neurons in the LT also receive information on the sodium concentration of the plasma sensed by visceral afferents via the nucleus tractus solitarius (NTS) and the parabrachial nucleus (PBN) (Gizowski & Bourque, 2018, Zimmerman et al., 2017).

In the SFO, neurons expressing neuronal nitric oxide synthase (nNOS) promote water ingestion while neurons expressing the vesicular GABA transporter (VGAT) inhibit water ingestion. These cells directly detect blood osmolarity and receive input from the vagus and trigeminal nerves that detect water ingestion. (Gizowski & Bourque, 2018).

The OVLT and MnPO have been implicated in thirst response, but specific cell types involved in drinking have only recently been identified (Zimmerman et al., 2017). The OVLT contains 13 transcriptomic cell classes, including 8 neuronal classes (Pool et al., 2020). While all neuronal types responded to water deprivation, cell types responded differently to osmotic vs hypovolaemic thirst, suggesting distinct neuronal response to different thirst states (Pool et al., 2020). OVLT neurons expressing the angiotensin 1A receptor ($OVLT^{Agtr1a}$) are activated during thirst and induce drinking (Leib et al., 2017). MnPO neurons expressing Adcyap1 receive input from the SFO and OVLT, are activated during thirst, and induce drinking (Leib et al., 2017).

Neurons in the OVLT, SFO, and MnPO send projections to the thalamus which then projects to the anterior cingulate cortex (ACC) and insular cortex (IC) to direct appropriate physiological responses to maintain fluid homeostasis (Denton et al., 1999, Farrell et al., 2006, Robinson & Mishkin, 1968, Pastuskovas et al., 2003). ACC and IC are considered the sites where thirst perception is generated (Egan et al., 2003, Gizowski & Bourque, 2018). The ACC and IC are activated upon thirst, and activity in both regions rapidly declines after drinking water to satiation (Egan et al., 2003, Farrell et al., 2011, Pastuskovas et al., 2003). Additionally, activation of ACC and IC rapidly produces drinking (Denton et al., 1999, Farrell et al., 2006, Robinson & Mishkin, 1968, Pastuskovas et al., 2003).

Thirst regulation in insects

Very little is known about water ingestion regulation in *Drosophila*. Most studies have focused on factors that regulate water reabsorption in the Malpighian tubules, analogous to mammalian kidneys (de Haro et al., 2010; Liu et al., 2015; Paluzzi et al., 2014; Terhzaz et al., 2012). Recent studies have identified the first hormone described to regulate water ingestion in *Drosophila*, the ion transport peptide (ITP). ITP is a

functional analog of vasopressin; it promotes water ingestion and water reabsorption from the hindgut (Gáliková et al., 2018). ITP expression is elevated under osmotic stress (high Na^+ diet). Overexpression of ITP in sated flies, causes flies to drink, while knockdown of ITP in dehydrated flies decreases their drive to drink (Gáliková et al., 2018). ITP producing neurons are located in the brain and ventral nerve cord, and include 6 different cell types (Gáliková et al., 2018). There is currently no known receptor for ITP, therefore its mechanism to induce drinking and water reabsorption from the gut remains unclear.

Another recent study identified Janu neurons whose activity promotes thirsty water seeking, but has a limited effect on drinking (Landayan, et al., 2021). There are four Janu neurons in the central brain: two GABAergic neurons (Janu-GABA) and two Janu-AstA neurons, which secrete neuropeptide Allatostatin A. Janu-GABA are interneurons located in the subesophageal zone (SEZ), the taste and feeding center of the fly brain. Janu-AstA are projection neurons that innervate the SEZ and the superior medial protocerebrum, the endocrine center of the fly brain (Landayan, et al., 2021). Janu-AstA neurons communicate with NPF neurons that express the AstA-R2 receptor. AstA-R2 knockdown in NPF neurons reduced water intake as well as water seeking. While Janu-AstA activity has a limited effect on drinking, AstA signaling in NPF neurons regulates both water seeking and water ingestion (Landayan, et al., 2021).

Coordination of hunger and thirst

Coordination of hunger and thirst in mammals

The nervous system must be able to balance competing needs in order to enact the appropriate physiological response. Recent studies have found communication between thirst promoting and hunger promoting neurons in the SFO and the arcuate nucleus of the hypothalamus. Activation of AgRP neurons of the arcuate nucleus of the hypothalamus increases feeding. Interestingly, authors found that activating these neurons also decreased water ingestion (Burnett et al., 2016). Furthermore, nNOS expressing neurons in the SFO also modulate both drinking and feeding. nNOS expressing cells in the SFO are activated upon dehydration and promote drinking. Inhibition of nNOS expressing cells in the SFO reduced drinking and increased food ingestion (Zimmerman et al., 2016). This is thought to be due to dehydration induced anorexia, where animals will feed less when thirsty. Because nNOS expressing neurons in the SFO are activated upon dehydration, silencing these neurons should increase feeding. This suggests that thirst sensing cells promote water ingestion and inhibit food ingestion, while hunger sensing cells do the opposite (Jourjine, 2017).

Studies in mammals have found that hunger signals can modulate activity of neurons in thirst sensing regions of the brain. The SFO, one of the nuclei involved in thirst regulation, is glucose sensitive (Medeiros et al., 2012). Using patch clamp electrophysiological experiments, researchers found a population of glucose inhibited neurons that depolarized to decreases in glucose concentration and hyperpolarized to increases in glucose concentration. They also found a population of glucose activated

SFO neurons that depolarized to increases in glucose concentration and hyperpolarized to decreases in glucose concentration (Medeiros et al., 2012). This suggests that the SFO may integrate blood osmolarity with blood glucose concentration to regulate ingestion.

The SFO has a high density of amylin receptors. Amylin is a peptide that is co-secreted with insulin from the pancreas upon food intake, and negatively regulates food intake (Rink et al., 1993). Scientists found that SFO neurons were excited upon amylin perfusion (Riediger et al. 1999). This excitatory effect was blocked upon application of the amylin receptor antagonist AC-187. Additionally, amylin subcutaneous injections increased water intake in rats (Riediger et al. 1999). Authors suggest that amylin release after feeding functions to stimulate prandial drinking (Riediger et al. 1999).

The SFO also expresses the ghrelin receptor (Pulman et al., 2006). Ghrelin is a peptide secreted from the gut and brain which stimulates food intake (Cowley et al., 2003, Kojima et al., 1999). Using voltage-clamp electrophysiology, Pulman et al. found that ghrelin activates a population of SFO neurons (Pulman et al., 2006). Since previous studies found that amylin, an inhibitor of food intake, activates SFO neurons, authors tested SFO neurons' response to amylin and ghrelin. Authors found SFO neurons that respond only to amylin, and only to ghrelin, but none that respond to both peptides (Pulman et al., 2006). Additionally, ghrelin has been found to reduce water intake, but only under certain conditions (Hashimoto et al., 2007, Mietlicki et al., 2009). This suggests that ghrelin and amylin may work together to regulate water intake in response to feeding. Because the SFO nucleus is found outside of the blood-brain barrier, it is strategically located to sense multiple circulating factors. This suggests the SFO as a possible integration site to other hypothalamic nuclei that control nutrient intake (Ferguson, 2014).

Coordination of hunger and thirst in insects

ITP functions as an anti-diuretic hormone that increases water ingestion, reduces food intake, and increases metabolic rate (Gáliková et al., 2018, Gáliková & Klepsatel, 2022). Recent studies found that ITP interacts with the AKH pathway (Gáliková & Klepsatel, 2022). ITP promotes AKH secretion from the CC cells and transcription of the AKH receptor. Authors found that ITP’s glycemic effect is AKH dependent, as overexpression of ITP increased glucose in the hemolymph in a standard but not in an AKH-deficient background (Gáliková & Klepsatel, 2022). However, other metabolic processes are AKH independent. AKH does not mediate the reduction in food intake, as overexpression of ITP still decreases food intake in AKH-deficient backgrounds. Similarly, ITP RNAi increased food intake independently of the presence of AKH (Gáliková & Klepsatel, 2022). Therefore, ITP’s mechanism to reduce food intake remains unclear.

Janu-AstA, but not Janu-GABA, were found to increase water seeking behavior and inhibit feeding behavior (Landayan et al., 2021). AstA knockdown in Janu neurons increases sucrose ingestion. Janu-AstA neurons are upstream of NPF neurons that express the AstA-R2 receptor. NPF neurons release NPF, which signals nutrient

release from organ storage and food intake (Brown et al., 1999, Lee et al., 2004). Therefore, Janu-AstA neurons likely inhibit the NPF neurons. This is consistent with previous findings that AstA functions as an inhibitory neurotransmitter in mammals (Lechner et al., 2002). Interestingly, AstA signaling in NPF neurons modulates both sugar and water ingestion, as AstA-R2 knockdown in NPF neurons increases sugar ingestion and decreases water ingestion (Landayan et al., 2021).

Recent studies from the Scott lab have identified a single pair of neurons called Interoceptive Subesophageal zone Neurons (ISNs) in D. melanogaster that detect both hunger and thirst signals to oppositely regulate sucrose and water ingestion (Jourjine, Mullaney et al., 2016). ISNs are activated by the endogenous signal of nutrient deprivation AKH through the AKH receptor. ISN activation increases sucrose ingestion and requires the AKH receptor, suggesting that AKH activates the ISNs to promote sucrose ingestion. ISNs sense water abundance through Nanchung (Nan), a Transient receptor potential (TRP) channel which acts as molecular sensor of hemolymph osmolarity. Jourjine et al. showed that ISNs hyperpolarize in response to increases in osmolarity, which correlates with water deprivation, and depolarize when osmolarity decreases. These responses to changes in osmolarity require Nan expression. Accordingly, activation of ISNs decreases water ingestion, while silencing ISNs increases water ingestion. These results suggest that thirst signals, such as increased hemolymph osmolarity, inhibit ISNs to promote water ingestion. Thus, ISNs intrinsically detect hunger and thirst signals to regulate sucrose and water ingestion. Hunger, detected through the AKHR, activates the ISNs to promote sucrose ingestion and inhibit water ingestion. Thirst, detected through Nan, inhibits the ISNs to promote water ingestion and inhibit sugar ingestion.

How the ISN neural activity achieves these bidirectional effects on ingestion remains unclear. To investigate how the ISNs transform internal nutrient detection into changes in feeding behaviors, we examined the neural network downstream of the ISNs. Using the fly brain connectome, intersectional genetic approaches, *in vivo* functional imaging, and behavioral assays we identified a neural circuit downstream of the ISNs that regulates sugar and water ingestion. We identified a novel neuron which we named Bilateral T-shaped neuron (BiT), that is postsynaptic to the ISNs and oppositely regulates sugar and water ingestion. BiT downstream neurons include insulin producing cells (IPCs) and the uncharacterized CCHamide-2 receptor isoform RA (CCHa2R-RA) neurons, and crustacean cardioactive peptide (CCAP) neurons. We found that BiT, IPCs, and CCAP neurons reciprocally regulate sugar and water ingestion, while CCHa2R-RA neurons only regulate water ingestion. Together, our work reveals that the ISNs communicate with the neuroendocrine center of the fly brain and regulate the activity of a large number of neurons that transmit or receive peptidergic signals of nutritive state to bidirectionally regulate sugar and water ingestion.

Chapter 2: Hunger- and thirst-sensing neurons modulate a neuroendocrine network to coordinate sugar and water ingestion

SUMMARY

Consumption of food and water is tightly regulated by the nervous system to maintain internal nutrient homeostasis. Although generally considered independently, interactions between hunger and thirst drives are important to coordinate competing needs. In *Drosophila*, four neurons called the Interoceptive Subesophageal zone Neurons (ISNs) respond to intrinsic hunger and thirst signals to oppositely regulate sucrose and water ingestion. Here, we investigate the neural circuit downstream of the ISNs to examine how ingestion is regulated based on internal needs. Utilizing the recently available fly brain connectome, we find that the ISNs synapse with a novel cell type, Bilateral T-shaped neuron (BiT), that projects to neuroendocrine centers. *In vivo* neural manipulations revealed that BiT oppositely regulates sugar and water ingestion. Neuroendocrine cells downstream of ISNs include several peptide-releasing and peptide-sensing neurons, including insulin producing cells (IPC), crustacean cardioactive peptide (CCAP) neurons, and CCHamide-2 receptor isoform RA (CCHa2R-RA) neurons. These neurons contribute differentially to ingestion of sugar and water, with IPCs and CCAP neurons oppositely regulating sugar and water ingestion, and CCHa2R-RA neurons modulating only water ingestion. Thus, the decision to consume sugar or water occurs via regulation of a broad peptidergic network that integrates internal signals of nutritional state to generate nutrient-specific ingestion.

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INTRODUCTION

The survival of an organism depends on its ability to coordinate nutrient ingestion with internal nutrient abundance in order to meet its metabolic needs. The nervous system acts as an internal nutrient abundance sensor to drive ingestion in nutrient-deprived states and inhibit ingestion in nutrient-replete states to restore homeostasis (Gizowski & Bourque, 2018, Jourjine, 2017, Sternson, 2013, Qi et al., 2021, Yoshinari et al., 2021). Although generally considered independently, recent studies have demonstrated that interactions between hunger and thirst signals coordinate competing needs (Burnett et al., 2018, Cannell et al., 2016, Jourjine, Mullaney et al., 2016, Watts & Boyle, 2010, Zimmerman et al., 2016).

In mammals, regulation of hunger and thirst drives likely occurs through interactions between food and water ingestion circuits (Eiselt et al., 2021). In the arcuate nucleus of the hypothalamus, neurons that express the agouti-related peptide (AgRP) and neuropeptide Y promote food ingestion while neurons that express pro-opiomelanocortin inhibit food ingestion (Aponte et al., 2011, Graham et al., 1997, Sternson, 2013). These neurons can detect circulating ghrelin, glucose, insulin, and leptin secreted from peripheral organs, in addition to receiving input from the gut through the vagus nerve (Sternson, 2013). In the subfornical organ, neurons expressing neuronal nitric oxide synthase (nNOS) promote water ingestion while neurons expressing the vesicular GABA transporter inhibit water ingestion. These cells directly detect blood osmolality and receive input from the gut via the vagus nerve and from the

mouth via the trigeminal nerve (Gizowski & Bourque, 2018, Zhang et al., 2022). Interestingly, activation of AgRP neurons decreases water ingestion and inhibition of nNOS expressing cells increases food ingestion (Burnett et al., 2016; Zimmerman et al., 2016). This suggests that hunger sensing cells promote food ingestion and inhibit water ingestion, while thirst sensing cells do the opposite (Jourjine, 2017). However, the underlying circuit mechanisms that lead to this reciprocal coordination of hunger and thirst remain unexplored.

Because of its numerically less complex nervous system, complete connectome, and abundant genetic tools, *Drosophila* is an ideal organism in which to study the coordination of hunger and thirst (Pfeiffer et al., 2008). Like mammals, *Drosophila melanogaster* selectively consumes food when hungry and water when thirsty (Dethier, 1976, Gáliková et al., 2018, Landayan et al., 2021, Lin et al., 2014, Min et al., 2016, Yapici et al., 2016). Moreover, in *Drosophila*, two pairs of neurons, the Interoceptive Subesophageal zone Neurons (ISNs), directly integrate hunger and thirst signals to oppositely regulate sugar and water ingestion (Jourjine, Mullaney et al., 2016).

The ISNs express the adipokinetic hormone receptor (AKHR), a G-protein coupled receptor which binds to the glucagon-like peptide adipokinetic hormone (AKH), a hormone released from the corpora cardiaca during starvation that signals nutrient deprivation (Orchard, 1987, Gáliková et al., 2015). AKH increases ISN activity to drive sugar ingestion and reduce water ingestion. The ISNs also express the TRPV channel Nanchung, which senses changes in hemolymph osmolality. High hemolymph osmolality, such as that experienced during thirst, decreases ISN activity to promote water ingestion and inhibit sugar ingestion (Jourjine, Mullaney et al., 2016). How the ISNs achieve these effects on ingestion remains unclear.

To investigate how the ISNs transform internal nutrient detection into changes in feeding behaviors, we examined the neural network downstream of the ISNs. Using the fly brain connectome, intersectional genetic approaches, *in vivo* functional imaging, and behavioral assays, we identified a neural circuit downstream of the ISNs that regulates sugar and water ingestion. Our work reveals that the ISNs communicate with the neuroendocrine center of the fly brain and regulate the activity of a large number of neurons that transmit or receive peptidergic signals of nutritive state to bidirectionally regulate sugar and water ingestion.

RESULTS

The ISNs are peptidergic neurons that release dILP3

To examine how the ISNs reciprocally regulate sugar and water ingestion, we aimed to identify the neural circuit downstream of the ISNs. We first sought to identify which neurotransmitter the ISNs use to communicate with downstream neurons. We expressed RNAi against enzymes involved in neurotransmitter synthesis, vesicular transporters, and neuropeptides in the ISNs and monitored water ingestion in water deprived flies (Figure 1A). As decreasing activity of the ISNs increases water ingestion (Jourjine, Mullaney et al., 2016), we anticipated that an RNAi against the ISN neurotransmitter would decrease neurotransmission and increase water ingestion. Interestingly, in an RNAi screen of 18 common neurotransmitters and neuropeptides, only suppression of *Drosophila* insulin-like peptide 3 (dILP3) in the ISNs altered water ingestion (Figure 1A).

To confirm that dILP3 functions in the ISNs and to test whether it is involved in the reciprocal regulation of water and sugar ingestion, we expressed RNAi against dILP3 in the ISNs and measured sugar or water ingestion in water sated or thirsty flies respectively (Figure 1B). As an additional approach to reduce dILP3, we expressed an RNAi against a neuropeptide processing protease, *amontillado* (Siekhaus & Fuller, 1999), in the ISNs and tested sugar and water ingestion. We found that knockdown of either dILP3 or *amontillado* in the ISNs caused both a decrease in sugar ingestion and an increase in water ingestion (Figure 1B). This is the same phenotype that was previously reported in the ISNs upon loss of neurotransmission (Jourjine, Mullaney et al., 2016). These data argue that the ISNs are peptidergic neurons that release dILP3 and that one function of dILP3 is to promote sugar ingestion and inhibit water ingestion.

The ISNs synapse onto neurons that arborize in neuroendocrine and feeding centers

Drosophila has one insulin-like receptor (dInR), a tyrosine kinase type receptor homologous to the human insulin receptor, which binds dILP3 and six of the additional *Drosophila* insulin-like peptides (Brogiolo et al., 2001, Claeys et al., 2002, Clancy et al., 2001, Fernandez, et al., 1995, Grönke et al., 2010, Nässel & Broeck, 2016, Tatar et al., 2001). In adult flies, insulin signaling has been shown to regulate an array of physiological processes including metabolism, feeding, reproduction, and lifespan (Badisco et al., 2013, Biglou et al., 2021, Clancy et al., 2001, Nässel et al., 2013, Ohhara et al., 2018). Since dInR is ubiquitous and involved in many different processes (Chen et al., 1996, Garofalo, 2002, Veenstra et al., 2008), we could not leverage neurotransmitter receptor identity for postsynaptic neuron identification. We instead used the *trans*-Tango system (Talay et al., 2017), a genetic trans-synaptic tracer, to label neurons postsynaptic to the ISNs (Supp 1A). We expressed the *trans*-Tango ligand in the ISNs and its receptor panneurally. Binding of the ligand to its receptor induces GFP expression in the receptor-expressing cells and labels potential synaptic partners (Talay et al., 2017). *trans*-Tango labeling revealed numerous ISN postsynaptic arborizations in the subesophageal zone (SEZ), a brain region associated with taste processing and feeding circuits (Gordon & Scott, 2009, Scott et al., 2001, Wang et al., 2004), and along the median bundle to the superior medial protocerebrum (SMP), a neuroendocrine center (Hartenstein, 2006, Nässel & Zandawala, 2020) (Supp 1A). However, as many ISN candidate postsynaptic neurons were labeled, the morphology of individual neurons was unclear.

To comprehensively examine the postsynaptic partners of the ISNs, we employed the Full Adult Fly Brain (FAFB) volume, a whole-brain electron microscopy volume that provides synaptic resolution of all neurons in the fly brain (Zheng, Lauritzen et al., 2018). We manually reconstructed the ISNs using CATMAID (Li et al., 2019, Saalfeld et al., 2009) by tracing neuronal arbors from the pharyngeal nerve with large cell bodies in the SEZ. Due to the ISNs' unique morphology, with large cell bodies near the pharyngeal nerve and dense neurites in the flange that cross the midline, we used visual morphological comparison of the reconstructed ISNs in the FAFB volume (Figure 1C) and light microscopy images of *ISN-Gal4* (Figure 1D) to identify the ISNs. Once we had reconstructed the ISNs, we labeled presynaptic sites in the ISNs and postsynaptic

sites in other neurons based on known synapse active zone structure (Zhai & Bellen, 2004). We then reconstructed neurons that were postsynaptic to the ISNs.

Soon after we had reconstructed the four ISNs and several postsynaptic neurons in CATMAID, the FlyWire whole brain connectome of more than 80,000 reconstructed EM neurons became available (Dorkenwald et al., 2022, flywire.ai). Since FlyWire uses the FAFB volume, we used the coordinates of the ISNs we traced in CATMAID to locate them in FlyWire. Additionally, we compared a pointcloud generated from a registered light microscopy image of *ISN-Gal4* (Figure 1D) to the reconstructed ISNs in the FAFB volume (Figure 1C) to further confirm ISN identity. We identified neurons downstream of the ISNs (Fig 1E). We found that the ISNs have 104 postsynaptic partners with 5 or more synapses, comprising 9 morphological cell types (Supplementary Table 1, Supp 1C-K). These include known cell types (Cowboy, DSOG1, FLAa2, FLAa3/Lgr3 and the ISNs; Lee et al., 2020, Pool et al., 2014, Sterne et al., 2021, Yu et al., 2013) as well as many uncharacterized cell types. The ISN postsynaptic partners include projection neurons that project along the median bundle to the SMP (64 cells), local SEZ neurons (18 cells), ascending neurons with projections coming through the neck connective (10 cells), descending neurons with projections leaving through the neck connective (8 cells), and the ISNs themselves (4 cells). This connectivity is consistent with the connectivity determined by *trans*-Tango (Supp 1A). Overall, the ISN synaptic connectivity suggests that the hunger and thirst signals sensed by the ISNs are conveyed to a broad network, with the potential to coordinate feeding behaviors (SEZ neurons), nutrient status (SMP neuroendocrine centers), and movement or digestion (ascending and descending neurons). We note that, as neuropeptides may diffuse over long distances (van den Pol, 2012), ISN dILP3 release may also influence activity of additional neurons that are not synaptically connected to the ISNs.

The ISN postsynaptic neuron BiT reciprocally regulates sugar and water ingestion

As the majority of the ISN postsynaptic partners project to the SMP, we examined whether ISN communication to this region regulates neuroendocrine cells and/or influences feeding behavior. As a first step, we focused on an uncharacterized neuron that receives the most synaptic input from the ISN per single cell. We named this neuron Bilateral T-shaped neuron (BiT). BiT has its cell body in the SEZ and bilateral projections in the flange and SMP. It receives 7.4% of ISN synaptic output (301/4050 synapses) (Supp 1B and Supplementary Table 1). In turn, the ISNs are the main synaptic input to BiT, comprising 17% of BiT's synaptic input (301/1763 synapses). We generated a split-Gal4 line that labels BiT to study its function (Fig 2B). We screened over 20 AD-DBD combinations and found that *VT002073-Gal4.AD* and *VT040568-Gal4.DBD* specifically labeled BiT. We confirmed this by comparing a pointcloud generated from a registered light microscopy image of *BiT split-Gal4* (Fig 2B) with the reconstructed BiT in the FAFB volume (Fig 2A).

To test whether the ISNs are functionally connected to BiT, we conducted *in vivo* functional imaging experiments in which we activated the ISNs while simultaneously monitoring BiT's neural activity. We expressed the light activated cation channel Chrimson in the ISNs and the voltage sensor ArcLight in BiT (Fig 2C) (Jin et al., 2012, Klapoetke et al., 2014). In one experiment, we applied two consecutive 2s stimulations

(Fig 2D) to test whether the response was reproducible. In another experiment, we applied a longer 30s stimulation (Fig 2E) to ensure we captured the full response to ISN stimulation, since dILPs can act over longer time scales (Sudhakar et al., 2020). In both experiments, we found that stimulating the ISNs increased ArcLight fluorescence in BiT, demonstrating that BiT became hyperpolarized (Fig 2D-E). Oscillation in BiT's response during the 30s stimulation (Fig 2E) is due to oscillations in the LED stimulation paradigm. Thus, increased activity in the ISNs inhibits BiT.

Next, we tested whether BiT modulates sugar or water ingestion. We measured total ingestion time of sugar or water while activating or inhibiting BiT. We found that acute optogenetic activation of BiT decreased sugar ingestion and increased water ingestion (Fig 2F). Moreover, reducing synaptic transmission in BiT using nSynaptobrevin (nSyb) RNAi caused increased sugar ingestion and decreased water ingestion (Fig 2G). These data demonstrate that BiT is both necessary and sufficient to regulate sugar and water ingestion. Furthermore, we find that the activation and silencing phenotypes for BiT are opposite to the ISN phenotypes, consistent with the conclusion of our calcium imaging studies that the ISNs inhibit BiT. These findings reveal that the coordination of sugar and water ingestion is maintained downstream of the ISNs.

These studies demonstrate that BiT activity reciprocally regulates sugar and water ingestion, similar to the ISNs. Hunger signals (i.e. adipokinetic hormone) activate the ISNs, causing the ISNs to inhibit BiT, which in turn increases sugar ingestion. On the other hand, thirst signals (i.e. high hemolymph osmolality) inhibit the ISNs, releasing ISN inhibition onto BiT, causing an increase in water ingestion (Fig 2H). Strikingly, although BiT is only one ISN downstream neuron, its activity increases and decreases are sufficient to coordinate both sugar and water ingestion, suggesting that it is a critical node in the ISN network.

BiT downstream partners include neuroendocrine cells that convey nutritional status

To examine how BiT coordinates sugar and water ingestion, we investigated the neural circuit downstream of BiT using the FlyWire connectome (Fig 3). The FAFB connectivity revealed that BiT has 93 postsynaptic partners. Unlike the ISNs' downstream partners, which only innervate the SMP and SEZ, BiT postsynaptic partners reach more brain regions including the superior lateral protocerebrum (SLP), fan shaped body (FB), lobula, SMP, and SEZ. This suggests that the hunger and thirst signals detected by the ISNs are conveyed by BiT to widely regulate brain activity.

Many of the BiT postsynaptic partners arborize in both the SEZ and SMP, suggesting that they might coordinate nutritional status and feeding. Several BiT targets transmit or receive peptidergic signals of nutrient state. For example, BiT postsynaptic partners include insulin producing cells (IPCs), FLAa3/Lgr3 neurons, and neurons labeled by the *CCHa2R-RA-Gal4* line (Deng et al., 2019) (Fig 3, Supplementary Table 2). IPCs are a well-studied cell type that release dILP2, dILP3 and dILP5, regulate glucose uptake, and influence many physiological processes including feeding (Nässel et al., 2013, Ohhara et al., 2018). FLAa3/Lgr3 neurons detect dILP8 and influence sugar ingestion (Meissner et al., 2016, Yeom et al., 2021, Yu et al., 2013). CCHa2 and its receptor CCHa2R have been shown to participate in feeding regulation and regulate

insulin signaling, although the function of CCHa2R-RA neurons has not been examined (Deng et al., 2019, Ida et al., 2012, Ren et al., 2015, Sano et al., 2014, Shahid et al., 2021). Thus, BiT synapses onto many neuroendocrine neurons, possibly enabling integration of the hunger and thirst signals sensed by ISNs with diverse nutrient state signals.

IPCs regulate sugar and water ingestion

The IPCs integrate multiple signals of nutrient status and regulate feeding and metabolism (Nässel & Zandawala, 2020). We found that the ISNs are connected to the IPCs via BiT. BiT is the main synaptic input into IPCs, making up 25% of the IPCs' synaptic input (442/1735) and IPCs receive 25% of BiT's synaptic output (442/1742) (Fig 3, Table 2). We tested whether BiT is functionally connected to the IPCs by optogenetically stimulating BiT and monitoring activity in IPCs using the calcium sensor GCaMP6s (Chen et al., 2013). We found that BiT inhibits IPCs (Supp 3A-B), consistent with neurotransmitter predictions (Eckstein et al., 2020) that BiT uses glutamate, which can act as an inhibitory neurotransmitter in *Drosophila* (Liu et al., 2013).

To test whether IPCs modulate ingestion of sucrose or water under conditions that reveal ISN behavioral phenotypes, we measured ingestion time of sucrose or water while acutely activating the IPCs. We found that acute activation of IPCs increased sucrose ingestion and decreased water ingestion (Supp 3E). These results are consistent with one study (Sudhakar et al., 2020) but differ from other studies showing that acute IPC activation limits ingestion of sucrose or food (Nässel et al., 2015, Wang et al., 2020). IPCs integrate many signals and release multiple peptides (Sano et al., 2015, Söderberg et al., 2012, Ohhara et al., 2017, Wang et al., 2020), suggesting that differences in these behavioral results may, in part, stem from differences in the current nutritional state sensed by the IPCs. While further experiments are needed to elucidate how IPCs coordinate nutrient state and ingestion under different conditions, our results show that BiT regulates IPC activity, and that IPC activity coordinates both sugar and water ingestion.

CCHa2R-RA neurons regulate water ingestion downstream of BiT

A number of studies indicate that CCHa2 and its receptor, CCHa2R, promote food intake and appetite in various insects, including blowflies (Ida et al*.,* 2012), aphids (Shahid et al., 2021), and *Drosophila* (Ren et al*.,* 2015). BiT synapses with CCHa2R-RA neurons, four neurons with cell bodies in the SEZ and arbors in the flange and pars intercerebralis (PI) (Fig 4B). BiT is the dominant input onto CCHa2R-RA neurons, comprising 94% of CCHa2R-RA presynaptic sites (171/181 synapses). CCHa2R-RA neurons receive the most output from BiT per single cell comprising 13% of BiT's output (228/1742 synapses). To investigate whether BiT's synaptic input to CCHa2R-RA neurons regulates ingestion, we examined the functional connectivity between BiT and CCHa2R-RA neurons and the behavioral phenotypes associated with CCHa2R-RA neurons.

We monitored activity in CCHa2R-RA neurons with the calcium indicator GCaMP6s upon optogenetic stimulation of BiT; however, we did not observe a response in CCHa2R-RA neurons (Supp 4D-E). As BiT likely inhibits CCHa2R-RA neurons, it is possible that we were unable to detect an inhibitory response in CCHa2R-

RA neurons using a calcium sensor. We therefore monitored activity of CCHa2R-RA neurons upon optogenetic stimulation of the ISNs, as the ISNs should activate CCHa2R-RA neurons given that the ISNs inhibit BiT (Fig 4C). Indeed, we found that CCHa2R-RA neurons showed robust calcium responses upon ISN stimulation (Fig 4D-E), demonstrating that these neurons are functionally connected to the ISNs, likely via BiT inhibition.

To test if CCHa2R-RA neurons regulate sugar or water ingestion, we manipulated activity in these neurons and measured ingestion of sugar or water. We found that activation of CCHa2R-RA neurons decreased water ingestion but did not change sugar ingestion (Fig 4F). Moreover, inhibiting neurotransmission in CCHa2R-RA neurons increased water ingestion (Fig 4G), but did not change sucrose ingestion relative to *CCHa2R-RA-Gal4* controls. These behavioral experiments demonstrate that peptide-sensing neurons downstream of the ISNs regulate water ingestion. The finding that CCHa2-RA neurons recapitulate the water ingestion phenotypes of the ISNs but not sugar ingestion phenotypes suggests that the ISNs activate different arrays of peptidergic neurons that contribute differentially to ingestion of specific nutrients.

CCAP neurons are downstream of the ISNs and reciprocally regulate sugar and water ingestion

In a separate effort to find neurons that are postsynaptic to the ISNs, we tested whether neurons that had previously been implicated in ingestion were functionally connected to the ISNs. We conducted pilot *in vivo* functional imaging experiments monitoring the activity of candidate neurons with GCaMP7b while optogenetically stimulating the ISNs. We found one set of peptidergic neurons, the crustacean cardioactive peptide (CCAP) neurons, that were activated upon ISN optogenetic stimulation (Fig 5D-E).

CCAP neurons have been shown to regulate feeding behavior in adult *Drosophila* as loss of CCAP in these neurons reduced sucrose ingestion (Williams et al., 2020). To directly test if CCAP neural activity modulates sugar or water ingestion, we acutely manipulated the activity of CCAP neurons and measured ingestion of sugar or water. We found that activation of CCAP decreased water ingestion and increased sugar ingestion (Fig 5F). To test whether CCAP neurons are necessary for sugar and water ingestion, we reduced CCAP neurotransmission with nSyb RNAi, and measured ingestion of sugar or water. We found that silencing CCAP neurons decreased sugar ingestion and increased water ingestion (Fig 5G), demonstrating that CCAP neurons reciprocally regulate sugar and water ingestion, similar to the ISNs.

Although CCAP neurons are functionally connected to the ISNs, their synaptic connectivity is indirect. We identified the CCAP neurons in the FAFB volume (Fig 5A) and found weak connections between CCAP neurons and ISN synaptic partners: Cowboy (5 synapses), VESa1 (22 synapses), and a novel neuron we named Bilateral T-shaped neuron 2, based on its anatomical similarities to BiT (37 synapses). In addition, the ISN third-order neuron CCHa2R-RA neurons provide 26 synapses onto CCAP neurons (Supplementary Table 3). This connectivity suggests that CCAP neurons are part of the broad network that receives ISN input (Fig 5H). Moreover, the reciprocal regulation of sugar and water ingestion by CCAP neurons argues that multiple

peptidergic neurons downstream of the ISNs cooperate to coordinate ingestion of sugar versus water based on specific need.

DISCUSSION

In this study, we report that the ISNs communicate hunger and thirst states to a complex neural network that reaches several brain regions to regulate sugar and water ingestion (Fig 6). The ISNs synapse with neurons that project to higher brain neuroendocrine centers, including BiT, a novel neuron that reciprocally regulates sugar and water ingestion. Several peptide-releasing and peptide-sensing neurons known to regulate feeding behavior also receive ISN signals, providing the capacity to integrate hunger and thirst signals with many internal signals of nutritional need. These peptidergic neurons, connected to the ISNs via interneurons, contribute differentially to ingestion of sugar and water, with IPC and CCAP neurons reciprocally regulating sugar and water ingestion and CCHa2R-RA neurons modulating water ingestion. Thus, our work argues that the coordinated regulation of a peptidergic network weighs nutrient needs to generate nutrient-specific ingestion.

The ISNs influence activity of several brain regions involved in feeding and nutrient homeostasis to coordinate sugar and water ingestion

Previous studies showed that the ISNs sense the hunger signal AKH and changes in hemolymph osmolality associated with thirst to correspondingly alter ISN neural activity. Increased ISN activity promotes sugar ingestion and decreases water ingestion, and decreased ISN activity decreases sugar ingestion and increases water ingestion (Jourjine, Mullaney et al., 2016). Here, we investigated how ISN activity reciprocally regulates sugar and water ingestion according to internal needs by examining the neural network modulated by the ISNs.

We found that the ISNs synapse with 100 neurons, including projection neurons that arborize in neuroendocrine centers, SEZ interneurons, and ascending and descending neurons that likely innervate the ventral nerve cord. The majority of the ISN synaptic partners are projection neurons that send arbors via the median bundle to the SMP, a neuroendocrine center (Hartenstein, 2006). This includes the cell type BiT characterized in this study that reciprocally regulates sugar and water ingestion. Local SEZ neurons downstream of the ISNs include DSOG1, which are GABAergic and inhibit consumption (Pool et al., 2014), consistent with the notion that ISN activity directly influences feeding motor programs. In addition, eight uncharacterized descending neurons are downstream of the ISNs, suggesting that they may coordinate feeding with other motor behaviors, such as locomotion or digestion. While the number of ISN postsynaptic partners precludes comprehensive functional and behavioral analysis, the restricted number of brain regions that are direct targets of the ISNs (SMP, SEZ, and possibly ventral nerve cord) is consistent with ISN activity directly regulating neuroendocrine centers and feeding behavior.

We characterized the pathway from the second-order BiT projection neuron that oppositely regulates sugar and water consumption. We found that BiT has 93 synaptic partners, including IPCs which are known to modulate food intake (Nässel & Zandawala, 2020), FLAa3/Lgr3 which have been implicated in regulating ingestion (Laturney et al., 2022, Meissner et al., 2016, Nässel et al, 2013, Yeom et al., 2021), and

neurons labeled by the CCHa2R-RA-Gal4 (Deng et al., 2019) which we found regulate water ingestion. BiT downstream neurons innervate several neuropils including the SEZ, SMP, SLP, fan shaped body, and lobula. Therefore, hunger and thirst signals sensed by the ISNs fan out to modulate multiple brain regions via BiT. We speculate that the broad reach of the ISNs serves to modulate different behaviors such as sleep, reproduction, and locomotion based on the hunger or thirst state of the fly.

Communication between peptidergic neurons coordinates ingestion

Our studies demonstrate that multiple peptidergic neurons participate in regulation of sugar and water ingestion. We find that dILP3 RNAi or *amontillado* RNAi expression in the ISNs recapitulates the ISN loss-of function phenotype, arguing that the ISNs themselves are peptidergic and utilize dILP3 as the neurotransmitter that conveys hunger and thirst signals. The ISNs have increased activity upon AKH detection or low osmolality (hunger signals) (Jourjine, Mullaney et al., 2016), arguing that increased dILP3 release from the ISNs drives sucrose ingestion and limits water ingestion in hungry flies to maintain homeostasis. This conversion of an AKH signal to a dILP3 signal resembles findings in *Drosophila* larvae, where circulating AKH binds to the AKH receptor on IPCs to release dILP3 and promote sucrose consumption (Kim & Neufeld, 2015, Palovcik et al., 1984).

