Inhibitory Control in the *Drosophila melanogaster* Feeding Circuit

by

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Abstract

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Feeding behavior is essential for achieving metabolic homeostasis and is critical for survival. Animals adjust their food intake based on their physiological needs and food availability. How sensing deprivation signals and detection of taste gets converted to feeding behaviors remains poorly understood. Here I use the genetically tractable model organism *Drosophila melanogaster* to examine the neural mechanisms underlying feeding decisions. The genetic conservation of molecular signaling pathways, the simpler nervous system and the powerful genetic tools make it an excellent system to explore the organization and logic behind brain circuits controlling food intake.

This thesis investigates the neuronal mechanisms underlying inhibition in the *Drosophila* feeding circuit. Here I describe the identification of 4 GABA-ergic interneurons in the *Drosophila* brain that establish a central feeding threshold which is required for any taste and satiety dependent feeding decisions. I show that these neurons control consumption in an activity dependent manner. Inactivation of these cells results in indiscriminate and excessive ingestion, independent of taste quality or nutritional state. Conversely, acute activation of these neurons significantly reduces consumption of water and nutrients. I show that their output is acutely required to express any feeding preference and that these neurons are not regulated by taste processing pathways or satiety signals. This work reveals a new layer of inhibitory control in insect feeding circuits that is required to suppress a latent state of unrestricted and nonselective consumption. Furthermore I identify the recurrent nerve as a peripheral source of post-ingestive inhibition of nutrient intake in *Drosophila* and show that the two feeding inhibitory mechanisms are distinct and independent of each other. The work presented here opens the door to analyzing how central and peripheral inhibition regulates feeding behaviors in *Drosophila melanogaster*.

To my family

‘Reason is, and ought only to be the slave of the passions, and can never pretend to any other office than to serve and obey them.’

David Hume (1739), ‘*A Treatise of Human Nature*’

Table of Contents

Chapter 1: Introduction ... 1

1. Physiological homeostasis and regulation of feeding behavior ... 2

2. Food intake regulation in *Drosophila melanogaster* ... 3

2.1. Motor-action patterns in feeding behavior of an adult *Drosophila melanogaster* ... 4

2.2. Quantification of feeding behaviors ... 5

3. Circuits and signaling systems controlling feeding behavior ... 7

3.1. Sensory systems ... 8

3.2. Need and satiety sensing systems ... 10

3.4. Central effector pathways ... 16

Chapter 2: Four GABA-ergic interneurons act as an essential brake on feeding in Drosophila melanogaster ... 20

1. Summary ... 21

2. Introduction ... 22

3. Results ... 23

4. Conclusions ... 28

5. Materials and Methods ... 29

Transgenic flies ... 29

Immunohistochemistry ... 29

Behavior ... 29

Mosaic Analysis of 98-Gal4 ... 30

Electrophysiology ... 30

Labelling neurons by photoactivation ... 31

RNAi screen ... 31

2-photon lesions of the recurrent and medial abdominal nerves ... 31

Electrical stimulation of Recurrent Nerve ... 32

Acknowledgements ... 32

6. Figure Legends ... 33

7. Figures ... 38

Chapter 3: Discussion 58
Inhibitory control in the *Drosophila* feeding circuit 59
DSOG1 interneurons as a novel layer of inhibitory control 59
DSOG1 upstream regulators and downstream targets 60
Gut derived post-ingestive feedback 61
DSOG1 and plasticity in the feeding circuit 62
Future directions 62
Figure legends 64
Figures 65
References 66

List of Abbreviations

Anatomy

AHL – adult hemolymph

AL – antennal lobe

CC – corpus cardiacum

FB – fat body

GRN – gustatory receptor neuron

mAN – medial abdominal nerve

mNSCs – medial neurosecretory cells in pars intercerebralis

ORN – olfactory receptor neuron

PI – pars intercerebralis

RN – recurrent nerve

SOG – suboesophageal ganglion

Genes, peptides and transgenic tools

dInR – *Drosophila* insulin like peptide

CCK – cholecystokinin

ChaT – choline acetyltransferace

CRZ – corazonin

DA – dopamine

DDC – dopa decarboxylase

dILP – *Drosophila* insulin like peptide

DSK – drosulfakinin

GAD1 **–** glutamic acid decarboxylase

GFP – green fluorescent protein

HDC **–** histidine decarboxylase

NPF – neuropeptide F

NPFR – neuropeptide F receptor

nSyb – synaptobrevin

PA-GFP – photoactivatable GFP

sNPF – short neuropeptide F

SYT – synaptotagmin

TH – tyrosine beta-hydroxylase

TDC2 – tyrosine decarboxylase

TPH – tryptophane hydroxylase

VGAT – vesicular GABA transporter

VGluT – vesicular Glutamate transporter

VMAT – vesicular monoamine transporter

<u>Other</u>

CAFÉ assay – capillary feeder assay

KCl – potassium chloride

NaCl – sodium chloride

PER – proboscis extension reflex

HRP – horse radish peroxidase

JAK/STAT – janus kinase/signal transducer and activator of transcription signaling cascade

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First and foremost I would like to thank my supervisor Prof. Kristin Scott who has been an exemplary thesis adviser and scientific mentor. I have learned an enormous amount from her during my time in her lab about science and how to run a successful enterprise. Her dedication, deep concern for the quality of work and ability to provide all technical means to keep the lab at the bleeding edge of science has made the Scott lab an exceptional environment to do research. I'm very fortunate to have had the opportunity to pursue an exciting scientific problem of high personal interest here and am grateful for Kristin's advice and support throughout my time in her lab.

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eventually learn from them how thirst, hunger, taste, sexual drive, memory and ultimately behavior are generated by the tiny fruit fly brain.

I couldn't have made it this far without the support of my family, particularly my mother Linda Pool, who has been supporting my endeavors with regular dispatches of Estonian sweets, but on a more serious note has taught me the importance of grit and perseverance in pursuing one's goals. I would also like to thank my partner Helen Põldsam whose unwavering support throughout grad school has made the years at Berkeley a joyous and enjoyable period of my life. Furthermore I would also like to thank my father Christian Weiss for continued enthusiasm and support.

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Chapter 1:
Introduction

1. Physiological homeostasis and regulation of feeding behavior

Animals are constantly challenged by their environment. The ability to counteract these challenges and maintain a stable internal physiology is essential for survival. This was recognized early on by the French physiologist Claude Bernard who maintained that "all the vital mechanisms . . . have only one object, that of preserving constant the conditions of life in the internal environment." The set of physiological processes aimed to preserve and maintain the stability of the internal milieu came later to be known as homeostasis (Cannon, 1929). A number of metabolic parameters are under homeostatic control including macronutrients such as carbohydrates, lipids and amino acids relevant for energy homeostasis, as well as micronutrients important for hydromineral balance and other physiological functions (Woods et al., 1998).

Animals have evolved sophisticated feedback mechanisms to monitor and rectify metabolic imbalances. In vertebrates and many invertebrate species the nervous system plays a central role in coordinating metabolic and physiological responses to biochemical perturbations and mobilizing feeding behaviors for the eventual restoration of homeostasis. How the nervous system coordinates feeding behavior with internal requirements and external sensory information is an area of active investigation.

Many of the mechanisms underlying energy homeostasis and regulation of food intake were first described in mammals. The molecular logic underlying the chemical senses involved in feeding was first worked out in rodents (Adler et al., 2000; Buck and Axel, 1991; Chandrashekar et al., 2006). Leptin, the canonical "adiposity signal", that communicates systemic energy reserves to the central nervous system was also identified in mammals (Zhang et al., 1994) as were multiple meal regulating satiety signals (Gibbs et al., 1973; Murphy and Bloom, 2006). Furthermore, early lesioning studies identified brain regions that are responsible for promoting feeding and satiety (Stellar, 1954). Recently, these studies have been followed up with more sophisticated tools for analyzing circuit structure and function (Sternson, 2013). Despite rapid progress, we have limited understanding of many fundamental questions in food intake regulation. How are needs represented in the brain and how do these representations gate or trigger feeding behaviors? How is taste information integrated with relevant signals of physiological deprivation? How do need sensing systems determine the behavioral valence of an external sensory stimulus? The excessive complexity of the mammalian endocrine and nervous systems has become limiting in efforts to understand these problems and has warranted the use of simpler model systems.