The ISNs modulate activity in many neuroendocrine cells, potentially causing widespread changes in peptide release (Nässel & Zandawala, 2022, Schlegel et al., 2016). We find that ISN activation increases activity of CCAP neurons and CCHa2R-RA neurons, and BiT activation decreases the activity of IPCs. CCAP neurons are orexigenic and communicate to CCAP receptor cells, including IPCs (Zhang et al., 2022) and a subpopulation of neuropeptide F (NPF) neurons (Williams et al., 2020). While this is the first study that characterizes the CCHa2R-RA neurons, the knockin Gal4 line that labels the CCHa2R-RA neurons was generated for the RA isoform of CCHa2 receptor, suggesting that these neurons respond to CCHa2, a peptide produced in the midgut and brain that increases appetite (Deng et al., 2019, Ida et al., 2012, Reiher et al., 2011, Ren et al., 2015). Therefore, CCHa2R-RA neurons potentially integrate the hunger and thirst signals from the ISNs with CCHa2 signals from the gut. IPCs are central regulators of appetite and metabolism, receive multiple direct and indirect signals of nutrient status, and release dILP2, dILP3, and dILP5 (Nässel & Zandawala, 2020). Our finding that the ISNs communicate with multiple peptidergic systems argues that hunger and thirst signals sensed by the ISNs are integrated with other nutritive state signals for a global assessment of the current nutritional demands of the animal.

Sugar and water ingestion remain coordinated downstream of the ISNs

Multiple neurons downstream of the ISNs bidirectionally regulate both sugar and water ingestion, arguing that they bias ingestion based on nutrient need. By studying the activation and silencing phenotypes associated with CCAP neurons, we show that acute activation promotes sugar ingestion and limits water ingestion, while silencing these neurons has the opposite effects. These findings are consistent with and expand upon previous studies showing that CCAP neurons promote feeding (Selcho et al, 2018, Williams et al., 2020). IPCs have a more complex role in regulating ingestion, with

several studies showing that their acute activation limits ingestion of sucrose or food (Nässel et al., 2015, Semaniuk and Gospodaryov et al., 2018, Wang et al., 2020) and other studies suggesting the opposite (Sudhakar et al., 2020). We find that under the specific conditions of our assay, acute activation of IPCs promotes sucrose ingestion and limits water ingestion. We suspect that differing findings upon IPC manipulation may stem from differences in the deprivation state of the fly, the behavioral assay, the type and timing of neural manipulation, and the food source. As IPCs receive multiple internal state signals, it is possible that activation phenotypes depend on the current state of IPC modulation set by the internal state of the fly.

Overall, we show that the hunger and thirst signals detected by the ISNs influence a network of peptidergic neurons that act in concert to prioritize ingestion of specific nutrients based on internal needs. We hypothesize that multiple internal state signals are integrated in higher brain regions such that combinations of peptides and their actions signify specific needs to drive ingestion of appropriate nutrients. Thus, our work sheds light on neural circuit mechanisms that translate internal nutrient abundance cues into the coordinated regulation of sugar and water ingestion.

MATERIALS AND METHODS

Key resources table		
Drosophila strains	**Source or Reference**	**Identifier**
UAS-nSynaptobrevin RNAi	Bloomington Drosophila Stock Center	BDSC 31983
UAS-dcr2	Bloomington Drosophila Stock Center	BDSC 24650
UAS-Trh RNAi	Bloomington Drosophila Stock Center	BDSC 25842
UAS-ChAT RNAi	Bloomington Drosophila Stock Center	BDSC 25856
UAS-Tbh RNAi	Bloomington Drosophila Stock Center	BDSC 27667
UAS-Hdc RNAi	Bloomington Drosophila Stock Center	BDSC 26000
UAS-VMAT RNAi	Bloomington Drosophila Stock Center	BDSC 31257
UAS-GAD1 RNAi	Bloomington Drosophila Stock Center	BDSC 28079
UAS-DDC RNAi	Bloomington Drosophila Stock Center	BDSC 27030
UAS-DVGlut RNAi	Bloomington Drosophila Stock Center	BDSC 27538
UAS-sNPF RNAi	Bloomington Drosophila Stock Center	BDSC 25867
UAS-VGAT RNAi	Bloomington Drosophila Stock Center	BDSC 41958
UAS-TDC2 RNAi	Bloomington Drosophila Stock Center	BDSC 25871
UAS-dILP1 RNAi	Bloomington Drosophila Stock Center	BDSC 32861
UAS-dILP2 RNAi	Bloomington Drosophila Stock Center	BSC 32475
UAS-dILP3 RNAi	Bloomington Drosophila Stock Center	BSC 31492

UAS-dILP4 RNAi	Bloomington Drosophila Stock Center	BDSC 33682
UAS-dILP5 RNAi	Bloomington Drosophila Stock Center	BDSC 31378
UAS-dILP6 RNAi	Bloomington Drosophila Stock Center	BDSC 33684
UAS-dILP7 RNAi	Bloomington Drosophila Stock Center	BDSC 32862
UAS-amon RNAi	Bloomington Drosophila Stock Center	BDSC 29009
ISN-Gal4 (VT011155-Gal4)	FlyLight, Janelia Research Campus	Fly Light ID 54404
ISN-LexA (GMR34G02-LexA)	Bloomington Drosophila Stock Center	BDSC 54138
UAS-myrGFP.QUAS-mtdTomato-3xHA; trans-Tango	Bloomington Drosophila Stock Center	BDSC 77124
VT002073-Gal4.AD	Bloomington Drosophila Stock Center	BDSC 71871
VT040568-Gal4.DBD	Bloomington Drosophila Stock Center	BDSC 72902
UAS-csChrimson.mVenus	Bloomington Drosophila Stock Center	BDSC 55134
LexAop-ChrimsonR.mCherry	Gift from Jayaraman Lab	
UAS-ArcLight	Bloomington Drosophila Stock Center	BDSC 51056
Empty split	Bloomington Drosophila Stock Center	BDSC 79603
ppk28-Gal4	Cameron et al 2010.	BDSC 93020
Gr5a-Gal4	Chyb et al 2003.	BDSC 57592, 57591
CCha2R-RA-Gal4	Bloomington Drosophila Stock Center	BDSC 84603
LexAop-CsChrimson.tdTomato (III)	Bloomington Drosophila Stock Center	BDSC 82183
UAS-GCaMP6s (III)	Bloomington Drosophila Stock Center	BDSC 42749
20XUAS-GCaMP7b	Bloomington Drosophila Stock Center	BDSC 79029
CCAP-Gal4 (II)	Bloomington Drosophila Stock Center	BDSC 25685
CCAP-Gal4 (III)	Bloomington Drosophila Stock Center	BDSC 25686
CCHa2R-RA-LexA	Bloomington Drosophila Stock Center	BDSC 84363
dILP2-LexA	Li and Gong 2015.	

Fly husbandry

All experiments and screening were carried out with adult *D. melanogaster* females reared on standard cornmeal-agar-molasses medium, at 25°C, 65-70% humidity, on a 12 hr light: 12 hr dark cycle. Flies used in optogenetic assays were reared on food containing 0.25mM all-trans-retinal (Sigma-AldrichSigna-Aldrich) in darkness, before and after eclosion.

Temporal consumption assay (TCA)

Flies were anesthetized using CO_2 and then fixed to a glass slide with nail polish. Flies recovered for 2 hours in a humidified box, if testing for sucrose ingestion, or in a desiccated box with Drierite, if testing for water ingestion. Immediately before testing for sucrose ingestion, flies were given water until they no longer responded to 3 consecutive presentations. In testing, flies were presented with the tastant (water or 1M sucrose) 10 times and consumption time was manually recorded.

In vivo calcium imaging

Calcium imaging studies were carried out as described in Shiu, Sterne et al. (2022). Mated female flies were dissected for calcium imaging studies 5-14 days post-eclosion. Flies were briefly anesthetized with ice and placed in a custom plastic holder at the neck to isolate the head from the rest of the body. The head was then immobilized using UV glue, the proboscis was immobilized using wax, and the esophagus was cut to provide unobstructed imaging access to the SEZ. All flies imaged were sated. *In vivo* calcium imaging with optogenetic activation was performed in a 2-photon microscope using a Scientifica Hyperscope with resonant scanning, a piezo drive, and a 20x water immersion objective (NA = 1.0) with 1.8-3x digital zoom, depending on the cell type imaged. Calcium responses were recorded with a 920 nm laser and optogenetic stimulation was achieved with a 660 nm LED. 2s LED stimulation paradigm: 20s off, 2s on, 30s off, 2s on, 30s off. 30s LED stimulation paradigm: 20s off, (1s on, 1s off) x 15, 60s off. For the 2s LED stimulation, 80 stacks of 20 z slices of 4-5 µm were acquired at 0.667 Hz. For the 30s stimulation, 125 stacks of 20 z slices of 4-5 µm were acquired at 0.667 Hz. Analysis was done on max-z projections of the 20 z slices. $\%\Delta F/F = 100*((Ft-F0)/F0)$, where Ft is the fluorescence of the Neuron ROI - the Background ROI at each timepoint and F0 is the mean Ft for the 23 time points prior to stimulus onset. Quantification was carried out in GraphPad Prism. A mean fluorescence intensity for LED off and LED on was calculated for each fly. For the 2s LED stimulation, mean intensity for LED off was calculated for 5 timepoints immediately before LED exposure and mean intensity for LED on was calculated for 5 timepoints during LED exposure. For the 30s stimulation, mean intensity for LED off was calculated for 28 timepoints immediately before LED exposure and mean intensity for LED on was calculated for 28 timepoints during LED exposure. Paired t-test or paired Wilcoxon test was performed.

Immunohistochemistry

All brain and CNS dissections and immunostaining (unless directly addressed) were carried out as described (https://www.janelia.org/project-team/flylight/protocols, 'IHC-Anti-GFP') substituting the below antibodies and eschewing the pre-embedding fixation steps. Ethanol dehydration and DPX mounting was carried out as described (https://www.janelia.org/project-team/flylight/protocols, 'DPX Mounting').
Primary antibodies:

- mouse α-Brp (nc82, DSHB, University of Iowa, USA) at 1:40
- chicken α-GFP (Invitrogen, A10262) at 1:1000
- rabbit α-dsRed (Takara, Living Colors 632496) at 1:1000

Secondary antibodies:

- goat α-mouse AF647 (Invitrogen, A21236) at 1:500
- goat α-chicken AF488 (Life Technologies, A11039) at 1:1000

- goat α-rabbit AF568 (Invitrogen, A21236) at 1:1000

Images were acquired with a Zeiss LSM 880 NLO AxioExaminer with Airyscan and Coherent Chameleon Vision or Zeiss LSM 780 Laser Scanning Confocal Microscope at the Berkeley Molecular Imaging Center with a Plan-Apochromat 20x/1.0 W, 40x W, 40x/1.4 oil, or 63x/1.4 oil objective. Images were prepared in Fiji.

Electron microscopy neural reconstructions and connectivity

Neurons were reconstructed in a serial section transmission electron volume (Full Adult Female Brain, Zheng and Lauritzen et al., 2018) using the CATMAID software (Saalfeld et al., 2009). Fully manual reconstructions were generated by following the branches of the neuron and marking the center of each branch, thereby creating a 'skeleton' of each neuron. In addition to fully manual reconstructions, segments of an automated segmentation (Li et al., 2019) were proofread and expanded to generate complete reconstructions. In addition to the skeleton tracing, new chemical synapses were also annotated as previously described (Zheng and Lauritzen et al. 2018). Downstream synaptic targets of the ISNs and BiT were then traced out from these additional locations using both manual and assisted tracing techniques as described above. Neurons traced in CATMAID, including ISNs and BiT, were all located in Flywire (flywire.ai), which uses the same EM electron microscopy dataset (Zheng and Lauritzen et al. 2018). To identify synaptic partners, we used connectome annotation versioning engine (CAVE, Buhmann et al. 2021, Heinrich et al. 2018) using a cleft score cutoff of 50 to generate synapses of relatively high confidence (Heinrich et al. 2018; Baker et al., 2022). FAFB neural reconstructions were visualized using NAVis (Copyright 2018, Philipp Schlegel), which is based on natverse (Bates et al., 2020).

BiT split-Gal4 generation

We created a color depth max intensity projection (CDM) mask of BiT reconstructed EM skeleton and used CDM mask searching (Otsuna et al., 2018) to find enhancers whose expression patterns seemed to include the desired cell type using MCFO (Nern et al., 2015) screening of subsets of the Janelia Research Campus and Vienna Tile Gal4 collections. Construction of stable split-Gal4 lines was performed as previously described (Dionne et al., 2018, Sterne et al., 2020). Immunohistochemistry and confocal imaging was used to determine successful split-Gal4 combinations.

Identification of GAL4 lines from EM reconstructions

Visual inspection of Gal4 collections was used to determine cell type. Images of potential Gal4 lines were skeletonized in FIJI, converted into .swc format using natverse (Bates et al., 2021), and uploaded to Flywire using the Flywire Gateway. This generated pointclouds that were used to identify the neurons of interest. As Flywire permits exhaustive searching of neurons in an area, we examined all neurons in the region of interest to conclusively identify our neuron of interest.

Statistical analysis

Statistical tests were performed in GraphPad Prism. For all group comparisons, data was first tested for normality with the KS normality test (alpha = 0.05). If all groups passed then groups were compared with a parametric test, but if at least one group did

not pass, groups were compared with a non-parametric version. All statistical tests, significance levels, and number of data points (N) are specified in the figure legend. All datasets from optogenetic behavior assays were normalized within each genotype. To generate this normalized dataset, data from females within the no light condition was averaged, creating a “no-light mean” for each genotype. This value was subtracted from each individual female within the light condition of the corresponding genotype. This dataset was then graphed, and statistical analyses were performed as outlined above.

ACKNOWLEDGEMENTS
Members of the Scott lab provided contributions to experimental design, data analysis, and manuscript preparation. This work was supported by NIH R01GM128209 (K.S.), R01GM128209 Diversity Supplement (A.G.S.), UC LEADs fellowship (A.D.T.) and International Fellowship for CONICET Researchers (G.P.). Neuronal reconstruction for this project took place in a collaborative CATMAID environment in which 27 labs are participating to build connectomes for specific circuits. Development and administration of the FAFB tracing environment and analysis tools were funded in part by National Institutes of Health BRAIN Initiative grant 1RF1MH120679-01 to Davi Bock and Greg Jefferis, with software development effort and administrative support provided by Tom Kazimiers (Kazmos GmbH) and Eric Perlman (Yikes LLC). We thank Peter Li, Viren Jain and colleagues at Google Research for sharing the automatic segmentation (Li et al., 2019). Tracing in Cambridge was supported by Wellcome Trust (203261/Z/16/Z) and ERC (649111) awards to G. Jefferis. Neurons were also reconstructed and proofread in FlyWire, where we also identified pre- and postsynaptic partners. We acknowledge the Princeton FlyWire team and members of the Murthy and Seung labs for development and maintenance of FlyWire (supported by BRAIN initiative grant MH117815 to Murthy and Seung). In addition to our tracing efforts in CATMAID and proofreading in FlyWire, the following labs greatly contributed to the proofreading in FlyWire of the neurons characterized in this study: Murthy and Seung Labs (76.06%), Jefferis Lab (13.57%) and Jinseop Kim Lab (7.21%). We are appreciative of the proofreading contributed by other labs including the Anderson, Bock, Dacks, Dickson, Huetteroth, Pankratz, Seeds/Hampel, Selcho, Simpson, Waddell, Wilson and Wolf Labs, and Janelia tracers (Supplementary Table 1, Supplementary Table 2, Supplementary Table 3). Vivek Jayaraman provided unpublished fly lines used in this study. We thank Stefanie Engert for tracing two ISNs in CATMAID, Zepeng Yao for identifying CCHa2R-RA neurons, Phil Shiu for identifying DSOG1 neurons, and Amanda Abusaif for her tracing and proofreading efforts. Confocal imaging experiments were conducted at the CRL Molecular Imaging Center, RRID:SCR_017852, supported by NSF DBI-1041078 and the Helen Wills Neuroscience Institute. We thank Holly Aaron and Feather Ives for microscopy training and support.

AUTHOR CONTRIBUTIONS
A.G.S. and K.S. conceived and designed the study and wrote the manuscript. A.G.S performed the FAFB circuit building and cell identification, BiT split-Gal4 creation, functional imaging experiments, immunohistochemistry, and data analysis. G.P. performed behavior experiments and BiT split-Gal4 creation. N.J performed the ISN neurotransmitter RNAi screen. A.D.T. traced ISNs and BiT in CATMAID.

FIGURES

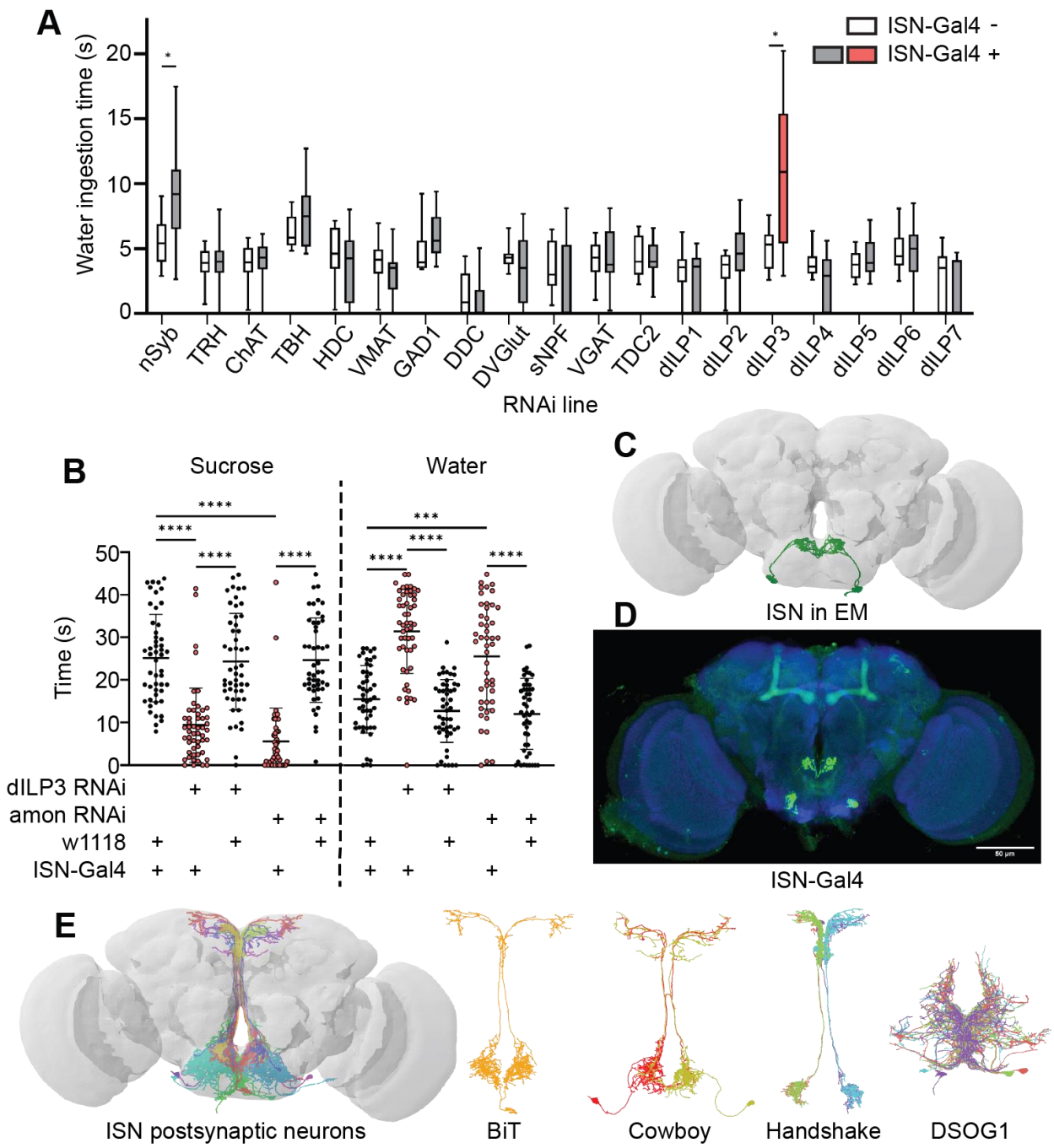


Figure 1. ISNs relay information to the Pars Intercerebralis
(A) Temporal consumption assay screen for water ingestion using RNAi targeting different neurotransmitter pathways. UAS-RNAi + or - ISN-Gal4. RNAi against: nSynaptobrevin (nSyb), tryptophan hydroxylase (TRH), choline acetyltransferase (ChAT), tyrosine beta-hydroxylase (TBH), histamine decarboxylase (HDC), vesicular monoamine transporter (VMAT), glutamic acid decarboxylase 1 (GAD1), dopa decarboxylase (DDC), *Drosophila* vesicular glutamate transporter (DVGlut), short

neuropeptide F (sNPF), vesicular GABA transporter (VGAT), Tyrosine decarboxylase 2 (TDC2), *Drosophila* insulin like peptide 1 (dILP1), *Drosophila* insulin like peptide 2 (dILP2), *Drosophila* insulin like peptide 3 (dILP3), *Drosophila* insulin like peptide 4 (dILP4), *Drosophila* insulin like peptide 5 (dILP5), *Drosophila* insulin like peptide 6 (dILP6), *Drosophila* insulin like peptide 7 (dILP7). Represented are the mean, and the 10-90 percentile; data was analyzed using Kruskal-Wallis test, followed by multiple comparisons against the RNAi control; p-values were adjusted using False Discovery Rate. n=8-39 animals/genotype except nSyb positive control (70-72). (B) Temporal consumption assay for 1M sucrose or water using RNAi targeting dILP3 or amontillado in ISNs. Sucrose assay: Kruskal-Wallis test followed by Dunn's multiple comparison tests against ISN control and respective RNAi control. Water assay: ANOVA, Šídák's multiple comparison test to ISN control and respective RNAi control. n=48-52 animals/genotype. (C) ISNs reconstruction from FAFB volume. (D) Light microscopy image of ISN-Gal4 registered to JFRC2010. (E) ISN postsynaptic neurons based on synapse predictions using FAFB volume (Zheng et al. 2018) and connectome annotation versioning engine (CAVE, Buhmann et al. 2021, Heinrich et al. 2018). Left: 10 postsynaptic neurons, right: postsynaptic neurons BiT, Cowboy, Handshake and DSOG1. *p<0.05, ***p<0.001, ****p<0.0001

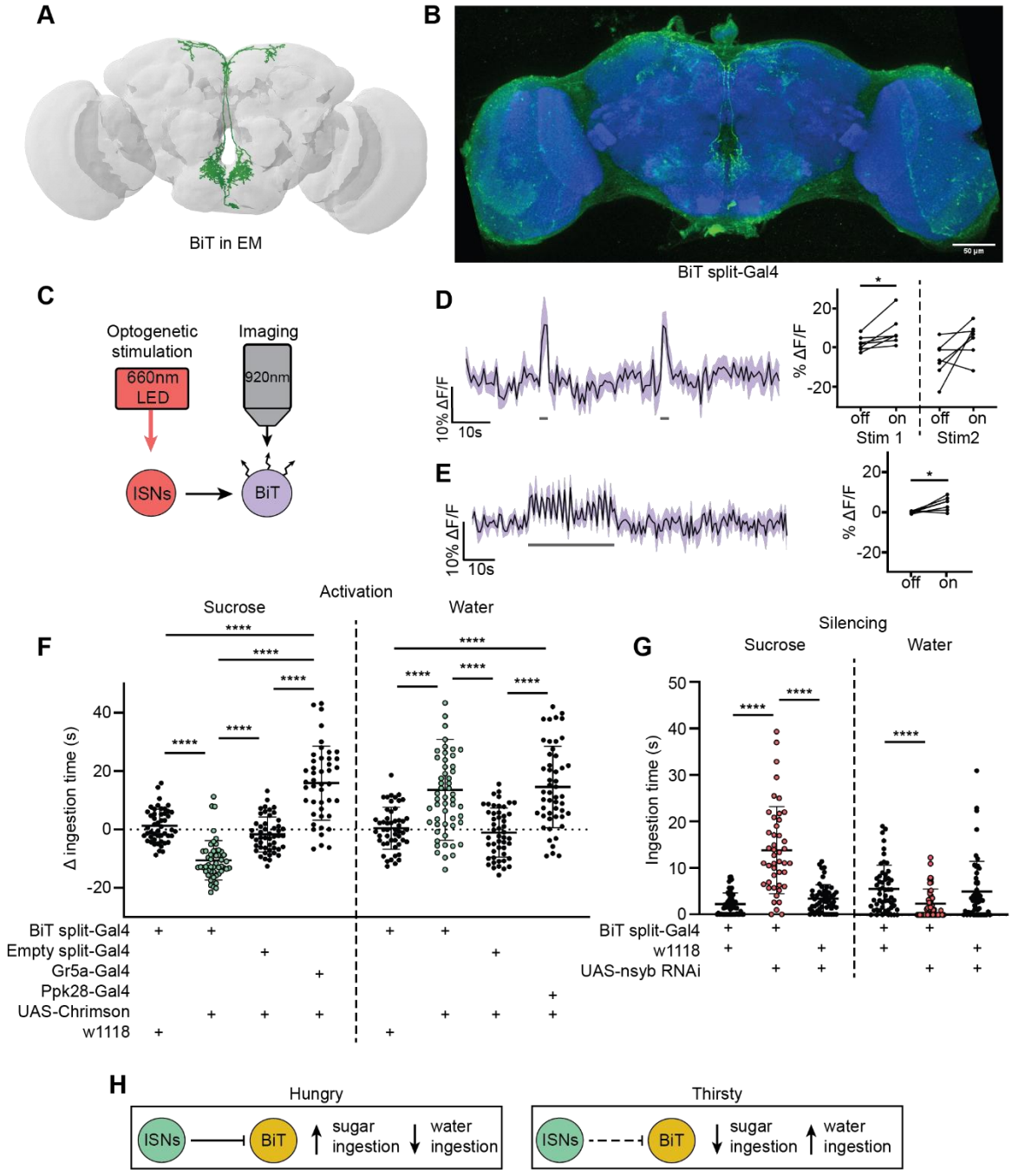


Figure 2. ISNs inhibit BiT, which oppositely regulates sugar and water ingestion
(A) BiT neuron reconstruction from FAFB dataset. (B) Light microscopy image of BiT split-Gal4. (C) Experimental setup for in vivo voltage imaging. We expressed the light sensitive ion channel Chrimson in the ISNs and optogenetically stimulated them with 660nm LED. We expressed the voltage sensor ArcLight in BiT and imaged it with a 2 photon microscope. (D) ArcLight response of BiT soma to 2s optogenetic stimulation of the ISNs or (E) 30s optogenetic stimulation of the ISNs. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents

one fly. Paired Wilcoxon and paired t-test (Stim 2, p=0.07). n=7 flies. (F) Temporal consumption assay for 1M sucrose or water during acute optogenetic activation of BiT with Chrimson. Ingestion time of females exposed to light normalized to dark controls of indicated genotype. Sucrose: Kruskal-Wallis test with Dunn's multiple comparison test. Water: One-way ANOVA with Holm-Šídák multiple comparison test. n=44-54 animals/genotype.(G) Temporal consumption assay for 1M sucrose or water using RNAi targeting nSyb in BiT. Kruskal-Wallis with Dunn's multiple comparison test. n=45-57 animals/genotype. (H) Neural model for BiT coordination of sucrose and water intake. Dashed lines indicate inactive synapses. *p<0.05, ***p<0.001, ****p<0.0001

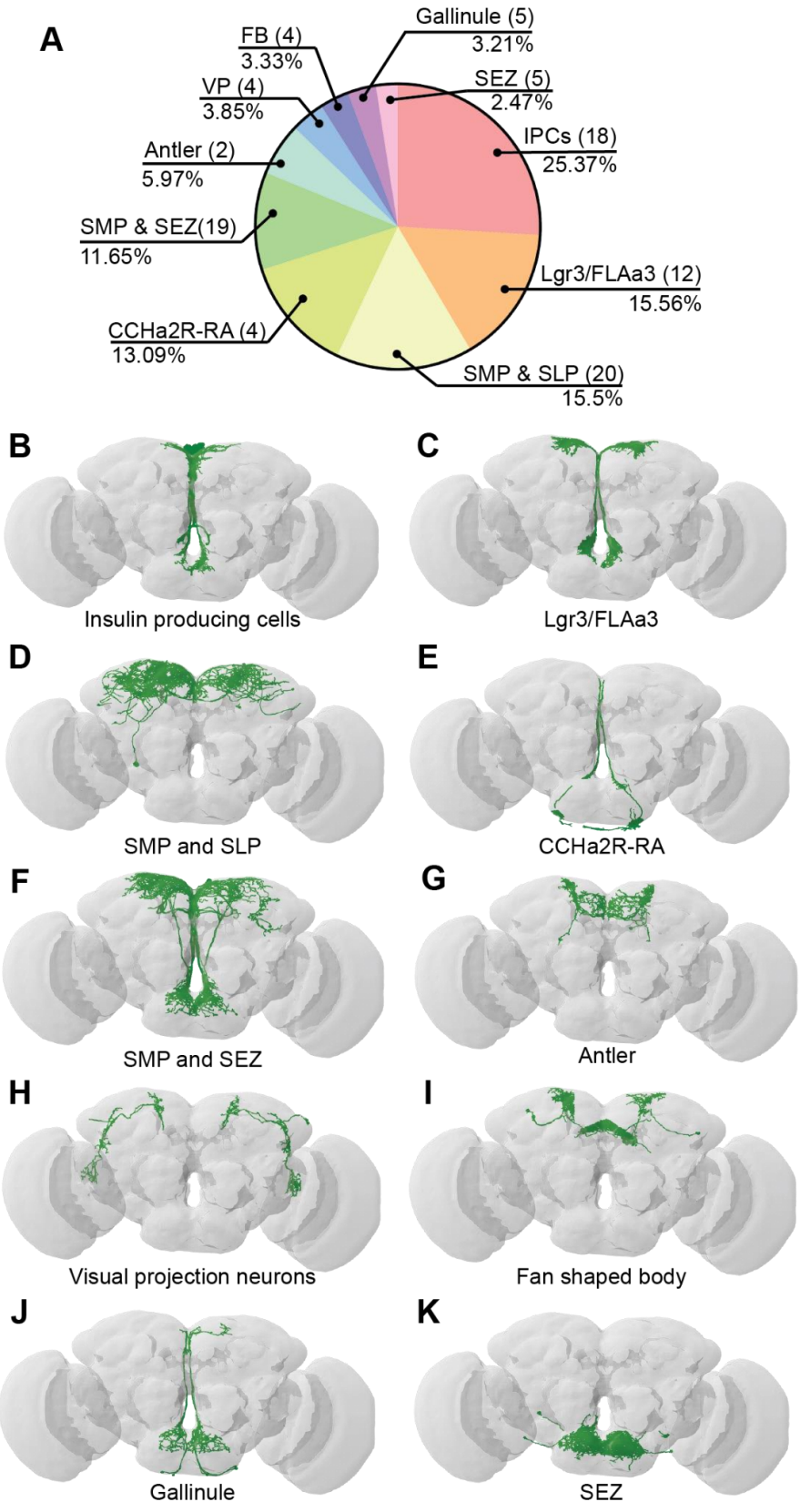

A
FB (4)
3.33%
Gallinule (5)
3.21%
VP (4)
3.85%
SEZ (5)
2.47%
IPCs (18)
25.37%
Antler (2)
5.97%
SMP & SEZ(19)
11.65%
Lgr3/FLAa3 (12)
15.56%
CCHa2R-RA (4)
13.09%
SMP & SLP (20)
15.5%
B
Insulin producing cells
C
Lgr3/FLAa3
D
SMP and SLP
E
CCHa2R-RA
F
SMP and SEZ
G
Antler
H
Visual projection neurons
I
Fan shaped body
J
Gallinule
K
SEZ

Figure 3. BiT postsynaptic neurons include neuroendocrine cells
(A) Distribution of synaptic output from BiT divided by cell class or brain region. Total of 1742 synapses from BiT and 93 postsynaptic partners. IPCs (18 neurons) receive 25.37% of all BiT output, Lgr3/FLAa3 (12 neurons) 15.56%, SMP and SLP (20 neurons) 15.5%, CCHa2R-RA (4 neurons) 13.09%, SMP and SEZ (19 neurons) 11.65%, Antler (2 neurons) 5.97%, visual projections (4 neurons) 3.85%, fan shaped body (4 neurons) 3.33%, Gallinule (5 neurons) 3.21%, SEZ (5 neurons) 2.47%. Only postsynaptic partners with 5 or more synapses were considered for this analysis. Reconstruction of IPCs (B), Lgr3/FLAa3 neurons (C), neurons innervating the SMP and SLP (D), CCHa2R-RA neurons (E), neurons innervating the SMP and SEZ (F), Antler neurons (G), visual projection neurons (H), neurons innervating the fan shaped body (I), Gallinule neurons (J), and neurons innervating the SEZ (K).

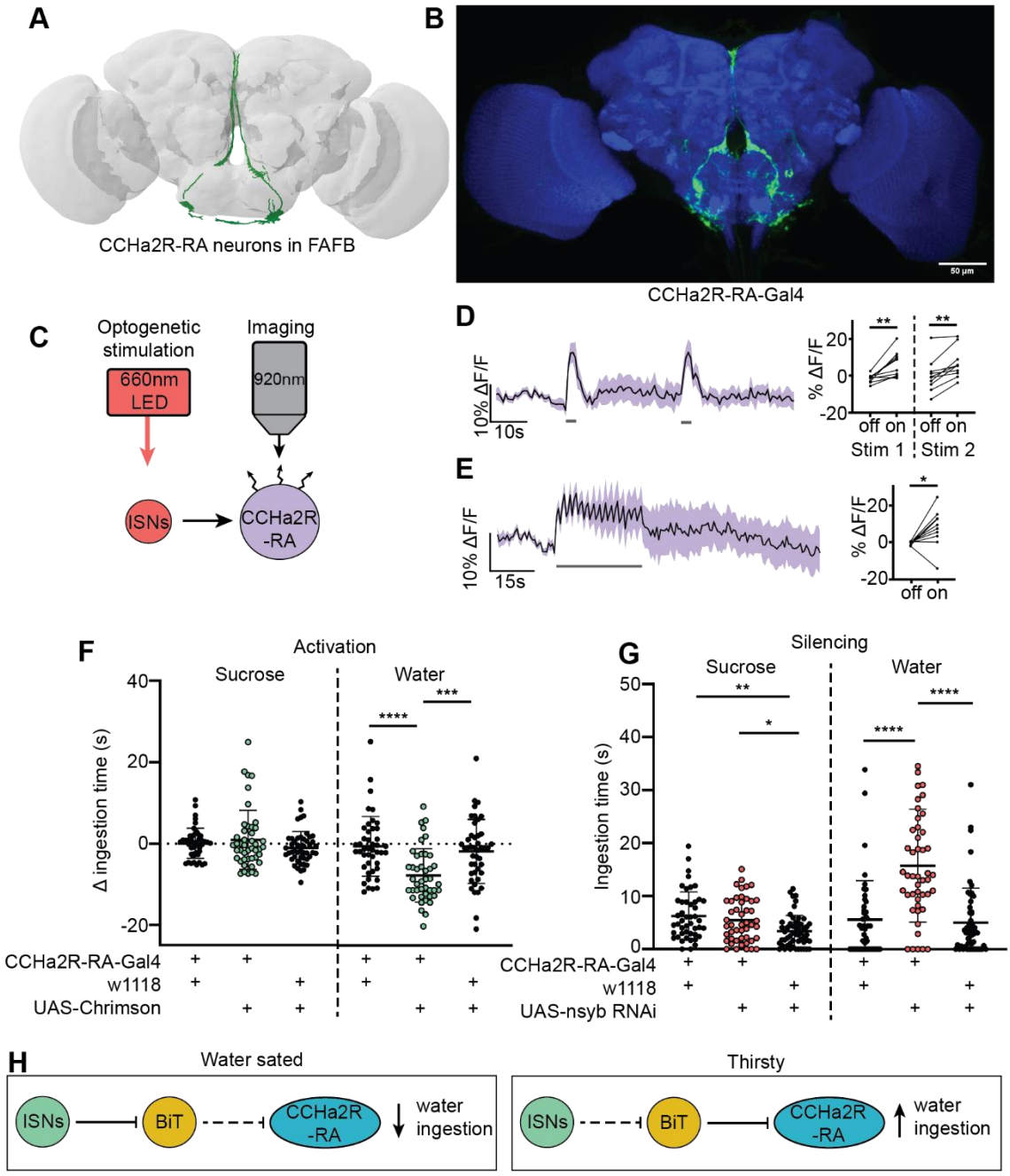


Figure 4. CCHa2R-RA neurons regulate water but not sugar ingestion and are likely inhibited by BiT

(A) CCHa2R-RA neurons reconstruction from FAFB dataset. (B) Light microscopy image of CCHa2R-RA-Gal4. (C) Experimental setup for *in vivo* calcium imaging. We expressed the light sensitive ion channel Chrimson in the ISNs and optogenetically stimulated them with 660nm LED. We expressed the calcium sensor GCaMP in the CCHa2R-RA neurons and imaged them with a 2-photon microscope. (D) Calcium responses of CCHa2R-RA neurites in SEZ to 2s optogenetic stimulation of the ISNs or (E) 30s optogenetic stimulation of the ISNs. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean

fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired t-test and paired Wilcoxon test. n=10 flies. (F) Temporal consumption assay for 1M sucrose or water during acute optogenetic activation of CCHa2R-RA neurons with Chrimson. Ingestion time of females exposed to light normalized to dark controls of indicated genotype. Sucrose: Kruskal-Wallis with Dunn's multiple comparison test. Water: One-way ANOVA with Holm-Šídák multiple comparison test. n=42-47 animals/genotype. (G) Temporal consumption assay for 1M sucrose or water using RNAi targeting nSyb in CCHa2R-RA neurons. Kruskal-Wallis with Dunn's multiple comparison test. n=45-54 animals/genotype. (H) Neural model for CCHa2R-RA regulation of water intake. Dashed lines indicate inactive synapses. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