In this thesis, I explore how the nervous system coordinates feeding behaviors to attain homeostasis using the genetically tractable model organism *Drosophila melanogaster*. During the past two decades, the common fruit fly has emerged as a promising model system to dissect the neural basis of feeding decisions. Many of the endocrine and neuropeptide systems that control feeding in mammals have been conserved in the fruit fly (Baker and Thummel, 2007; Melcher et al., 2007). Furthermore, rapid development of genetic, behavioral and physiological tools has made it a lucrative organism to study molecular and cellular mechanisms underlying behavior (Venken et al., 2011).

2. Food intake regulation in *Drosophila melanogaster*

Insects, as other animals, are systems geared to maintain homeostasis. To that end, they employ a diverse arsenal of feeding behaviors to survive in their respective ecological niches. Feeding strategies can vary considerably within and across species – for example in the silk moth *Bombyx mori* feeding is confined to the larval stage with no food intake in the adult phase of its life cycle (Wanner and Robertson, 2008). Feeding can be either intermittent and tightly regulated as in most fly species in the adult stage or be subject to very little regulation and almost continuous as in larval fruit flies (Gelperin, 1971a; Melcher et al., 2007). Several insect species including the black blowfly *Phormia regina*, desert locust *Schistocerca gregaria* and the moth *Manduca sexta* have served as important model systems in studying food intake regulation in invertebrates (Chapman and de Boer, 1995).

In recent decades, however, the common fruit fly has rivalled the traditional models and has rapidly become the model species of choice to study neural basis of behavior due to its powerful genetic tools. The fly nervous system has four to five orders of magnitude fewer neurons than any mammalian model system, being comprised of approximately 200,000 neurons (Heisenberg, 2001). A major advantage of the *Drosophila* brain is that many neurons are uniquely identifiable between animals, which makes functional analysis of circuits tractable (Olsen and Wilson, 2008). More recently, the dissemination of large collections of Gal4 genetic driver lines (Jenett et al., 2012) have enabled reliable genetic targeting and manipulation of almost any combination of neurons in the nervous system, opening up the functional dissection of what was once deemed "an impenetrable interneuron jungle" (Gelperin, 1971a). Despite the relative numerical simplicity of its brain, *Drosophila* boasts a complicated behavioral repertoire and displays plasticity in many behavioral programs - feeding among them.

The adult fruit fly is an intermittent feeder that adjusts its food intake based on physiological state, recent feeding history, reproductive requirements, food availability, food quality and competing behavioral priorities (Carvalho et al., 2006; Edgecomb et al., 1994; Mann et al., 2013; Melcher and Pankratz, 2005; Ribeiro and Dickson, 2010). Since the need for hydromineral balance and dietary requirement for sugars, amino acids and fats are essentially universal, the basic logic behind feeding regulation is likely to be conserved. Significant genetic conservation has been described in the regulation of metabolism and feeding with orthologous peptidergic signaling systems regulating glucose homeostasis and food intake (Baker and Thummel, 2007; Leopold and Perrimon, 2007; Melcher et al., 2007). Although chemosensory receptors mediating olfaction and taste detection are not genetically homologous to mammalian counterparts, the functional organization of these systems is conserved (Scott, 2005; Vosshall and Stocker, 2007). Predictably *Drosophila* feeding is also regulated by molecular and cellular mechanisms with no obvious homology to vertebrates (Hergarden et al., 2012; Lee et al., 2004). Despite the wealth of data that has been gathered during the past few decades, how these signaling systems achieve the behavioral aspects of feeding regulation is still relatively unclear.

How is plasticity achieved in fruit fly feeding? Measuring behavioral thresholds to appetitive substances has been historically used in both vertebrates as well as invertebrates to tease apart the mechanisms underlying food intake regulation (Dethier, 1976). Nutritional state is by far the most influential parameter influencing the behavioral

thresholds to food. One of the hallmarks of regulation by the internal state is a decrease in food acceptance threshold that correlates with deprivation time. In *Drosophila* and related insect species, the behavioral sensitivity to different carbohydrate concentrations can vary by three to five orders of magnitude as a function of starvation time (Inagaki et al., 2012; Marella et al., 2012; Minnich, 1929). Feeding thresholds are subject to rapid modulation during and after feeding with a number of factors implicated to underlie this phenomenon including a decay in a central excitatory drive, volumetric factors from gut distention, adaptation of chemosensory neurons, nutrient and humoral factors in hemolymph communicating negative feedback to the brain (Gelperin, 1971a). The molecular and cellular processes underlying feeding plasticity and their relative contribution to food intake regulation is actively pursued. To understand regulation of feeding, it is important to obtain a rigorous quantitative description of the organization of feeding behavior. Previous attempts to achieve that are reviewed below.

2.1. Motor-action patterns in feeding behavior of an adult *Drosophila melanogaster*

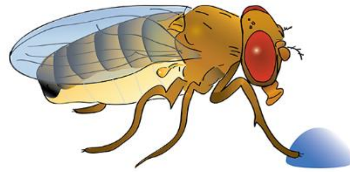
Akin to other species (Dethier, 1976) feeding behavior in *Drosophila* is comprised of a series of modules or fixed action patterns (Figure 1). In a food deprived state, an adult fruit fly will forage (I) to locate potential food sources. This is achieved either by elevating its locomotion to increase the probability of encountering food or by taxing towards food predicting odorant sources (Lee and Park, 2004; Root et al., 2011). Encountering a palatable food source will lead to food engagement (II) by chemosensory detection triggered cessation of locomotion. While the fly walks, chemosensory bristles on tarsi are sampling the environment and an appropriate chemosensory signal will terminate the food search phase of the behavior. The same stimulation of tarsal chemosensory bristles will trigger meal initiation (III) with the fly extending its proboscis to the food source. Stimulation of chemo- and mechanosensory bristles on the labellum (sensory organ at the tip of the proboscis) will trigger and sustain ingestion (IV). Physical and endocrine signals generated during the drinking bouts will terminate the meal and preclude further attempts to feed (V). The same post-ingestive signals enable the fly to disengage from the food source (VI) by reactivating its locomotion.

Physiological deprivation changes the probability of generating all of the feeding sub-programs. Periods of starvation will trigger foraging in an apparent propensity to tax towards food predicting odorants (Beshel and Zhong, 2013; Root et al., 2011) and increased locomotion (Hergarden et al., 2012; Lee and Park, 2004), increased probability of initiating meals (Inagaki et al., 2012), prolonged consumption bouts and decreased probability of meal termination (Al-Anzi et al., 2010; Edgecomb et al., 1994). These behaviors also depend on the intensity of appetitive sensory signals available to the animal, with higher concentrations of nutrients leading to more reliable rates of meal initiation and ingestion volume (Edgecomb et al., 1994; Inagaki et al., 2012; Marella et al., 2012). Increasing food intake is contingent on successful sequential execution of each of these steps. Conversely in a sated state, the probability of observing any of these behaviors is very low. The clear modular organization of feeding behaviors in the fruit fly provides an opportunity to precisely operationally define terms such as hunger, satiety and specific

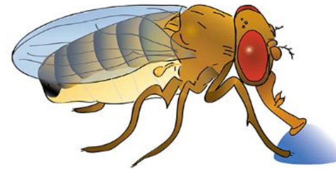
appetites in terms of deprivation time and production of particular feeding behavioral patterns.

1. Foraging

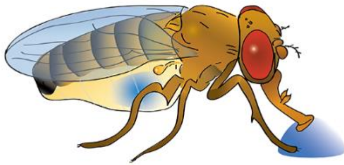
2. Food engagement



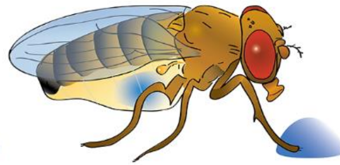
3. Meal Initiation



4. Consumption/ ingestion



5. Meal termination



6. Food Disengagement

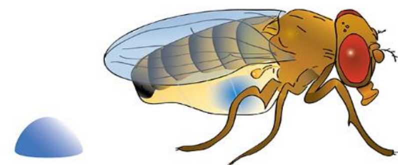


Figure 1.1. Motor-action patterns in the feeding behavioral repertoire in *Drosophila melanogaster*.