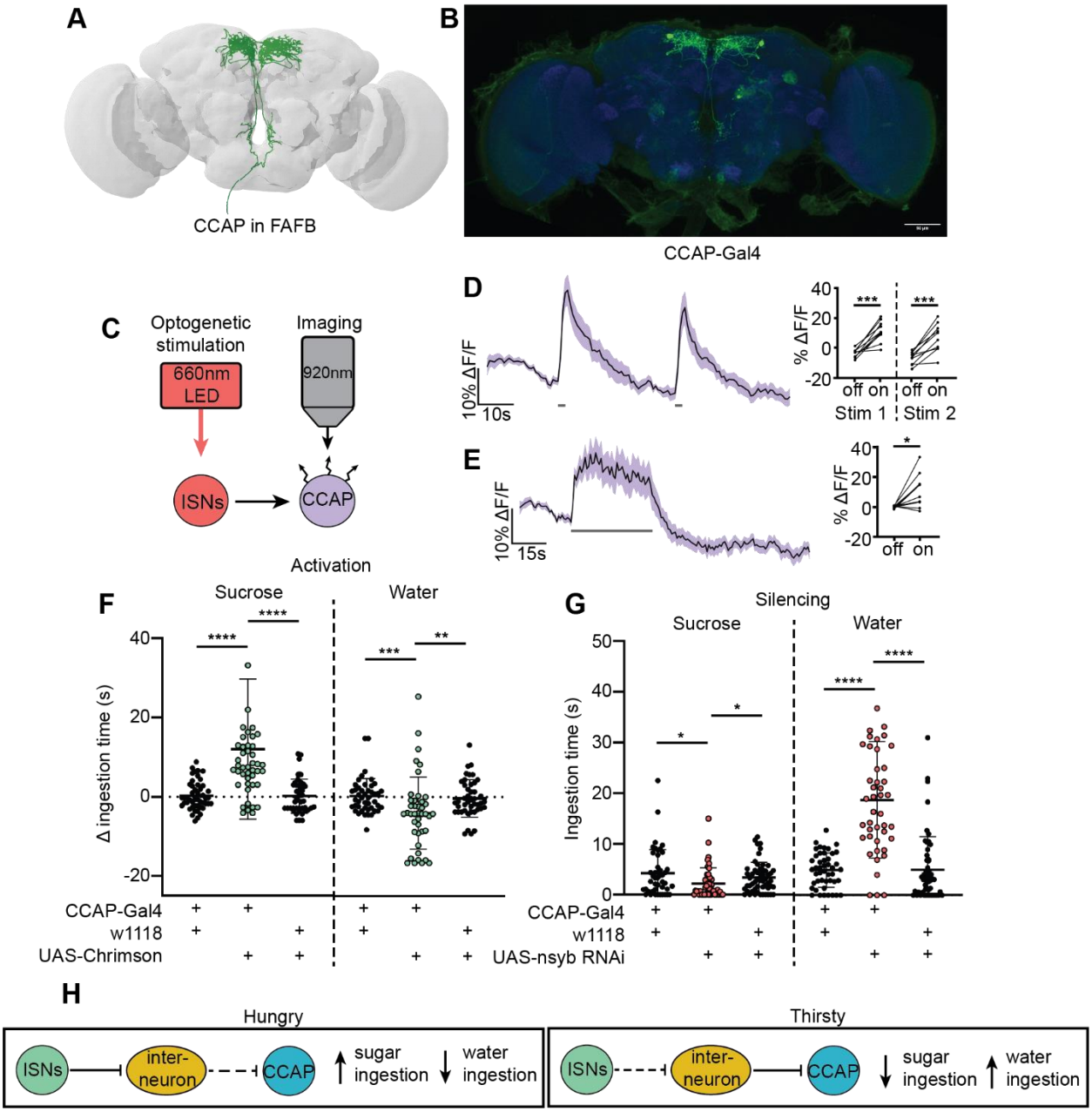


Figure 5. CCAP neurons are downstream of the ISNs and oppositely regulate sugar and water ingestion

(A) CCAP neurons reconstruction from FAFB dataset. (B) Light microscopy image of CCAP-Gal4. (C) Experimental setup for *in vivo* calcium imaging. We expressed the light sensitive ion channel Chrimson in the ISNs and optogenetically stimulated them with 660nm LED. We expressed the calcium sensor GCaMP in the CCAP neurons and imaged them with a 2-photon microscope. (D) Calcium response of CCAP neurites to 2s optogenetic stimulation of the ISNs or (E) 30s optogenetic stimulation of the ISNs. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired t-test. n=10 flies. (F) Temporal

consumption assay for 1M sucrose or water during acute optogenetic activation of CCAP neurons with Chrimson. Ingestion time of females exposed to light normalized to dark controls of indicated genotype. Sucrose: Kruskal-Wallis with Dunn's multiple comparison test, Water: One-way ANOVA with Holm-Šídák multiple comparison test. n=42-48 animals/genotype. (G) Temporal consumption assay for 1M sucrose or water using RNAi targeting nSyb in CCAP neurons. Kruskal-Wallis with Dunn's multiple comparison test. n=45-54 animals/genotype. (H) Neural model for CCAP coordination of sugar and water intake. Dashed lines indicate inactive synapses. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

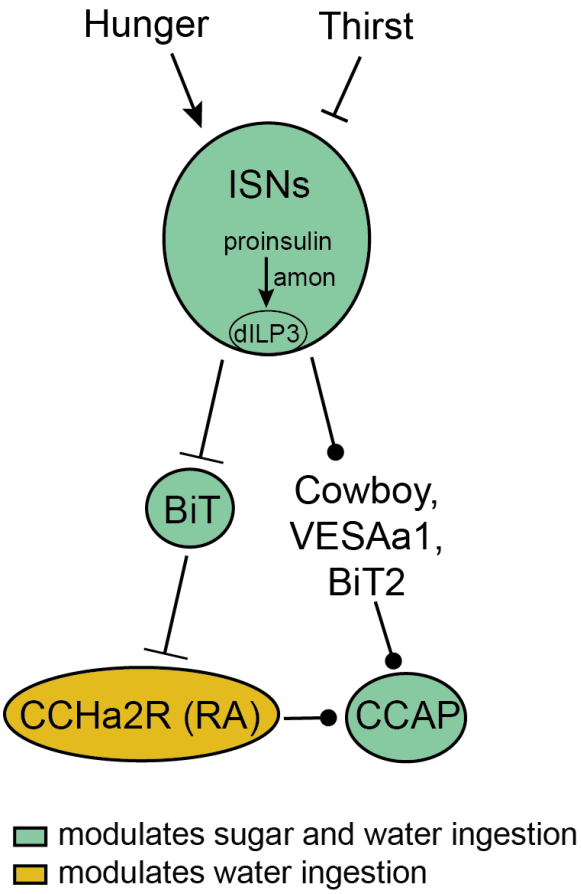


Figure 6: ISN regulation of sugar and water ingestion model
Hunger signals activate the ISN while thirst signals inhibit the ISNs. ISNs use dILP3 as a neurotransmitter and require amontillado (amon) for neuropeptide processing. ISN activity inhibits BiT, which in turn inhibits CCHa2R-RA neurons. CCAP neurons are downstream of the ISNs, connected via Cowboy, VESAa1, BiT2 and CCHa2R-RA neurons. BiT activity inhibits sugar ingestion and promotes water ingestion. CCAP activity promotes sugar ingestion and inhibits water ingestion. CCHa2R-RA activity inhibits water ingestion.

SUPPLEMENTAL MATERIALS

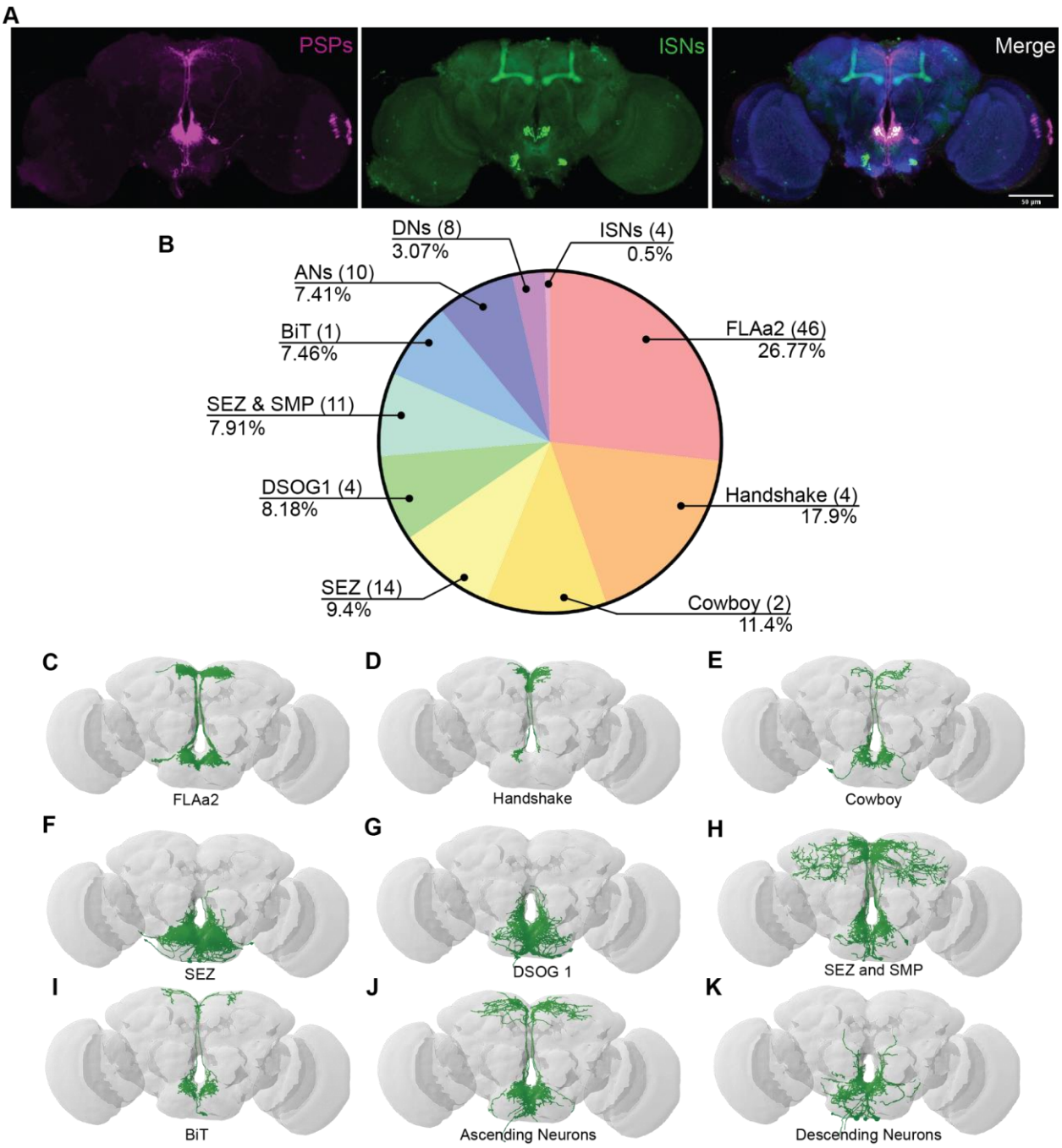


Figure S1. ISN postsynaptic partners labeled by trans-Tango and EM, Related to Fig 1

(A) Expression of trans-Tango ligand in the ISNs (green) and postsynaptic partners (PSPs) (magenta). Nc82 staining in blue. (B) Distribution of synaptic output from the ISNs divided by cell class or brain region. Total of 4050 synapses from the ISNs and 104 postsynaptic partners. FLAa2 (46 neurons) receive 26.77% of all ISN output, Handshake (4 neurons) 17.9%, Cowboy (2 neurons) 11.4%, neurons located in the

subesophageal zone (SEZ) (14 neurons) 9.04%, DSOG1 (4 neurons) 8.18%, neurons with neurites in the subesophageal zone and superior medial protocerebrum (SEZ & SMP) (11 neurons) 7.91%, BiT (1 neuron) 7.46%, Ascending neurons (ANs) (10 neurons) 4.41%, Descending neurons (DNs) (8 neurons) 3.07%, and ISNs (4 neurons) 0.5%. Only postsynaptic partners with 5 or more synapses were considered for this analysis. Reconstruction of FLAa2 neurons (C), Handshake neurons (D), Cowboy neurons (E), neurons innervating the SEZ (F), DSOG1 neurons (G), neurons innervating the SEZ & SMP (H), BiT (I), ascending neurons (J), descending neurons (K).

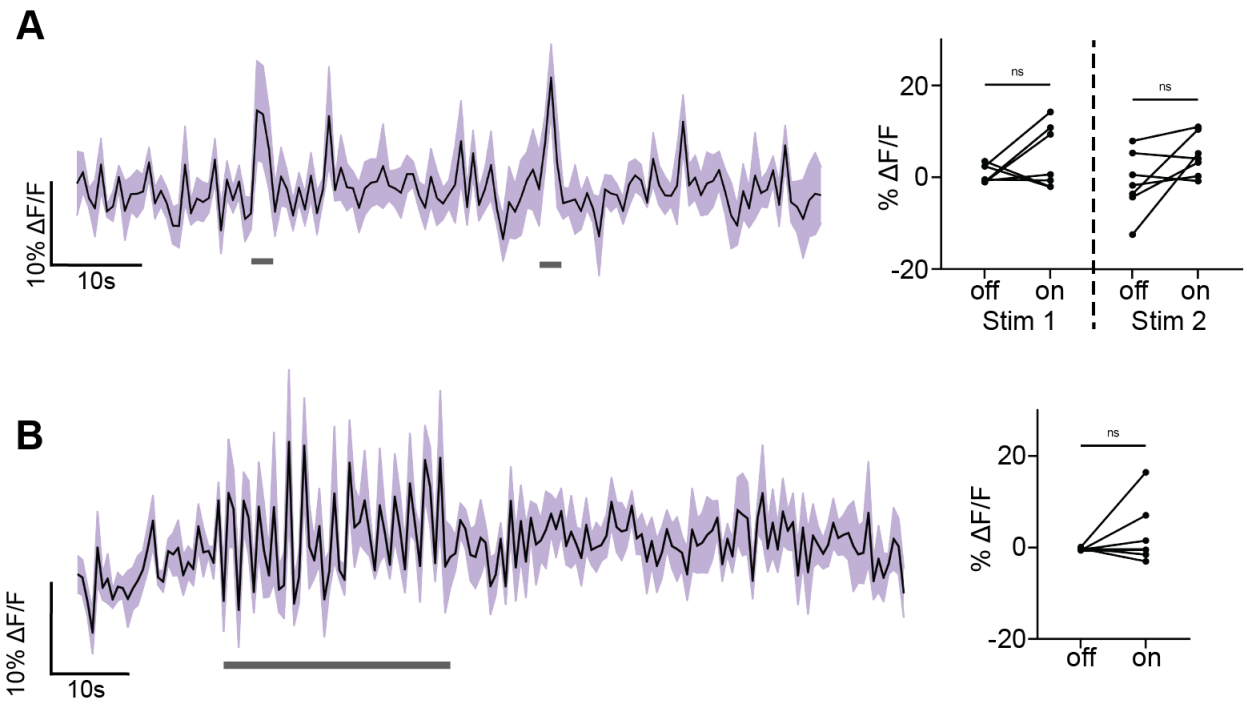
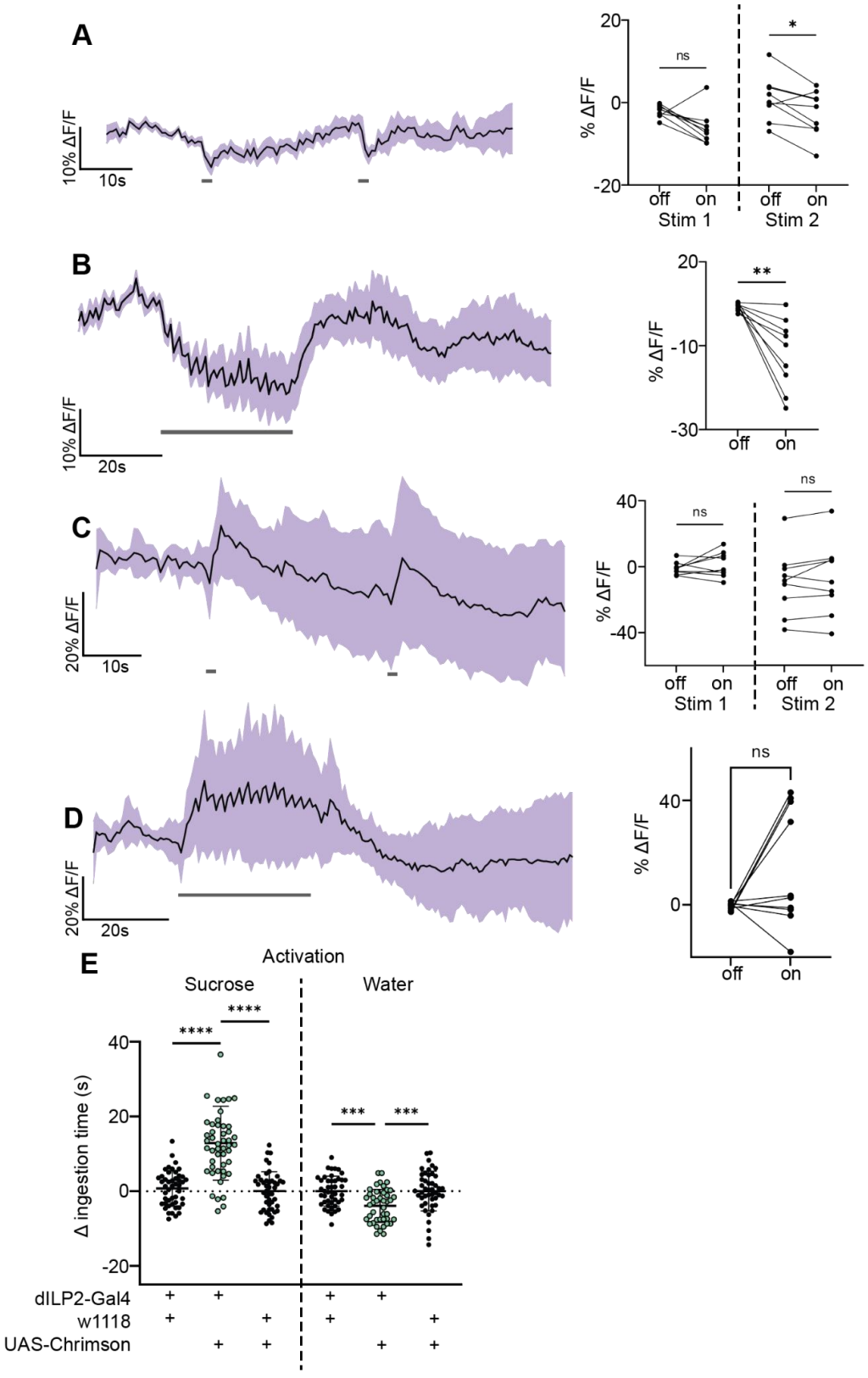


Figure S2. BiT genetic control functional imaging, Related to Fig 2
Genetic controls: we expressed the light sensitive ion channel Chrimson without the ISN-LexA driver and exposed the brains to 660nm LED. We expressed the voltage sensor ArcLight in BiT and imaged it with a 2-photon microscope. (A) ArcLight response of BiT soma to 2s LED exposure or (B) 30s LED exposure. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired Wilcoxon or paired t-test. n=7 flies.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E
Activation
Sucrose
Water
****
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40
20
0
-20
Δ ingestion time (s)
dILP2-Gal4
w1118
UAS-Chrimson

Figure S3. IPC response to BiT stimulation, Related to Fig 3
A and B: We expressed the light sensitive ion channel Chrimson in BiT and optogenetically stimulated it with 660nm LED. We expressed the calcium sensor GCaMP in the IPCs and imaged them with a 2-photon microscope. (A) Calcium response of IPC somas to 2s optogenetic activation of BiT or (B) 30s optogenetic activation of BiT. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired Wilcoxon test or paired t-test. n=9 flies. C and D: Genetic controls: we expressed the light sensitive ion channel Chrimson without the BiT-split Gal4 driver and exposed the brains to 660nm LED. We expressed the calcium sensor GCaMP in the IPCs and imaged them with a 2-photon microscope. (C) Calcium response of IPC somas to 2s LED exposure or (D) 30s LED exposure. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired t-test. n=9-10 flies. (E) Temporal consumption assay for 1M sucrose or water during acute optogenetic activation of IPCs with Chrimson. Ingestion time of females exposed to light normalized to dark controls of indicated genotype. One-way ANOVA with Holm-Šídák multiple comparison test. n=43-49 animals/genotype. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

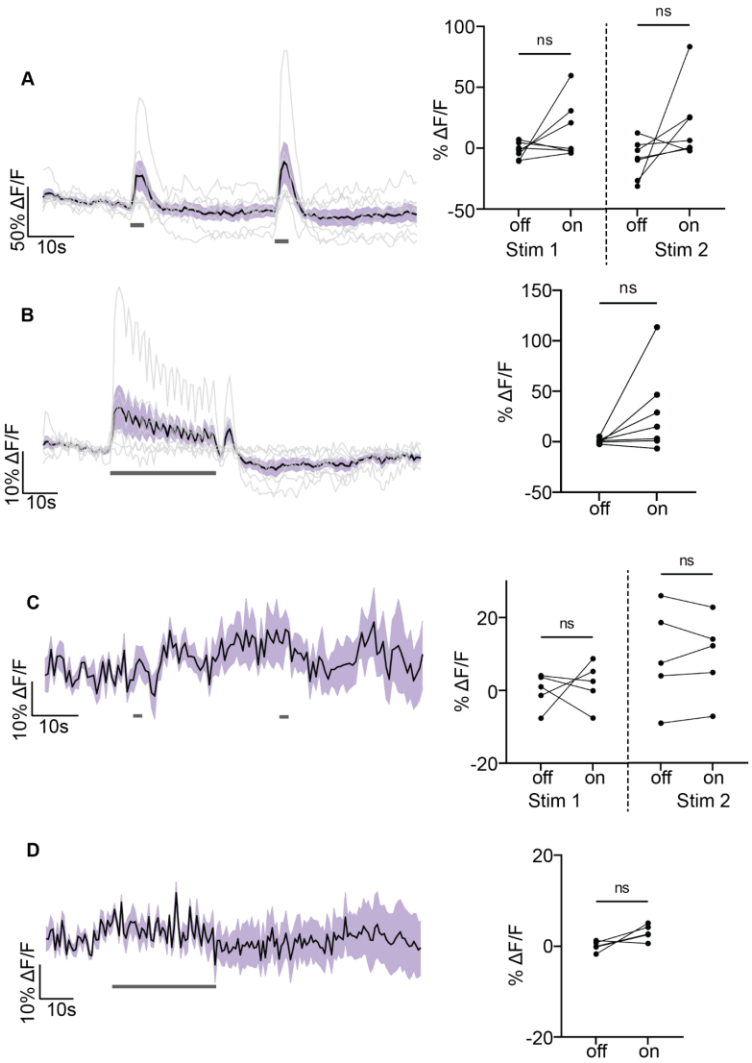


Figure S4. CCHa2R-RA genetic controls functional imaging and response to BiT optogenetic stimulation, Related to Fig 4

A and B: Genetic controls: we expressed the light sensitive ion channel Chrimson without the ISN-LexA driver and exposed the brains to 660nm LED. We expressed the calcium sensor GCaMP in the CCHa2R-RA neurons and imaged them with a 2-photon microscope. (A) Calcium response of CCHa2R-RA SEZ neurites to 2s LED exposure or (B) 30s LED exposure. Left: Scatter plot shows mean +/- SEM of all flies imaged, with individual traces in gray, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired Wilcoxon test and paired t-test. n=7 flies. C and D: We expressed the light sensitive ion channel Chrimson in BiT and optogenetically stimulated it with 660nm LED. We expressed the calcium sensor GCaMP in the CCHa2R-RA neurons and imaged them with a 2-photon microscope. (C) Calcium response of CCHa2R-RA somas to 2s optogenetic stimulation of BiT or (D) 30s optogenetic stimulation of BiT. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired t-test. n=5 flies.

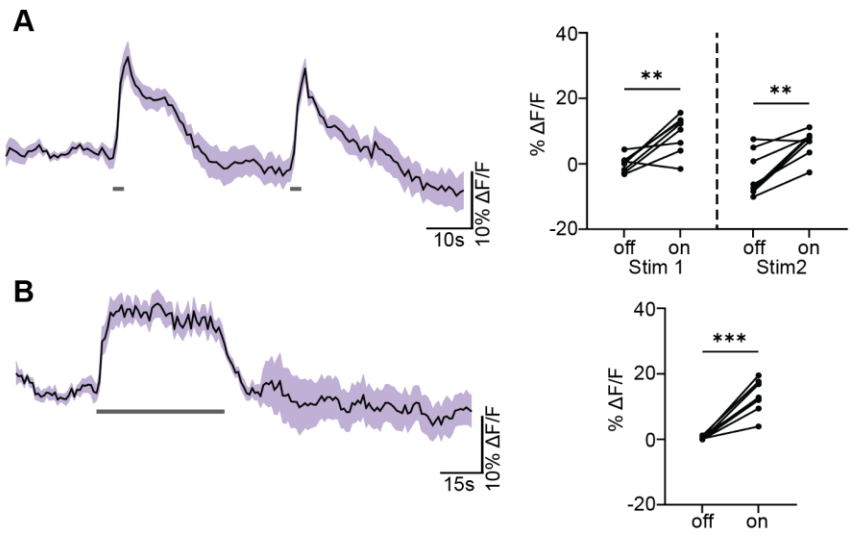


Figure S5. CCAP respond to ISN activation using another CCAP-Gal4 driver, Related to Fig 5

We expressed the light sensitive ion channel Chrimson in the ISNs and optogenetically stimulated them with 660nm LED. We expressed the calcium sensor GCaMP in the CCAP neurons and imaged them with a 2-photon microscope. (A) Calcium response of CCAP neurites to 2s optogenetic stimulation of the ISNs or (B) 30s optogenetic stimulation of the ISNs. Left: Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. Right: Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired t-test. n=8 flies. **p<0.01, ***p<0.001

Table S1: ISN postsynaptic partners

	Cell type / Classification	Flywire ID	Synapses from ISN 720575940619731393	Synapses from ISN 720575940628363855	Synapses from ISN 720575940625627932	Synapses from ISN 720575940624153528	Total synapses from 4 ISNs	Total synapses per cell type	Tracing contributions (number of edits)
1	FLAa2	720575940627559623	19	20	29	25	93		Jefferis Lab: Irene Salgarella (3), Varun Sane (1).
2	FLAa2	720575940613754833	19	32	21	19	91		Murthy and Seung Labs: James Hebditch (1), Ben Silverman (1), remer tancontian (1). Jefferis Lab: Bhargavi Parmar (4).
3	FLAa2	720575940621782057	18	14	21	7	60		Murthy and Seung Labs: Nash Hadjerol (6).
4	FLAa2	720575940623605180	10	10	14	14	48		Dickson Lab: Alisa Poh (1). Murthy and Seung Labs: Austin T Burke (16), Shirleyjoy Serona (1).
5	FLAa2	720575940625218590	7	16	11	10	44		Jefferis Lab: Irene Salgarella (8), Arti Yadav (1).
6	FLAa2	720575940620994292	7		23	14	44		Jefferis Lab: Irene Salgarella (1), Greg Jefferis (1), Philipp Schlegel (1), Bhargavi Parmar (1). Murthy and Seung Labs: Ben Silverman (1), Kendrick Joules Vinson (15), Joshua Bañez (2).
7	FLAa2	720575940632134483	17	6	13	6	42		Jefferis Lab: Imaan Tamimi (1), Arti Yadav (1). Murthy and Seung Labs: James Hebditch (2), Nash Hadjerol (1).
8	FLAa2	720575940619283553	11	10	9	12	42		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Austin T Burke (14).

9	FLAa2	720575940620306300	21		5	12	38		Murthy and Seung Labs: Austin T Burke (1), James Hebditch (2). Jefferis Lab: Arti Yadav (1).
10	FLAa2	720575940611991830	15	11	5		31		Jefferis Lab: Irene Salgarella (2). Murthy and Seung Labs: Austin T Burke (11).
11	FLAa2	720575940626512881	7		14	10	31		Murthy and Seung Labs: Mendell Lopez (10).
12	FLAa2	720575940621680264	8		10	16	34		Jefferis Lab: Anjali Pandey (1). Murthy and Seung Labs: Austin T Burke (12), J. Anthony Ocho (3).
13	FLAa2	720575940637381466	11	5		12	28		Jefferis Lab: Irene Salgarella (4). Murthy and Seung Labs: Zairene Lenizo (1).
14	FLAa2	720575940632695904	6	11	10		27		Jefferis Lab: Irene Salgarella (4), Rashmita Rana (1).
15	FLAa2	720575940631570636		6	14	6	26		Murthy and Seung Labs: Austin T Burke (5), Shirleyjoy Serona (3), Mendell Lopez (45).
16	FLAa2	720575940621605964	6		12	8	26		Seung Lab: Zhihao Zheng (1). Jefferis Lab: Irene Salgarella (9), Márcia Santos (2).
17	FLAa2	720575940637151807	8	5	13		26		Seung Lab: Zhihao Zheng (4). Jefferis Lab: A. Javier (2), Dickson Lab: Alisa Poh (3). Murthy and Seung Labs: James Hebditch (2), regine salem (1).
18	FLAa2	720575940622161032	15	9			24		Jefferis Lab: Irene Salgarella (4). Murthy and Seung Labs: Nash Hadjerol (8), Joshua Bañez (14).
19	FLAa2	720575940612087922	6		12	6	24		Jefferis Lab: Irene Salgarella (5). Murthy and Seung Labs: Austin T Burke (2), Nash Hadjerol (5).
20	FLAa2	720575940623752741	12	5	5		22		Jefferis Lab: Irene Salgarella (5).
21	FLAa2	720575940643191575		6	15		21		Murthy and Seung Labs: Mendell Lopez (11). Jefferis Lab: Arti Yadav (1).
22	FLAa2	720575940635191438		12		8	20		Jefferis Lab: Irene Salgarella (3). Murthy and Seung Labs: Zairene Lenizo (2).
23	FLAa2	720575940610624206	8		12		20		Jefferis Lab: Irene Salgarella (4). Murthy and Seung Labs: remer tancontian (2), J. Dolorosa (1).
24	FLAa2	720575940644751651	6	6	6		18		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Doug Bland (19).
25	FLAa2	720575940623989245		7		7	14		Jefferis Lab: Irene Salgarella (5). Murthy and Seung Labs: remer tancontian(1), Austin T Burke (1), Ben Silverman (2).
26	FLAa2	720575940623231527			9	5	14		Jefferis Lab: Yijie Yin (3). Dickson Lab: Alisa Poh (1). Murthy and Seung Labs: James Hebditch (1), Mendell Lopez (6).
27	FLAa2	720575940604764862	7		6		13		Jefferis Lab: Irene Salgarella (2). Murthy and Seung Labs: Mendell Lopez (1).
28	FLAa2	720575940628857850			13		13		Wolf Lab: fred wolf (1). Jefferis Lab: Arti Yadav (1), Yijie Yin (4). Murthy and Seung Labs: Mendell Lopez (3), remer tancontian (1).
29	FLAa2	720575940638900469	6		6		12		Jefferis Lab: Irene Salgarella (4).
30	FLAa2	720575940619398872	12				12		Jefferis Lab: A. Javier (1), Chitra Nair (1). Dickson Lab: Alisa Poh (13). Murthy and Seung Labs: Zairene Lenizo (4).
31	FLAa2	720575940631044141	6		6		12		Murthy and Seung Labs: James Hebditch (2). Jefferis Lab: Arti Yadav (1).
32	FLAa2	720575940638996413		5	7		12		Dickson Lab: Alisa Poh (8). Murthy and Seung Labs: Kyle Patrick Willie (2), Shirleyjoy Serona (3), Kendrick Joules Vinson (1), Joshua Bañez (1), James Hebditch (1). Jefferis Lab: Arti Yadav (1), Bhargavi Parmar (1).
33	FLAa2	720575940624735565	6		5		11		Dickson Lab: Alisa Poh (1). Jefferis Lab: Varun Sane (1), Arti Yadav (1). Murthy and Seung Labs: Ryan Willie (24), Rey Adrian Candilada (1).
34	FLAa2	720575940622266748		5		5	10		Jefferis Lab: Rashmita Rana (1), Zeba Vohra (1). Murthy and Seung Labs: Shirleyjoy Serona (15).

35	FLAa2	720575940644327022		5	5		10		Murthy and Seung Labs: James Hebditch (2). Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: J. Dolorosa (7).
36	FLAa2	720575940637002724	8				8		Jefferis Lab: Imaan Tamimi (1), Irene Salgarella (5). Murthy and Seung Labs: Darrel Jay Akiatan (2), J. Anthony Ocho (18).
37	FLAa2	720575940612348438				8	8		Jefferis Lab: Anjali Pandey (1). Murthy and Seung Labs: Zairene Lenizo (2), Kendrick Joules Vinson (4), Rey Adrian Candilada (3).
38	FLAa2	720575940623063847	8				8		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Shirleyjoy Serona (17).
39	FLAa2	720575940616872337		7			7		Jefferis Lab: Rashmita Rana (1). Murthy and Seung Labs: Ben Silverman (8), Kendrick Joules Vinson (6), Kyle Patrick Willie (1).
40	FLAa2	720575940626982165		6			6		Seung Lab: Zhihao Zheng (4). Jefferis Lab: Irene Salgarella (4), Yijie Yin (2). Murthy and Seung Labs: Nash Hadjerol (8).
41	FLAa2	720575940619403435		5			5		Jefferis Lab: Marta Costa (3), Irene Salgarella (1), Anjali Pandey (1), Varun Sane (18).
42	FLAa2	720575940626742532			5		5		Jefferis Lab: Marta Costa (3), Irene Salgarella (1), Anjali Pandey (1), Varun Sane (18).
43	FLAa2	720575940630393427			5		5		Jefferis Lab: Irene Salgarella (9).
44	FLAa2	720575940616510425		5			5		Dickson Lab: Alisa Poh (5). Murthy and Seung Labs: Rey Adrian Candilada (1).
45	FLAa2	720575940634776511			5		5		Jefferis Lab: Imaan Tamimi (2), Arti Yadav (1). Dickson Lab: Alisa Poh (2). Murthy and Seung Labs: Mendell Lopez (8), Ariel Dagohoy (19), Joshua Bañez (2), Ben Silverman (1).
46	FLAa2	720575940605589378				5	5	1080	Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Austin T Burke (15), Zairene Lenizo (1).
47	Handshake	720575940651601910	54	51	51	35	191		Jefferis and Waddell Labs: Joseph Hsu (1). Wolf Lab: fred wolf (1). Scott Lab: Amanda Abusaif (2). Murthy and Seung Labs: Ariel Dagohoy (2), J. Anthony Ocho (2), Shirleyjoy Serona (13). Jefferis Lab: Sangeeta Sisodiya (23).
48	Handshake	720575940626449158	46	46	42	37	171		Jefferis and Waddell Labs: Joseph Hsu (1). Scott Lab: Amanda Abusaif (1).
49	Handshake	720575940618791515	45	51	49	38	183		Wolf Lab: fred wolf (2). Scott Lab: Amanda Abusaif (3).
50	Handshake	720575940622839786	39	56	44	38	177	722	Jefferis and Waddell Labs: Joseph Hsu (1). Jefferis Lab: Laia Serratosa (1), Rashmita Rana (1). Dickson Lab: Alisa Poh (1). Murthy and Seung Labs: Kyle Patrick Willie (4), remer tancontian (5), Austin T Burke (8). Itisha Joshi (15).
51	Cowboy L fragment	720575940611730674	72	95	75	59	301		Simpson Lab: Li Guo (7). Jefferis Lab: Yijie Yin (2), Siqi Fang (3). Scott Lab: Amanda González-Segarra (7), Amanda Abusaif (1). Murthy and Seung Labs: J. Anthony Ocho (2), Mendell Lopez (1), Shaina Mae Monungolh (7), Ben Silverman (1), Nash Hadjerol (1), remer tancontian (1), Miguel Albero (2), Rey Adrian Candilada (2).
52	Cowboy R	720575940624514492	36	51	38	34	159	460	Jefferis and Wilson Labs: Laia Serratosa Capdevila (6). Jefferis Lab: Yijie Yin (6), Katharina Eichler (2), Zeba Vohra (18). Scott Lab: Amanda Abusaif (6). Murthy and Seung Labs: J. Dolorosa (1), Austin T Burke (10), Darrel Jay Akiatan (13)
53	SEZ: GNG.GNG. 2607	720575940633170969	37	30	33	24	124		Murthy and Seung Labs: Ryan Willie (32), Joshua Bañez (1), remer tancontian (1), Nash Hadjerol (1). Jefferis Lab: Katharina Eichler (1), Dhwani Patel (4), Griffin Badalemente (2), Dharini Sapkal (1).
54	SEZ: PRW.GNG. 9	720575940616167730	35	19	40	21	115		Jefferis Lab: Arti Yadav (1), Griffin Badalemente (2), Dharini Sapkal (5), Yashvi Patel (2), Bhargavi Parmar (1). Scott Lab: Amanda González-Segarra (3). Murthy and Seung Lab: Mendell Lopez (12).
55	SEZ: PRW.PRW .166	720575940622732253	5	12	17		34		Jefferis Lab: Rashmita Rana (1), Zeba Vohra (4). Murthy and Seung Labs: Shirleyjoy Serona (55), Austin T Burke (1). Kim Lab: Chan Hyuk Kang (1).
56	SEZ: PRW.PRW .214	720575940625178768	8	6		17	31		Jefferis Lab: Rashmita Rana (1), Sangeeta Sisodiya (1). Murthy and Seung Labs: Ben Silverman (7), Shirleyjoy Serona (23), Austin T Burke (1), Zairene Lenizo (4), Kyle Patrick Willie (2).