2.2. Quantification of feeding behaviors

The basic unit of feeding is a meal which is defined as an uninterrupted bout of consumption (Dethier and Gelperin, 1967). Total food intake is a function of meal size and meal frequency, with meal size depending on meal duration and ingestion rate (Brobeck, 1955; Chapman and de Boer, 1995). In *Drosophila,* the rate of ingestion is relatively constant making meal duration the major meal variable (Manzo and Scott, 2012). The parameters of meal size and frequency in turn depend on the expression of feeding motor action patterns, with meal frequency being determined by foraging, food engagement and meal initiation rates while meal size depends on ingestion time and probability of meal termination. Adjustments to these parameters allow animals to maintain a stable energy balance. It is important to note, however, that an increase in one of the food intake parameters e.g. ingestion duration does not necessarily result in an overall increase in food intake as there may be compensatory changes in other feeding behaviors such as meal

initiation probability (Al-Anzi et al., 2010). This underscores the importance of quantifying both the overall food intake as well as individual feeding motor action patterns.

A rich selection of assays have been developed to quantify feeding. Many assays allow evaluation of overall food intake irrespective of behavioral steps leading up to consumption. The easiest and one of the earliest assays developed relied on mixing food with an inert food dye or radioactive tracer and quantifying food intake by qualitative evaluation or spectroscopic/radiographic measurements of the consumed food marker (Carvalho et al., 2005; Min and Tatar, 2006; Tanimura et al., 1982). These strategies however require sacrificing flies and affording only population measures. An alternative to this approach is the capillary feeder (CAFÉ) assay, that was initially developed for larger insects and was recently adapted for *Drosophila* (Dethier, 1976; Ja et al., 2007). In this case, either a single or a population of flies are allowed free access to a capillary containing a nutritive substance while displacement of the solution in the capillary is measured as a proxy for food intake. Although drinking from a capillary requires some adjustment and is subject to distortion by evaporation, the method has proven popular (Masek and Scott, 2010; Stafford et al., 2012). Other proxies for food intake such as measuring dissected crop volume after a feeding episode have been employed but have not found wider use (Melcher and Pankratz, 2005).

Similarly, many assays are available to quantify individual feeding motor programs. Several single fly and population assays have been developed to quantify foraging including beam crossing assays for activity measurements as well as olfactory traps, 4 field olfactory preference assays and a single dish video-tracking assay (Beshel and Zhong, 2013; Ditzen et al., 2008; Lee and Park, 2004; Root et al., 2011). Proboscis extension reflex assay is widely used to quantify meal initiation rates (Dethier, 1976; Gordon and Scott, 2009; Wang et al., 2004). Quantification of the probability of extending the mouthparts in response to stimulation of gustatory receptor neurons enables easy and scalable evaluation of the feeding thresholds without the accompanying consumption. Several assays have been developed to selectively monitor ingestion. Temporal consumption assay where flies are allowed to consume to satiety with drinking time being monitored is easy and scalable (Cameron 2010). More laborious solutions involving the CAFÉ assay and direct monitoring of feeding events have been used to selectively quantify consumption, but are restrictive due to time and labor intensity (Ja et al., 2007; Wong et al., 2009). No standardized assay currently exists to quantify food engagement.

On top of changes to the behavioral repertoire and gross intake, energy homoeostasis can also be altered by changes in efficiency of nutrient uptake that is not necessarily reflected in food input/output balance (Wong et al., 2008). Therefore, biochemical assays are employed to quantify triglycerides and carbohydrates as a proxy for internal energy reserves (Al-Anzi et al., 2009; Meunier et al., 2007). Finally, a range of assays have been developed to investigate food preference and memory functions related to nutritional state that are instrumental in studying feeding choices (Ja et al., 2007; Krashes et al., 2009; Tanimura et al., 1982).

3. Circuits and signaling systems controlling feeding behavior

With the exception of peripheral sensory neurons and motor neurons, little is known about the neural circuits generating feeding behaviors. The modular organization of feeding behavior is likely to be reflected in the underlying circuit organization. Each of the feeding fixed action patterns is achieved by virtue of a simple sensory-motor transformation: during foraging, odorant cues drive activity in populations of olfactory sensory neurons that is read out and converted into directed movement towards the appetitive odor source; food engagement and meal initiation is driven by stimulation of chemosensory sensilla on tarsi leading to cessation of locomotion and activation of motorneurons that coordinate proboscis extension respectively; ingestion results from ongoing activity in labellar gustatory and mechanosensory neurons that is required to maintain activity in motorneurons innervating the ingestion mediating cibarial pump muscles (Dethier, 1976; Rajashekhar and Singh, 1994a). These subcircuits provide the scaffolding that is necessary to contextualize the myriad of neuromodulatory and regulatory systems impinging on the feeding system. Currently, we do not know the full sensory-motor circuit for a single feeding motor action pattern. With the advent of whole brain Ca^{2+} imaging and anatomical circuit tracing tools, rapid progress in mapping these circuits is to be anticipated (Ahrens et al., 2013; Datta et al., 2008).

Anatomic work as well as molecular genetic approaches have started to shed light on some of the key circuit nodes involved in feeding regulation. Early genetic screens and candidate approaches identified both transcriptional as well as molecular signaling pathways that influence food intake (Al-Anzi et al., 2010; Hergarden et al., 2012; Lee et al., 2004; Melcher and Pankratz, 2005; Wu et al., 2005a; Zinke et al., 1999), albeit often with low anatomical resolution and no clear causal cells for behavior. Excitingly, screens involving perturbations of neuronal activity and careful functional studies have recently started to yield single neurons with distinct roles in feeding (Flood et al., 2013; Mann et al., 2013; Marella et al., 2012; Miyamoto et al., 2012). Despite these advances, there are many things about feeding regulation that we do not understand. How are specific hungers detected and represented in the brain, how is taste information integrated with need sensing and satiety signals, how are the thresholds for appetitive behaviors established and how are they gated and triggered?

What is required to achieve a circuit level understanding of feeding? Identification of causal links between sensory input, circuit activity and behavioral outputs stipulates identification of individual neurons making up the circuit, defining the signals that drive activity in these neurons and elucidating how activity in these cells contributes to behavior and describing the synaptic, cellular and circuit level mechanisms that underlie the transformation carried out by the circuit (Clark et al., 2013; Olsen and Wilson, 2008). Although the field is still far from even knowing all the circuit components, the complexity of the fruit fly brain and the rapid development of new tools makes the ambition of a complete circuit level description of feeding regulation a realistic goal. Below, I will review the current knowledge of circuits underlying *Drosophila* feeding with an emphasis on identifying the circuit nodes that are likely targets of modulation to achieve plasticity in food intake.

3.1. Sensory systems

The fruit fly relies predominantly on its chemical senses to locate and evaluate potential food sources in the external environment. Olfaction meditates detection of long range volatile chemical cues whereas gustatory receptor neurons mediate contact chemoreception that gates acceptance and rejection behaviors based on the chemical composition of encountered substances (Stocker, 1994). As sensory neurons are easily accessible, they are one of the best described elements of the feeding circuit.

3.1.1. Olfaction

Detection of odorants is mediated by olfactory receptor neurons (ORNs) that reside in the antennae or maxillary palps. Each ORN expresses an odorant binding receptor protein belonging to either Olfactory Receptor (OR) or Ionotropic Receptor (IR) family of genes (Benton et al., 2009; Vosshall et al., 1999). ORNs expressing the same receptor project to a dedicated glomerulus in the antennal lobe (AL) thus segregating odor information both anatomically as well as functionally. Projection neurons (PNs) make up the second order neurons in the olfactory circuit. Most PNs innervate a single glomerulus and project in turn to higher order brain centers – the mushroom body (MB) and the lateral horn (Vosshall and Stocker, 2007). The MB is a dedicated structure for associative learning where odor information gets paired with unconditioned stimuli to form odor associations to approach and avoidance behaviors (de Belle and Heisenberg, 1994). Odorants that predict food cues obtain an attractive behavioral valence in a nutritional state dependent manner (Krashes et al., 2009). The lateral horn, in contrast, has been suggested to mediate experience independent odor recognition (Tanaka et al., 2004).

Olfactory cues are either innately attractive/repulsive or can obtain a behavioral valence as a result of associative learning or a change in physiological state (Gerber et al., 2009). For example, ORNs expressing *Or56a* detect geosmin an odorant produced by microbes harmful to *Drosophila*, which drives innate odor avoidance (Stensmyr et al., 2012). Conversely, many odorant cues, such as apple cider vinegar and complex fruit odorant mixes, are innately attractive to *Drosophila* and trigger increased food seeking responses in starved states (Beshel and Zhong, 2013).

There is significant evidence that food intake particularly at the level of foraging is already regulated at the level of ORNs. Root and colleagues showed that ORNs expressing *Or42b* are required for apple cider vinegar driven food search behavior. Crucially starvation-derived behavioral sensitivity to cider vinegar was mediated by sNPF signaling that increases presynaptic release in *Or42b* cells in a starvation-dependent manner. Interestingly, the OSNs driving the approach behavior are both the source as well as the target of sNPF signaling. Starvation-dependent plasticity is achieved by downregulating sNPFR expression in OSNs by a reduction of a global insulin signal that correlates with satiety (Root et al., 2011). This is intriguing as dILP release has been shown to correlate with starvation (Geminard et al., 2009; Ikeya et al., 2002) and sNPF was previously demonstrated to promote food intake (Lee et al., 2004). However, an independent study found no evidence of increased sNPFR expression in starved olfactory sensory organs

(Farhadian et al., 2011). It remains to be determined whether the same mechanism is responsible for other hunger state-dependent food seeking responses.

3.1.2. Taste

The taste system is the sensory modality most intimately involved in feeding decisions. Taste detection is tightly coupled to innate behaviors leading to either food acceptance or rejection (Yarmolinsky et al., 2009). The fruit fly uses chemosensory sensilla on its legs, proboscis and internal mouthparts to evaluate the nutritional content and safety of potential food sources. Each taste sensillum is innervated by 2 – 4 modality specific gustatory receptor neurons (GRNs) (Scott, 2005; Stocker, 1994). Modality specificity of GRNs is achieved by non-overlapping expression of several families of receptor genes. Appetitive substances such as carbohydrates and fatty acids are detected by a population of GRNs that expresses members of the Gr family of gustatory receptor neurons including *Gr5a*, *Gr64a-f* and others (Masek and Keene, 2013; Thorne et al., 2004; Wang et al., 2004). Bitter compounds are detected by GRNs expressing a different subset of Gr genes including *Gr66a* and others. Water-sensitive GRNs detect low osmolarity by expressing *ppk28* - a member of the Deg/ENaC family of ion channels (Cameron et al., 2010). The *Drosophila* taste system also detects carbonation, reproductive pheromones and salinity (Fischler et al., 2007; Thistle et al., 2012; Zhang et al., 2013). Behavioral evidence also suggests that amino acids are sensed by the taste system however the molecular basis for this remains elusive (Ribeiro and Dickson, 2010). Although the receptor genes are distinct, the functional principles underlying the organization of the taste system in insects and vertebrates appear to be conserved (Scott, 2005). GRNs of a particular modality project to distinct locations in the central nervous system forming modality and organ specific taste maps (Wang et al., 2004). The second order neurons receiving input from GRNs have currently not been identified.

Evidence from locusts and blowflies suggests that metabolic state controls the sensitivity of GRN responses to sugars and amino acids which indicates that feeding thresholds are at least in part established already at the taste sensory neuron level (Abisgold and Simpson, 1988; Amakawa, 2001; Simpson et al., 1991). There is conflicting evidence whether similar starvation state dependent modulation of GRN sensitivity occurs in *Drosophila* (Inagaki et al., 2012; Meunier et al., 2007). Inagaki and colleagues recently demonstrated that while GRN tuning to sugars does not change with starvation, the evoked presynaptic Ca^{2+} influx at the first order taste relay and consequently the presynaptic release probability correlates with deprivation time. The authors showed that dopaminergic signaling through the DopEcR receptor in GRNs is responsible for the increased presynaptic Ca^{2+} responses in GRN terminals and that DA signaling in GRNs is required to reduce feeding thresholds after short but not long term starvation (Inagaki et al., 2012). Although DA clearly modulates meal initiation rates in *Drosophila*, the source of this signaling remains elusive. An independent study recently identified a single DA neuron in the SOG that controls meal initiation probability in an activity dependent manner. However its effects seem to be mediated by a different DA receptor (Marella et al., 2012). Even though GRNs sensitivity appears to be adjusted to the nutritional state in a number of

insect species, the contribution of this modulation to the overall feeding threshold is likely to be limited (Dethier, 1976; Gelperin, 1966).

3.2. Need and satiety sensing systems

What are the internal signals that the central nervous system keeps track of to adjust food intake? Evidence from both vertebrates as well as insects suggests that animals have evolved multiple feedback mechanisms to monitor the physiological status of the organism (Miyamoto et al., 2013). Recently, a number of studies have convincingly shown that fruit flies are capable of detecting caloric content of food independently of taste and to adjust their feeding preferences accordingly (Burke and Waddell, 2011; Dus et al., 2011; Fujita and Tanimura, 2011; Stafford et al., 2012). Insects can clearly distinguish between imbalances in carbohydrate and amino acid metabolism as well as hydromineral balance (Dethier and Evans, 1961; Ribeiro and Dickson, 2010; Simpson et al., 1991).

There are four strategies for how the nervous system could keep track of the metabolic needs of an organism: (a) it could directly monitor the level of nutrients in circulation by specialized interoceptive neurons; (b) the nervous system could read out an endocrine cue secreted by a peripheral nutrient sensing tissue that communicates available energy reserves; (c) the nervous system could monitor the physicochemical properties of the post-ingestive load such as detecting the distention of the digestive tract; (d) the physiological state may be monitored by assessing energy availability locally by means of cell autonomous fuel sensing mechanisms in a population of food intake regulating interneurons. Excitingly, significant progress has been made recently in describing the molecular and cellular underpinnings of these processes in *Drosophila*, which is reviewed below.

3.2.1. Peripheral nutrient sensing

In *Drosophila* several endocrine organs such as corpus cardiacum (CC) and the fat body (FB) are responsible for coupling metabolism and systemic growth to nutritional state through signals in the hemolymph (Leopold and Perrimon, 2007). Whether CC and fat body-derived signals directly influence feeding decisions in adult *Drosophila* however is currently much less clear.

The fat body combines the functions of mammalian adipose tissue and liver (Buch and Pankratz, 2009). It monitors available carbohydrate, amino acid and fat levels and modifies physiology by secreted humoral signals (Geminard et al., 2009; Rajan and Perrimon, 2012). Interestingly, some of those signals directly control activity and insulin release in medial neurosecretory cells (mNSCs) in pars intercerebralis (PI) in the brain which as a population have previously been shown to exercise limited effects on food intake (Söderberg et al., 2012; Wu et al., 2005a). The fat body expresses and secretes the cytokine Unpaired 2 (Upd2) proportionally to available carbohydrate and fat reserves. Increased Upd2 levels activate the JAK/STAT signal transduction pathway through its cognate receptor Dome in central GABA-ergic neurons downregulating their activity. As these inhibitory neurons synapse onto *Drosophila* insulin like peptides (dILPs) secreting

mNSCs, increased levels of Udp2 result in increased dILP release proportionally to the nutritional state. Although Upd2 bears all the hallmarks of an 'adiposity signal', such as mammalian leptin that suppresses feeding proportionally to organismal lipid reserves, knocking down Upd2 in the fat body does not increase nutrient consumption in adult flies. This raises the possibility that there are other signaling mechanisms that link feeding behavior to peripheral nutrient sensing (Rajan and Perrimon, 2012). Available amino acid levels in the fat body as detected by TOR signaling were similarly shown to correlate with dILP release from mNSCs through an unidentified secreted humoral signal. However the effect of blocking amino acid sensing in the fat body on food intake has not been explored (Geminard et al., 2009). Although blocking a FB based amino acid catabolic pathway in larvae has been shown to perturb their feeding behavior, extrapolating larval findings to adults should be done with caution (Zinke et al., 1999).

Corpus cardiacum plays an important role in glucose homeostasis. CC cells depolarize and secrete the adipokinetic hormone (AKH) under conditions of energy deprivation. Depolarization of CC cells is regulated by a potassium channel that is sensitive to the intracellular ADP/ATP ratio. AKH mobilizes stored energy reserves by triggering lipolysis, glycogenolysis and trehalose release in the FB (Kim and Rulifson, 2004). Manipulation of activity in CC cells however has yielded either no or only modest feeding phenotypes related to foraging behavior (Gruber et al., 2013; Lee and Park, 2004; Marella et al., 2012). Mutation of the AKHR receptor however was shown to reduce post-starvation feeding by three fold although steady state food intake appears to be normal (Bharucha et al., 2008). As AKH has profound effects on circulating nutrients, it is important in the future to dissociate the central effects of AKH signaling on feeding behavior from the metabolic consequences of AKH signaling in peripheral tissues.