57	SEZ: PRW.GNG.6	720575940614736290	8	8	5	5	26		Murthy and Seung Labs: Ryan Willie (1), Ben Silverman (1), J. Anthony Ocho (37), Nash Hadjerol (2). Jefferis Lab: Chitra Nair (1), Yashvi Patel (1).
58	SEZ: PRW.GNG.12	720575940619042427			8		8		Seeds Hampel Lab: Patricia Pujols (7). Murthy and Seung Labs: Zairene Lenizo (4), Rey Adrian Candilada (1), remer tancontian (1), Kendrick Joules Vinson (1). Jefferis Lab: Bhargavi Parmar (5).
59	SEZ: PRW.PRW.146	720575940621490721				6	6		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: J. Anthony Ocho (2), Ben Silverman (3), Shaina Mae Monungolh (1), Kyle Patrick Willie (4).
60	SEZ: GNG.GNG.1075	720575940620627803	5				5		Jefferis Lab: Shanice Bailey (3), Rashmita Rana (1), Dhwani Patel (1). Murthy and Seung Labs: J. Anthony Ocho (1).
61	SEZ: PRW.PRW.42	720575940613120721			5		5		Murthy and Seung Labs: Nash Hadjerol (9). Kim Lab: hanetwo (1).
62	SEZ: PRW.PRW.62	720575940615052812			5		5		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Austin T Burke (1), Zairene Lenizo (2), Rey Adrian Candilada (1), Szi-chieh Yu (1). Jefferis Lab: Dharini Sapkal (1).
63	SEZ: PRW.PRW.199	720575940624532408			5		5		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Jay Gager (9). Itisha Joshi (2).
64	SEZ: FLA_L.FLA_L.47	720575940631299602			5		5		Jefferis Lab: Katharina Eichler (10), Laia Serratosa (1), Marina Gkantia (11), Bhargavi Parmar (28), Dharini Sapkal (10). Murthy and Seung Labs: J. Anthony Ocho (1), Zairene Lenizo (4), Shirleyjoy Serona (41), Darrel Jay Akiatan (1). Scott Lab: Amanda Abusaif (2). Itisha Joshi (5).
65	SEZ: PRW.PRW.120	720575940619866059			5		5		Jefferis Lab: Yijie Yin (1), Rashmita Rana (1), Zeba Vohra (10). Murthy and Seung Labs: Michelle Pantujan (1), Zairene Lenizo (1), regine salem (88), Rey Adrian Candilada (1), remer tancontian (3)
66	SEZ: TRdm	720575940612921571			5		5	379	Murthy and Seung Labs: Doug Bland (5). Jefferis Lab: Imaan Tamimi (1), Laia Serratosa (1), Griffin Badalemente (1), Dhwani Patel (15).
67	DSOG 1	720575940617291323	32	33	36	22	123		Seeds Hampel Lab: Katharina Eichler (10). Jefferis and Wilson Labs: Laia Serratosa Capdevila (2). Jefferis Lab: A. Javier (8), Katharina Eichler (1), Yijie Yin (1), Dharini Sapkal (47), Arzoo Diwan (6), Dhara Kakadiya (7), Zeba Vohra (9), Dhwani Patel (10), Yashvi Patel (1). Scott Lab: Amanda Abusaif (21). Pankratz Lab: Damian Demarest (1). Murthy and Seung Labs: remer tancontian (6), J. Dolorosa (54), Kendrick Joules Vinson (3), Shaina Mae Monungolh (2), Zairene Lenizo (2). Itisha Joshi (5)
68	DSOG 1	720575940623529610	28	27	32	31	118		Seeds Hampel Lab: Katharina Eichler (3). Jefferis and Wilson Labs: Laia Serratosa Capdevila (8). Jefferis Lab: Irene Salgarella (3), Yijie Yin (2), Zeba Vohra (7), Arti Yadav (16), Bhargavi Parmar (25), Chitra Nair (39), Dhara Kakadiya (4). Scott Lab: Amanda Abusaif (12). Murthy and Seung Labs: Celia D (1), remer tancontian (2), Austin T Burke (4), Zairene Lenizo (11), Shaina Mae Monungolh (26), Rey Adrian Candilada (1), Shirleyjoy Serona (24), J. Anthony Ocho (3). Itisha Joshi (6)
69	DSOG 1	720575940623338281	19	14	8	8	49		Seeds Hampel Lab: Katharina Eichler (8). Jefferis Lab: Siqi Fang (14), Katharina Eichler (4), Irene Salgarella (2), Griffin Badalemente (7), Nidhi Patel (3), Zeba Vohra (15), Dhwani Patel (10), Chitra Nair (8), Yashvi Patel (15), Dharini Sapkal (19), Arti Yadav (4). Murthy and Seung Labs: Rey Adrian Candilada (6), Shaina Mae Monungolh (45). Kim Lab: Chan Hyuk Kang (2). Scott Lab: Amanda Abusaif (12).
70	DSOG 1	720575940644666660	13	5	17	5	40	330	Seeds Hampel Lab: Katharina Eichler, Steven Calle, Lucia Kmecova, Alexis E Santana Cruz. Murthy and Seung Labs: remer tancontian, Zairene Lenizo.
71	SEZ & SMP: FLA_L.SMP_L.27	720575940626500362	19	5	18	8	50		Jefferis Lab: Greg Jefferis (2). Dickson Lab: Alisa Poh (1). Murthy and Seung Labs: Shirleyjoy Serona (25), regine salem (17), Mendell Lopez (1), Shaina Mae Monungolh (2).

72	SEZ & SMP: VESa1	720575940632951597	12		19	16	47		Murthy and Seung Labs: Austin T Burke (72), Sarah Morejohn (1), Kyle Patrick Willie (11), James Hebditch (6), Doug Bland (5), Nash Hadjerol (39), Ben Silverman (7), J. Dolorosa (3), Zairene Lenizo (48), Mendell Lopez (9), Shaina Mae Monungolh (5), Rey Adrian Candilada (7), regine salem (80), remer tancontian (19), Darrel Jay Akiatan (13), Joshua Bañez (112), Ariel Dagohoy (9), Kendrick Joules Vinson (27), Miguel Albero (4), Shirleyjoy Serona (89), Michelle Pantujan (14), J. Anthony Ocho (11). Jefferis and Wilson Labs: Laia Serratosa Capdevila (6). Jefferis Lab: Marlon Blanquart (1), Imaan Tamimi (5), Yijie Yin (3), Irene Salgarella (3), Varun Sane (4), Griffin Badalemente (21), Philipp Schlegel (9), Dharini Sapkal (35), Chitra Nair (34), Arzoo Diwan (8), Zeba Vohra (24), Anjali Pandey (2), Dhara Kakadiya (48), Bhargavi Parmar (10), Kaushik Parmar (1), Arti Yadav (10), Yashvi Patel (12), Greg Jefferis (1). Janelia Tracers: Tansy Yang (38). Selcho Lab: Mareike Selcho (1).
73	SEZ & SMP: FLA_L.SMP_L.32	720575940627436554	12	16	6	12	46		Jefferis Lab: Greg Jefferis (1), Christophe Dunne (1), Bhargavi Parmar (1). Murthy and Seung Labs: Mendell Lopez (1), Joshua Bañez (2).
74	SEZ & SMP: FLA_R.SMP_R.32	720575940645920052	7		17	13	37		Jefferis and Wilson Labs: Laia Serratosa Capdevila (1). Wolf Lab: fred wolf (1). Huetteroth Lab: Wolf Huetteroth (1). Murthy and Seung Labs: Zairene Lenizo (6), Shirleyjoy Serona (29), Ariel Dagohoy (28), Shaina Mae Monungolh (11). Jefferis Lab: Márcia Santos (1).
75	SEZ & SMP: BiT2	720575940621662332	7	7	14	8	36		Murthy and Seung Labs: Austin T Burke (47), Joshua Bañez (9), Kyle Patrick Willie (16). Jefferis Lab: Imaan Tamimi (12), Yijie Yin (2), Bhargavi Parmar (2), Dharini Sapkal (1). Scott Lab: Amanda Abusaif (14).
76	SEZ & SMP: FLA_R.SMP_R.30	720575940637780969	5	5	10	14	34		Jefferis Lab: Arti Yadav (1), Dhwani Patel (5). Murthy and Seung Labs: Shirleyjoy Serona (3), Ryan Willie (2), Kyle Patrick Willie (2), Rey Adrian Candilada (1).
77	SEZ & SMP: Lgr3/FLAa3	720575940626452879	8	15	9		32		Jefferis Lab: Yijie Yin (1), Anjali Pandey (1). Murthy and Seung Labs: James Hebditch (1), Mendell Lopez (21), Austin T Burke (3), Joshua Bañez (3), Kendrick Joules Vinson (4).
78	SEZ & SMP: SMPpv2	720575940623701768		6	9		15		Jefferis Lab: Greg Jefferis (1), Yijie Yin (2), A. Javier (3), Irene Salgarella (5). Scott Lab: Amanda Abusaif (1). Murthy and Seung Labs: Szi-chieh Yu (15), Zairene Lenizo (9).
79	SEZ & SMP: SMPpv2	720575940628310275		7		5	12		Jefferis and Wilson Labs: Laia Serratosa Capdevila (9). Jefferis Lab: Katharina Eichler (9), Imaan Tamimi (5), Irene Salgarella (4), Philipp Schlegel (39). Murthy and Seung Labs: James Hebditch (1), Rey Adrian Candilada (10).
80	SEZ & SMP: Lgr3/FLAa3	720575940629764906		5			5		Jefferis and Waddell Labs: Joseph Hsu (3). Murthy and Seung Labs: Austin T Burke (14). Jefferis Lab: Anjali Pandey (1).
81	SEZ & SMP: Gallinule	720575940618926757				5	5	319	Jefferis and Waddell Labs: Joseph Hsu (8). Jefferis Lab: Yijie Yin (4), Imaan Tamimi (1), Rashmita Rana (2). Murthy and Seung Labs: Mendell Lopez (2).
82	BiT	720575940610708430	71	74	93	63	301	301	Jefferis Lab: Yijie Yin (3), A. Javier (1), Imaan Tamimi (22), Varun Sane (2), Griffin Badalemente (1), Dhara Kakadiya (1), Arti Yadav (6). Murthy and Seung Labs: Claire McKellar (1), Michelle Pantujan (2), Austin T Burke (2), James Hebditch (1), J. Anthony Ocho (420), Rey Adrian Candilada (1), Nash Hadjerol (4). Wes Murfin (1). Scott Lab: Amanda Abusaif (30).
83	Ascending neuron: FLA_R.PRW.5	720575940625841241	20	6	16	27	69		Seeds Hampel Lab: Steven Calle (1). Jefferis Lab: Katharina Eichler (8). Kim Lab: Chan Hyuk Kang (2). Murthy and Seung Labs: M Sorek (2), J. Anthony Ocho (1), Zairene Lenizo (1).
84	Ascending neurons: PRW.FLA_L.15	720575940633548128		29	33	5	67		Jefferis and Wilson Labs: Laia Serratosa Capdevila (11). Scott Lab: Zepeng Yao (1), Amanda Abusaif (2), Amanda González-Segarra (5), Rey Adrian Candilada (4).
85	Ascending neuron: FLA_L.FLA_L.25	720575940621599741	19	18		5	42		Jefferis and Waddell Labs: Joseph Hsu (3). Jefferis and Wilson Labs: Laia Serratosa Capdevila (3).

86	Ascending neuron: PRW.SMP_L.30	720575940635527092	8	5	14	11	38		Seeds Hampel Lab: Katharina Eichler (27). Jefferis and Wilson Labs: Laia Serratosa Capdevila (15). Jefferis Lab: Katharina Eichler (1), Varun Sane (1), Irene Salgarella (1), Bhargavi Parmar (10), Chitra Nair (40), Sangeeta Sisodiya (18), Zeba Vohra (4), Bhargavi Parmar (10), Dharini Sapkal (1). Murthy and Seung Labs: Zairene Lenizo (5), J. Anthony Ocho (3), Nash Hadjerol (5), Mendell Lopez (3), Joshua Bañez (4), regine salem (1), Rey Adrian Candilada (1), Kendrick Joules Vinson (3), Ariel Dagohoy (8), Kyle Patrick Willie (4), Doug Bland (31), Ryan Willie (1). Itisha Joshi (25)
87	Ascending neuron: FLA_R.PRW.2	720575940611548273	10	11	5		26		Jefferis and Wilson Labs: Laia Serratosa Capdevila (11). Murthy and Seung Labs: Mendell Lopez (1), Zairene Lenizo (2), Kyle Patrick Willie (4).
88	Ascending neuron: PRW.PRW.304	720575940631295506	7		9		16		Jefferis and Wilson Labs: Laia Serratosa Capdevila (3). Murthy and Seung Labs: Shaina Mae Monungolh (2), Nash Hadjerol (7). Jefferis Lab: Dhwani Patel (1).
89	Ascending neuron: PRW.PRW.343	720575940636263543	5		8		13		Seung Lab: Zhihao Zheng (1). Jefferis Lab: Katharina Eichler (1), Dharini Sapkal (5). Murthy and Seung Labs: Michelle Pantujan (2), Nash Hadjerol (7), Shaina Mae Monungolh (1). Dickson Lab: Alisa Poh (2).
90	Ascending neuron: PRW.PRW.137	720575940620833901			6	6	12		Murthy and Seung Labs: James Hebditch (9), Austin T Burke (11), Ben Silverman (1), Nash Hadjerol (3), Mendell Lopez (18), Ryan Willie (1), regine salem (3), Joshua Bañez (6), Rey Adrian Candilada (5), Zairene Lenizo (7), Darrel Jay Akiatan (3). Jefferis and Wilson Labs: Laia Serratosa Capdevila (3). Jefferis Lab: Katharina Eichler (10), Sangeeta Sisodiya (1), Dharini Sapkal (1), Bhargavi Parmar (37), Chitra Nair (17). Seeds Hampel Lab: Alexis E Santana Cruz (1).
91	Ascending neuron: PRW.PRW.247	720575940627304424				10	10		Jefferis Lab: Dharini Sapkal (1). Murthy and Seung Labs: Shirleyjoy Serona (16), Zairene Lenizo (1), Rey Adrian Candilada (3).
92	Ascending neuron: FLA_L.GNG.5	720575940617662950			6		6	299	Jefferis Lab: Katharina Eichler. Murthy and Seung Labs: Ben Silverman.
93	Descending neuron: PRW.GNG.16	720575940621328048	7	16	15	9	47		Jefferis and Wilson Labs: Laia Serratosa Capdevila (3). Jefferis Lab: Katharina Eichler (1), Arti Yadav (1), Dharini Sapkal (1), Dhwani Patel (1), Bhargavi Parmar (31). Murthy and Seung Labs: Austin T Burke (1), Mendell Lopez (1), Shaina Mae Monungolh (7), Ariel Dagohoy (1). Itisha Joshi (5).
94	Descending neuron: FLA_L.GNG.3	720575940610610841	9			5	14		Jefferis and Wilson Labs: Laia Serratosa Capdevila (14). Jefferis Lab: A. Javier (9), Yijie Yin (3), Katharina Eichler (1), Rashmita Rana (1).
95	Descending neuron: FLA_L.NO_OUT.7	720575940625587325	7		5		12		Jefferis Lab: Philipp Schlegel (1), Katharina Eichler (15), Paul Brooks (45). Jefferis and Wilson Labs: Laia Serratosa Capdevila (3). Selcho Lab: Mareike Selcho (7). Kim Lab: Usb (1).
96	Descending neuron: GNG.GNG.1079	720575940620668609			12		12		Seeds Hampel Lab: Katharina Eichler (2), Stefanie Hampel (1). Jefferis and Wilson Labs: Laia Serratosa Capdevila (1). Jefferis Lab: Griffin Badalemente (1), Dhwani Patel (30), Dharini Sapkal (18). Murthy and Seung Labs: regine salem (1).
97	Descending Neuron: Gumdrop	720575940630697078	5			6	11		Jefferis and Wilson Labs: Laia Serratosa Capdevila (21). Jefferis Lab: Katharina Eichler (30), Imaan Tamimi (1), Yashvi Patel (5), Arti Yadav (10), Zeba Vohra (23), Bhargavi Parmar (15). Murthy and Seung Labs: Nash Hadjerol (5). Itisha Joshi (8).
98	Descending neuron: FLA_L.NO_OUT.5	720575940619576001	6		5		11		Jefferis and Wilson Labs: Laia Serratosa Capdevila (3). Jefferis Lab: Katharina Eichler (17), Paul Brooks (28), Imaan Tamimi (1), Yijie Yin (8). Murthy and Seung Labs: Michelle Pantujan (1), J. Dolorosa (1), Ben Silverman (14).
99	Descending neuron	720575940644812398		6	5		11		Jefferis and Wilson Labs: Laia Serratosa Capdevila (4). Jefferis Lab: A. Javier (24), Katharina Eichler (4), Rashmita Rana (1), Varun Sane (1). Selcho Lab: Mareike Selcho (8). Murthy and Seung Labs: Joshua Bañez (1).

100	Descending neuron: GNG.GNG.391	720575940612692889	6				6	124	Jefferis and Wilson Labs: Laia Serratosa Capdevila (2). Jefferis Lab: Katharina Eichler (2), Zeba Vohra (1), Yashvi Patel (2). Murthy and Seung Labs: Shirleyjoy Serona (253), Darrel Jay Akiatan (5).
101	ISN	720575940625627932	5	6		5	16		Scott Lab: Alexander Edward Del Toro BSc (1). Jefferis Lab: Arti Yadav (1). Kim Lab: Hyungjun Choi (183), Chan Hyuk Kang (26), hanetwo (5). Murthy and Seung Labs: Ariel Dagohoy (2), Rey Adrian Candilada (3).
102	ISN	720575940619731393		5		5	10		Jefferis Lab: Katharina Eichler (1). Murthy and Seung Labs: Claire McKellar (1), Nash Hadjerol (2), Celia D (1), regine salem (332), Joshua Bañez (286), Kendrick Joules Vinson (8), J. Anthony Ocho (1). Scott Lab: Amanda González-Segarra (3), Alexander Edward Del Toro BSc (1). Kim Lab: Keehyun Park (6), hanetwo (19).
103	ISN	720575940628363855			5		5		Kim Group: Hyungjun Choi (29), Chan Hyuk Kang (25), hanetwo (3). Murthy and Seung Labs: Shaina Mae Monungolh (5), Joshua Bañez (1), Shirleyjoy Serona (126), Zairene Lenizo (1).
104	ISN	720575940624153528			5		5	20	Scott Lab: Amanda González-Segarra (1). Jefferis Lab: Anjali Pandey (1), Varun Sane (1). Kim Lab: Keehyun Park (1), hanetwo (22). Murthy and Seung Labs: Nash Hadjerol (12), Zairene Lenizo (4), remer tancontian (1).
								4034	

Table S2: BiT postsynaptic neurons

	Name	Neuron ID	Synapses from BiT	Total synapses from BiT per cell type	Tracing contributions (number of edits)
1	IPC	720575940650527222	54		Jefferis Lab: Imaan Tamimi (2), Anjali Pandey (1). Murthy and Seung Labs: Austin T Burke (4).
2	IPC	720575940603765280	44		Maimon Lab: Gaby Maimon (1). Jefferis Lab: Katharina Eichler (2). Murthy and Seung Labs: Austin T Burke (3).
3	IPC	720575940620932045	39		Jefferis Lab: Imaan Tamimi (2), Laia Serratosa (7). Murthy and Seung Labs: Austin T Burke (6).
4	IPC	720575940612923390	33		Jefferis Lab: Imaan Tamimi (1), Laia Serratosa (6), Markus Pleijzier (1), Rashmita Rana (1). Murthy and Seung Labs: Ryan Willie (1).
5	IPC	720575940625379859	30		Jefferis Lab: A. Javier (4), Irene Salgarella (2), Laia Serratosa (9). Murthy and Seung Labs: Austin T Burke (4).
6	IPC	720575940622897639	28		Seung Lab: Zhihao Zheng (1), Jefferis Lab: A. Javier (1). Dacks Lab: Andrew Dacks (1). Murthy and Seung Labs: Austin T Burke (7).
7	IPC	720575940643539566	26		Jefferis Lab: Imaan Tamimi (3), A. Javier (7), Laia Serratosa (4), Rashmita Rana (1). Murthy and Seung Labs: Austin T Burke (13).
8	IPC	720575940620628957	25		Jefferis Lab: Imaan Tamimi (1), A. Javier (6). Murthy and Seung Labs: Austin T Burke. (18)
9	IPC	720575940623081400	22		Jefferis Lab: Markus Pleijzier (5). Murthy and Seung Labs: Austin T Burke (18).
10	IPC	720575940631884883	22		Dacks Lab: Andrew Dacks (1). Jefferis Lab: Imaan Tamimi (1), Laia Serratosa (8). Murthy and Seung Labs: Austin T Burke (7). Itisha Joshi (2).
11	IPC	720575940628363820	22		Jefferis Lab: Imaan Tamimi (1). Dacks Lab: Andrew Dacks (1). Murthy and Seung Labs: Austin T Burke (6). Scott Lab: Meghan Laturney (1).
12	IPC	720575940624064295	22		Dacks Lab: Andrew Dacks (1). Jefferis Lab: Imaan Tamimi (3), Laia Serratosa (10), Anjali Pandey (1). Murthy and Seung Labs: Austin T Burke (5).
13	IPC	720575940628199802	21		Jefferis Lab: Markus Pleijzier (1), A. Javier (1), Laia Serratosa (9), Philipp Schlegel (1). Murthy and Seung Labs: Austin T Burke (1). Wolf Lab: Fred Wolf (1).
14	IPC	720575940623586284	17		Jefferis Lab: Imaan Tamimi (1), Márcia Santos (4). Murthy and Seung Labs: Austin T Burke (66).

15	IPC	720575940618694827	14		Jefferis Lab: A. Javier (3), Imaan Tamimi (1), Laia Serratosa (12), Anjali Pandey (1).
16	IPC	720575940614623455	10		Murthy and Seung Labs: James Hebditch (4), Nash Hadjerol (1), Doug Bland (6), Joshua Bañez (6). Jefferis Lab: Philipp Schlegel (1).
17	IPC	720575940611254681	7		Jefferis Lab: Markus Pleijzier (2), A. Javier (3), Imaan Tamimi (9). Murthy and Seung Labs: Austin T Burke (13). Kim Lab: Minsik Yun (2).
18	IPC	720575940615911380	6	442	Jefferis Lab: A. Javier (6), Laia Serratosa (6), Philipp Schlegel (1). Murthy and Seung Labs: Ryan Willie (1).
19	Lgr3/FLAa3	720575940626983185	36		Jefferis Lab: Irene Salgarella (10). Murthy and Seung Labs: James Hebditch (5), Kyle Patrick Willie (4), Rey Adrian Candilada (1), Kendrick Joules Vinson (11), Zairene Lenizo (2), Nash Hadjerol (4).
20	Lgr3/FLAa3	720575940620201084	32		Jefferis Lab: Irene Salgarella (7), Laia Serratosa (4). Murthy and Seung Labs: James Hebditch (4).
21	Lgr3/FLAa3	720575940613895934	31		Jefferis Lab: Irene Salgarella (10), Dharini Sapkal (2), Dhwani Patel (12). Murthy and Seung Labs: James Hebditch (2), Zairene Lenizo (2), Joshua Bañez (8).
22	Lgr3/FLAa3	720575940629696763	30		Jefferis Lab: Anjali Pandey (1). Murthy and Seung Labs: James Hebditch (4), Nash Hadjerol (6), Ben Silverman (1), Darrel Jay Akiatan (2).
23	Lgr3/FLAa3	720575940644053271	19		Jefferis Lab: Anjali Pandey (1), Christopher Dunne (11). Murthy and Seung Labs: Zairene Lenizo (1).
24	Lgr3/FLAa3	720575940630329903	19		Jefferis Lab: Irene Salgarella (15). Murthy and Seung Labs: James Hebditch (3).
25	Lgr3/FLAa3	720575940616987426	19		Murthy and Seung Labs: James Hebditch (4). Jefferis Lab: Anjali Pandey (8), Christopher Dunne (4), Sangeeta Sisodiya (1), Dharini Sapkal (4).
26	Lgr3/FLAa3	720575940639420032	19		Jefferis Lab: Irene Salgarella (16). Murthy and Seung Labs: James Hebditch (4), Zairene Lenizo (1).
27	Lgr3/FLAa3	720575940614714299	18		Jefferis Lab: Irene Salgarella (7). Murthy and Seung Labs: Nash Hadjerol (1).
28	Lgr3/FLAa3	720575940632879842	18		Scott Lab: Zepeng Yao (17). Jefferis Lab: Arti Yadav (1), Bhargavi Parmar (6). Murthy and Seung Labs: J. Anthony Ocho (1), Austin T Burke (1), Zairene Lenizo (2).
29	Lgr3/FLAa3	720575940626179658	15		Jefferis Lab: Irene Salgarella (10).
30	Lgr3/FLAa3	720575940626327070	15	271	Murthy and Seung Labs: Austin T Burke (1), James Hebditch (4), Ariel Dagohoy (4), Nash Hadjerol (2). Janelia tracers: Tansy Yang (2). Jefferis Lab: Irene Salgarella (8), Laia Serratosa (3). Scott Lab: Zepeng Yao (3). Pankratz Lab: Damian Demarest (2).
31	SMP & SLP: SMP_L.SMP_L.276	720575940617650203	45		Jefferis and Waddell Labs: Joseph Hsu (2). Murthy and Seung Labs: Kyle Patrick Willie (32). Jefferis Lab: Laia Serratosa (1), Anjali Pandey (1), Yijie Yin (4).
32	SMP & SLP: SMP_R.SMP_R.278	720575940619352198	44		Murthy Lab: Lucas Encarnacion-Rivera (1), Bock Lab: Davi Bock (4). Jefferis Lab: Yijie Yin (13), Greg Jefferis (15).
33	SMP & SLP: SMPpv1; right	720575940631373869	38		Jefferis and Waddell Labs: Joseph Hsu (2). Jefferis Lab: Irene Salgarella (1). Murthy and Seung Labs: J. Dolorosa (2), Kyle Patrick Willie (4).
34	SMP & SLP: SMPpv1; left	720575940633754292	26		Jefferis Lab: Yijie Yin (2). Murthy and Seung Labs: Ben Silverman (1).
35	SMP & SLP: SMP_R.SMP_R.808	720575940635354981	12		Murthy and Seung Labs: Zairene Lenizo (1), Nash Hadjerol (24), remer tancontian (5). Jefferis Lab: Anjali Pandey (1).
36	SMP & SLP: SMPpd1; left	720575940627327750	12		Jefferis Lab: Rashmita Rana (1). Murthy and Seung Labs: James Hebditch (3), remer tancontian (1).
37	SMP & SLP: SMP_R.SMP_R.70	720575940611332722	11		Jefferis Lab: Yijie Yin (1), Rashmita Rana (1). Murthy and Seung Labs: Kyle Patrick Willie (10.
38	SMP & SLP: SMPpd1; left	720575940619099558	10		Jefferis Lab: A. Javier (2), Arti Yadav (1).
39	SMP & SLP: SMP_R.SMP_R.742	720575940631589407	9		Murthy and Seung Labs: Austin T Burke (17), James Hebditch (2), Kendrick Joules Vinson (1). Jefferis Lab: Anjali Pandey (1).
40	SMP & SLP: SLPal1	720575940618140283	8		Jefferis Lab: A. Javier (3). Murthy and Seung Labs: Nash Hadjerol (1), Ariel Dagohoy (1), James Hebditch (2).

41	SMP & SLP: SMPpd2	720575940619949556	7		Jefferis Lab: Irene Salgarella (3), Anjali Pandey (1).
42	SMP & SLP: SMP_R.SMP_R.757	720575940632526547	6		Murthy and Seung Labs: Austin T Burke (1), Kyle Patrick Willie (6), Mendell Lopez (10), Doug Bland (24), remer tancontian (6). Jefferis Lab: Anjali Pandey (1).
43	SMP & SLP: SMP_L.SMP_L.706	720575940629298679	6		Murthy Lab: Lucas Encarnacion-Rivera (3). Jefferis Lab: A. Javier (1), Varun Sane (8), Arti Yadav (1). Murthy and Seung Labs: Kendrick Joules Vinson (7), remer tancontian (2).
44	SMP & SLP: SMP_R.SMP_R.906	720575940640456923	6		Jefferis Lab: Philipp Schlegel (3), Yijie Yin (1), Varun Sane (6), Anjali Pandey (1). Selcho Lab: Mareike Selcho (17). Murthy and Seung Labs: Mendell Lopez (19), Kyle Patrick Willie (1).
45	SMP & SLP: DM3; right	720575940621647498	5		Jefferis Lab: A. Javier (1), Yijie Yin (1).
46	SMP & SLP: DM3_canonical	720575940619899668	5		Jefferis Lab: Yijie Yin (16). Murthy and Seung Labs: Nash Hadjerol (1), Kyle Patrick Willie (1).
47	SMP & SLP: SMP_R.SMP_R.677	720575940629901307	5		Jefferis Lab: Yijie Yin (2), Chitra Nair (1), Varun Sane (3). Murthy and Seung Labs: Zairene Lenizo (2).
48	SMP & SLP: SLP_L.LH_L.5	720575940616707545	5		Jefferis and Wilson Labs: Laia Serratosa Capdevila (1). Wes Murfin (4). Jefferis Lab: Yijie Yin (3), Varun Sane (1), Dhara Kakadiya (2), Chitra Nair (4). Murthy and Seung Labs: Doug Bland (3), Kendrick Joules Vinson (23), Joshua Bañez (10), Rey Adrian Candilada (1).
49	SMP & SLP: SLP_R.SLP_R.557	720575940629163419	5		Murthy and Seung Labs: Austin T Burke (3). Jefferis Lab: Tomke S (14), Griffin Badalemente (4), Yijie Yin (1), Varun Sane (4), Dhwani Patel (1).
50	SMP & SLP: SMP_L.SMP_L.578	720575940626063688	5	270	Murthy and Seung Labs: Austin T Burke (13).
51	CCHa2R (RA)	720575940638190133	65		Murthy and Seung Labs: Claire McKellar (1). Jefferis Lab: Anjali Pandey (1), Laia Serratosa (117), Dhwani Patel (3). Scott Lab: Amanda Abusaif (1), Zepeng Yao (27). Kim Lab: hanetwo (1).
52	CCHa2R (RA)	720575940615181910	64		Jefferis Lab: Anjali Pandey (1). Scott Lab: Amanda Abusaif (12). Murthy and Seung Labs: Michelle Pantujan (41), Ryan Willie (459).
53	CCHa2R (RA)	720575940629642460	50		Jefferis and Waddell Labs: Joseph Hsu (1). Murthy and Seung Labs: Claire McKellar (12), Ryan Willie (1), Shirleyjoy Serona (1), Doug Bland (296), J. Anthony Ocho (3). Jefferis Lab: Arti Yadav (1). Kim Lab: Hyungjun Choi (74).
54	CCHa2R (RA)	720575940611411162	49	228	Murthy and Seung Labs: Claire McKellar (2), Nash Hadjerol (2), Zairene Lenizo (1). Jefferis Lab: Irene Salgarella (3). Scott Lab: Amanda Abusaif (11). Kim Lab: hanetwo (14),Chan Hyuk Kang (2).
55	SMP & SEZ: ADM09	720575940628462927	48		Anderson Lab: Altyn Rymbek (2). Jefferis Lab: Imaan Tamimi (6), Laia Serratosa (10), Rashmita Rana (1), Sangeeta Sisodiya (1). Murthy and Seung Labs: James Hebditch (1), Joshua Bañez (1), Mendell Lopez (58), Zairene Lenizo (2), J. Dolorosa (1). Kim Lab: Minsik Yun (1).
56	SMP & SEZ: pCd1?	720575940618057095	22		Jefferis Lab: A. Javier (1), Irene Salgarella (1). Murthy and Seung Labs: Austin T Burke (23)
57	SMP & SEZ: pCd1?	720575940604332460	12		Seung Lab: Zhihao Zheng (1). Jefferis and Wilson Labs: Laia Serratosa Capdevila (1). Jefferis: A. Javier (1), Bhargavi Parmar (1). Murthy and Seung Labs: Austin T Burke (13).
58	SMP & SEZ: ADM09p	720575940642910152	12		Scott Lab: Zepeng Yao (6). Murthy and Seung Labs: Austin T Burke (3), Shaina Mae Monungolh (1). Jefferis Lab: Yijie Yin (4), Marina Gkantia (10).
59	SMP & SEZ: DM3	720575940626005330	11		Janelia tracers: Tansy Yang (5). Jefferis Lab: Yijie Yin (3). Murthy and Seung Labs: Austin T Burke (7).
60	SMP & SEZ: Dh44	720575940618579505	10		Jefferis Lab: Imaan Tamimi (1), A. Javier (4), Rashmita Rana (1). Murthy and Seung Labs: Austin T Burke (13), remer tancontian (7).
61	SMP & SEZ: pCd1?	720575940645882420	10		Jefferis and Waddell Labs: Joseph Hsu (1). Murthy and Seung Labs: Austin T Burke (18), Joshua Bañez (1).
62	SMP & SEZ: pCd1?	720575940638671219	10		Jefferis Lab: A. Javier (3), Irene Salgarella (2), Tomke S (2), Varun Sane (5). Murthy and Seung Labs: Claire McKellar (2), remer tancontian (5), Nash Hadjerol (7).
63	SMP & SEZ: pCd1?	720575940622119861	8		Jefferis Lab: Irene Salgarella (2), Varun Sane (5), Yijie Yin (2). Murthy and Seung Labs: Austin T Burke (21).
64	SMP & SEZ: DM2_dorsal	720575940629754588	8		Anderson Lab: Altyn Rymbek (1). Jefferis Lab: Yijie Yin (11), Varun Sane (4). Murthy and Seung Labs: Austin T Burke (1), Joshua Bañez (1).

65	SMP & SEZ: SMPpv2	720575940621362044	7		Jefferis and Wilson Labs: Laia Serratosa Capdevila (1). Jefferis Lab: Yijie Yin (34), Irene Salgarella (1), Imaan Tamimi (7), Chitra Nair (1). Murthy and Seung Labs: Shirleyjoy Serona (1), Austin T Burke (1).
66	SMP & SEZ: FLA_R.NO_OUT.6	720575940623184567	7		Jefferis Lab: Katharina Eichler (1), Philipp Schlegel (1). Murthy and Seung Labs: Austin T Burke (1), Ben Silverman (2).
67	SMP & SEZ: pCd1?	720575940638719907	7		Jefferis and Waddell Labs: Joseph Hsu (1). Murthy and Seung Labs: Austin T Burke (1), Kyle Patrick Willie (6). Wes Murfin (29).
68	SMP & SEZ: pCd1?	720575940612572054	6		Seung Lab: Zhihao Zheng (1). Jefferis Lab: Irene Salgarella (6). Murthy and Seung Labs: Austin T Burke (37).
69	SMP & SEZ: DM2	720575940608174894	5		Anderson Lab: Altyn Rymbek (1), Jefferis and Waddell Labs: Joseph Hsu (14).
70	SMP & SEZ: DM2	720575940635196334	5		Jefferis Lab: Irene Salgarella (6). Janelia tracers: Tansy Yang (11). Murthy and Seung Labs: Austin T Burke (7).
71	SMP & SEZ: pMP5/DM2	720575940628732610	5		Murthy and Seung Labs: Austin T Burke (4), remer tancontian (5). Jefferis Lab: Irene Salgarella (3), Yijie Yin (22). Janelia tracers: Tansy Yang (2).
72	SMP & SEZ: SMP_R.FLA_L.29	720575940636113264	5		Jefferis Lab: Greg Jefferis (1), Laia Serratosa (11), Anjali Pandey (1). Dickson Lab: Alisa Poh (1). Murthy and Seung Labs: Nash Hadjerol (11).
73	SMP & SEZ: pCd1?	720575940638285184	5	203	Murthy and Seung Labs: Austin T Burke (3). Wes Murfin (9). Jefferis Lab: Laia Serratosa (4), Varun Sane (4).
74	Antler L	720575940622998967	54		Murthy and Seung Labs: Austin T Burke (17), Zairene Lenizo (4), Darrel Jay Akiatan (1), Shirleyjoy Serona (12), Shaina Mae Monungolh (2). Jefferis Lab: Yijie Yin (3).
75	Antler R	720575940631226439	50	104	Murthy and Seung Labs: Austin T Burke (17), Rey Adrian Candilada (3). Jefferis Lab: Varun Sane (7), Yijie Yin (5).
76	visual projection: SMP_R.SMP_R.201	720575940616608837	23		Jefferis Lab: Irene Salgarella (1), Rashmita Rana (1), Griffin Badalemente (4). Murthy and Seung Labs: J. Anthony Ocho (12), J. Dolorosa (1).
77	visual projection: SMP_R.SMP_R.698	720575940630512711	17		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Doug Bland (19).
78	visual projection: SMP_L.SMP_L.880	720575940636320063	15		Murthy and Seung Labs: J. Dolorosa (26). Jefferis Lab: Rashmita Rana (1).
79	visual projection: SMP_L.SMP_L.362	720575940620022960	12	67	Kim Lab: Dustin Garner (2). Jefferis Lab: Yijie Yin (16). Murthy and Seung Labs: Ben Silverman (5), remer tancontian (1).
80	Fan shaped body: DL1_dorsal; left	720575940626612254	29		Jefferis Lab: Rashmita Rana (1), Zeba Vohra (1). Murthy and Seung Labs: Austin T Burke (3), Kyle Patrick Willie (1).
81	Fan shaped body: FBl1-2	720575940642476448	17		Murthy and Seung Labs: Jay Gager (1), James Hebditch (4), Kendrick Joules Vinson (1). Jefferis Lab: Varun Sane (6), Yijie Yin (3).
82	Fan shaped body: FB.FB.473	720575940619615936	7		Jefferis Lab: Imaan Tamimi (1), A. Javier (1), Laia Serratosa (3), Arti Yadav (1), Sangeeta Sisodiya (7).
83	Fan shaped body: DL1	720575940613052200	5	58	Jefferis Lab: Varun Sane (3), Griffin Badalemente (1). Murthy and Seung Labs: Shaina Mae Monungolh (1).
84	Gallinule	720575940617054621	21		Jefferis Lab: Anjali Pandey (1). Murthy and Seung Labs: Mendell Lopez (1), Joshua Bañez (2).
85	Gallinule	720575940630349905	18		Seeds Hampel Lab: Lucia Kmecova (1). Huetteroth Lab: Wolf Huetteroth (3). Jefferis Lab: Laia Serratosa (3).
86	Gallinule	720575940629913130	7		Murthy and Seung Labs: Zairene Lenizo (12).
87	Gallinule	720575940620228449	5		Jefferis Lab: Yijie Yin (1). Murthy and Seung Labs: remer tancontian (10), Zairene Lenizo (1). Itisha Joshi (4).
88	Gallinule	720575940633711028	5	56	Murthy and Seung Labs: Claire McKellar (6)
89	SEZ: PRW.PRW.213	720575940625172016	16		Jefferis and Waddell Labs: Joseph Hsu (14). Murthy and Seung Labs: Darrel Jay Akiatan (31), Doug Bland (23), Austin T Burke (1), Ryan Willie (3), remer tancontian (1).
90	SEZ: PRW.GNG.43	720575940630664556	8		Jefferis Lab: Marta Costa (1), Sangeeta Sisodiya (2). Seeds Hampel Lab: Alexis E Santana Cruz (2). Murthy and Seung Labs: Zairene Lenizo (3), Darrel Jay Akiatan (5), Ariel Dagohoy (1), Rey Adrian Candilada (2), Joshua Bañez (1), Nash Hadjerol (6), remer tancontian (1).