The feeding phenotypes produced by manipulating known peripheral metabolic signaling pathways involved in global energy homeostasis have been relatively subtle. It remains to be determined whether the effects of peripheral nutrient sensing mechanisms on feeding are communicated by as yet undiscovered signaling mechanisms or whether FB and CC have only a limited effect on coordinating behavior.

3.2.2. Central nutrient sensing

The nutritional state of the organism can also be monitored directly in the central nervous system by dedicated interoceptive neurons (Sternson, 2013). As hemolymph composition reflects the nutritional state and recent feeding history of the animal, hemolymph metabolites could serve as a basis for feedback to regulate food intake and feeding decisions. Circulating carbohydrates have been shown to be detected in mammalian brains and have also been proposed to regulate feeding in insects (Burdakov et al., 2005; Chapman and de Boer, 1995). The main circulating carbohydrate in insects is trehalose. In many insects, trehalose levels change as a function of starvation state and would thus be an appropriate signal for feeding regulation (Thompson, 2003). Whether circulating carbohydrates directly control feeding thresholds has been much less clear. Early work in blowflies suggested that direct injection of various carbohydrates to hemolymph had no effect on regulating feeding thresholds (Dethier and Evans, 1957; Evans, 1961; Hudson, 1958). A more contemporary study has recently contradicted these

findings showing that injection of trehalose to the blowfly hemocoel does alter meal initiation probability (Amakawa 2001). The results of these classic experiments however should be interpreted with caution as it is difficult to distinguish between direct and indirect effects of altering levels of circulating carbohydrates.

There is conflicting data on whether trehalose levels change during starvation in *Drosophila* (Meunier et al., 2007; Miyamoto et al., 2012) and no molecular mechanism for direct central trehalose detection has been uncovered. However Miyamoto and colleagues recently identified a small population of fructose-sensitive cells in the central brain that detects circulating fructose levels in a Gr43a dependent manner (Miyamoto et al., 2012). Although fructose is present in only very small quantities in *Drosophila* hemolymph, its levels increase three to tenfold after a carbohydrate meal. Importantly, activity in Gr43a positive brain cells influences feeding in a satiety state dependent manner. In fed states, it reduces intake of nutritious sugars whereas in deprived states activity in protocerebral Gr43a positive cells is required to discriminate nutritious foods in a taste independent manner. Intriguingly *Gr43a* belongs to the Gr family of gustatory receptors that apart from taste tissues are widely expressed throughout the organism including in tissues implicated in nutrient sensing (Park and Kwon, 2011; Thorne and Amrein, 2008). It is feasible that other members of the Gr family detect additional parameters of energy homeostasis either directly in the CNS or in peripheral tissues. In light of macronutrient sensing cells in the mammalian brain, it is likely that insect nervous systems have similarly evolved internal chemosensory mechanisms to directly monitor levels of circulating amino and fatty acids (Oomura et al., 1975; Wang et al., 2006).

Alternatively to central nutrient sensing by dedicated interoceptive sensory neurons in the brain, feeding can also be regulated by cell autonomous mechanisms for detecting energy availability in interneurons modulating feeding behavior. In mammals, manipulating the activity of mTOR (mammalian Target of Rapamycin), a serine-threonine kinase that signals proportionally to nutrient availability, in subpopulations of hypothalamic orexigenic and anorexic neurons has been shown to affect bulk food intake (Cota et al., 2006). A similar mechanism has been shown to influence feeding decisions in *Drosophila* larvae where manipulating the activity of dS6K – a nutritional state sensitive kinase downstream of dTOR – either in insulinergic mNSCs or neurons expressing the receptor for the orexigenic peptide NPF was shown to regulate larval feeding decisions (Wu et al., 2005a, 2005b). Manipulating dTOR signaling pan-neuronally in the adult fruit fly brain has been shown to influence food choice but the cellular substrates for this effect are unknown (Ribeiro and Dickson, 2010).

3.2.3 Gut derived post-ingestive food intake regulation

In most vertebrate and invertebrate species systemic homeostasis is not restored during the course of a meal. However the feeding thresholds are rapidly modulated during and shortly after food ingestion and remain elevated for an extended duration (Dethier, 1976; Woods et al., 1998). This indicates that feeding thresholds are at least in part established by signals emanating from the digestive tract.

The *Drosophila* digestive tract is similar to that of other dipterans. The main compartments of the alimentary tract are foregut, midgut and hindgut. In addition to the

main digestive tract, the flies also have a crop, which is an elastic storage organ emanating from the foregut immediately anterior to the proventriculus (foregut/midgut junction). The crop is filled with fluid at the time of feeding and emptied slowly by propagating its contents periodically to the midgut which is the primary site for digestion and nutrient absorption. The fruit fly digestive system is only partially innervated: part of the foregut, the crop and the anterior midgut is innervated by the hypocerebral ganglion that receives input and projects to the brain via the recurrent nerve (Fig. 1.2). The hindgut is innervated through projections carried in the abdominal nerves emanating from the abdominal ganglion (Lemaitre and Miguel-Aliaga, 2013).

No experimental dissection of gut derived feedback mechanisms on food intake regulation has currently been reported in *Drosophila*. However classic experiments on feeding regulation in the black blowfly *Phormia regina* implicated two key neural mechanisms that provide acute post-ingestive negative feedback to feeding. These structures include the recurrent nerve, that connects the visceral nervous system to the central brain, and the medial abdominal nerve, that emanates from the abdominal ganglion and arborizes throughout the abdomen (see Fig. 1.2).

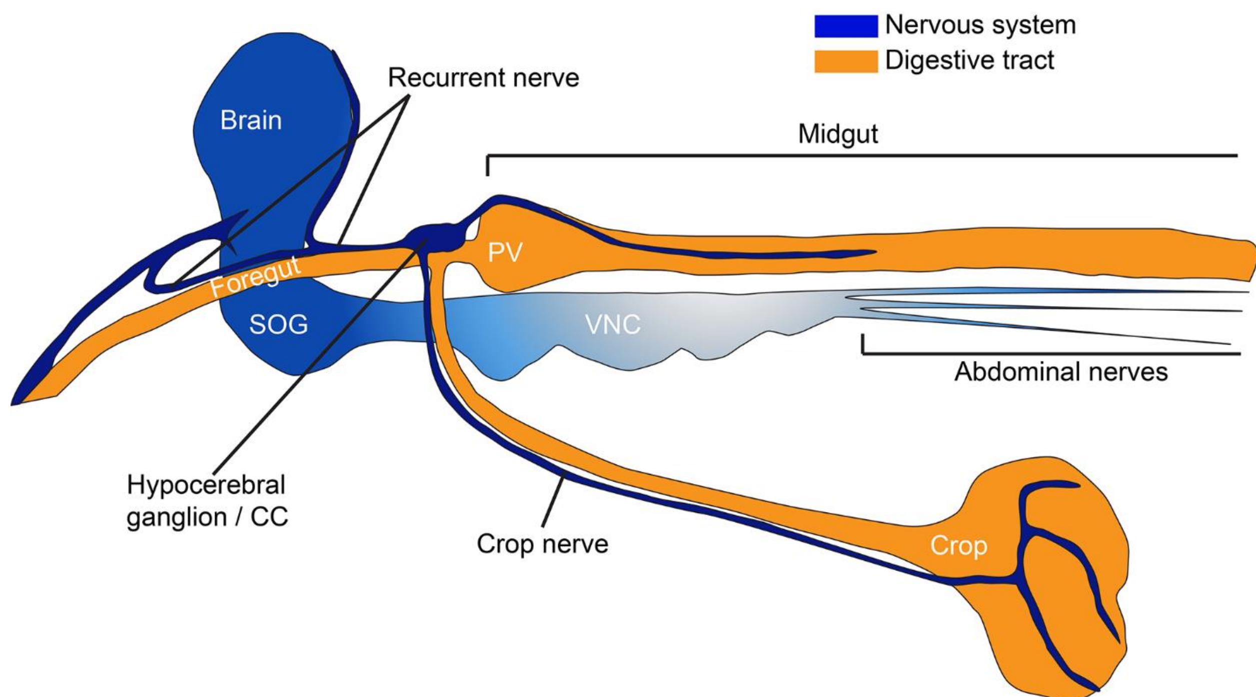


Figure 1.2. Stomatogastric nervous system and the digestive tract in *Drosophila melanogaster*. CC – neuroendocrine organ corpus cardiacum; PV – proventriculus, the transition zone between foregut and midgut; VNC – ventral nerve cord; SOG – suboesophageal ganglion.

3.2.3.1. Recurrent nerve

The search for the physiological mechanism responsible for the elevation of the feeding threshold after a carbohydrate meal in blow flies yielded a number of surprising observations. The elevation of circulating carbohydrates had been ruled out earlier by direct injections of trehalose and other carbohydrates to the hemocoel (Dethier and Evans, 1957). The contribution of some post-meal humoral signal secreted to the circulation was eliminated by two key experiments. First, injection of hemolymph from a freshly fed fly to starved flies failed to elevate the feeding threshold (Dethier and Bodenstein, 1958). Moreover, experiments with parabiotic flies where the circulation of two flies were surgically combined exposing each fly to the humoral and metabolic signals stemming from the other fly argued against a post-meal humoral signal in feeding regulation. When the paired flies were starved and only one fly was allowed to feed, then the feeding thresholds were elevated only in the fed fly with no effects on meal initiation probability in the unfed parabiotic fly (Dethier et al., 1965).

These preliminary experiments pointed toward the digestive tract as the site for meal threshold regulation. Dethier and colleagues tested whether sugar solutions in different compartments of the digestive tract could be sufficient to regulate feeding probability. Through a series of elegant experiments, they were able to show that the presence of food in the crop, midgut as well as the hindgut is dispensable for food intake modulation (Dethier and Bodenstein, 1958; Dethier and Evans, 1957). However, the only section of the digestive tract that had a reliable effect on feeding thresholds upon exposure to food was a part of the foregut in front of the proventriculus. As this portion of the alimentary canal is innervated by the hypocerebral ganglion, the authors sought to denervate this region by severing the recurrent nerve, which connects the hypocerebral ganglion to the central brain. This manipulation resulted in a dramatic overconsumption of sugar solutions leading to continuous attempts to feed and extreme bloating (Dethier and Bodenstein, 1958). These results demonstrate that feedback from the foregut carried by the RN is a major source of feeding inhibition in insects regulating both meal initiations as well as overall food intake. It is necessary to specify that the recurrent nerve carries fibers from several populations of neurons including neuroendocrine projections to and from the mNSCs as well as projections from the hypocerebral ganglion to the SOG. Follow up experiments with RN lesions in front of the brain convincingly showed that the fibers connecting the HCG with the SOG carry the pertinent food intake regulating negative feedback (Dethier and Gelperin, 1967).

Belzer carried out a careful characterization of the RN lesion in derived overconsumption phenotype and showed that severing the recurrent nerve leads to an overconsumption of carbohydrate and protenacious solutions as well as solutions containing low concentrations of salt. Interestingly, the flies don't consume 1M NaCl solution and that in choice situations RN lesioned flies strongly prefer to overfeed on carbohydrate solutions, demonstrating that the consumption phenotype is subject to modulation by the taste system (Belzer, 1978). There is conflicting evidence whether RN lesioning leads to abnormal water consumption (Dethier and Evans, 1961; Dethier and Gelperin, 1967; Evans and Browne, 1960).

The RN lesioning derived hyperphagia raises a number of questions about the nature of negative feedback carried by the recurrent nerve. Is the negative feedback specific for a particular nutrient or tastant class or is this a proprioceptive mechanosensory signal? Only two studies by a single author have addressed this question in detail supporting the latter scenario. Behavioral evidence shows that the only parameter relevant for feeding thresholds is the duration of crop emptying with no significant contribution by osmolarity, nutritional value or the type of carbohydrate consumed (Gelperin, 1966). This led the author to conclude that the negative feedback carried by the RN stems from detecting the foregut stretch that is accompanied by crop emptying. Indeed, electrophysiological recordings from the recurrent nerve revealed two units that responded to foregut peristalsis and artificial pressure application to the digestive tract. The source of this activity was assumed to stem from two bipolar neurons in the recurrent nerve based on visual inspection, but no data was provided (Gelperin, 1967).

3.2.3.2. Abdominal nerve

The role of abdominal nerve system in feeding was first identified in follow up studies of another hyperphagic blowfly phenotype. Curiously, severing the cervical connective that connects the brain and the thoracic ganglion in the blowfly was shown earlier to cause extreme hyperphagia which sometimes led to bursting of the abdomen (Dethier and Gelperin, 1967; Nunez, 1964). Dethier and colleagues dissected putative neural pathways underlying this effect and found that severing the abdominal nerves, that stem from the abdominal ganglion and form a network of projection in the abdomen, led to a fourfold increase in carbohydrate drinking times, suggesting that abdominal nerves carry some sort of negative feedback to central feeding circuits (Dethier and Gelperin, 1967). Gelperin characterized the abdominal nerve network in greater detail and narrowed down the feeding regulatory function to neurites in the medial abdominal nerve (mAN) and the first two branching points after the nerve leaves the abdominal ganglion. Nerve recordings from mAN revealed that projections in this structure contain stretch sensitive units that respond with elevated activity in response to stretching the nerve or increasing abdominal pressure during feeding. Visual inspection of the nerve revealed a group of 4-8 cells at the branching points of the mAN that were assumed to underlie these observations (Gelperin, 1971b). Based on these data, the abdominal nerve network was proposed to sense increased distention of the abdomen that is produced by crop filling during a meal. Although intriguing, these findings have currently not been independently confirmed.

The RN and mAN lesioning experiments in the blowfly seem to suggest a surprisingly simple model for feeding regulation in insects whereupon a state of satiety and elevated feeding thresholds are maintained by inhibitory proprioceptive feedback from the foregut and abdomen.

3.2.4. Osmosensation

Internal osmosensory mechanisms have not been explored in *Drosophila melanogaster*. However, several genes relevant to osmoregulation have recently been identified. External osmosensation is mediated by members of the ppk family of TRP

channels. *ppk28* is expressed in gustatory receptor neurons and acts as an osmosensitive water taste receptor (Cameron et al., 2010; Chen et al., 2010). Furthermore, two ppk channels *water witch* and *nanchung* have been implicated in external hygrosensation detecting humidity and dry air respectively (Liu et al., 2007). Cognigni and colleagues recently identified a role for the neuropeptide leucokinin in fluid homeostasis. Activation of leucokinin-Gal4 positive neurons promotes diuresis whereas inactivation compromises it with extreme fluid accumulation outside the digestive tract resulting from prolonged perturbation of leucokininergic cells. Gene knockdown experiments suggest that diuresis relevant leucokinin receptor (LKR) expressing cells are located outside the nervous system, whereas the source of leucokinin is neuronal (Cognigni et al., 2011).

Studies in the blowfly suggest that water consumption is independently regulated from nutrient intake (Dethier and Evans, 1961). Hemolymph pressure has been proposed to be the key determinant for water responsiveness as injections of both hyper- and hypotonic solutions led to reduced water consumption while bleeding leads to increased drinking. This argues that instead of osmotic or ionic composition of hemolymph, the water drinking decisions are driven by volumetric factors (Dethier and Evans, 1961; Evans, 1961). There is conflicting evidence on the role of stomatogastric nervous system in water intake regulation, with some reports arguing that recurrent nerve lesions lead to overconsumption of water (Dethier and Evans, 1961; Evans and Browne, 1960) and yet other reports claiming the contrary (Dethier and Gelperin, 1967). A systematic study of the neural basis of osmoregulation in *Drosophila* is warranted to clarify these contradictory findings.

3.4. Central effector pathways

Out of all the food intake regulating systems, central mechanisms integrating taste and satiety related information and producing feeding behaviors remain the most poorly understood. Despite early recognition that central mechanisms are major determinants for setting feeding thresholds, the lack of experimental tools precluded detailed analysis for a long time (Gelperin, 1967). *Drosophila* as a model system has now overcome these problems and has started to yield significant insight into central circuits governing food intake in the past few years.

3.4.1. Neuromodulatory systems

A dozen neurotransmitter and neuromodulatory systems in *Drosophila* have been implicated to date in food intake regulation. Many of the signaling systems appear to be functionally conserved throughout evolution including orthologs for mammalian peptidergic signals tachykinin, cholecystokinin, neuropeptide Y, Neuromedin U and insulin. Analysis of their function however has been significantly compromised by the lack of basic sensory motor circuits that these systems are likely to act on. Nevertheless, a few interesting conclusions can be drawn based on the currently available data.