91	SEZ: PRW.PRW.312	720575940632055521	7		Murthy and Seung Labs: J. Dolorosa (3), Shirleyjoy Serona (103). Jefferis Lab: Arti Yadav (1).
92	SEZ: PRW.PRW.82	720575940616860758	6		Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Joshua Bañez (7), Zairene Lenizo (3), Doug Bland (12), J. Dolorosa (2), remer tancontian (1).
93	SEZ: PRW.PRW.164	720575940622457579	6	43	Jefferis Lab: Arti Yadav (1). Murthy and Seung Labs: Michelle Pantujan (2), Shirleyjoy Serona (3).
				1742	

Table S3: CCAP presynaptic partners

Name	Flywire ID	synapses from BiT2 (720575940621662332)	synapses from VESa1 (720575940632951597)	synapses from CCHa2R-RA (720575940621942021)	synapses from CCHa2R-RA (720575940627765903)	synapses from Cowboy (720575940611730674)	Total synapses	Tracing contributions (number of edits)
CCAP	7205759406461609 48	18	8	9	5	0	40	Jefferis Lab: Greg Jefferis (15), Zeba Vohra (17), A. Javier (14), Siqi Fang (9), Varun Sane (6), Dhara Kakadiya (4). Jefferis and Wilson: Laia Serratosa Capdevila (1). Murthy and Seung Labs: Michelle Pantujan (1), Shaina Mae Monungolh (1), Rey Adrian Candilada (2), regine salem (1), Nash Hadjerol (1), Joshua Bañez (1).
CCAP	7205759406211489 93	19	14	5	7	5	50	Murthy and Seung Labs: Austin T Burke (5), Rey Adrian Candilada (1), J. Anthony Ocho (8), Nash Hadjerol (26), Joshua Bañez (4), Ryan Willie (2). Jefferis Lab: A. Javier (28), Imaan Tamimi (1), Katharina Eichler (11), Mendell Lopez (80). Jefferis and Wilson Labs: Laia Serratosa Capdevila (2), Varun Sane (1). Janelia tracers: Tansy Yang (1).
Total synapses per cell type		37	22	14	12	5	90	

Table S4: Fly genotypes in figures

Figure	Short Genotype	Full Genotype
1A	nSyb	w1118 ; UAS-dcr2/+ ; UAS-nSynaptobrevin RNAi (attP2)/+
1A	nSyb	w1118 ; UAS-dcr2/+; UAS-nSynaptobrevin (attP2)/VT011155-Gal4 (attP2)
1A	TRH	w1118 ; UAS-dcr2/+ ; UAS-Trh RNAi (attP2)/+
1A	TRH	w1118 ; UAS-dcr2/+ ; UAS-Trh RNAi (attP2)/VT011155-Gal4 (attP2)
1A	ChAT	w1118 ; UAS-dcr2/+ ; UAS- ChAT RNAi (attP2)/+
1A	ChAT	w1118 ; UAS-dcr2/+ ; UAS- ChAT RNAi (attP2)/VT011155-Gal4 (attP2)
1A	TBH	w1118 ; UAS-dcr2/+ ; UAS-Tbh RNAi (attP2)/+
1A	TBH	w1118 ; UAS-dcr2/+ ; UAS-Tbh RNAi (attP2)/VT011155-Gal4 (attP2)

1A	HDC	w1118 ; UAS-dcr2/+ ; UAS-Hdc RNAi (attP2)/+
1A	HDC	w1118 ; UAS-dcr2/+ ; UAS-Hdc RNAi (attP2)/VT011155-Gal4 (attP2)
1A	VMAT	w1118 ; UAS-dcr2/+; UAS-VMAT RNAi (attP2)/+
1A	VMAT	w1118 ; UAS-dcr2/+ ; UAS-VMAT RNAi (attP2)/VT011155-Gal4 (attP2)
1A	GAD1	w1118 ; UAS-dcr2/+; UAS-GAD1 RNAi (attP2)/+
1A	GAD1	w1118 ; UAS-dcr2/+ ; UAS-GAD1 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	DDC	w1118 ; UAS-dcr2/+ ; UAS-DDC RNAi (attP2)/+
1A	DDC	w1118 ; UAS-dcr2/+ ; UAS-DDC RNAi (attP2)/VT011155-Gal4 (attP2)
1A	DVGlut	w1118 ; UAS-dcr2/+ ; UAS-DVGlut RNAi (attP2)/+
1A	DVGlut	w1118 ; UAS-dcr2/+ ; UAS-DVGlut RNAi (attP2)/VT011155-Gal4 (attP2)
1A	sNPF	w1118 ; UAS-dcr2/+ ; UAS-sNPF RNAi (attP2)/+
1A	sNPF	w1118 ; UAS-dcr2/+ ; UAS-sNPF RNAi (attP2)/VT011155-Gal4 (attP2)
1A	VGAT	w1118 ; UAS-dcr2/+ ; UAS-VGAT RNAi (attP2)/+
1A	VGAT	w1118 ; UAS-dcr2/+ ; UAS-VGAT RNAi (attP2)/VT011155-Gal4 (attP2)
1A	TDC2	w1118 ; UAS-dcr2/+ ; UAS-Tdc2 RNAi (attP2)/+
1A	TDC2	w1118 ; UAS-dcr2/+ ; UAS-Tdc2 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	dILP1	w1118 ; UAS-dcr2/+ ; UAS-dILP1 RNAi (attP2)/+
1A	dILP1	w1118 ; UAS-dcr2/+ ; UAS-dILP1 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	dILP2	w1118 ; UAS-dcr2/+ ; UAS-dILP2 RNAi (attP2)/+
1A	dILP2	w1118 ; UAS-dcr2/+ ; UAS-dILP2 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	dILP3	w1118 ; UAS-dcr2/+ ; UAS-dILP3 RNAi (attP2)/+
1A	dILP3	w1118 ; UAS-dcr2/+ ; UAS-dILP3 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	dILP4	w1118 ; UAS-dcr2/+ ; UAS-dILP4 RNAi (attP2)/+
1A	dILP4	w1118 ; UAS-dcr2/+ ; UAS-dILP4 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	dILP5	w1118 ; UAS-dcr2/+ ; UAS-dILP5 RNAi (attP2)/+
1A	dILP5	w1118 ; UAS-dcr2/+ ; UAS-dILP5 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	dILP6	w1118 ; UAS-dcr2/+ ; UAS-dILP6 RNAi (attP2)/+
1A	dILP6	w1118 ; UAS-dcr2/+ ; UAS-dILP6 RNAi (attP2)/VT011155-Gal4 (attP2)
1A	dILP7	w1118 ; UAS-dcr2/+ ; UAS-dILP7 RNAi (attP2)/+
1A	dILP7	w1118 ; UAS-dcr2/+ ; UAS-dILP7 RNAi (attP2)/VT011155-Gal4 (attP2)
1B	dILP3 RNAi	w1118 ; + ; UAS-dILP3 RNAi (attP2)/+
1B	dILP3 RNAi	w1118 ; + ; UAS-dILP3 RNAi (attP2)/VT011155-Gal4 (attP2)

1B	Amon RNAi	w1118/w* ; + ; UAS-amon RNAi (attP2)/+
1B	Amon RNAi	w1118/w* ; + ; UAS-amon RNAi (attP2)/VT011155-Gal4 (attP2)
1B	ISN-Gal4	w1118 ; + ; VT011155-Gal4 (attP2)/+
1D	ISN-Gal4 > tdT	w1118/w* ; UAS-myrGFP.QUAS-mtdTomato-3xHA/+; trans-Tango/VT011155-Gal4 (attP2)
2B,2F	BiT split-Gal4 > Chrimson	w1118, UAS-IVS-CsChrimson.mVenus (attP18)/+; VT002073-Gal4.AD (attP40)/+; VT040568-Gal4.DBD (attP2)/+
2D, 2E	ISN > Chrimson, BiT > ArcLight	13XLexAop2-IVS-p10-ChrimsonR-mCherry (attP18)/w1118; GMR34G02-LexA (attP40)/VT002073-Gal4.AD (attP40); VT040568-Gal4.DBD (attP2)/UAS-ArcLight (attP2)
2F, 2G	BiT split-Gal4	w1118; VT002073-Gal4.AD (attP40)/+; VT040568-Gal4.DBD (attP2)/+
2F	Empty split-Gal4 > Chrimson	w1118, UAS-IVS-CsChrimson.mVenus (attP18); p65-AD.empty (attp40)/+; GAL4-DBD.empty (attP2)/+
2F	Gr5a-Gal4 > Chrimson	w1118, UAS-IVS-CsChrimson.mVenus (attP18); Gr5a-Gal4/+; Gr5a-Gal4/+
2F	ppk28-Gal4 > Chrimson	w1118, UAS-IVS-CsChrimson.mVenus (attP18); + ; ppk28-Gal4/+
2G	BiT split-Gal4 > nSyb RNAi	w1118; VT002073-Gal4.AD (attP40)/+; VT040568-Gal4.DBD (attP2)/UAS-nSynaptobrevin RNAi (attP2)
2G, 4G, 5G	nSyb RNAi	w1118; +; UAS-nSynaptobrevin RNAi (attP2)/+
4B, 4F	CCHa2R (RA) > Chrimson	w1118, UAS-IVS-CsChrimson.mVenus (attP18)/w1118; TI{2A-GAL4}CCHa2-R[2A-A.GAL4]/+; +
4D, 4E	ISN > Chrimson, CCHa2R (RA) > GCaMP	w1118; GMR34G02-LexA (attP40)/ TI{2A-GAL4}CCHa2-R[2A-A.GAL4]; LexAop-Chrimson,UAS-GCaMP6s/TM2
4F, 4G	CCHa2R (RA)	w1118; TI{2A-GAL4}CCHa2-R[2A-A.GAL4]/+; +
4F, 5F	UAS-Chrimson	w1118, UAS-IVS-CsChrimson.mVenus (attP18)/w1118; +; +
4G	CCHa2R (RA) > nSyb RNAi	w1118; TI{2A-GAL4}CCHa2-R[2A-A.GAL4]/+; UAS-nSynaptobrevin RNAi (attP2)/+
5B, 5F	CCAP > Chrimson	w1118, UAS-IVS-CsChrimson.mVenus (attP18)/w1118; CCAP-Gal4/+; +
5D, 5E	ISN > Chrimson, CCAP > GCaMP	w1118, 13XLexAop2-IVS-p10-ChrimsonR-mCherry (attP18)/w1118; GMR34G02-LexA (attP40)/CCAP-Gal4; 20XUAS-IVS-jGCaMP7b(VK00005)/+
5F, 5G	CCAP	w1118; CCAP-Gal4/+; +
5G	CCAP > NSyb RNAi	w1118; CCAP-Gal4/+; UAS-nSynaptobrevin RNAi (attP2)/+
Supp 1A	ISN > transTango	w*/w1118 ; UAS-myrGFP.QUAS-mtdTomato-3xHA/+; trans-Tango/VT011155-Gal4 (attP2)
Supp 2A,B	BiT genetic control	w1118, 13XLexAop2-IVS-p10-ChrimsonR-mCherry (attP18)/w1118; VT002073-Gal4.AD (attP40)/+; VT040568-Gal4.DBD (attP2)/UAS-ArcLight (attP2)
Supp 3A,B	BiT > Chrimson, IPC > GCaMP	10XUAS-syn21-Chrimson88-tdT3.1 (attP18), LexAop2-syn21-opGCaMP6s suHw (attP8)/w1118; TI{2A-lexA::GAD}CCHa2-R[2A-A.lexA]/VT002073-Gal4.AD (attP40); VT040568-Gal4.DBD (attP2)/+

Supp 3C,D	IPC genetic control	10XUAS-syn21-Chrimson88-tdT3.1 (attP18), LexAop2-syn21-opGCaMP6s suHw (attP8)/w1118; dILP2-LexA/CyO; TM2/TM3, Sb
Supp 4A,B	CCHa2R (RA) genetic control	w1118; TI{2A-GAL4}CCHa2-R[2A-A.GAL4]/CyO; LexAop-Chrimson,UAS-GCaMP6s/TM2
Supp 4C,D	BiT > Chrimson, CCHa2R (RA) > GCaMP	10XUAS-syn21-Chrimson88-tdT3.1 (attP18), LexAop2-syn21-opGCaMP6s suHw (attP8)/w1118; TI{2A-lexA::GAD}CCHa2-R[2A-A.lexA]/VT002073-Gal4.AD (attP40); VT040568-Gal4.DBD (attP2)/+
Supp 5	ISN > Chrimson, CCAP > GCaMP	13XLexAop2-IVS-p10-ChrimsonR-mCherry (attP18)/w1118; GMR34G02-LexA (attP40)/+ ; 20XUAS-IVS-jGCaMP7b(VK00005)/CCAP-Gal4

Chapter 3: Characterization of neurons involved in ingestion

SUMMARY

All organisms need to consume external nutrients to sustain their metabolic needs. In particular, water and sugar ingestion are vital to animal survival. Ingestion of sugar and water are tightly regulated to maintain homeostasis by signals of internal nutrient levels such as hunger, thirst and satiety signals. Although generally considered separately, interactions between hunger and thirst signals are important to coordinate competing needs. *Drosophila melanogaster* have two pairs of neurons called Interoceptive Subesophageal zone Neurons (ISNs) located in the Subesophageal Zone (SEZ) that detect both hunger and thirst signals to oppositely regulate sucrose and water ingestion. To elucidate how the ISNs coordinate sugar and water ingestion, we performed anatomical and behavioral studies to identify neurons downstream of the ISNs. We identified 30 candidate ISN postsynaptic neurons, based on anatomical proximity, from a novel SEZ split-Gal4 collection that provides genetic access to uncharacterized neurons in the SEZ. We tested their role in ingestion and identified several neurons that regulate sugar and water ingestionn. One set of neurons, Aster, bidirectionally regulates sugar and water ingestion, similar to the ISNs. A second set of neurons, Horseshoe, decreases both sugar and water ingestion, and a third, Cowboy, likely promotes sugar ingestion. Activating the ISNs and measuring neural activity in these candidate neurons revealed that they were not downstream of the ISNs. However, we found that several cell types were downstream of gustatory sensory neurons including Aster, possibly Horseshoe, and another cell type Gallinule. Further study of these cell types will prove useful to reveal how they regulate sugar and/or water ingestion.

INTRODUCTION

The survival of an organism depends on its ability to coordinate external nutrient ingestion with internal nutrient abundance in order to meet its metabolic needs. The nervous system acts as an internal nutrient abundance sensor to drive ingestion in nutrient-deprived states and inhibit ingestion in nutrient-replete states. Interoceptive Subesophageal zone Neurons (ISNs) in *D. melanogaster* detect both hunger and thirst signals to oppositely regulate sucrose and water ingestion (Jourjine, Mullaney, et al., 2016). The ISNs are located in the Subesophageal Zone (SEZ), with neurites in the flange region of the SEZ. ISNs are activated by the endogenous signal of nutrient deprivation adipokinetic hormone (AKH) through the AKH receptor. ISNs sense water abundance through Nanchung, a Transient receptor potential channel which acts as molecular sensor of hemolymph osmolarity. Hunger, detected through the AKH receptor, activates the ISNs to promote sucrose ingestion. Thirst, detected through Nanchung, inhibits the ISNs to promote water ingestion. Thus, ISNs intrinsically detect hunger and thirst signals to regulate sucrose and water ingestion.

To elucidate how the ISNs coordinate sugar and water ingestion, we performed an anatomical screen to identify neurons downstream of the ISNs. We screened the recently released SEZ Split-Gal4 collection using a computational approach based on neurite proximity (Sterne et al., 2021, Otsuna et al., 2018). The SEZ Split-Gal4 collection provides precise genetic access to over 100 uncharacterized cell types in the

SEZ (Sterne et al., 2021). We identified 30 candidate ISN postsynaptic neurons from this collection that had neurites in close proximity to the ISNs. Behavioral tests revealed several neurons that are involved in sugar and water ingestion. *In vivo* functional imaging revealed that these neurons are not downstream of the ISNs, but some are downstream of sensory neurons. Further research characterizing these neurons and their neural circuit will help us understand how sugar and water ingestion is coordinated and how sensory input is integrated to drive feeding.

RESULTS

Aster coordinates sugar and water ingestion

In an effort to identify neurons downstream of the ISNs, we used a computational approach based on neurite proximity to identify candidate postsynaptic partners of the ISNs. We compared images of the ISNs with images of over 100 cell types in a novel SEZ split-Gal4 collection, that allows genetic access to single cell types in the SEZ (Sterne et al., 2021). We identified 30 neurons from the SEZ split-Gal4 collection that had neurites that overlapped with ISN neurites.

ISN activation leads to increased sugar ingestion and decreased water ingestion. Therefore, we predicted that neurons downstream of the ISNs should also modulate sugar or water ingestion. To determine if the candidate ISN postsynaptic neurons were involved in sugar or water ingestion, we measured total ingestion time of sugar or water while activating the candidate neurons (Fig 1). We expressed the light activated cation channel Chrimson in the candidate neurons and acutely activated the neurons using a laser (Klapoetke et al., 2014). We found that optogenetic activation of SEZ-125 decreased sugar ingestion, SEZ-117, SEZ 177, SEZ-184, SEZ-435 increased sugar ingestion, SEZ-122 increased water ingestion, and SEZ-154, SEZ-297, SEZ-324, SEZ-416, SEZ-446 decreased water ingestion. Interestingly, we found that activation of SEZ-90 decreased both water and sugar ingestion, and SEZ-408 increased sugar ingestion, and decreased water ingestion. The latter is the same ingestion phenotype observed when the ISNs are activated (Jourjine, Mullaney, et al., 2016). SEZ-408 labels a neuron class called Aster (Fig 2), two pairs of neurons that innervate the flange region of the SEZ (Sterne et al., 2021). Double labeling of the ISNs and Aster suggest that neurites of both neurons overlap (Fig 2C). Segmenting the images to more clearly visualize the neurites further suggests that Aster and ISNs are synaptic partners (Fig 2D).

We performed preliminary tests to determine if Aster is in close proximity to the ISNs *in vivo* using GFP Reconstitution Across Synaptic Partners (GRASP) (Fig 2E-G). In this method, two complementary fragments of GFP are expressed on the plasma membrane of different cells, resulting in GFP fluorescence along membrane contacts between the two cells (Feinberg et al., 2008). We preliminarily found that the background subtracted mean fluorescence in the flange region of the SEZ, where the Aster and ISN neurites are located, was 0.487 and in the background region of interest it was 0.243. While we did not collect enough data points to measure significance, the increased GRASP signal at the flange region of the SEZ suggests that ISNs and Aster come in contact and might be synaptically connected.

To determine if the ISNs are presynaptic to Aster, we conducted *in vivo* functional imaging experiments in which we activated the ISNs while simultaneously monitoring Aster's neural activity (Fig 3A). We expressed Chrimson in the ISNs and the Calcium sensor GCaMP6f in Aster (Chen et al., 2013). We applied three consecutive 2s stimulations to test whether the response was reproducible. We found that the ISNs do not stimulate Aster (Fig 3B, C), and are likely not upstream of Aster. However, because we found that ISNs silence postsynaptic neuron BiT, it is possible that the ISNs also inhibit Aster. Therefore, it is possible that we did not capture an inhibitory response using a calcium sensor.

It is possible that candidates are involved in the sensorimotor feeding circuit and receive input from gustatory neurons. To determine if candidates receive gustatory information, we conducted *in vivo* functional imaging experiments in which we activated sugar sensory neurons Gr5a (Fig 3D) or water sensory neurons ppk28 (Fig 3G) while simultaneously monitoring Aster's neural activity. We found that optogenetic activation of sugar sensory neurons decreased activity in Aster, but this response was not reproducible across simulations (Fig 3E, F). Optogenetic activation of water sensory neurons also decreased activity in Aster neurons. This response was reproduced in two out of three stimulations (Fig 3H, I). It is possible that a longer interstimulus period would allow for calcium concentrations to return to baseline, potentially necessary to detect consecutive responses in Aster. Regardless, these studies argue that sugar and water sensory neurons silence Aster neurons.

Cowboy and Gallinule neurons receive sensory input and regulate feeding

We next investigated if SEZ-90, which decreased sugar and water ingestion (Fig 1) was downstream of the ISNs. SEZ-90 labels Cowboy and Horseshoe neurons (Fig 4A). Interestingly, SEZ-324, which labels only Horseshoe neurons (Fig 4C) also decreases water ingestion, and shows a trend for decreased sugar ingestion, although it is not significant (Fig 1). Meanwhile, Cowboy neurons, in addition to Gallinule neurons, are also labeled by SEZ-177 (Fig 4B), which increased sugar ingestion, and had no effect on water ingestion (Fig 1). Gallinule neurons are also labeled by SEZ-113, which did not alter sugar or water ingestion (Fig 1), suggesting that the sucrose phenotype driven by SEZ-177 is due to Cowboy and not Gallinule neurons.

To elucidate the connectivity between the ISNs and Horseshoe and Cowboy neurons, we conducted *in vivo* functional imaging experiments in which we optogenetically activated the ISNs while simultaneously monitoring Horseshoe and Cowboy neural activity, using SEZ-90, SEZ-324, and SEZ-177 (Fig 5). We applied three consecutive 2s LED stimulations to test whether the response was reproducible. We found that ISN stimulation did not alter neural activity of any of the neurons labeled in SEZ-90, SEZ-324, and SEZ-177.

Since we found that these neurons are involved in ingestion, we tested whether these neurons are involved in the sensorimotor feeding circuit. We conducted *in vivo* functional imaging experiments in which we activated sugar sensory neurons Gr5a (Fig

6) while simultaneously monitoring Horseshoe and Cowboy's neural activity. We found that SEZ-90, which labels Horseshoe and Cowboy, and SEZ-324, which only labels Horseshoe, did not respond to Gr5a stimulation (Fig 6A-F).

Since activation of SEZ-177 caused an increase in sugar ingestion, we tested if this line responds to Gr5a stimulation. We found that optogenetic stimulation of Gr5a increased neural activity in SEZ-177 (Fig 6G-I). SEZ-177 labels Cowboy and gallinule neurons, both of which have neurites in the SEZ, the region of the brain we were imaging. To determine if Cowboy or Gallinule responded to sugar sensory neurons, we performed functional imaging experiments in a line that only labels Gallinule neurons, SEZ-178. Optogenetic stimulation of sugar sensory neurons increased neural activity in Gallinule neurons (Fig 6J-L). This suggests that Gallinule and not Cowboy neurons responded to sensory activation in SEZ-177. Interestingly, activation of Gallinule neurons did not alter sugar ingestion (Fig 1), suggesting that although these neurons respond to sugar taste, they are not sufficient to elicit sugar feeding.

To determine if these neurons receive information from water sensory neurons, we conducted *in vivo* functional imaging experiments in which we activated water sensory neurons ppk28 (Fig 7) while simultaneously monitoring Horseshoe and Cowboy's neural activity. We found that SEZ-90, which labels Horseshoe and Cowboy, did not respond to ppk28 optogenetic stimulation (Fig 7A-C). However, SEZ-324, which only labels Horseshoe, seemed to be activated upon the first trial (Fig 7D-F). These conflicting data might be due to the differences in expression patterns of these two drivers. SEZ-90 also contains Cowboy neurites, which might not respond to water sensory neurons, and therefore, any neural activity in Horseshoe might be hard to detect.

Since SEZ-177 responded to sugar sensory neurons, we tested if this line is also activated by water sensory neurons. We found that optogenetic stimulation of ppk28 water sensory neurons increased neural activity in SEZ-177 (Fig 7G-I). To determine if Cowboy or Gallinule responded to water sensory neurons, we performed functional imaging experiments in a line that only labels Gallinule neurons, SEZ-178. Optogenetic stimulation of water sensory neurons increased neural activity in Gallinule neurons (Fig 7J-L). This suggests that Gallinule and not Cowboy neurons responded to water sensory activation in SEZ-177. Interestingly, activation of Gallinule neurons did not alter water ingestion (Fig 1), suggesting that although these neurons respond to water taste, they are not sufficient to elicit water ingestion. Thus, Gallinule neurons are activated by both sugar and water sensory neurons.

DISCUSSION

Aster

We found that Aster neurons, innervating the SEZ, coordinate sugar and water ingestion. When activated, flies increase sugar ingestion and decrease water ingestion. It will be interesting to perform silencing experiments in the future to determine whether silencing leads to a decrease in sugar ingestion and an increase in water ingestion, similar to ISN silencing phenotypes. We found that these neurons are likely inhibited by

sugar and water sensory neuron activation. Previous studies showed that ISNs do not receive sensory information, therefore Aster neurons are uniquely situated to integrate sensory information. As Aster innervates the same brain region as the ISNs, it will also be interesting to explore if Aster similarly integrates interoceptive signals in addition to sensory signals to coordinate sugar and water ingestion.

Horseshoe neurons
We found that Horseshoe neurons, located in the SEZ, decrease ingestion of both sugar and water ingestion. These neurons do not receive interoceptive information from the ISNs. These neurons do not receive sugar sensory information, however whether they receive water sensory information is inconclusive. It is possible that these neurons are bitter sensing neurons that function to inhibit feeding (French et al., 2015). However, further experiments are needed to test this hypothesis.

Cowboy neurons
We found that Cowboy neurons, which contain neurites in the SEZ and superior medial protocerebrum (SMP), likely drive an increase in sugar feeding. Cowboy neurons likely do not respond to sugar or water sensory neurons. Creating a split-Gal4 line that only labels Cowboy neurons might prove very useful in elucidating Cowboy's role in feeding.

Gallinule neurons
We found that Gallinule neurons, which contain neurites in the SEZ and superior medial protocerebrum (SMP), are activated by sugar and water sensory neurons. However, activation of Gallinule neurons is not sufficient to elicit an ingestion phenotype for either water or sugar. Gallinule neurons have been found to synapse onto dopaminergic neurons that synapse onto the mushroom body, the learning and memory center of the fly brain (Aso et al., 2014, Li et al., 2020). Gallinule neurons therefore might function to convey taste information to memory centers.

With the availability of the connectome, it would be useful to identify the synaptic inputs and outputs of Aster, Horseshoe, Cowboy, and Gallinule neurons in the connectome. While we have identified Aster, Cowboy, and Gallinule neurons in the connectome we have not performed a comprehensive characterization of their synaptic input and outputs. We have yet to identify Horseshoe in the connectome. By identifying and characterizing pre- and postsynaptic neurons we might get a better understanding of how these neurons are involved in sugar and water ingestion. It will also be interesting to see if any of these neurons are connected to the same neurons we have identified in the ISN circuit.

MATERIALS AND METHODS

Key resources table		
Drosophila strains	**Source or Reference**	**Identifier**
SEZ-44	Janelia FlyLight SEZ Split-Gal4 collection	SS29437

SEZ-89	Janelia FlyLight SEZ Split-Gal4 collection	SS31063
SEZ-90	Janelia FlyLight SEZ Split-Gal4 collection	SS31067
SEZ-94	Janelia FlyLight SEZ Split-Gal4 collection	SS31341
SEZ-113	Janelia FlyLight SEZ Split-Gal4 collection	SS32120
SEZ-117	Janelia FlyLight SEZ Split-Gal4 collection	SS32394
SEZ-122	Janelia FlyLight SEZ Split-Gal4 collection	SS32423
SEZ-125	Janelia FlyLight SEZ Split-Gal4 collection	SS47275
SEZ-132	Janelia FlyLight SEZ Split-Gal4 collection	SS31386
SEZ-143	Janelia FlyLight SEZ Split-Gal4 collection	SS32736
SEZ-154	Janelia FlyLight SEZ Split-Gal4 collection	SS32091
SEZ-162	Janelia FlyLight SEZ Split-Gal4 collection	SS32066
SEZ-177	Janelia FlyLight SEZ Split-Gal4 collection	IS34088
SEZ178	Janelia FlyLight SEZ Split-Gal4 collection	SS34089
SEZ-182	Janelia FlyLight SEZ Split-Gal4 collection	SS34732
SEZ-184	Janelia FlyLight SEZ Split-Gal4 collection	SS34750
SEZ-232	Janelia FlyLight SEZ Split-Gal4 collection	SS37818
SEZ-262	Janelia FlyLight SEZ Split-Gal4 collection	SS39040
SEZ-297	Janelia FlyLight SEZ Split-Gal4 collection	SS39890
SEZ-324	Janelia FlyLight SEZ Split-Gal4 collection	SS41397
SEZ-349	Janelia FlyLight SEZ Split-Gal4 collection	SS42603
SEZ-350	Janelia FlyLight SEZ Split-Gal4 collection	SS42606
SEZ-363	Janelia FlyLight SEZ Split-Gal4 collection	SS43335
SEZ-396	Janelia FlyLight SEZ Split-Gal4 collection	SS44899
SEZ-403	Janelia FlyLight SEZ Split-Gal4 collection	SS44937
SEZ-408	Janelia FlyLight SEZ Split-Gal4 collection	SS45680
SEZ-416	Janelia FlyLight SEZ Split-Gal4 collection	SS45730
SEZ-426	Janelia FlyLight SEZ Split-Gal4 collection	SS45898
SEZ-435	Janelia FlyLight SEZ Split-Gal4 collection	SS45915
SEZ-446	Janelia FlyLight SEZ Split-Gal4 collection	SS45940
Empty split	Bloomington Drosophila Stock Center	BDSC 79603
ISN-LexA (GMR34G02-LexA)	Bloomington Drosophila Stock Center	BDSC 54138
ppk28-LexA	Cameron et al., 2010.	

Gr5a-LexA	Gordon & Scott, 2009.	
UAS-csChrimson.mVenus	Bloomington Drosophila Stock Center	BDSC 55134
UAS-GCaMP6f	Bloomington Drosophila Stock Center	BDSC 52869
20XUAS-GCaMP7b	Bloomington Drosophila Stock Center	BDSC 79029
LexAop-ChrimsonR.mCherry	Gift from Jayaraman Lab	
LexAop-CsChrimson.tdTomato	Bloomington Drosophila Stock Center	BDSC 82183
GRASP	Bloomington Drosophila Stock Center	BDSC 64314

Fly husbandry

All experiments and screening were carried out with adult *D. melanogaster* females reared on standard cornmeal-agar-molasses medium, at 25°C, 65-70% humidity, on a 12 hr light: 12 hr dark cycle. Flies used in optogenetic assays were reared on food containing 0.25mM all-trans-retinal (Sigma-AldrichSigna-Aldrich) in darkness, before and after eclosion.

Temporal consumption assay (TCA)

Flies were anesthetized using CO_2 and then fixed to a glass slide with nail polish. Flies recovered for 2 hours in a humidified box, if testing for sucrose ingestion, or in a desiccated box with Drierite, if testing for water ingestion. Immediately before testing for sucrose ingestion, flies were given water until they no longer responded to 3 consecutive presentations. In testing, flies were presented with the tastant (water or 1M sucrose) 10 times and consumption time was manually recorded.

***In vivo* calcium imaging**

Calcium imaging studies were carried out as described in Shiu, Sterne et al. (2022). Mated female flies were dissected for calcium imaging studies 5-14 days post-eclosion. Flies were briefly anesthetized with ice and placed in a custom plastic holder at the neck to isolate the head from the rest of the body. The head was then immobilized using UV glue, the proboscis was immobilized using wax, and the esophagus was cut to provide unobstructed imaging access to the SEZ. All flies imaged were sated. *In vivo* calcium imaging with optogenetic activation was performed in a 2-photon microscope using a Scientifica Hyperscope with resonant scanning, a piezo drive, and a 20x water immersion objective (NA = 1.0) with 1.8-3x digital zoom, depending on the cell type imaged. Calcium responses were recorded with a 920 nm laser and optogenetic stimulation was achieved with a 660 nm LED. 2s LED stimulation paradigm: 20s off, 2s on, 30s off, 2s on, 30s off. 30s LED stimulation paradigm: 20s off, (1s on, 1s off) x 15, 60s off. For the 2s LED stimulation, 80 stacks of 20 z slices of 4-5 µm were acquired at 0.667 Hz. For the 30s stimulation, 125 stacks of 20 z slices of 4-5 µm were acquired at 0.667 Hz. Analysis was done on max-z projections of the 20 z slices. $\%\Delta F/F = 100*((Ft-F0)/F0)$, where Ft is the fluorescence of the Neuron ROI - the Background ROI at each timepoint and F0 is the mean Ft for the 23 time points prior to stimulus onset. Quantification was carried out in GraphPad Prism. A mean fluorescence intensity for LED off and LED on was calculated for each fly. For the 2s LED stimulation, mean

intensity for LED off was calculated for 5 timepoints immediately before LED exposure and mean intensity for LED on was calculated for 5 timepoints during LED exposure. For the 30s stimulation, mean intensity for LED off was calculated for 28 timepoints immediately before LED exposure and mean intensity for LED on was calculated for 28 timepoints during LED exposure. Paired t-test or paired Wilcoxon test was performed.

Immunohistochemistry

All brain and CNS dissections and immunostaining (unless directly addressed) were carried out as described (https://www.janelia.org/project-team/flylight/protocols, 'IHC-Anti-GFP') substituting the below antibodies and eschewing the pre-embedding fixation steps. Ethanol dehydration and DPX mounting was carried out as described (https://www.janelia.org/project-team/flylight/protocols, 'DPX Mounting').

Primary antibodies:

- mouse α-Brp (nc82, DSHB, University of Iowa, USA) at 1:40
- chicken α-GFP (Invitrogen, A10262) at 1:1000
- rabbit α-dsRed (Takara, Living Colors 632496) at 1:1000

Secondary antibodies:

- goat α-mouse AF647 (Invitrogen, A21236) at 1:500
- goat α-chicken AF488 (Life Technologies, A11039) at 1:1000
- goat α-rabbit AF568 (Invitrogen, A21236) at 1:1000

Images were acquired with a Zeiss LSM 880 NLO AxioExaminer with Airyscan and Coherent Chameleon Vision or Zeiss LSM 780 Laser Scanning Confocal Microscope at the Berkeley Molecular Imaging Center with a Plan-Apochromat 20x/1.0 W, 40x W, 40x/1.4 oil, or 63x/1.4 oil objective. Images were prepared in Fiji.

GRASP measurement

GRASP fluorescence intensity = GRASP mean fluorescence/nc82 mean fluorescence. Neurites ROI was drawn around the flange region of the SEZ, where the ISN and Aster neurites are located. Background ROI was drawn around and SEZ region that did not include the flange.

Statistical analysis

Statistical tests were performed in GraphPad Prism. For all group comparisons, data was first tested for normality with the KS normality test (alpha = 0.05). If all groups passed then groups were compared with a parametric test, but if at least one group did not pass, groups were compared with a non-parametric version. All statistical tests, significance levels, and number of data points (N) are specified in the figure legend.

AUTHOR CONTRIBUTIONS

A.G.S. and K.S. conceived and designed the study. A.G.S performed the experiments, data analysis, and wrote the manuscript. K.S. supervised the study.

FIGURES

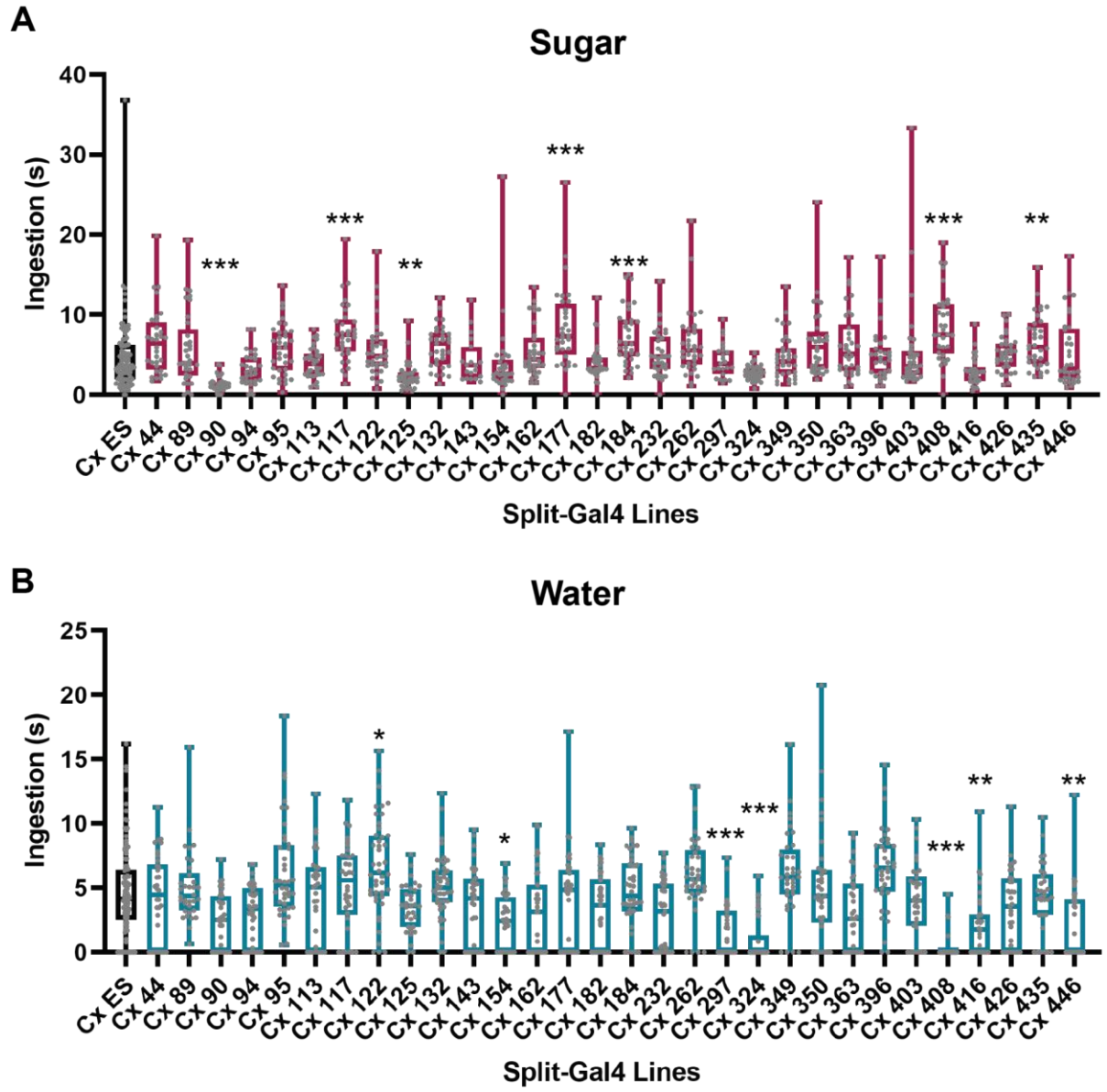


Figure 1. Behavioral screen of SEZ-Split Gal4 candidate ISN postsynaptic neurons

Temporal consumption assay screen for sugar (A) and water (B) ingestion. Represented are the mean, and the 10-90 percentile; data was analyzed using Kruskal-Wallis, followed by multiple comparisons against the Chrimson Empty split-Gal4 control (Cx ES) and corrected with Dunn's multiple comparison test to adjust p-value for multiple comparisons. n=19-44 animals/genotype, except Cx ES control, n=100-111. *p<0.05, **p<0.01,***p<0.001.