Drosophila neuropeptide F (dNPF), a homolog of the mammalian orexigenic peptide NPY, was first shown to have a significant role in larval feeding. Pan-neuronal

overexpression of dNPF led to an extension of the continuous feeding phase of larval development whereas perturbation of dNPF signaling reduced the propensity to feed on hard to access or tainted foods and results in early cessation of the larval feeding phase (Wu et al., 2003, 2005b). The feeding on normal foods, however, was unaffected by manipulations of the dNPF system. Interestingly, dNPF expression is high in continuously feeding larval stages and is downregulated upon cessation of feeding at the end of the 3rd instar phase (Wu et al., 2003). In adult fruit fly, dNPF signaling appears to have a more nuanced role in food intake regulation showing specific effects for particular feeding motor action patterns. dNPF neurons appear to be required for starvation induced foraging by showing increased preference for food odorants (Beshel and Zhong, 2013) but are dispensable for post-starvation derived increased meal initiation (Marella et al., 2012) and compensatory food intake (Al-Anzi et al., 2010). Overactivation of dNPF neurons in adults however appears to parallel larval phenotypes with increased intake of tainted foods following a period of starvation (Hergarden et al., 2012). Although Beshel and colleagues narrowed down the foraging relevant dNPF signal to 4 neurons in the brain, the source and target for the rest of the feeding phenotypes of dNPF signaling are yet to be determined.

Drosophila insulin like peptides (dILPS) play an important yet complicated role in regulating feeding behaviors which is confounded by their role in carbohydrate metabolism. The fruit fly has 8 different insulin like peptides with only one described insulin receptor (dInR) (Baker and Thummel, 2007; Colombani et al., 2012). In mammals, insulin has a complex physiological role in energy homeostasis. Although insulin acts as an adiposity signal in the brain suppressing food intake, perturbing insulin function will not lead to obesity despite hyperphagic symptoms in case of type 1 diabetes, since insulin is required peripherally for macronutrient uptake to tissues (Woods et al., 1998). In *Drosophila,* insulin signaling has a similarly convoluted record. Root and colleagues showed that an increase in insulin signaling from an unspecified source decreased sensitivity to food odorants by modulating presynaptic vesicle release in a subset of ORNs (Root et al., 2011). In larvae, pan-neuronal overexpression of dILP2 and dILP4 but not dILP3 led to reduction in feeding rate (Wu et al., 2005a). In contrast to these reports, there is a myriad of papers reporting insulin signaling having no discernable effects on various aspects of feeding behavior (Broughton et al., 2010; Cognigni et al., 2011; Marella et al., 2012; Wong et al., 2009). A more careful dissection of the role of different insulin peptides and their targets is warranted to clarify its role in food intake regulation.

Hugin is a homolog of the mammalian anorexigenic hormone neuromedin U, which appears to specifically regulate food engagement with novel or tainted foods by repressing the latency of initiating food intake (Melcher and Pankratz, 2005). Hugin however has no effect on meal initiation rates nor post-starvation compensatory food intake (Al-Anzi et al., 2010; Marella et al., 2012). Although a large cluster of huginergic neurons reside in the SOG, the causal subsets for this regulation are unknown.

Leucokinin and the leucokinin receptor (LKR) appear to specifically regulate meal size. Genetic and neural activity based perturbations of leucokininergic signaling led to increased meal volumes, however curiously this is compensated by changes in meal frequency rendering leucokinin signaling neutral with regard to overall caloric intake (Al-Anzi et al., 2010).

The source of the modulatory signal as well as the targets with most of the neuromodulatory systems implicated in feeding are either poorly defined or completely unknown. This is partially the case since the behavioral consequences of manipulating the modulatory systems often yield graded phenotypes which make finding the causal neurons problematic. There are however two notable exception to this rule.

Marella and colleagues recently showed that dopaminergic signaling is required for starvation derived increase in meal initiation probability. Through clonal analysis, they were able to localize this dopaminergic function to a single cell in the SOG – the TH-VUM neuron. This cell's baseline activity increases proportionally to nutrient deprivation time with high activity levels being sufficient to trigger meal initiation (Marella et al., 2012).

The second notable exception, where the source of neuromodulation is known, was recently reported by Söderberg and colleagues. They investigated the function of drosulfakinin (DSK) which is an ortholog of the canonical mammalian satiety signal cholecystokinin (Söderberg et al., 2012). DSK is expressed in several distinct clusters within the central nervous system, but the authors were able to show that manipulating DSK expression solely in a small subset of the medial neurosecretory cells co-expressing dILPS, yielded considerable nutrient intake phenotypes. Perturbing DSK expression in mNSCs increases post-starvation food intake by 40% and increases the probability of consuming tainted food, suggesting that similarly to mammals DSK signaling in fruit flies has an important role in regulating nutrient satiety. This finding is also intriguing as this may explain some of the feeding phenotypes previously ascribed to dILPs that are coexpressed in the same cells.

Quite a few feeding related neuromodulatory systems in *Drosophila* like allatostatin, sNPF and corazonin do not have vertebrate orthologs. Allatostatin was recently shown to control meal initiation rates and consumption but not foraging in an activity-dependent manner. Activation of allatostatin neurons leads to an 80% reduction in post-starvation food intake and suppression of meal initiation rates, while inactivation of allatostatinergic neurons leads flies to feed on tainted nutrient solutions (Hergarden et al., 2012). Furthermore, sNPF was shown to influence short latency food intake in fed states shown with pan-neuronal over-expression and RNAi based knockdown experiments (Lee et al., 2004). Finally, stimulation of corazonin, another insect specific neuropeptide, yielded a profound 3.5 fold increase in post-starvation nutrient intake (Hergarden et al., 2012). The causal neurons for all of these systems are unknown.

Taken together based on currently available data neuromodulatory systems appear to fine tune the expression of subsets of feeding motor action patterns.

3.4.2. Interneurons in feeding circuits

Despite decades of research, very few interneurons with a clearly defined role in feeding regulation have been characterized to date. A few recent studies, however, have been illuminating with regard to localizing specific functions to discrete circuit nodes.

Flood and co-authors identified a putative feeding command neuron (FDG-neuron) in the SOG that is labeled by NP883-Gal4 line (Flood et al., 2013). Activation of neurons labeled by this genetic marker results in spontaneous production of a variety of feeding motor programs including meal initiations, consumption of nutrients and water, cessation

of movement and postural adjustments for feeding. Inactivation of this line profoundly reduced meal initiation rates to carbohydrates, which led the authors to suspect that the line might contain feeding command neurons. Through clonal analysis, the authors identified a pair of interneurons in the SOG that were reported to trigger meal initiation upon ectopic activation and show increased sweet taste triggered activity selectively in starved states, arguing for a functional role in regulating food intake. It remains to be determined whether these neurons drive any other feeding subprograms apart from meal initiation. Nevertheless, the notion that there are command neuron populations that act as gatekeepers for various feeding behaviors is intriguing.

Another pair of interneurons that profoundly influences meal initiation rates was recently identified in the ventral nerve cord (Mann et al., 2013). PERin neurons appear to be activated by mechanosensory input from the legs and suppress meal initiation in response to locomotion. Inactivation of these cells results in constitutive proboscis extension. Curiously despite the profound effect on meal initiation, these neurons don't seem to influence ingestion in any detectable way, thus mediating suppression to only a single feeding subprogram.

These two sets of neurons together with the dopaminergic TH-VUM, Gr43a+ fructose sensing cells in the protocerebrum, and drosulfakinin secreting mNSCs in pars intercerebralis are the only central neurons with well defined roles in food intake regulation. Although a handful of studies have characterized sparse Gal4 lines with notable behavioral phenotypes upon activation and inactivation, the precise cellular substrate for these phenotypes needs to be clearly demonstrated. The advent of global Ca^{2+} imaging and improving genetic circuit tracing tools are certain to yield many more central components in the feeding circuit in the near future.

3.4.3. Motor Neurons

The motor neurons responsible for a subset of feeding behaviors including meal initiation and suction have been worked out in great detail. The movement of mouthparts and suction is controlled by 12 pairs of muscles in the fly head (Miller, 1950). 1 – 3 pairs of motorneurons control each of these muscles and have been anatomically characterized by Golgi stains and HRP fills (Rajashekhar and Singh, 1994a). These initial findings have now been corroborated by elegant molecular genetic approaches verifying the functional roles of motorneurons underlying the suction and proboscis extension subprograms of feeding (Gordon and Scott, 2009; Manzo and Scott, 2012; Tissot et al., 1998). Apart from the observation that sensory neurons do not appear to directly synapse onto motor output neurons (Gordon and Scott, 2009), nothing is known about the identity of cells that govern activity in these motor neuron populations.