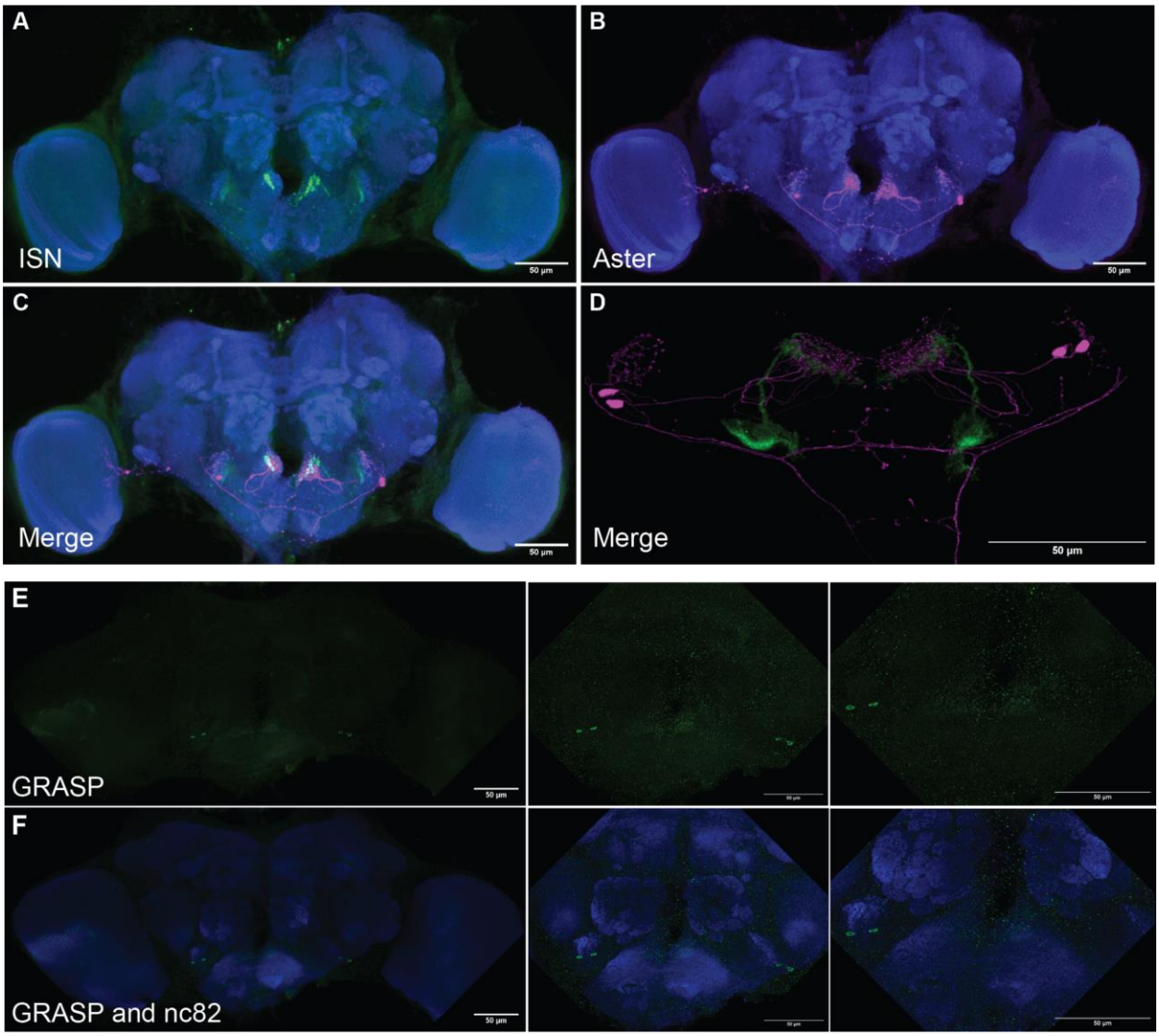


Figure 2. Aster neurites are close to ISN neurites *in vivo*
Immunohistochemistry labeling ISNs (A), Aster (B), and merge of both (C, D). magenta=RFP, green=GFP, blue=nc82 staining.
GRASP signal between ISNs and Aster (E), and merge of GRASP with background staining (F). green=GRASP, blue=nc82 neuropil staining.

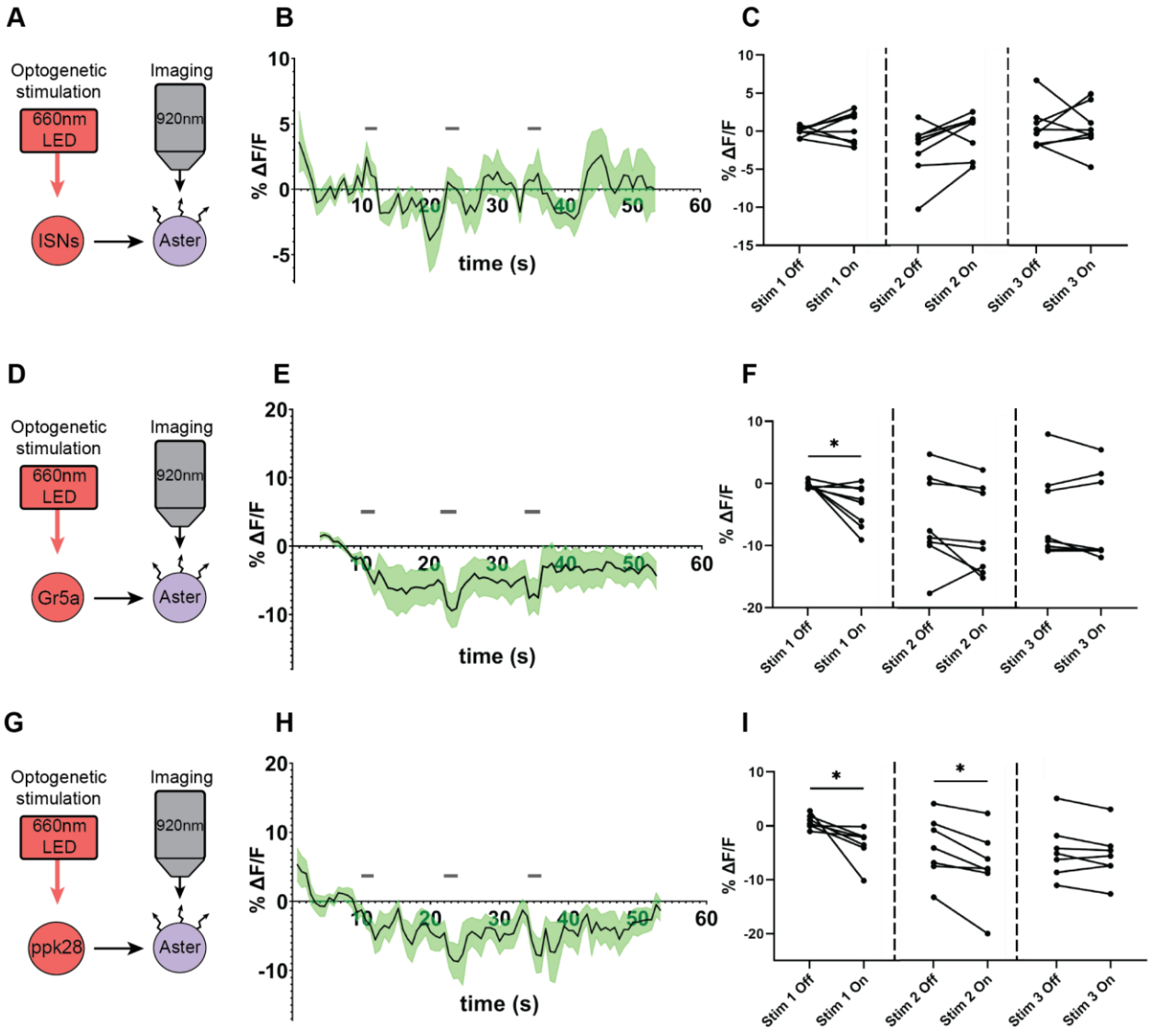


Figure 3. Aster *in vivo* functional imaging with ISNs and sensory neurons
(A, D, G) Experimental setup for *in vivo* calcium imaging. We expressed the light sensitive ion channel Chrimson in the ISNs (A), Gr5a neurons (D) or ppk28 neurons (G) and optogenetically stimulated them with 660nm LED. We expressed the fluorescent calcium sensor GCaMP in Aster and imaged it with a 2-photon microscope.
(B, E, I) GCaMP response of Aster neurites to three consecutive 2s optogenetic stimulation of the ISNs (B), Gr5a neurons (E) or ppk28 neurons (H). Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation.
(C) Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired Wilcoxon test. n=7-8 flies. *p<0.05

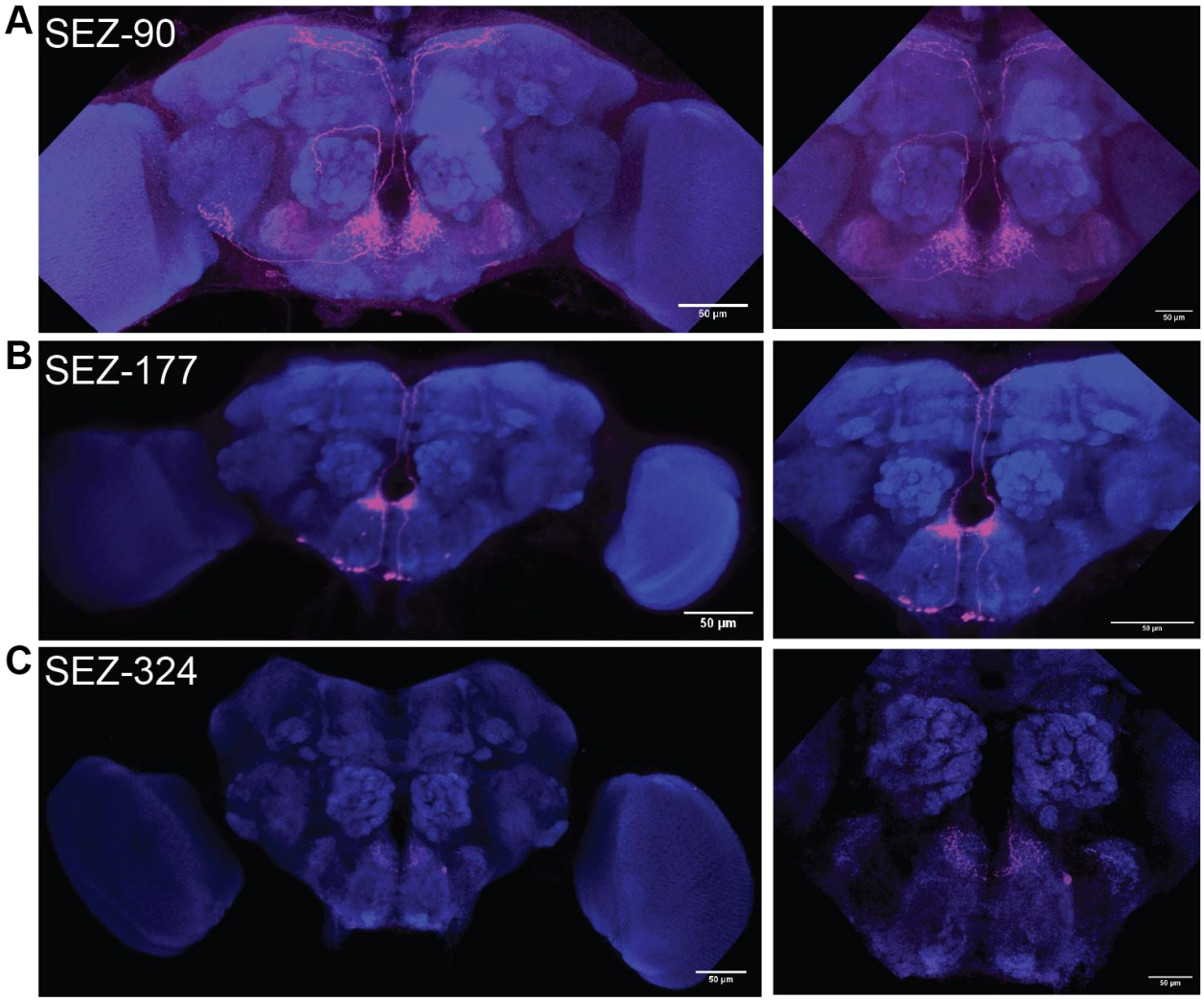


Figure 4. Morphology of Horseshoe, Cowboy and Gallinule neurons
Immunohistochemistry labeling SEZ-90, which contains Horseshoe and Cowboy neurons (A), SEZ-177 which contains Cowboy and Gallinule neurons (B), and SEZ-324 which contains Horseshoe neurons (C). Left imaged with 20X objective and right imaged with 40X objective. magenta=RFP, blue=nc82 neuropil staining.

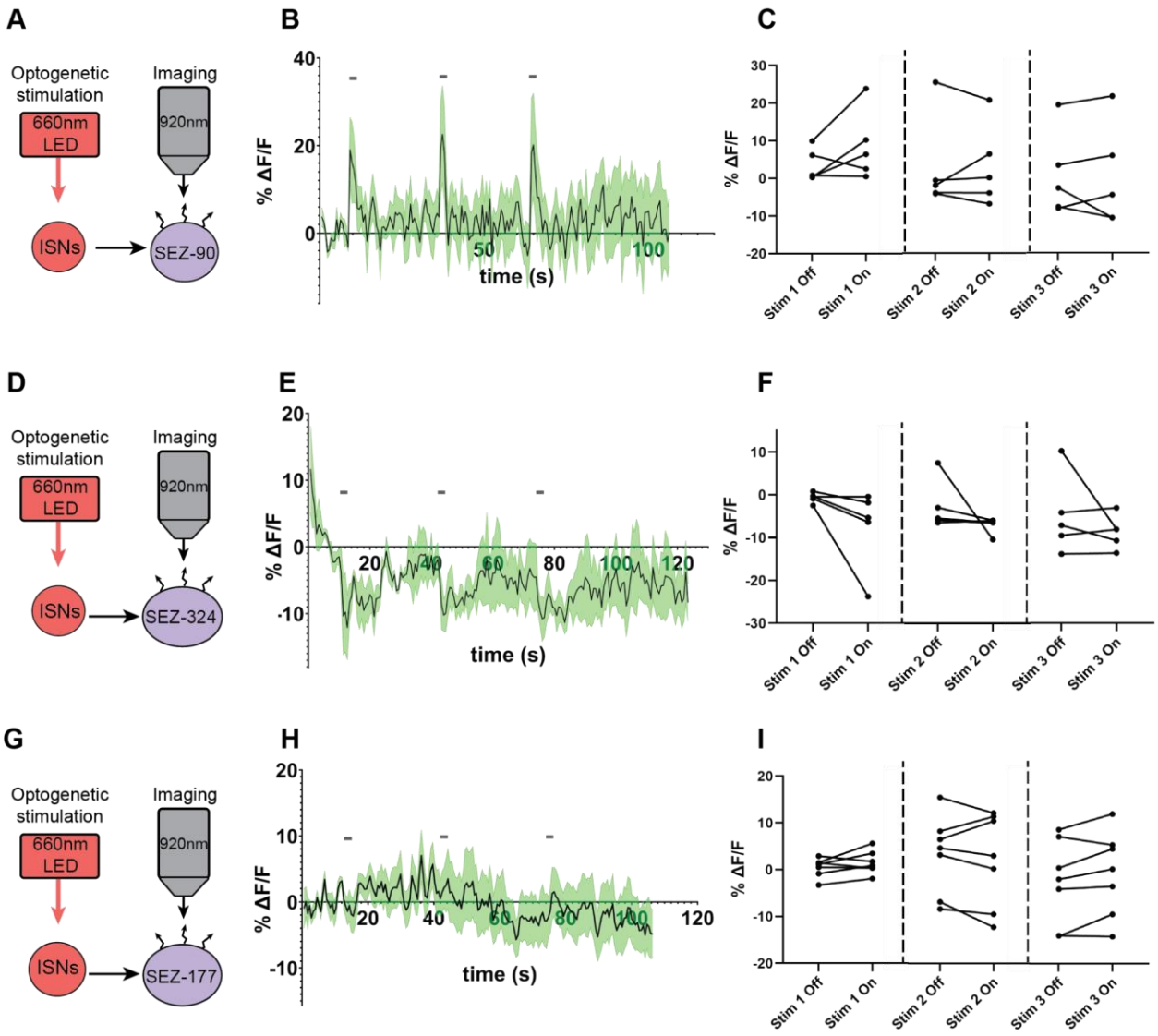


Figure 5. Horseshoe, Cowboy and Gallinule *in vivo* functional imaging with ISNs
(A, D, G) Experimental setup for *in vivo* calcium imaging. We expressed the light sensitive ion channel Chrimson in the ISNs and optogenetically stimulated them with 660nm LED. We expressed the fluorescent calcium sensor GCaMP in SEZ-90 (A), SEZ- 324 (D), or SEZ-177 (G) and imaged them with a 2-photon microscope.
(B, E, H) GCaMP response of SEZ-90 (B), SEZ-324 (E) or SEZ 177 (H) neurites to three consecutive 2s optogenetic stimulation of the ISNs. Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation.
(C, F, I) Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired Wilcoxon test. n=5-7 flies.

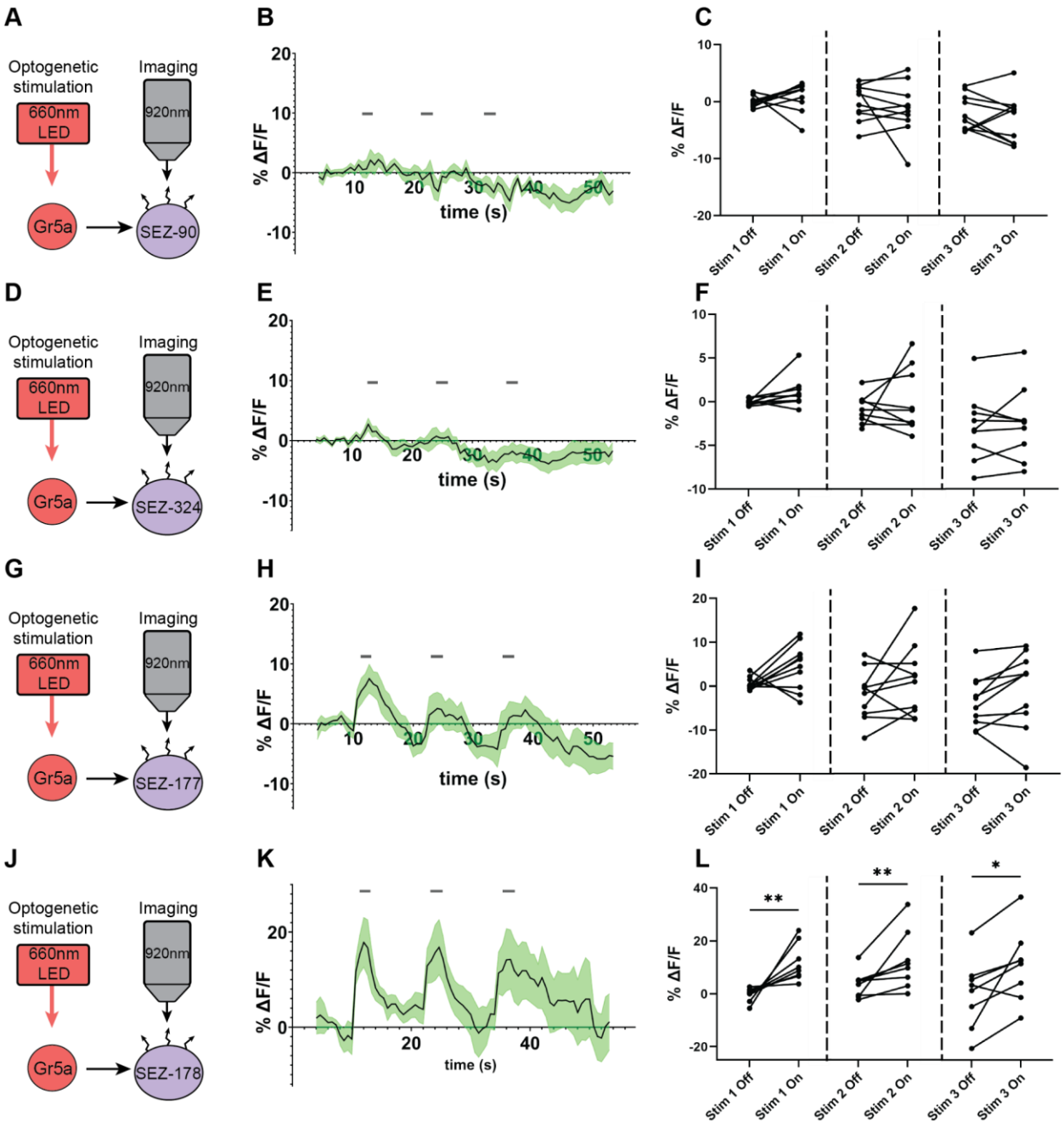


Figure 6. *in vivo* functional imaging with sugar sensory neuron activation
(A, D, G, J) Experimental setup for *in vivo* calcium imaging. We expressed the light sensitive ion channel Chrimson in the Gr5a neurons and optogenetically stimulated them with 660nm LED. We expressed the fluorescent calcium sensor GCaMP in SEZ-90 (A), SEZ- 324 (D), SEZ-177 (G), or SEZ-178 (J) and imaged them with a 2-photon microscope. (B, E, H, K) GCaMP response of SEZ-90 (B), SEZ-324 (E), SEZ 177 (H) or SEZ-178 (K) neurites to three consecutive 2s optogenetic stimulation of the Gr5a neurons. Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. (C, F, I, L) Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired Wilcoxon test. n=8-10 flies. *p<0.05, **p<0.01.

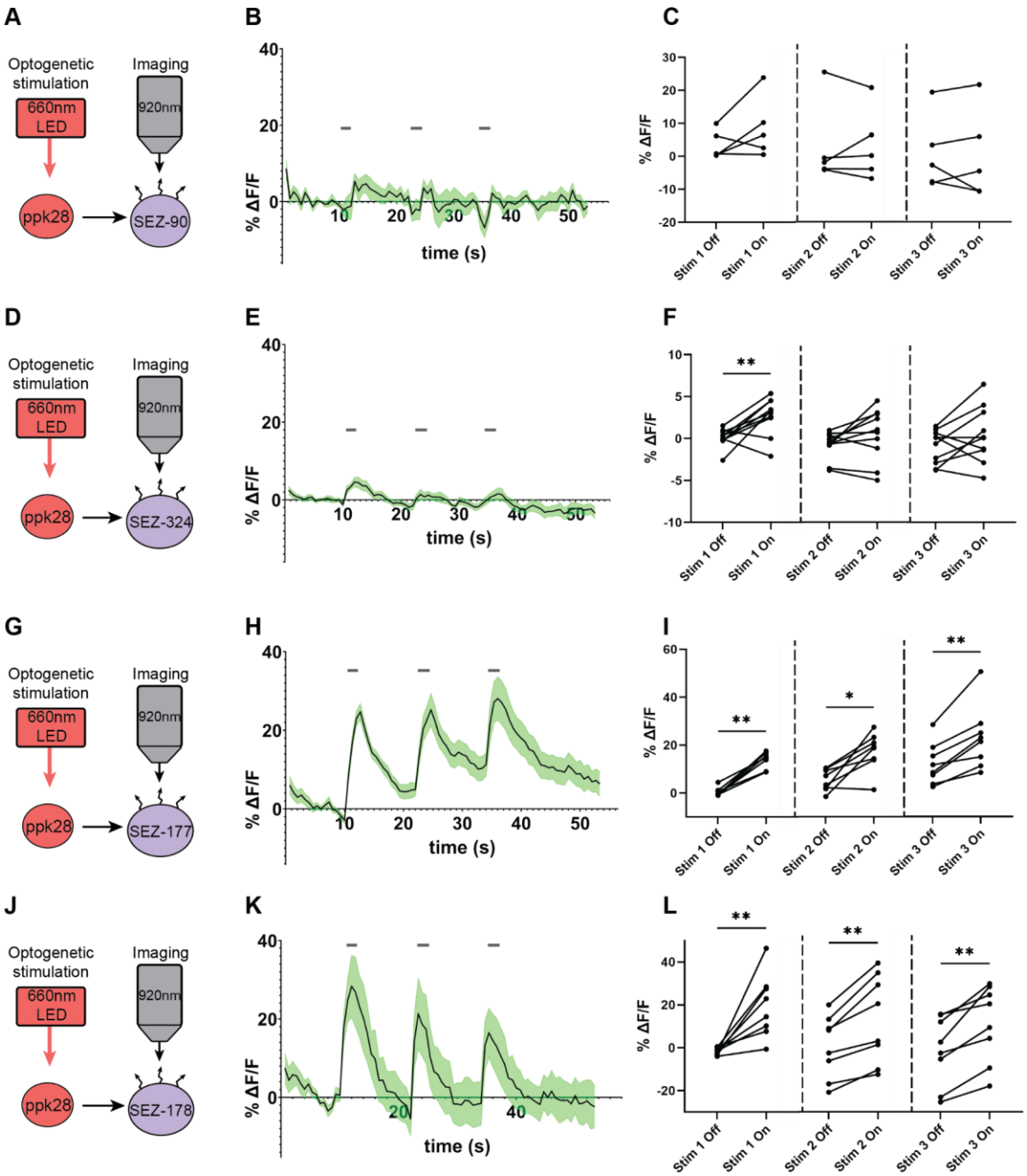


Figure 7. *in vivo* functional imaging with water sensory neuron activation
(A, D, G, J) Experimental setup for *in vivo* calcium imaging. We expressed the light sensitive ion channel Chrimson in the ppk28 neurons and optogenetically stimulated them with 660nm LED. We expressed the fluorescent calcium sensor GCaMP in SEZ-90 (A), SEZ- 324 (D), SEZ-177 (G), or SEZ-178 (J) and imaged them with a 2-photon microscope. (B, E, H, K) GCaMP response of SEZ-90 (B), SEZ-324 (E), SEZ 177 (H) or SEZ-178 (K) neurites to three consecutive 2s optogenetic stimulation of the ppk28 neurons. Scatter plot shows mean +/- SEM of all flies imaged, gray bars represent LED stimulation. (C, F, I, L) Quantification of mean fluorescence intensity before stim (off) and during stim (on), each dot represents one fly. Paired Wilcoxon test. n=7-10 flies. *p<0.05, **p<0.01.

Chapter 4: Design, implementation, and evaluation of a qualifying exam preparation program for STEM PhD programs

SUMMARY

The qualifying exam (QE), also known as the preliminary exam, is one of the two major evaluations in graduate programs. It is usually administered in the second or third year of the program and is meant to evaluate if a student is prepared to undertake an individual thesis project. Preparation for the qualifying exam is often daunting, as many programs do not have structured guidelines and it is the the first evaluation of its kind that many students encounter. We developed and implemented a pilot for the Inclusive Excellence in Quals Prep (IEQP) program, which was designed to provide mentorship, community, and academic support for students from diverse backgrounds as they prepared for their QE. The program focused on three STEM PhD programs at the University of California, Berkeley, which have similar QE formats. The main components for IEQP included pairing students with graduate student mentors, academic and wellness workshops, and community building events. Here, we evaluated the program's effect on the pilot cohort of 11 students. The most beneficial component of the program, as per student feedback, was peer mentorship. Overall, we saw an increase in students' perception of their preparedness, QE-related skills, the support received from their advisors, and the agency they felt over their proposed work. We conclude that the pilot program was successful as it increased the structure around QE prep and provided students with mentors in their field, and we recommend broad implementation of similar programs to enhance equity in graduate education.

INTRODUCTION

Scholars from certain ethnic and gender groups are severely underrepresented in STEM fields. In comparison to the U.S. population, Women, Black, Hispanic, and American Indian/Alaska Native scientists are underrepresented in STEM. Racially minoritized groups comprise 16.9% of doctoral degree recipients in STEM (US Census Bureau, 2019). Multiple factors contribute to the lack of diversity in academia, with retention of students from minoritized groups being a crucial, often overlooked, component (Liu, et al., 2019, Turner, 2002). There are many stages at which retention can be affected, especially around major evaluations or program requirements. There are two major evaluation components in PhD programs: the qualifying exam (QE) or preliminary exam early in the program, and the dissertation defense at the end of the program. Here, we identified a way to increase retention of students from minoritized groups in STEM by creating a program that addresses QE preparation.

The goal of a QE is to demonstrate that the student has knowledge in their field of study and is prepared to undertake independent research. Once a student passes their QE, they advance to candidacy, after which they carry out thesis research until their committee deems they are ready to defend their dissertation and obtain the PhD (Fisher et al., 2019, Manus et al., 1992). A student usually has 1-3 attempts to pass their QE; if they do not pass, then they are dismissed from the doctoral program.

The QE can vary greatly depending on the graduate institution, and even within the same institution, as different departments may have different QE formats. For example,

some QEs are a 2-3 hr oral examination, which may also include a written component. The committee can examine the student's knowledge on their proposed research, topics related to their field of study, and more general topics not related to their field of study (UC Davis Graduate Studies). Meanwhile, some QEs are a written examination spanning several days, where the student answers questions provided by their committee. These questions can cover general topics to topics in the student's specialization (Graduate Support Office, University of South Florida).

In the Helen Wills Neuroscience Institute (HWNI), Molecular and Cell Biology (MCB), and Plant and Microbial Biology (PMB) programs at UC Berkeley, the QE is administered by a committee of four professors. The exam consists of writing and orally defending a thesis proposal and presenting a separate breadth requirement: answering general questions about Neuroscience in HWNI, answering questions about major papers in the field in MCB, and writing a proposal on another topic not related to your research in PMB.

While the QE is a crucial point in the scientific development of a graduate student, it can be very difficult to navigate for most students and can be particularly isolating for students from minoritized backgrounds (Lopez, 2022). Further, students receive varying levels of support from their graduate programs and advisors, which was the case for students who participated in this program. Because, for most students, the QE is drastically different from exams they experienced during their undergraduate studies, and for many students it is the first time writing a proposal of the required caliber, students are often left not knowing how to best prepare for their QE. Due to a lack of courses in most graduate programs dedicated to developing the skills needed to succeed in the QE, students often find it difficult to define milestones to measure their preparedness for the QE. The entire process is part of a hidden curriculum (Calarco, 2020) where students often need to know to ask faculty to communicate their expectations, making the process inequitable and more challenging for students from minoritized backgrounds. In addition, the COVID-19 pandemic has made community building and peer mentorship difficult, compounding the difficulties described above.

We identified the need for a program dedicated to preparing students for their QE. When designing the curriculum for this program, the lack of literature on this topic proved challenging. In 2015, the National Science Foundation Alliance for Graduate Education and the Professoriate California Alliance (Berkeley, Caltech, Stanford, UCLA) conducted a wide-ranging survey of graduate students across STEM to pinpoint ways to improve the success of PhD students, and eventually increase diversity in STEM leadership positions. This study found that women and minority students were most likely to publish at rates comparable to their male majority peers if they (1) felt that they were prepared for their graduate courses and (2) were enrolled in a structured PhD program. Student distress levels were also reduced if they were provided clear expectations for completing the PhD program (Fisher et al., 2019). Based on this study, we focused on creating a structured program with clear expectations and milestones, that developed the skills assessed during the QE and would increase students' self-

perceived preparedness. This would greatly benefit all graduate students but could be particularly advantageous for students of minoritized communities in academia.

Here, we describe the creation and assessment of the Inclusive Excellence in Quals Preparation program (IEQP) at University of California, Berkeley. The aim of the IEQP program is to prepare graduate students for their qualifying exam by establishing structured goals, building a sense of community, providing a support system, and creating an environment where students feel valued and capable of achieving their goals. To our knowledge, a program dedicated to support graduate students' preparation for the QE has not been previously reported.

METHODS

The Inclusive Excellence in Quals Prep (IEQP) program was developed as a pilot program to support graduate students in their qualifying exam preparation process. The first iteration of the program was focused on second-year graduate students in the Plant and Microbial Biology (PMB) and Molecular and Cell Biology (MCB) programs at the University of California (UC) Berkeley.

Curriculum design

The program was designed by two graduate students, one in PMB and one in MCB, two faculty members, similarly from PMB and MCB, and the director of the Office of Graduate Diversity. Curriculum design drew largely from our own experiences in these two programs and graduate students' collective experiences.

The main goals of the IEQP program were to prepare graduate students for their qualifying exam by building a sense of community, providing a support system, and creating an environment where students feel valued and capable of achieving their goals. As such, the core principles addressed by the IEQP curriculum (Fig 1) were academic preparation, community building, and mentorship.

Academic preparation was addressed by panels and workshops led by invited speakers (from UC Berkeley and elsewhere), which covered topics such as strategies to read research articles efficiently, crafting and giving an elevator pitch, and what to talk about when meeting with committee members. These topics were specifically addressed because they are often overlooked and not incorporated into graduate curricula despite their importance (academia's hidden curriculum) (Apple & King, 1977). Additionally, a subset of workshops was focused on wellness, resilience, and stress management. These topics were crucial to the IEQP curriculum because students frequently find QE preparation very stressful, and we wanted to provide them with tools to help address anxiety during this stressful time and throughout their graduate education.

We paired each student in the program with an older, post-QE graduate student mentor from the same program. Mentors formally engaged with their mentees through monthly one-on-one "coffee hours" where they discussed their progress and wellness. The program directors suggested some guide topics and questions for each of these

meetings that would match the mentees' preparation stage. This peer mentoring gave students the opportunity to engage with older graduate students in their program that had shared interests and represented an additional source of support for each program participant. Additionally, mentors and mentees interacted informally during social events hosted by the IEQP program directors. These social events included monthly dinners, as well as a kickoff event and an end-of-program celebration, and were meant to encourage mentees to build affinity as a cohort, and provide opportunities for informal interactions which were missing during the COVID-19 lockdown.

Additionally, each student was required to have at least one practice exam in the month preceding their qualifying exam. This practice exam consisted of a mock examination led by trainees with expertise in each student's research area. In most cases, practice exam participants were trainees in the laboratory of each student's committee members. To decrease IEQP mentees' workload, the program directors coordinated these practice exams.

A detailed curriculum containing lesson plans and full descriptions is included in the supplementary materials.

Timeline

Students in the target programs normally take their qualifying exam late in the Spring semester of their second year. For this reason, the program's timeline was designed to support participants through this semester. The curriculum was designed during the summer months, invited speakers were contacted and confirmed between the Summer and Fall semesters. The program started officially in December 2021 with one introductory workshop, and weekly workshops were held from mid-January through the first week of April. The month of April was dedicated to practice exams.

Recruitment

Mentee recruitment. Graduate students were recruited via a general message sent by each program's graduate advisor to the cohort of second-year students. Additionally, we encouraged students to apply to the program through one-on-one conversations. Students applied through a form that collected information regarding their identity and research interests. We received 13 applications (7 MCB, 4 PMB, 2 HWNI) and admitted 11 students to the pilot IEQP cohort. Criteria for selection were (1) availability during the semester, and (2) self-proclaimed sense of preparedness for the exam (students that felt least prepared were prioritized). Care was taken to ensure that students belonging to underrepresented groups were represented in the mentee cohort.

Mentor recruitment. We recruited potential mentors at the same time that we recruited mentees. The graduate student directors and faculty advisors personally reached out to graduate students in the PMB, MCB, and HWNI programs that were in their third year or above to invite them to participate as mentors in the program. We focused on inviting people with previous experience with mentorship, and we ensured that students from underrepresented groups were represented in the mentor cohort. Prospective mentors

filled out an interest form where they detailed their research interests and indicated their availability for the Spring semester.

Pairing mentors and mentees. *We* received 18 mentor interest forms and carefully selected pairs of mentors and mentees with the following criteria in mind: (1) Mentor and mentee should be in the same program, (2) They should have similar or relevant research experience, (3) They should not be in the same lab to expand mentees' access to the graduate student community. Mentors were notified once they were matched to a mentee, and they were required to attend an inclusive mentorship workshop before they officially interacted with their mentees.

Program evaluation

We evaluated the program via a series of surveys, including a pre-program and end-of-program survey for mentees (Table 1), workshop surveys for mentees, and monthly check-in forms for mentors.

RESULTS

The program consisted of a cohort of 11 mentees from the PMB, MCB, and HWNI doctoral programs. We administered a survey at the beginning of the program (n=10) to evaluate the mentees' preparedness, wellbeing, wellness, and sense of community support, and a second survey at the end of the program (n=11) to assess the progress of mentees and the program's impact.

When comparing the results of the pre- and post-program surveys, there are some categories where the effects of general QE preparation cannot be disentangled from the effects of the IEQP program. In general, students' QE preparedness and the support received from their advisors improved throughout the semester, as did their confidence in writing, critical reading, presentation, and communication skills. In some cases, the program may have influenced the observed improvements, but these are factors for which we would expect an increase regardless. We also observed an increase in metrics of sense of belonging in graduate school, and while some students attributed this to their participation in IEQP, the process of preparing for their QE and eventually passing it may have also contributed to feelings of belonging. We make this distinction throughout the results.

Demographics

One mentee identified as Black or African, four Hispanic, Latinx, or Spanish Origin, two Middle Eastern or North African, one Native American or Alaska Native, two South Asian, and three White (Fig 2A). 10% of mentees reported being genderfluid or genderqueer, 20% men, 10% non-binary, and 60% women. 40% of mentees reported being part of LGBTQ+ community (Fig 2B). 40% of mentees reported having a physical, mental, or learning/cognitive disability. 30% of mentees identified as international students. 40% of mentees identified as first-generation students (Fig 2C), and no mentees reported having parents that had obtained a Ph.D. (Fig 2D).

QE preparedness
We assessed the mentees self-perceived preparedness for the QE before and after completion of the IEQP program (Fig 3). The scale was 1 = Strongly Disagree; 2 = Disagree; 3 = Neutral; 4 = Agree; 5 = Strongly Agree. Nine mentees had written a research proposal before participating in the IEQP program. Overall, mentees disagreed that they knew what they needed to do to prepare for the QE (2.5 score) and felt neutral that they could pass the QE (3.1 score) before participating in the program.

We saw an increase in agreement when comparing the following statements before and after the program: I feel prepared to take my qualifying exam, I am confident in my writing and editing skills, I am confident in my science presentation skills, I am confident in my ability to interpret research articles in my field, I am confident when communicating with faculty. Most mentees disagreed or felt neutral about these statements before the program and agreed after the program. Overall, the skills they honed throughout the workshops increased the mentees' self-perceived preparedness at the time of taking their QE, but additional preparation outside of the program likely also contributed to this effect

Community support
We found that after the completion of the IEQP program mentees increased discussion of their proposal with their PI, felt their PI provided useful input on their proposal, and felt increased agency over the content of their QE proposal (Fig 4). We believe that weekly check-in with mentees during the workshops likely increased the number of times the mentees met with their PI and thus increased their sense of agency over their proposal, and sense of usefulness of the meetings. However, we cannot disentangle the effects that simply studying for the QE had on increasing the mentees' agency over their proposal and discussions with their PI about the proposal. We found that mentees asked more members outside of UC Berkeley (friends, family) for help regarding the QE but asked fewer members of UC Berkeley for help regarding the QE (Fig 4). This is likely because the IEQP program already provided mentees with an extensive network of UC Berkeley members to help students with their QE. Additionally, mentees did not report an increase in asking lab members for help with the proposal, or discussions about topics other than the research proposal with lab members or PI (Fig 4).

Two of the three participating departments provided a document with guidelines for the QE while one department did not provide students any guidelines for the QE. 80% of mentees said their department organized a panel or seminar about the QE. However, mentees felt that they did not receive training similar to that covered in IEQP workshops in their program's curriculum. While the departments provided guidelines on the structure of the exam and a timeline of when documents need to be submitted, departments failed to provide guidelines for how to prepare for the exam.

When asked to rank sources of support at the end of the program, mentees felt most supported by their IEQP mentor, followed by their PI and Lab members, followed by IEQP directors and faculty advisors, followed by friends and family, and people at UC Berkeley outside of their lab (Fig 5).

Career Goals

When asked if the QE impacted their career goals, five mentees said no, three said yes, and three said maybe. Some of the responses as to why the QE impacted their career goals:

“I felt that writing the proposal was difficult, so I am not sure if I want to continue in academia which requires writing grants a lot.”

“It made me deeply consider how much I want to complete my PhD.”

“Realized academia is a grind with too much work and too little pay; I don't want to do a postdoc. I'd rather go somewhere that values my time more highly.”

“I don't think I want to feel this continuous scrutiny for the rest of my life…”

While the goal of the QE is to prepare students for graduate school, we found that it has a deep impact on students’ self-perceived scientific identity. Unfortunately, students from minoritized backgrounds in academia may be more susceptible to the QE’s impact on self-perceived scientific identity.

Four mentees said participating in IEQP impacted their career goals. One mentee wrote “IEQP has made me more confident about my place in STEM. Having people like me (Latinx, minorities, etc.) prepare and support me for the QE was very beneficial.” Another mentee wrote “I realized that I don't actually love benchwork compared to reading the literature and coming up with ideas/testable hypotheses. I think I'd like a more social career that allows me to help mentor and manage other scientists, but without doing the primary bench work myself.

Wellness

We found that after the completion of the IEQP program mentees increased their feelings of belonging in graduate school, were better equipped to deal with stress, and felt they could overcome hardship (Fig 6). However, we do not know if mentees also sought help to deal with stress outside of the IEQP program, which might have contributed to these results. We found that feelings that they can complete their PhD and that their degree is preparing them for the type of career that they want decreased slightly (Fig 6). We believe that these decreases might be due to a change in career choice, as we saw that the QE affected some students’ career choice.

DISCUSSION

We designed and implemented the Inclusive Excellence in Quals Prep program at the University of California, Berkeley. The pilot cohort consisted of eleven students, and we based our assessment of the program on their feedback through surveys and on our experience as program directors and faculty advisors. Ten out of eleven students succeeded at their QE on the first attempt, and one student will be retaking it the next semester. Overall, we saw an increase in students’ perception of preparedness, QE-related skills, the support received from their advisors, and the agency they felt over their proposed work. When asked about community support, 11/11 students stated

feeling supported by their IEQP mentors. While mentees felt varied levels of support from their community, all mentees agreed that they felt supported by their IEQP mentor.

Our main takeaway is that peer mentorship was the most successful part of the Inclusive Excellence in Quals Prep program. The peer mentorship aspect of IEQP increased mentees' community in their programs, as many of them had not interacted much with older graduate students due to the COVID-19 pandemic. Having an older graduate student mentor provided mentees with support and guidance, an outlet for stress and anxiety, an external source of feedback for their proposal. Further, having a peer mentor meant students had someone to hold them accountable without judgment. After this first iteration of IEQP, we recommend that peer mentors are retained as a crucial part of the program. This program component provided significant support for mentees and allowed more advanced graduate students to practice their mentorship skills and get compensated for their work. If we had to choose only one program component to retain, it would be peer mentorship.

While the participating graduate programs provide some training on soft skills such as a class on giving scientific talks, the breadth of training provided by IEQP through academic-focused workshops is not provided by these programs' curricula. Students are generally expected to acquire these skills indirectly but teaching them in a structured, hands-on manner proved to be beneficial for students. However, we believe that the skills taught through IEQP workshops are crucial at all stages of a graduate career and should be taught earlier in graduate programs. We suggest skills such as giving a chalk talk, giving an elevator pitch, skimming papers efficiently, and stress management be integrated into the first-year curriculum or taught as part of a program for first-year students.

Students expressed that they would have enjoyed having more structured 'group study time' to allow them to work on their proposals. With transitioning workshops to be part of the first-year curriculum, IEQP can be more focused on providing students a structured, guided space to work towards their QE preparation goals with their peers, while building community and fostering accountability. By addressing core skills during graduate students' first year, we recommend only maintaining workshops that are QE specific in future iterations of IEQP, such as how to effectively communicate with committee members, planning a quals study strategy, and proposal specific chalk talk and elevator pitch practices. Further, we recommend that these workshops be led by the IEQP community: program directors, faculty advisors, or mentors. We noticed that bringing in new people to lead weekly workshops disrupted the process of community building.

Finally, while some mentees relied heavily on IEQP as their main source of mentorship for QE prep, others had advisors that were very involved in the process. In the future, we recommend more communication between each mentee's advisor and the program directors in order to determine the best ways to support each student to make QE prep more equitable. This can be addressed by communicating with advisors at the beginning of the year to inform them that their mentee is a program participant, sharing the program curriculum, and the expected timeline. Additionally, communication

channels should be open throughout the duration of the program. This can be addressed by communicating with advisors at the beginning of the year to inform them that their mentee is a program participant, sharing the program curriculum, and the expected timeline. Additionally, communication channels should be open throughout the duration of the program.

When asked whether the QE impacted their career goals, three students answered yes and three answered maybe. They expressed reconsidering whether they want to complete their PhD and whether they want to stay in academia. While the QE provides an opportunity for scientific development, the high rigor and scrutiny associated with the QE, compounded with a lack of structure and poorly stated expectations can have a negative effect on students' confidence and scientific identity. The QE, as it is employed in many programs, represents an antiquated way to measure student's success and preparation, as it seems that it is meant to filter students out rather than prepare them for an independent research project (Purakgazi et al., 2017). Graduate academic programs should evaluate if their QE format is designed to achieve the stated goal of preparing students to undertake an independent research project (Yamamoto, 2023). Additionally, if programs do not provide the tools and training required for student's QE success, each student is dependent on their advisor's and committee's involvement in their QE preparation, which does not ensure an equitable experience across each program (Malcom & Parik, 2023). Providing the proper training to encourage student QE success represents one of many necessary steps in creating more accessible and equitable environments in STEM PhD programs.

In conclusion, the Inclusive Excellence in Quals Prep program was successful in its first year. A small cohort size that spanned three departments provided the opportunity to pilot the program and collect sufficient feedback to inform our recommendations for future iterations of the program and for implementation by others. The IEQP program provided students with the necessary structure, skill development, and mentorship during their QE prep. We hope that with the establishment of this model program at UC Berkeley, graduate programs will incorporate similar training into their curriculum to support graduate students throughout their QE preparation and ensure their success in graduate school.

FIGURES

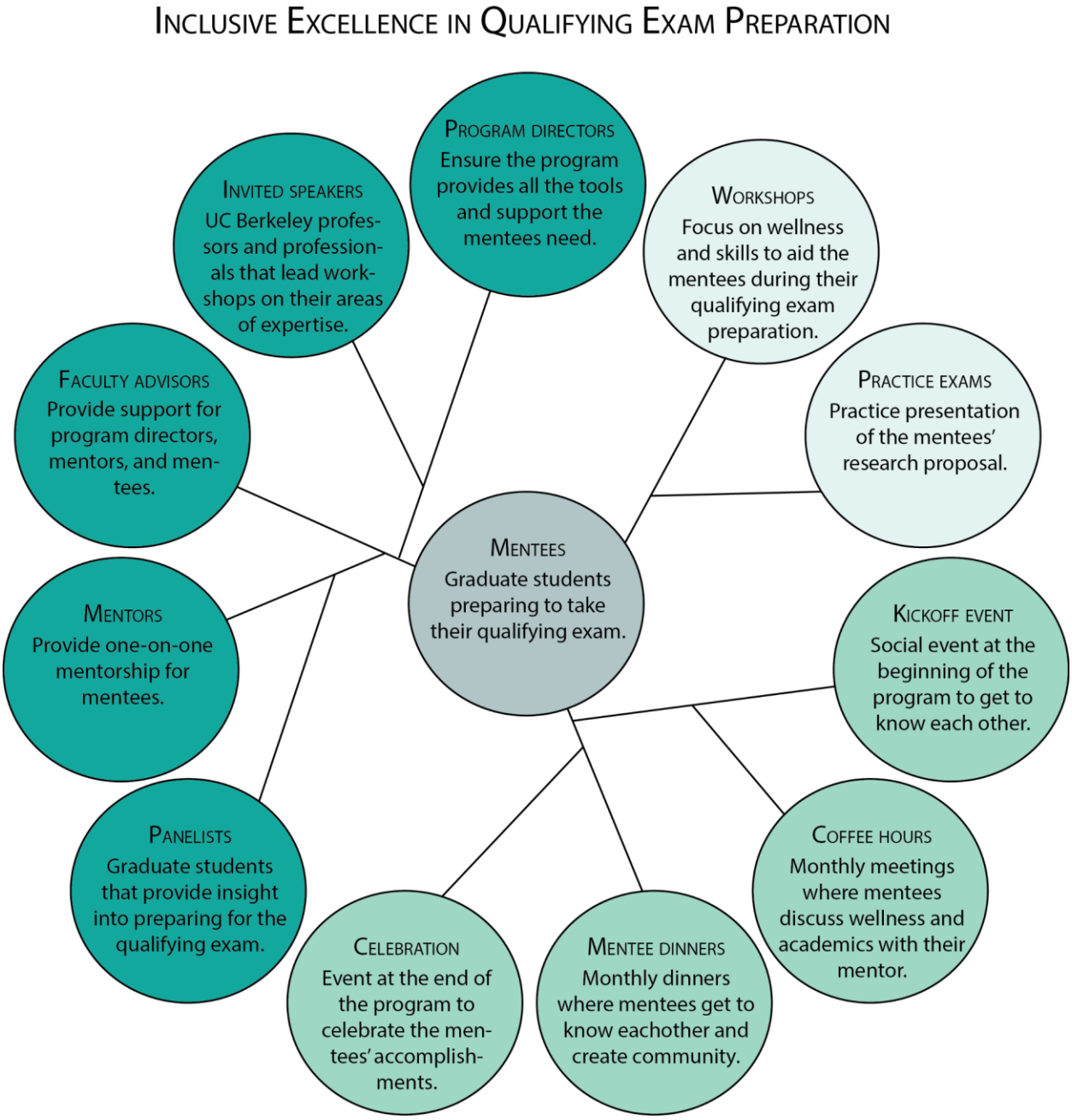


Figure 1. Curriculum map for the Inclusive Excellence in Quals Prep program
The curriculum was designed around the core goals of academic preparation and community support. The academic preparation component consisted of workshops and practice exams, while the community support component consisted of coffee hours with mentors, monthly dinners, a kickoff event and a final celebration. Additionally, the IEQP community consisted of mentees, mentors, faculty advisers, and program directors.

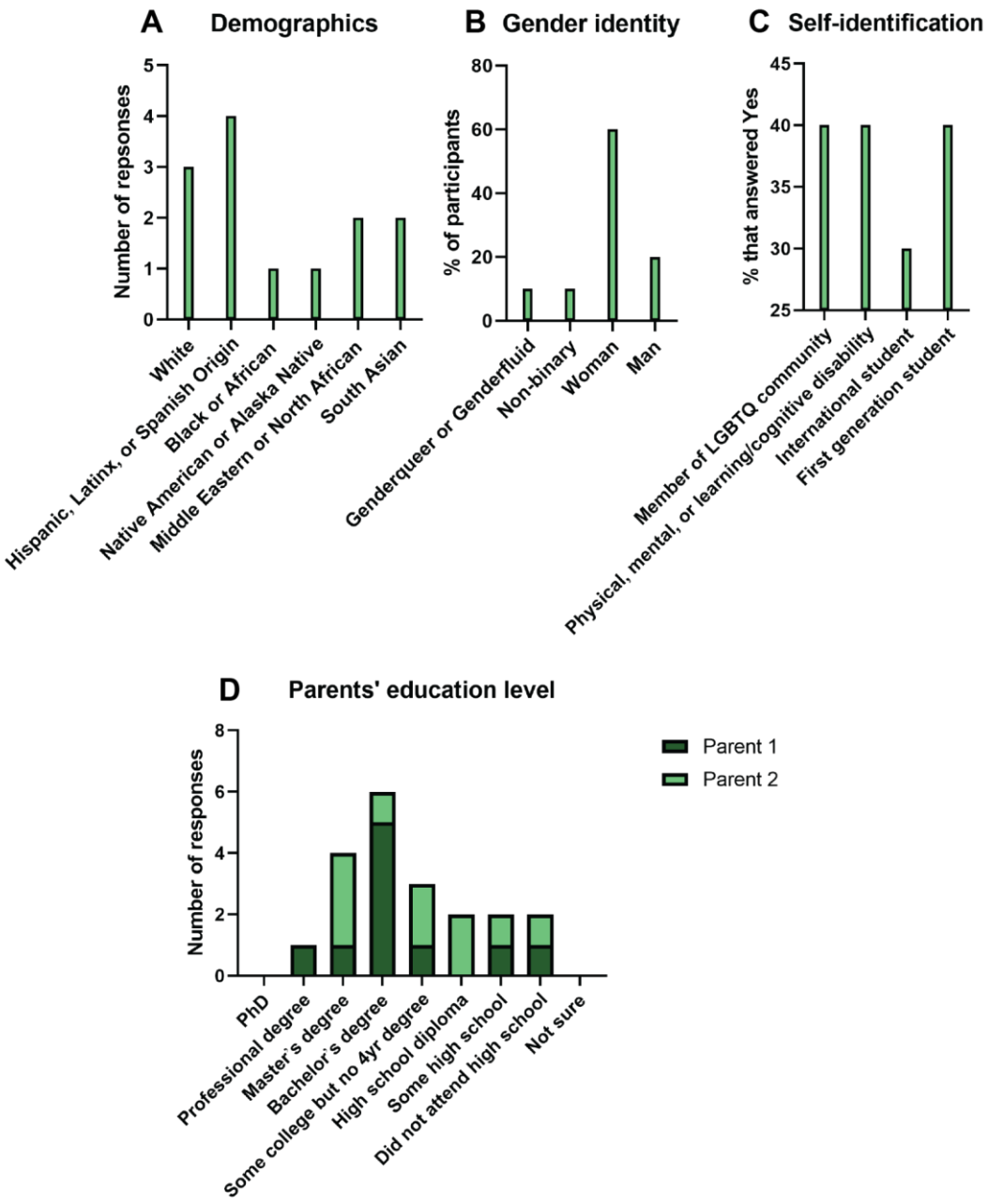


Figure 2. Demographic information for participants

(A) In a survey distributed before the start of the program, students selected all ethnicities that they identified with, (B) the gender they identified with, (C) all categories they identified with, (D) and parents' educational level.

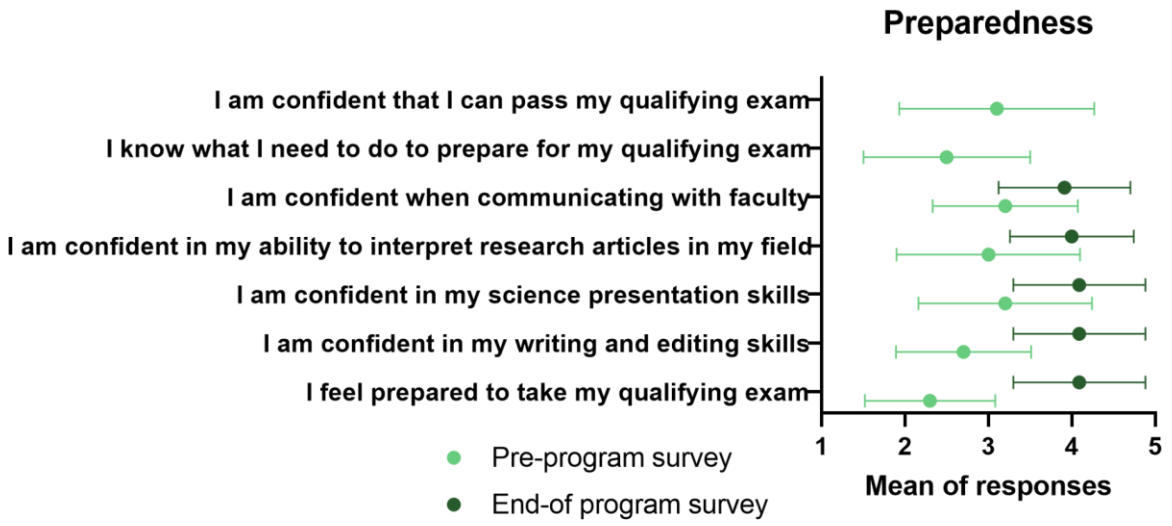


Figure 3. Academic preparation
Students reported their self-perceived academic preparation before the start of the program, light green, and after the program ended, dark green.

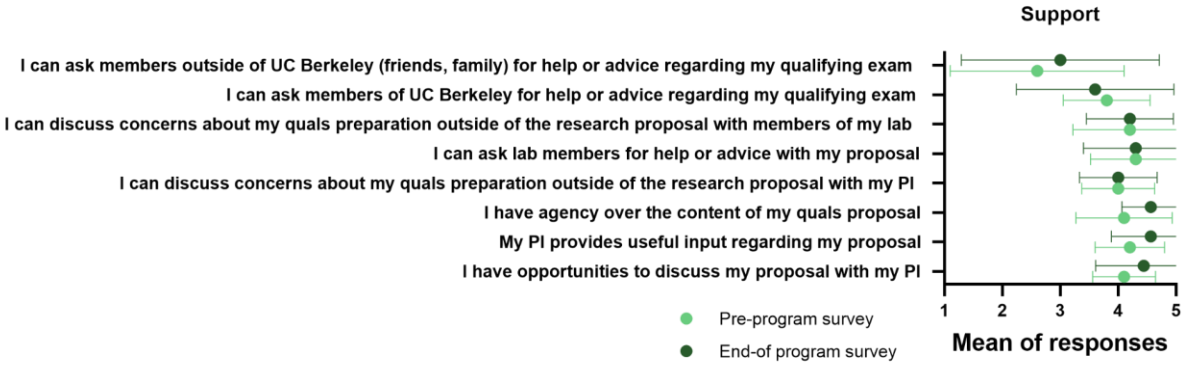


Figure 4. Support
Students reported the support received for qualifying exam preparation before the start of the program, light green, and after the program ended, dark green.

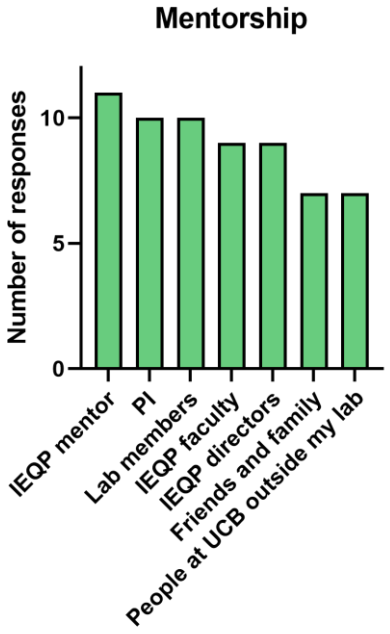


Figure 5. Mentorship
Students selected all the support received from various mentors during their qualifying exam preparation after the program ended.

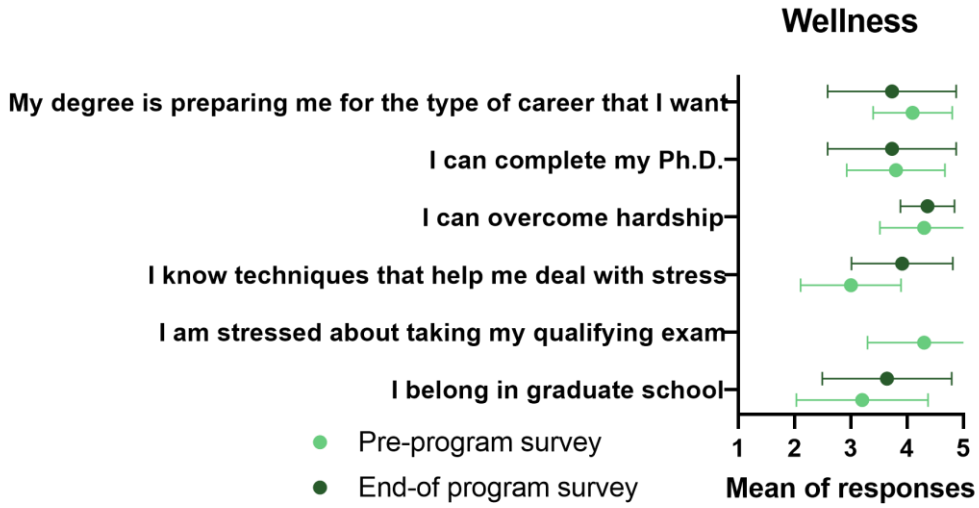


Figure 6. Wellness
Students reported the self-perceived wellness and belonging before the start of the program, light green, and after the program ended, dark green.

Table 1. Questions in the pre-program and end-of-program surveys.

No.	Category	Pre-program survey questions and answer choices	End-of-program survey questions and answer choices
1	Self-identification	Name	
2	Self-identification	Which category best describes you? (1) White; (2) Hispanic/Latinx; (3) Black or African; (4) Asian; (5) Native American or Alaska Native; (6) Middle Eastern or North African; (7) Pacific Islander; (8) Not listed above, please specify	
3	Self-identification	What is your gender identity? (1) Man; (2) Woman; (3) Trans man; (4) Trans woman; (5) Genderqueer/Nonbinary Gender; (6) Not listed above, please specify; (7) Prefer not to say	
4	Self-identification	Are you a member of the LGBTQ+ community?	
5	Self-identification	Are you a first generation student?	
6	Self-identification	Are you a student with a disability?	
7	Self-identification	Are you an international student?	
8	Self-identification	What are your parents' or caretakers' highest educational degree? (1) PhD, (2) Professional degree, (3) Masters' degree, (4) Bachelor's degree, (5) High school diploma, (6) Some high school, (7) Did not attend high school	
9	QE preparedness	Have you written a research proposal before? (1) Yes, (2) No	Did you pass your qualifying exam? (1) Yes, (2) No, (3) I haven't taken my qualifying exam yet
For questions 10 - 26: Indicate agreement or disagreement with each of the following statements. (1) Strongly disagree, (2) Disagree, (3) Neutral, (4) Agree, (5) Strongly agree			
10	QE preparedness	I feel prepared to take my qualifying exam	At the time of taking my qualifying exam, I felt prepared
11	QE preparedness	I know what I need to do to prepare for my qualifying exam	I am confident in my writing and editing skills

12	QE preparedness	I am confident that I can pass my qualifying exam	I am confident in my science presentation skills
13	QE preparedness	I am confident in my writing and editing skills	I am confident in my ability to interpret research articles in my field
14	QE preparedness	I am confident in my science presentation skills	I am confident when communicating with faculty
15	QE preparedness	I am confident in my ability to interpret research articles in my field	
16	QE preparedness	I am confident when communicating with faculty	
17	Community support	I can discuss my proposal with my PI	I frequently discussed my proposal with my PI
18	Community support	My PI provides useful input regarding my proposal	My PI provided useful input regarding my proposal
19	Community support	I have agency over the content of my quals proposal	I had agency over the content of my quals proposal
20	Community support	I can discuss concerns about my quals preparation outside of the research proposal with my PI	I discussed concerns about my quals preparation outside of the research proposal with my PI
21	Community support	I can ask lab members for help or advice with my proposal	I asked lab members for help or advice with my proposal
22	Community support	I can discuss concerns about my quals preparation outside of the research proposal with members of my lab	I discussed concerns about my quals preparation outside of the research proposal with members of my lab
23	Community support	I can ask members of UC Berkeley for help or advice regarding my qualifying exam	I asked members of UC Berkeley for help or advice regarding my qualifying exam
24	Community support	I can ask members outside of UC Berkeley (friends, family) for help or advice regarding my qualifying exam	I frequently asked members outside of UC Berkeley (friends, family) for help or advice regarding my qualifying exam
25	Community support	Indicate what type of support you have received from your department. Select all that apply: (1) My department provided a detailed document for the qualifying exam, (2) My department provided a workshop/panel/seminar about the qualifying exam, (3) My department didn't provide any guidance	I received training similar to that covered in IEQP workshops in my program's curriculum
26	Community support		I felt supported in my quals prep by the following people: (1) PI, (2) Lab members, (3) People at UC Berkeley outside my lab, (4)

			Friends/family, (5) My IEQP mentor, (6) IEQP faculty advisors, (7) IEQP directors
For questions 27-32: Indicate agreement or disagreement with each of the following statements. (1) Strongly disagree, (2) Disagree, (3) Neutral, (4) Agree, (5) Strongly agree			
27	Wellness, belonging, and stress management	I belong in graduate school	I belong in graduate school
28	Wellness, belonging, and stress management	I am stressed about taking my qualifying exam	I know techniques that help me deal with stress
29	Wellness, belonging, and stress management	I know techniques that help me deal with stress	I can overcome hardship
30	Wellness, belonging, and stress management	I can overcome hardship	
31	Career goals	I can complete my Ph.D.	I can complete my Ph.D.
32	Career goals	My degree is preparing me for the type of career that I want.	My degree is preparing me for the type of career that I want.
For questions 33-38: Select one, or complete with short answer			
33	Career goals	Indicate what type of career you wish to pursue (1) education: tenure track faculty, non-tenure track faculty, K-12 teacher; (2) non-faculty research: scientist in national lab, academic, industry setting; technical specialist; (3) other science: science communication, science outreach, science policy, patent lawyer, medical professional; (4) other: finance, accounting, marketing, sales, musician, self-employed; (5) undecided	Indicate what type of career you wish to pursue
34	Career goals	What are your career goals? short answer	What are your career goals? short answer
35	Career goals		Did participating in IEQP impact your career goals? Yes, No, Maybe
36	Career goals		If you answered yes to the above question, explain why: short answer

37	Career goals		Did the quals process impact your career goals? Yes, No, Maybe
38	Career goals		If you answered yes to the above question, explain why: short answer
For questions 39-48: Select all that apply: (1) Workshops; (2) Practice exams; (3) Community social events; (4) Interacting with mentor one-on-one; (5) Meeting with Rachel and Diana; (6) Other			
39	Program evaluation		Which program elements were most effective in terms of preparation for the experience of the oral exam?
40	Program evaluation		Which program elements were least effective in terms of preparation for the experience of the oral exam?
41	Program evaluation		Which program elements were the most effective in terms of writing the inside proposal?
42	Program evaluation		Which program elements were the least effective in terms of writing the inside proposal?
43	Program evaluation		Which program elements were the most effective in terms of stress management?
44	Program evaluation		Which program elements were the least effective in terms of stress management?
45	Program evaluation		Did your interactions with your peer mentor add a lot to your program experience, and how could they improve? Short answer
46	Program evaluation		Did your interactions with faculty directors (Rachel and Diana) add a lot to your program experience, and how could they improve? Short answer
47	Program evaluation		What was the hardest part of quals prep for you and how could the program have helped you meet this challenge better? Short answer
48	Program evaluation		Is there anything you would change in the program going forward? Short answer

SUPPLEMENTARY MATERIALS

Inclusive Excellence in Qualifying Exam Preparation Curriculum

Developed by Amanda González-Segarra and Zoila Alvarez-Aponte

STATEMENT OF INTENT

Motivation

In 2015, the National Science Foundation Alliance for Graduate Education and the Professoriate (NSF AGEP) California Alliance (Berkeley, Caltech, Stanford, UCLA) conducted a wide-ranging survey of graduate students across STEM to pinpoint ways to improve the success of PhD students, and eventually increase diversity in STEM leadership positions. Authors found that women and minority students were most likely to publish at rates comparable to their male majority peers if they felt that they were prepared for their graduate courses, were accepted by their colleagues, and were enrolled in a structured PhD program. They also found that stating what was expected of the students clearly, deeming that they were prepared for graduate level courses, and feeling accepted by their colleagues reduced student distress levels. (Fisher et al., 2019)

Aim

The aim of the Inclusive Excellence in Quals Preparation program is to prepare graduate students for their qualifying exam, build a sense of community, provide a support system, and create an environment where students feel valued and capable of achieving their goals.

Objectives

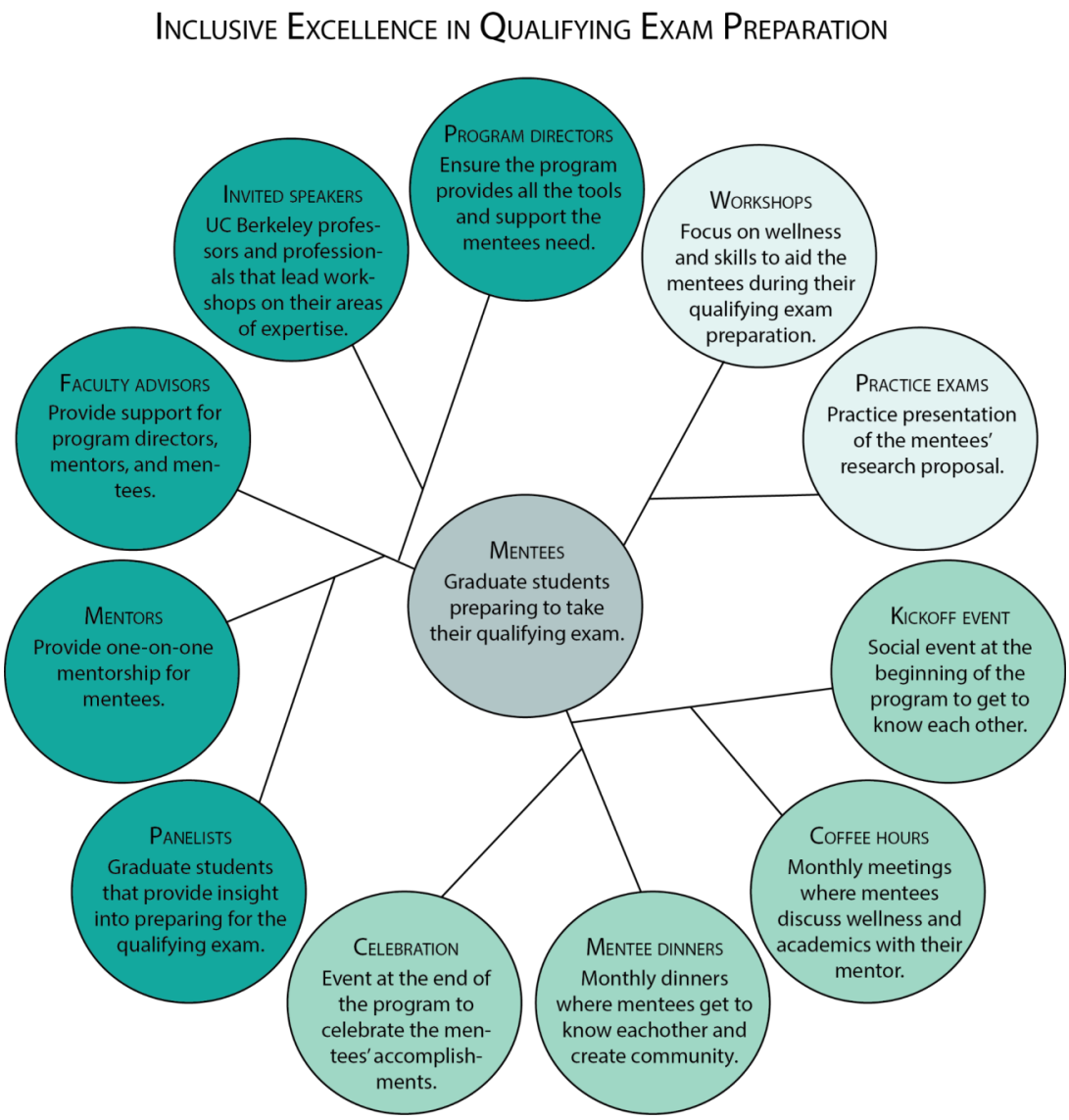
1. To pair every student with a mentor that will support them as they prepare for the qualifying exam.
2. To create a sense of community among the students.
3. To help students cope with imposter syndrome and learn resilience building techniques that will help them through graduate school.
4. To ensure every student is satisfied with their preparation at the time of taking their qualifying exam.
5. To strengthen students' time management, writing, editing, and presentation skills.
6. To contribute to the retention of graduate students from groups minoritized in STEM.

Broad outcomes

1. Students will write a research proposal.
2. Students will present their research proposal during their qualifying exam.
3. Students will successfully pass their qualifying exam.
4. Students will complete their PhD.
5. Students will feel capable of achieving their goals.
6. Students will increase their social capital.

STRUCTURE OF PROGRAM

The program will have two main components: an academic component that will focus on enhancing the mentees' operational skills and a social component that will focus on providing the mentees a strong support network. The academic component will involve: (1) workshops on topics such as creating a study plan, giving a chalk talk, and navigating imposter syndrome and (2) practice exams where the mentees will present their research proposal. The social component will involve: (1) a kickoff event so mentees get to know the program community, (2) coffee hours where the mentees will meet with their graduate student mentors, (3) mentee dinners where the mentees will strengthen bonds with each other, and (4) a celebration of the mentees' accomplishments at the end of the program.



OVERVIEW OF THE CURRICULUM

I. Statement of intent
    A. Through this pilot program, we intend to prepare 2nd year graduate students in the Molecular and Cell Biology (MCB) and Plant and Microbial Biology (PMB) departments for their qualifying exam and create an environment where students feel capable of achieving their goals.

II. Structure
    A. The program will have both academic and social activities to create a safe environment for the mentees to excel as they prepare for their qualifying exam. These will include workshops, practice exams, coffee hours, monthly dinners, a kickoff event, and a celebration at the end of the program.
    B. The mentees will have a support network throughout the program. The program will be composed of approximately 10 graduate student mentees, 2 graduate student leaders, 10 graduate student mentors, 3 program directors, invited speakers, and panelists.

III. Program community
    A. Mentees
    B. Graduate student leaders
    C. Graduate student mentors
    D. Program directors
    E. Invited speakers
    F. Student panelists
    G. Practice exam attendees

IV. Practice exams
    A. Each mentee will give one practice exam, which will be a chalk talk of the mentee's research proposal.
    B. Practice exams will take place during April. Each practice exam will run 2 hours.
    C. First year MCB and PMB graduate students from the Path to Professoriate program will be invited to attend the practice exams.

V. Social events
    A. Kickoff event
        1. Event at the beginning of the program in which mentees will get to know each other, their mentors, student leaders, and program directors.
    B. Coffee hours
        1. Monthly coffee hours where the mentees will meet with their graduate student mentors to talk about academic or wellness concerns in an informal setting.
        2. Coffee hours run through January-April.
    C. Mentee dinners
        1. Monthly dinners where mentees can get to know each other and create community.
        2. These will run through January-April.
    D. Mentor appreciation dinner

1. Dinner at the end of the semester to show mentors our appreciation for their commitment to the program.

E. Celebration
    1. Activity at the end of the semester to celebrate the mentees' accomplishments.

VI. Schedule
- A. The program will have weekly workshops starting the first week of December. There will be a holiday break for part of December and January, after which the program will resume until the end of May.

VII. Metrics
- A. Surveys for mentees at the start, end, and after 1 year of completing the program.
- B. Survey for mentors at the end of the program.

VIII. Budget:
- A. Stipend for graduate student leaders, graduate student mentors, and mentees.
- B. Gift card as a token of appreciation for panelists and practice exam attendees.
- C. Cost of tailored workshops, survey development and analysis.
- D. Monthly allowance for dinners and coffee hours.
- E. Snacks at practice exams, kickoff event, and celebration at the end of semester.

IX. Workshop lesson plans
- A. There will be a total of 12 workshops, covering topics of wellness and exam related skills. Every workshop will be facilitated by an invited speaker or panelists with some expertise on that topic.
- B. Workshops will be once a week and 1-2 hrs long. Workshops will run from December through April.
- C. Workshop topics:
    1. The qualifying exam experience
    2. Creating a sense of belonging
    3. Creating a study strategy for the qualifying exam
    4. Strategies for reading papers
    5. How to meet with your committee members
    6. Coping with imposter syndrome
    7. How to write the qualifying exam proposal
    8. How to write an abstract and specific aims
    9. Editing the proposal
    10. How to give an elevator pitch
    11. How to give a chalk talk
    12. Stress management and resilience

PROGRAM COMMUNITY

I. Mentees
   A. There will be approximately 10 mentees.
   B. Mentees should be second year graduate students enrolled in MCB or PMB programs that will be taking their qualifying exam in the Spring of their second year.
   C. Care should be taken to make sure that students belonging to minoritized groups in STEM are represented in the mentee cohort.
   D. Mentees are expected to attend all academic and social events.
   E. Mentees will complete tasks outside of the weekly workshops, including writing an abstract, specific aims, proposal, and elevator pitch.
   F. Mentees will give one practice exam and attend at least one other mentee's practice exam.
   G. Mentees will meet with each committee member at least once, with their PI at least twice, and give at least one practice exam with their lab before their qualifying exam.
   H. Participants will receive a stipend for their participation in the program.

II. Graduate student leaders
   A. There will be at least two graduate student leaders.
   B. Graduate student leaders must be enrolled in the MCB or PMB departments, be in their third year or beyond, and cannot be GSIs during the Spring to be considered for the position.
   C. Care should be taken to make sure that students belonging to minoritized groups in STEM are represented in the graduate student leader positions.
   D. Graduate student leaders are expected to commit to the role for 1 academic year.
   E. Graduate student leaders will lead at least one workshop, attend every workshop, attend the celebration, and at least one graduate student leader will be present at every practice exam.
   F. During the Fall semester, graduate student leaders will be in charge of helping select graduate student mentors, finalizing the lesson plans, scheduling speakers for every workshop, and finalizing the budget.
   G. During the Spring semester, graduate student leaders will be in charge of reserving rooms for each workshop, providing assistance to speakers during the workshops, sending out surveys, sending out email reminders, checking in on the graduate student mentors, bringing snacks for the practice exam, and setting up the celebration.
   H. Graduate student leaders will be the main point of contact for mentees, graduate student mentors, program directors, and invited speakers.
   I. Graduate student leaders will receive a stipend.

III. Graduate student mentors
   A. There will be approximately 10 mentors, one for every mentee.
   B. The graduate student mentor must be in the same Department as the mentee and be in their third year or beyond. The mentor will preferably be in a similar field of study as the mentee. Graduate student mentors cannot be GSIs during the Spring to be considered for the position.

C. Care should be taken to make sure that students belonging to minoritized groups in STEM are represented in the graduate student mentor cohort.
D. The graduate student mentor will provide support and advice to their mentee throughout the semester. Each graduate student mentor will have no more than one mentee.
E. The graduate student mentor will meet for a Coffee Hour with their mentee a minimum of 3 times throughout the Spring semester.
F. The graduate student mentor will edit their mentee's proposal, attend their mentee's practice exam, and will be responsible for taking notes during the practice exam.
G. The graduate student mentor will also attend at least one other mentee's practice exam and be available to serve as a facilitator in workshops if needed.
H. Graduate student mentors will receive a stipend.

IV. Program directors
A. There should be at least one program director representing MCB and one program director representing PMB.
B. Care should be taken to make sure that faculty belonging to minoritized groups in STEM are represented among the program directors.
C. Program directors will provide funding for the program, feedback on the lesson plans and curriculum before the start of the program, and select the graduate student mentees, mentors and leaders.
D. Program directors will lead one workshop, attend at least 2 more workshops, and attend the kickoff event and celebration at the end of the program.
E. Each mentee should meet with one program director for a Coffee Hour at least once before the end of the program.

V. Invited speakers
A. Invited speakers should have some expertise in the workshop they will be leading.
B. Invited speakers should adapt their presentation based on the program curriculum.
C. Care should be taken to make sure that speakers belonging to minoritized groups in STEM are represented in the speaker list.

VI. Panelists
A. Invited panelists should have some expertise in the panel topic.
B. Care should be taken to make sure that students belonging to minoritized groups in STEM are represented in the panelist list.
C. Panelists will receive a gift card as remuneration for each panel in which they participate.
D. Graduate student leaders and graduate student mentors cannot receive additional remuneration for participating in a panel.

VII. Additional practice exam attendees
A. Additional practice exam attendees will be senior graduate students or postdocs invited to participate in some practice exams. They should have some expertise in the mentees' research topic.

B. Practice exam attendees are expected to stay for the entirety of the practice exam, make questions during the presentation, and provide written feedback to the mentee on ways to improve their proposal or presentation.
C. Practice exam attendees will receive a gift card as remuneration per exam they attend.
D. Graduate student leaders, graduate student mentors, and mentees cannot receive an additional remuneration for attending a practice exam.

PRACTICE EXAMS

Practice exams will be carried out through the last four weeks of the program, in April. The purpose of practice exams is to simulate the qualifying exam. By the time of their practice exam, each student must have their final talk ready and should have shown it to their PI.

Guidelines

1. Each mentee will have one practice exam in the program. They should have at least one other practice exam with their lab.
2. Practice exams will be scheduled for 2 hours.
3. Each practice exam will consist of a mentee's proposal presentation and a feedback session.
4. There will be 4-5 attendees per practice exam including one graduate student leader, the mentee's graduate student mentor, another graduate student mentor, and 1-2 additional practice exam attendees. Care will be taken to ensure that participants in each practice exam are part of, or adjacent to, the IEQP program community. Attendees are expected to ask informed questions throughout the practice exam to help each student prepare.
5. Schedule of practice exams will be arranged according to the students' qualifying exam dates. Students should schedule their practice exam no later than two weeks before their qualifying exam.
6. First year graduate students from MCB and PMB will be invited to attend the practice exams.

Responsibilities

1. The mentee is responsible for having their talk ready for the practice exam and practicing their elevator speech beforehand.
2. The graduate student leader will be responsible for moderating the exam and bringing snacks.
3. The student's mentor will be responsible for taking notes of questions asked during the exam.
4. Every attendee will be responsible for asking questions throughout the exam and providing feedback to the student.

SOCIAL EVENTS

To create a sense of community among program participants, we will have several social events throughout the semester. The specifics of each series of social events are detailed below.

1. Kickoff event
    a. We will have an event in the first week of December to kick off the program. All program participants will be invited, this includes mentees, mentors, student leaders, program directors, speakers, and student panelists.
    b. The purpose is to give everybody an official welcome and an opportunity to meet each other and start building the IEQP community.
    c. The event will consist of casual interactions, and we will have an icebreaker-community building activity at the beginning of this event where everyone can share something personal about themselves and start getting to know each other.

2. Coffee hours
    a. Coffee hours will be a scheduled time for mentees to meet with their mentor or with a program director to discuss their needs and receive support.
    b. Each mentee will have at least 4 coffee hours throughout the Spring semester.
    c. At least one coffee hour should be with a program director, and the rest should be with the mentee's graduate student mentor.
    d. The topics to be discussed at each coffee hour are open for the student to decide, but some topics are suggested in the program schedule.

3. Mentee dinners
    a. The cohort of program mentees will have monthly dinners. These are meant to allow them to share time together and strengthen their sense of community.
    b. All mentees should attend each monthly dinner.
    c. The restaurant will be chosen by the mentees.

4. Mentor appreciation dinner
    a. Graduate student mentors, leaders and program directors will be invited to one dinner near the end of the program. The purpose of this dinner is to show appreciation for their commitment to the program.

5. Celebration
    a. We will have a casual celebration at the end of the program to celebrate the mentees' accomplishments.
    b. Everyone involved in the program, and members of the mentees' labs will be invited.

SCHEDULE

Week	Workshop	Speaker	Social Event
1. Dec 8	The qualifying exam experience	Grad student panel Moderator: Amanda	Kickoff event
2. Dec 15	Assembling a qualifying exam committee	Grad student panel Moderator: Zoila	
3. Jan 19	1. Creating a sense of belonging *Mentors invited* 2. Mentorship workshop for grad student mentors	UC Berkeley Psychology Clinic	Coffee hour - choosing committee members, scheduling the exam
4. Jan 26	Creating a study strategy for the qualifying exam	Zoila Alvarez-Aponte	Mentee dinner
5. Feb 2	Strategies for reading papers	Dr. Marla Feller	
6. Feb 9	How to meet with your committee members	Dr. Rachel Brem	
7. Feb 16	Coping with imposter syndrome *First-years and mentors invited*	Dr. Amy Honigman	Coffee hour - designing your proposal, exam preparation
8. Feb 23	How to write the qualifying exam proposal	Dr. Sheila McCormick	Mentee dinner
9. Mar 2	How to write an abstract and specific aims* *Mentors invited*	Dr. Brett Mensh	
10. Mar 9	Editing the proposal*	Amanda González-Segarra	
11. Mar 16	How to give an elevator pitch	Dr. Diana Bautista	Coffee hour- elevator pitch, what to expect on the day of exam

Mar 23	*Spring Break*		
12. Mar 30	How to give a chalk talk*	Dr. Britt Glaunsinger	Mentee dinner
13. Apr 6	1. Stress management and resilience *Mentors invited* 2. Practice exams *First-years invited*	Dr. Amy Honigman	
14. Apr 13	Practice exams *First-years invited*		Coffee hour-mental health, practice exams
15. Apr 20	Practice exams *First-years invited*		
16. Apr 27	Practice exams *First-years invited*		1. Mentor appreciation dinner 2. Mentee dinner
May 31			Celebration

* Denotes workshop which mentees will be required to bring prepared materials.

METRICS

To evaluate the success of the program and its impact on mentees, we will conduct four surveys, the goals of which are detailed below.

Survey mentees at the start of the semester

- Get an understanding of where mentees are in their qualifying exam preparation.
- Have a baseline for comparison at the end of the program. Evaluate students' confidence level regarding passing their qualifying exam, completing their PhD, achieving their goals, and their wellness (belonging, resilience stress, preparedness).

Survey mentees at the end of the semester

- Ask students to evaluate the program and its impact.
- Questions for comparison against baseline.
- Collect suggestions for the future of the program.
- Understand what was most challenging for students and identify steps that can be taken to provide more support.

Survey mentors at the end of the semester

- Collect feedback and suggestions for the future of the program.
- Understand what was most challenging for mentors and identify steps that can be taken to provide more support.
- Evaluate impact of the program on mentors.

Survey after 1yr of completing the program.

- Evaluate long-term effects of the program in terms of retention, mentees' confidence and wellness, and success in their graduate program.

BUDGET

Some funding for the IEQP program will come from the Office of Graduate Diversity, and some funding will be matched by the PMB and MCB departments.

The budget includes:

1. Stipend for mentees. (Spring semester)
2. Stipend for graduate student leaders. (Fall and Spring semesters)
3. Stipend for graduate student mentors. (Spring semester)
4. Payment for tailored workshops.
5. Token of appreciation for panelists.
6. Token of appreciation for practice exam attendees.
7. Kick-off event.
8. Coffee hour with graduate student mentor. 4 total coffee hours.
    a. This should cover coffee and snacks for both the mentee and graduate student mentor. A gift card with half the amount should be given to the mentee and one to the graduate student mentor.
9. Mentee monthly dinners, 4 total.
10. Mentor appreciation dinner.
11. Snacks at practice exams.
12. Celebration at the end of the program.

*Graduate student leaders, graduate student mentors, and mentees cannot receive an additional reward on top of their stipend.

WORKSHOP LESSON PLANS

I. The Qualifying Exam Experience

Workshop goals

1. Provide mentees insight on navigating the qualifying exam experience by hearing stories from fellow MCB and PMB graduate students.
2. Understand the guidelines for selection of the qualifying exam committee.
3. Learn strategies for selecting committee members and reaching out to them.

Workshop outline

1. Panel of MCB and PMB graduate students (3 students from each department) who will talk to the mentees about their qualifying exam experience.

Achievement

1. Mentees will get a better idea of what the qualifying exam is like and gain tips on how to prepare for the exam.
2. Mentees will assemble a list of potential committee members by end of the week and discuss it with their PI.

Panel Moderator tasks:

1. Send a survey to the mentees one week prior to the panel to collect questions they might have or the panelists. Make sure to incorporate these questions in the panel discussion.
2. Assign each question to two people, one of each department. Alternate panelists. This will hopefully avoid repetitiveness in responses.

Questions for the panel:

1. Panel introductions: Name, department, year in PhD, year you took quals, lab and brief description/overview of your research.
2. What was your qualifying exam like? (Was it in person, through zoom, or a hybrid, chalk talk or presentation, etc)
3. How did you go about choosing potential members for your QE committee? Did you talk to other grad students or your PI about potential committee members for your QE?
4. Once you chose your potential committee members, when and how did you approach them? When did you finalize your committee members? How did you set your exam date?
5. How did you organize your quals preparation? What helped you in the preparation process? Did you feel prepared before taking the exam?
6. How much support did you have from your PI, lab mates, and committee members? What were their roles and responsibilities?

7. How did you balance the amount of time spent on your research in comparison to the amount of time spent preparing for your exam, and time spent on self-care and hobbies?
8. Now that you've gone through the experience, what advice would you give your past self?

Leave 15 min at the end of the panel for any extra questions the mentees might have.

<u>Extra questions if there is time:</u>

9. How did you evaluate if the people you were considering would be good for your committee?
10. Did anyone say no to being on your committee, and how did you resolve that?
11. How helpful/supportive were your committee members? What were their roles as you prepared for your QE? Did you meet with them? If so, how many times and in general, what did you discuss with them?

II. Creating a Sense of Belonging

Workshop goals

1. Reinforce mentees' confidence in their preparation and ability to pass their Qualifying Exam.
2. Make mentees feel accepted.

Workshop outline

1. Welcome (15 min)
2. Community building (10 min)
3. Story Circle (15 min)
4. Micro-affirmations (10 min)
5. Closing (10 min)

Achievement

1. Mentees will reflect on what makes them feel that they belong.

Plan

1. Welcome
    A. Breathing exercise
        a. Invite participants to engage in an embodied practice of mindfulness and internal and external presence. You might say something like: "Exhale first. Breathe in deeply. Hold it for a moment. Exhale slowly." (pause) Now take 3 deep breaths on your own. When you breathe in, allow yourself to receive the air. As you exhale, relax completely."
    B. Community agreements
        a. Use and adapt these agreements to help set the tone, intentions, values and ethics of the learning environment:
            i. Recognize that we all carry wisdom.
            ii. Seek first to understand, then to be understood.
            iii. Choose a love-based response before a fearful one. Consider your own and others actions and comments from that perspective.
            iv. Be honest with yourself and others.
            v. Be brave. Allow yourself to respectfully voice your disagreements with others, even if it's uncomfortable.
    C. Speaking order
        a. Practice Speaking Order, one way to address and practice the reversal of systemic inequities introduced and held by white supremacy and patriarchy.
        b. To introduce Speaking Order, you might say something like: "We are going to practice shifting the power dynamics today. We will use Speaking Order as a way to reckon with our shared history of

imperialism and a white ruling class. Speaking Order will ask that anyone who self identifies as having been granted the most unearned power in our society (based on factors of race, gender, age, religion, ability, etc) will let others speak before weighing in. We ask that individuals internally self-identify and simply stay mindful throughout our time together of who takes up the most/least airtime in group discussions."

2. Community building
    A. Round one: Each participant is invited to share their name and a gesture, to which the other participants respond by repeating and mirroring back as wholebody "call and response".
    B. Round two: Share what belonging means to you with a gesture you associate with that issue. As with the previous round, the whole group repeats and mirrors as whole-body call and response.
3. Story Circle
    A. Divide participants in pairs or groups of 3. Each participant will take 5 minutes to share a story of a time they were included in a community and/or achieved a collective goal because someone took the time to understand and implement change through a system, policy, or new practice around their unique needs or the structural obstacles they face on a personal level.
    B. After each person shares, the other person mirrors back an exact word, phrase or gesture they heard the speaker say that was particularly moving. Move on to the next speaker.
    C. After all members of the group have shared their story, participants will discuss common events that made them feel included and ways in which they can make others feel included.
4. Micro-affirmations
    A. Divide participants in a different group of 2-3 people to discuss micro-affirmations. Take 2 min to read the definition of micro-affirmations.
    B. You can say something like "Micro- affirmations are tiny acts of opening doors to opportunity, gestures of inclusion and caring, and graceful acts of listening which occur wherever people wish to help others to succeed. Micro- affirmations lie in the practice of generosity, in consistently giving credit to others, in providing comfort and support when others are in distress. (Adapted from Rowe, 2008, p. 46).
    C. Tangible actions that can be applied to challenging and affirming experiences:
        a. Active listening, which focuses on hearing clearly what is being shared, and demonstrated through eye contact, open body posture,

summarizing statements, and/or asking qualifying questions to ensure understanding.

b. Recognizing and validating experiences involves elucidating the what, why, and how. It is helpful to delve deeper by identifying and validating the constructive behaviors a student demonstrated to manifest or respond to the experience, expressing care about the effect of the event, and demonstrating a willingness to think through a productive path forward.
c. Affirming emotional reactions through verbal acknowledgement that they have experienced something exciting, frustrating, hurtful, etc. enables the conversation to focus on turning those feelings toward actions that will empower, heal, and/or foster learning. (Adapted from Powell, Demetriou, & Fisher (2013))"

D. Allow the groups to interpret and discuss what they just heard.
E. Questions: Had you heard of micro-affirmations before? Do you use micro-affirmations in your daily life, both personal and professionally? Please explain why you do or don't use micro-affirmations.

5. Closing
   A. Bring everyone together in a circle to close. Ask participants to choose one word that describes a take-away from their learning today. Go around the circle and ask each participant to speak it into the circle. Thank the group for being together and sharing their experiences.

Adapted from the Othering and Belonging Institute at UC Berkeley.
Othering and Belonging Institute at UC Berkeley. Conference Curriculum. (Accessed September 3, 2021). https://conference.otheringandbelonging.org/2021-othering-belonging-conference/curriculum

Disclaimer: While creating a sense of belonging is not something that can be achieved in a single workshop, we can start to create an environment where students feel valued and capable of achieving their goals.

III. Creating a Study Strategy for the Qualifying Exam

Workshop goals

1. Build mentees' confidence in the qualifying exam preparation process.
2. Develop an individual preparation strategy that works for each student.

Workshop outline

3. Presentation (10 min)
4. Study strategy preparation (50 min)

Achievement

1. Mentees will create a study strategy and calendar.

Plan

1. Presentation
   Go over:
   A. Effective preparation techniques
      a. Selecting what papers to read
      b. Setting up a journal club
      c. Identifying key topics to review
   B. Time management techniques
      a. How to identify main hindrances to good time management and how to deal with them.
   C. Making a study calendar and setting deadlines:
      a. Finalize the qualifying exam committee
      b. Set the exam date
      c. Meet with each committee member at least once
      d. Have a finished abstract and specific aims page
      e. Have an outline of the proposal
      f. Start writing the proposal
      g. Schedule practice quals
      h. Send proposal to the committee
      i. Pass the qualifying exam!
2. Study strategy preparation
   A. Pass out a handout which mentees will fill in with the following information:
      a. Mentees will think about what study strategies have worked for them in the past. (5min)
      b. Mentees will determine the main topics they want to focus on when studying, make a list, and assign each a priority level. (10min)
      c. Mentees will think about how they want to organize the writing process and how much time they should dedicate to it. (10 min)
      d. Mentees will make a list of all the logistical tasks they need to complete before taking the QE. (5 min)

e. Mentees will prepare a calendar and include: (5 min)
    i. Logistics -- sending emails, meetings with committee members, etc.
    ii. Studying -- reading, practicing the talk, etc.
    iii. Writing -- set date when they will start writing, have different drafts ready, and have the finalized proposal

B. Assign mentees into pairs to share study strategies. (10min)

IV. Strategies for Reading Papers

Workshop goals

1. Advise students on how to determine which papers they should read to form the basis of their proposal.
2. Guide students on how to extract relevant information from a paper.

Workshop outline

1. Presentation on strategies to choose, read, and talk about a paper (15 min)
2. Hands-on activity: Find and discuss a paper (45 min)

Achievement

1. Mentees will apply the skills acquired from the presentation to choose, skim, and discuss a paper with a peer.

Plan

1. Presentation
   Go over:
   A. How to find seminal papers in your field
   B. How to decide if you should read the paper
   C. How to skim a paper (what aspects to focus on)
   D. Getting what you want from the paper (i.e. you don't have to read the whole thing if you are only interested in the methods)
   E. Determining the key takeaways of a paper: Why did the authors pursue this research question? How did authors try to answer the question? What did the authors find? What impact did their findings have on the field? How does this tie into my thesis project?
2. Hands-on activity: Find and discuss a paper
   A. Direct the mentees to find a paper related to their research, which they have not read yet. (10 min)
   B. Mentees will skim the paper and write the main takeaways from the paper. (20 min)
   C. Assign mentees into pairs where they will take turns discussing the papers. Remind them to focus on the key takeaways of the paper. (7min each student - 15 min)

V. How to Meet with your Committee Members

Workshop goals

1. Consider the best ways to communicate with committee members and coordinate meetings.
2. Discuss the most appropriate and useful questions to ask during your meetings.

Workshop outline

1. Presentation on meeting with committee members (30min)
2. Discussion (30min)

Achievement

1. Mentees will create a list of questions to ask their committee members.

Plan

1. Presentation
   Go over:
   A. Setting up meetings
      a. Communicating with your committee members via email.
      b. Communicating with committee members in person or via zoom.
   B. Topics to discuss with your committee members when you meet with them individually:
      a. Bring a printed overview of your proposal to discuss.
      b. Bring specific questions on some of the papers to discuss (MCB). Ask for paper recommendations (PMB).
      c. Discuss your committee members' expectations for your preparation and for the exam; and discuss the exam format with your chair.
      d. Ask if they want to see drafts of your proposal at some point or if they just wish to read the final version.
   C. Example questions:
      a. How will the exam be structured? Will most of the time be spent discussing my proposal? How much time should I prepare to spend on background information?
      b. When writing the proposal, should I go into a lot of detail describing the methods?
      c. What type of questions should I expect on my proposal, mostly focused on the details of the methods or on the expected results?
      d. When reading papers, what should I focus on? (this question only relevant for MCB) For PMB: Can you recommend readings on XYZ topics that could be useful for my preparation?
      e. Would it be okay if I used a powerpoint during the exam to display preliminary results or figures? (this question only relevant for PMB, if the exam is a chalk talk)

2. Discussion
    A. Go over your personal experiences
        a. Ask specific questions based on the expertise of each committee member.
        b. What questions should mentees ask? What questions should they not ask?
    B. Open the floor for student questions.

VI. Coping with Imposter Syndrome

Workshop goals

1. Define imposter syndrome and discuss factors that can affect imposter syndrome.
2. Discuss students' experiences with imposter syndrome.

Workshop outline

1. Presentation on imposter syndrome (20min)
2. Small group discussion (20min)
3. Whole group discussion (20min)

Achievement

1. Mentees will reflect on what messaging they have gotten that has made them feel like an imposter and what has helped them cope with imposter syndrome.

Plan

1. Presentation
   Go over:
   A. Imposter syndrome is real and important to address.
   B. Difference between imposter syndrome and self-doubt.
   C. Factors that can affect imposter syndrome, with a focus on BIPOC experiences.
   D. Power dynamics in academia, and its effects on imposter syndrome.
   E. Self compassion as a way to cope with imposter syndrome.
   F.
2. Small group discussion
   Divide attendees into groups of 2-3 in which they will discuss the following questions:
   A. Have you ever experienced imposter syndrome? If you feel comfortable, please share one experience where you felt like an imposter.
   B. If you have experienced imposter syndrome, what specific factors do you think led you to feel this way? What messaging have you experienced that has made you feel like an imposter? If you haven't experienced imposter syndrome, speculate on why.
   C. Have you ever attended a workshop that addresses imposter syndrome? Have you ever discussed imposter syndrome with a PI, a mentor or peers?
   D. What strategies, if any, have helped you cope with imposter syndrome?
   E. How can the department alleviate the enhanced imposter syndrome experienced by minoritized students?
3. Whole group discussion
   A. Invite attendees to share some of their discussion topics with all the attendees. Each small group will be encouraged to share with everyone

else, with one or multiple people per group sharing what they discussed. Try to keep each group’s discussions under 5 min so that every group gets a chance to share.

VII. How to Write the Qualifying Exam Proposal

Workshop goals

1. Describe department requirements (length, sections, deadline) for the written qualifying exam proposal.
2. Discuss effective techniques for the writing process.

Workshop outline

1. Presentation on writing the QE proposal (45 min)
2. Mentees will draft an outline of their proposal (15 min)

Achievement

1. Mentees will have a first draft of the proposal outline.

Plan

Before the workshop: Students should have discussed written proposal expectations with their committee chair and PI.

1. Presentation
   Go over:
   A. Departmental requirements
      a. Research proposal format
      b. Other requirements
   B. Effective writing methods
      a. Planning the writing process.
      b. Writing an outline.
      c. Asking older graduate students for their QE proposal and asking the PI or other lab members for grant proposals related to the research project.
      d. Developing figures for the proposal.
   C. Proposal outline
      a. Draft title
      b. Key background information points and related literature
      c. For each aim: hypotheses, key experiments, expected results, potential pitfalls and alternative approaches.
   D. Getting feedback on your proposal:
      a. When to send out drafts to the PI, committee, and peers for review.
2. Drafting outline of the QE proposal
   Mentees will work individually to develop an outline of their proposal. They will define the sections in their proposal and identify points to include in each section. This can include, but isn't limited to: (1) draft title, (2) key background information points and related literature, (3) for each aim: hypotheses, key experiments, expected results, potential pitfalls. They should send this outline to their mentor for review before the editing workshop.

VIII. How to Write an Abstract and Specific Aims

Workshop goals

1. Discuss what information should be included in an abstract for a broader scientific audience.

Workshop outline

1. Presentation detailing how to write an abstract and specific aims (20min)
2. One on one meetings with a writing consultant. (45min)

Achievement

1. Mentees will receive feedback so they can polish their abstract and specific aims.

Plan

Before the workshop: Send out a spreadsheet so that students can sign up for a time slot to meet with the writing consultant. Mentees must bring a draft of their abstract and specific aims that they have discussed with their PI.

1. Presentation
   Go over:
   A. Review the information to include in the abstract:
      a. Background on the field of study
      b. Discuss the overarching goal/question of the proposal
      c. Go over the hypothesis
      d. Discuss preliminary data
      e. Include the main experiments of the proposal and possible outcomes.
      f. Discuss what information will be gained with the completion of the proposal.
   B. How to structure your abstract and the use of underlining and bolding.
   C. How to write and structure your specific aims.
      a. The proposal should include 2-3 specific aims. These should be thought of as dividing your main proposal question into 2-3 different components which should be complimentary yet independent of each other.
      b. Specific aims can be formatted as questions or statements.
2. One on one meetings with a writing consultant
   A. Mentees will meet individually with a writing consultant who will give them feedback on their abstracts and specific aims.

Resource:

Mensh B, Kording K (2017) Ten simple rules for structuring papers. PLoS Comput Biol 13(9): e1005619. https://doi.org/10.1371/journal.pcbi.1005619

IX. Editing the Proposal

Workshop goals

1. Discuss best practices for reviewing others' proposals and giving constructive feedback.
2. Have an editing session where mentees edit their proposals together and provide constructive feedback to their peers.

Workshop outline

1. Presentation on how to review the proposal (10 min)
2. Editing session (50 min)

Achievement

1. Mentees will give and receive feedback on their proposal during the workshop.

Plan

Before the workshop: Students should have developed a draft of their proposal and shown it to their PI and program mentor before this workshop.

1. Presentation
   Go over:
   A. Guidelines for reviewing your peer's written work:
      a. Skim read the proposal before making comments
      b. What is the main question addressed by the research? Is it relevant and interesting?
      c. Is the text clear and easy to read?
      d. Does each aim address the main question posed?
      e. Are the aims independent of each other?
      f. If the paper includes tables or figures, what do they add to the paper? Do they aid understanding or are they superfluous?
      g. What did the author do well? What needs improvement?
      h. Does the abstract give you a good idea of the proposal? Is it too detailed/not detailed enough?
2. Editing session
   A. Assign mentees into pairs to read and edit each other's proposals. If students desire their reviewer to focus on a certain part of their proposal, they should communicate it. (40 min)
   B. Mentees will then discuss the feedback with their reviewer. (10 min)

X. How to Give an Elevator Pitch

Workshop goals

1. Discuss what information to include in an elevator pitch.
2. Write an elevator pitch.
3. Present an elevator pitch.

Workshop outline

1. Presentation on creating an elevator pitch (20 min)
2. Writing the elevator pitch (20 min)
3. Presenting the elevator pitch (20min)

Achievement

1. Mentees will write and present their elevator pitch.

Plan

1. Presentation
   Go over:
   A. What is an elevator pitch and why is it important? An elevator pitch is a short description of your research proposal tailored for a broad audience in your general field.
   B. What information should be included in an elevator pitch? The elevator pitch should be a more succinct abstract that should focus more on the why than the how.
   C. How to structure your elevator pitch. The elevator pitch should be somewhere around 3-5 min long.
   D. Practice, practice, practice!
2. Writing the elevator pitch
   A. Mentees will work individually to determine what key information from their proposal should be included in their elevator pitch.
   B. Mentees will write their elevator pitch and prepare to present it.
3. Presenting the elevator pitch
   A. Divide the cohort into groups of 2-3 mentees and one facilitator. Each mentee will present their elevator pitch and then receive feedback from the facilitator and other mentees in the group. Each mentee should take 7-10 min so that every student has an opportunity to present and receive feedback.

XI. How to Give a Chalk Talk

Workshop goal

1. Discuss helpful techniques for giving a chalk talk.

Workshop outline

1. Presentation on how to give a chalk talk (30 min)
2. Practice chalk talks (30 min)

Achievement

1. Mentees will give a chalk talk about a figure.

Plan

Before the workshop: Each student will prepare a data or methods figure and be prepared to present it during the workshop.

1. Presentation topics
   Go over:
   A. What should you draw and what should you describe?
   B. How to draw and talk at the same time.
   C. Prepping before committee members arrive: Can include drawing specific aims, hypothesis, model, or preliminary data.
   D. Practice, practice, practice!
2. Practice chalk talks
   A. Divide the mentees into pairs where each mentee will present their figure in a chalk talk format (10 min) followed by a short feedback session from their peer (5 min). After one mentee is done, the other mentee will present and receive feedback as well.

XII. Stress Management and Resilience

Workshop goals

1. Foster resilience among mentees by providing them with tools necessary to thrive through their qualifying exam process and in grad school in general.

Workshop outline

1. Presentation focused on psychological resilience and stress management (30 min)
2. Storytelling (20 min)
3. Stressball fight (10 min)

Achievement

1. Mentees will explore a time when they faced and overcame adversity.

Plan

1. Presentation
   Go over:
   A. Resilience, self-care, and mental health best practices
   B. Emotional skills & cognitive distortion
   C. Social connection & support system
   D. Feedback resilience
   E. Self-compassion
2. Storytelling
   A. Mentees will write a story of a time when they faced adversity and how they overcame it. They can choose to share it with their peers or keep it for themselves.
   B. This activity is for the storyteller. It will help the students recognize that they are resilient.
3. Stressball fight
   A. Direct everyone to take a piece of paper and anonymously write something that's causing them stress. They should then crumple up their piece of paper. Ask everyone to stand up and countdown to start a snowball fight with their peers. At the end of the snowball fight, each person should pick up one snowball and read what's on the paper.
   B. This activity will help students get out what is causing them stress, move around, as well as empathize with and feel seen by their peers.

COFFE HOUR DISCUSSION TOPICS

Coffee hour #1

1. Get to know each other. Where are you from? What hobbies do you have? What type of research interests you?
2. Discuss what expectations you have of each other. Establish what your preferred method of communication will be during the semester.
3. For the mentor: Who did you have on your committee? When did you take the exam? How did you prepare for the exam? What was taking the exam like? What did you do after taking the exam?
4. For the mentee: When are you thinking of taking the exam? Who are you considering for your committee? Are there faculty members, aside from your advisors, whose research work interests you and/or is relevant to your potential thesis project?

Coffee hour #2

1. For the mentor: How did you design your proposal? How much input did you get from your PI and committee members on your proposal? When did you start writing the proposal? What was your general workflow for writing the proposal?
2. For the mentee: Have you finalized your committee? Have you scheduled your exam? How is exam preparation going? Have you discussed the aims of your proposal with your PI and committee members? Have you received any writing materials from older grad students or your PI?

Coffee hour #3

1. For the mentor: How did you schedule practice exams? Who did you invite for practice exams? How did you prepare for practice exams? How do you reserve rooms?
2. For the mentee: How is the writing process going? Have you scheduled any practice exams? How are you balancing lab research/exam preparation/life?

Coffee hour #4

1. For the mentor: What to expect on the day of exam? What was it like after you passed your exam?
2. For the mentee: How are you doing physically and mentally? How are your practice exams going? Do you have anything fun planned for after you take your exam?

Ideas for community building activities to do during social events

- The playlist that we play during the event will consist of each person's favorite song and people can choose to share when their favorite song starts playing.
- Pass around a bag of M&Ms or Skittles (or something more COVID friendly) and tell people to grab as many as they want. At the end, we'll tell them they need to share one thing about themselves for every color that they have.

Chapter 5: Discussion

Implications

In this study, we identified a complex neuroendocrine network that receives information from the ISNs about hunger and thirst states to regulate sugar and water ingestion. We identified a novel neuron, which we named Bilateral T-shaped neuron (BiT). This neuron is inhibited by the ISNs, and bidirectionally regulates sugar and water ingestion. Activation of BiT leads to a decrease in sugar ingestion and an increase in water ingestion. We found that BiT postsynaptic neurons reach many brain areas: the SEZ, the feeding sensorimotor center, the SMP and SLP, endocrine centers, the lobula, which contain inputs into the visual system, and the fan-shaped body, involved in decision making, locomotion, visual processing, and sleep.

We identified neurons regulated by the ISNs that are known to be involved in ingestion: IPCs, Lgr3, CCAP, DSOG1. IPCs are central regulators of appetite, metabolism, feeding, fecundity, aggression, sleep, locomotion, and stress responses. IPCs receive multiple direct and indirect signals of nutrient status, including AKH, allatostatin-A, CCAP, glucose, leucokinin, and tachykinin. IPCs release dILP2, dILP3, dILP5, and drosulfakinin (Kim et al., 2015, Nässel & Zandawala, 2022, Zhang et al., 2022). Lgr3 and CCAP neurons have been shown to promote feeding (Laturney et al., 2022, Williams et al., 2020). DSOG1 neurons have been shown to act as tonic inhibitors of feeding to promote appropriate ingestion of appetitive nutrients (Pool et al., 2014).

We found that the ISNs release the neuropeptide dILP3. This is the first study to our knowledge that implicates dILP3 in water ingestion, and furthermore in the coordination of sugar and water ingestion. We found that many ISN postsynaptic neurons are also peptidergic, either releasing or responding to peptides. CCAP neurons release the CCAP peptide, which has previously been implicated in ingestion (Williams et al., 2020). CCHa2R-RA knockin line was made for the RA isoform of the CCHa2 receptor, suggesting that these neurons respond to CCHa2 peptide, released from the gut to increase appetite (Deng et al., 2019, Reiher et al., 2011). IPCs release dilp2, 3, and 5 and respond to the peptides NPF, CCAP and AKH (Kim & Neufeld, 2015, Yoshinari et al., 2021, Zhang et al., 2022). Lgr3 neurons contain the Lgr3 receptor which binds dILP8, an insulin-like peptide (Yeom et al., 2021).

We found that water and sugar ingestion remain coordinated downstream of the ISNs in some neurons but not all. BiT, IPCs, and CCAP neurons all bidirectionally regulate sugar and water ingestion, while CCHa2R-RA neurons only regulate water ingestion. This suggests that some neurons might preferentially regulate water vs sugar ingestion, while other neurons might be integrating competing needs to regulate appropriate ingestion of nutrients.

We also identified additional neurons, aster, that coordinate sugar and water ingestion that are not downstream of the ISNs. These neurons receive inhibitory input from sensory neurons. This suggests that other neurons might be integrating other interoceptive signals with sensory input to modulate sugar and water ingestion.

Future directions

These studies have shed light on how ISNs bidirectionally modulate sugar and water ingestion. However, several questions still remain. Firstly, the molecular mechanisms that lead to BiT activation have yet to be elucidated. Does dILP3, the ISN neurotransmitter, bind to the InR in BiT? If so, how does insulin signaling lead to neural inhibition in BiT? Additionally, these studies used EM connectivity data to identify ISN postsynaptic neurons, which relies on the presence of chemical synapses between neurons. However, peptides have been shown to diffuse over long distances to act on peripheral organs. Do the ISNs also act on peripheral organs or communicate with neurons trans-synaptically? These experiments prove difficult to conduct as the insulin receptor is the only known receptor of dILP3 and is ubiquitously expressed.

It would also be interesting to explore what other signals BiT might be receiving. Using the connectome to identify other BiT presynaptic neurons might prove helpful. Furthermore, identifying the BiT neurotransmitter might shed light on how BiT is bidirectionally modulating sugar and water ingestion. We predict that BiT uses an inhibitory neurotransmitter, based on the behavioral and functional imaging studies done with the CCHa2R-RA neurons. Additionally, EM volume predicts that BiT is a glutamatergic neuron, which has been shown to be inhibitory in Drosophila (Liu et al., 2013). Functionally testing whether glutamate is the inhibitory transmitter for BiT would be an interesting future direction.

Further studies of the IPCs are also required. We found that acute activation of the IPCs increased water ingestion, but decreased sugar ingestion. This goes against the dogma that insulin is used as a satiety signal (Nässel et al., 2015). While no previous studies have implicated IPCs in water ingestion, mutating IPCs has been shown to decrease feeding. Our findings might be due to differences in the experimental design. Furthermore, it has been suggested that there is heterogeneity among IPCs (Wang et al., 2020). Further exploration of IPC subtypes and their regulation of feeding might explain confounding results.

CCHa2R-RA neurons were created from a knockin that labels the RA isoform of the CCHa2R receptor (Deng et al., 2019). CCha2 is released from the gut and stimulates

appetite, suggesting a gut-to-brain connection (Reiher et al., 2011). It will be interesting to explore if CCHa2R-RA neurons indeed respond to CCHa2 released from the gut. While previous studies have shown that CCHa2 stimulates appetite (Reiher et al., 2011), we did not see a change in sugar ingestion when we acutely activated CCHa2R-RA neurons. It is possible that CCHa2, might act on another population of neurons to mediate feeding, and acts on CCHa2R-RA neurons to stimulate drinking. Additionally, it is possible that signals from the gut may act on CCHa2R-RA neurons to stimulate prandial thirst, the need for drinking water after a meal.

Further dissection of the ISNs connectivity to DSOG1 neurons should also prove informative. DSOG1 neurons were found to act as a tonic inhibition on all feeding in order to drive appropriate feeding of appetitive nutrients (Pool et al., 2014). We found that the ISNs activate DSOG1 neurons, however, ISNs are active in hungry flies in order to drive sugar ingestion. Therefore, it will be interesting to learn if the ISNs differentially modulate DSOG1 neural activity based on the hunger and thirst state of the fly. Does DSOG1 also receive information from other interoceptive neurons? Previous studies were not able to identify DSOG1 presynaptic neurons (Pool et al., 2014), therefore, using the EM connectome might prove useful to dissect the DSOG1 circuit.

Lastly, what happens when a fly is both hungry and thirsty? In a hungry fly, the ISNs are active and drive sugar ingestion, but in a thirsty fly, the ISNs are silenced and drive water ingestion. We predict that if a fly is both thirsty and hungry, the activity state of the ISNs will serve to prioritize thirst or hunger, with decreased activity signaling thirst and increased activity signaling hunger. Our studies argue that the ISNs signal the hunger and thirst state of the fly, which is then integrated with other peptide and hormonal signals of nutritional need to guide feeding decisions. Future investigation of the integration of these signals will shed light on how nutritional needs are monitored to direct consumption and achieve homeostasis.

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