Chapter 2:
Four GABA-ergic interneurons act as an essential brake on feeding in *Drosophila melanogaster*

1. Summary

Feeding is dynamically regulated by palatability of potential food sources and the energy requirements of the animal. How food consumption is controlled by extrinsic and intrinsic factors remains under intense investigation. We identified 4 GABA-ergic interneurons in the *Drosophila* brain that are required for any taste and satiety dependent feeding decisions. Inactivation of these cells results in indiscriminate and excessive consumption, independent of taste quality or nutritional state. Conversely, acute activation of these neurons significantly reduces consumption of water and nutrients. We show that their output is acutely required to express any feeding preference and that these neurons are not regulated by taste processing pathways or satiety signals. Thus, our study identifies four interneurons that impart an inhibitory tone on ingestive behavior. This inhibition suppresses a latent state of unrestricted and nonselective consumption and is a prerequisite for ingestion regulation by taste quality and physiological state.

2. Introduction

Animals regulate their food intake as a function of food availability and long term energy homeostasis. How sensing physiological deprivation signals and detection of taste gets converted to appetitive and consummatory feeding behaviors remains poorly understood (Morton et al., 2006; Sternson, 2013). Similarly to other animals *Drosophila melanogaster* adjusts its food intake to its internal metabolic needs and recent feeding history (Edgecomb et al., 1994). The fruit fly assesses the quality of potential food sources by distinct populations of sweet and bitter sensing gustatory receptor neurons that drive acceptance and rejection respectively (Wang et al., 2004). Many of the molecular signaling pathways that influence food intake in *Drosophila* have been conserved (Baker and Thummel, 2007). Despite rapid progress in our understanding of taste and feeding motor-output circuits the identity and logic of central circuits that convert taste and homeostatic signals into feeding motor-action patterns is unclear.

3. Results

To identify neurons that regulate consumption, we carried out a behavioral screen in which we inactivated neural subsets within the *Drosophila* central nervous system and monitored the effect on water consumption. An inwardly-rectifying potassium channel that prevents depolarization, Kir2.1 (Baines et al., 2001), was expressed in different neurons using a collection of Gal4 enhancer trap lines. A ubiquitous temperature-sensitive $Gal80^{ts}$ was used to repress Kir2.1 expression until adulthood and then Kir2.1 was induced by a two-day temperature-shift to inactivate $Gal80^{ts}$ (McGuire et al., 2004). Flies were allowed to consume water until they became unresponsive to further stimulation of legs an proboscis and total consumption time was monitored (Fig. 2.1a). Water-satiated controls consumed no water, whereas water-deprived controls increased intake in proportion to water deprivation time (Fig. 2.1b). 363 Gal4 lines from the INSITE collection (Gohl et al., 2011) were screened for water consumption under water-replete conditions upon neural inactivation. The majority of lines (349/363) drank water for less than ten seconds. Strikingly, the screen yielded six transgenic lines that continued to consume water for more than a minute (Fig. 2.1c), with assay termination at two minutes. The lack of water satiety in 6 Gal4 lines suggests that these lines mark neurons essential for consumption regulation.

Five of the six Gal4 lines were broadly expressed in the brain and ventral nerve cord (Fig. 2.2). One line, *98-Gal4*, showed sparse expression and was further characterized. Unlike wild-type water-deprived flies that consumed water for a brief period, selective inactivation of *98-Gal4* neurons caused a complete absence of water satiety, resulting in extreme bloating and regurgitation with continuous attempts to initiate meals despite tenfold increase in water intake (Fig 2.10a).

To examine whether the water overconsumption phenotype was similar to previously identified feeding phenotypes, we tested whether manipulation of identified neuropeptide/neurotransmitter systems altered water consumption (Al-Anzi et al., 2010; Alekseyenko et al., 2010; Bharucha et al., 2008; Cole et al., 2005; Colombani et al., 2003; Friggi-Grelin et al., 2003; Hergarden et al., 2012; Lee and Park, 2004; Li et al., 2000; Melcher and Pankratz, 2005; Nassel et al., 2008; Rulifson et al., 2002; Salvaterra and Kitamoto, 2001; Wen et al., 2005). Neither conditional inactivation with *UAS-Kir2.1*, *tub-Gal80^{ts}* or acute activation with the *UAS-dTRPA1* heat-activated cation channel (Hamada et al., 2008) elicited water overconsumption using these Gal4 lines (Fig. 2.1d), arguing that the *98-Gal4* behavioral phenotype is distinct from other feeding phenotypes and is unlikely to result from altered activity of neuromodulatory systems associated with feeding regulation.

The *98-Gal4* line labels 32 neurons in the brain, six neurons in the ventral nerve cord and approximately ten peripheral neurons projecting to the abdominal ganglion (Fig. 2.5a). Only a subset of these cells is likely to influence consumption. Eliminating neural expression of Kir2.1 by inclusion of *elav-Gal80* (Rideout et al., 2010) or *cha-Gal80* (Kitamoto, 2002) abolished water overconsumption, arguing that the phenotype has a neural basis (Fig. 2.3). Based on cell body locations and neurite projections, we were able to distinguish seven different morphological cell classes labeled by *98-Gal4* (Fig. 2.4). To determine the causal neurons for the insatiable phenotype, we used a molecular genetic approach to limit reporter expression by stochastic excision of *Gal80*, resulting in mosaic

expression of tetanus toxin C (Sweeney et al., 1995) (to inhibit synaptic transmission, TNT) and GFP in *98-Gal4* cell subsets (Gordon and Scott, 2009). Mosaic animals were tested for water consumption and categorized into two groups: animals in the 'insatiable' cohort (n=44) were behaviorally indistinguishable from *98-Gal4, UAS-TNT* flies, whereas 'wt' animals (n=105) consumed water for less than 5 seconds. Cells causal for the overconsumption phenotype were identified based on two criteria: (1) labeling and consequently silencing in all the 'insatiable' cohort brains and (2) underrepresentation in the 'wt' cohort.

Five of the seven cell types were silenced in less than half of the brains in either group with no significant overrepresentation in the 'insatiable' cohort, demonstrating that they do not underlie the overconsumption phenotype (Fig. 2.5b). Two populations of descending suboesophageal neurons (DSOG) were always present in the 'insatiable' group: DSOG1 (4 cells total) and DSOG2 cells (16 cells total) (Fig. 2.5b). The frequency of DSOG1 cells was overrepresented in the 'insatiable' cohort, whereas DSOG2 cells were labeled in all animals. In addition, the number of DSOG1 and DSOG2 cells labeled in the 'insatiable' cohort was greater than in the control group (Fig 2.5c). Eight of 44 'insatiable' brains had only DSOG1 and DSOG2 cells silenced, arguing that DSOG1 or DSOG2 influences consumption.

To further determine whether DSOG1 or DSOG2 cells are causal for the phenotype, we restricted expression to smaller subsets by screening for FLP enhancer trap lines (Bohm et al., 2010) that excised *tub>Gal80>* in *98-Gal4* subsets. This screen identified two informative FLP lines: *276B-FLP* restricted Gal4 to the four DSOG1 cells and *934-FLP* that restricted Gal4 to 10-12 DSOG2 cells (Fig. 2.5e). Exclusively silencing DSOG1 cells resulted in insatiable behavior similar to silencing all 98-Gal4 neurons, with animals consuming water in non-deprived states (Fig. 2d). Silencing DSOG2 cells did not cause overconsumption (Fig. 2.5d). These studies demonstrate that inactivation of the four DSOG1 cells is sufficient to elicit overconsumption.

Single-cell labeling of DSOG1 revealed the cell body in the ventral suboesophageal ganglion (SOG), with wide-field bilateral arborizations in the SOG and ventral nerve cord (VNC) (Fig. 2.5e). Labeling individual DSOG1 cells with a photoactivatable GFP showed that all DSOG1 cells have a similar morphology (Fig. 2.6). Single-cell clones showed dendrites (labeled with DenMark) in the SOG and axons (labeled with synaptotagmin-GFP) (Nicolai et al., 2010) in the SOG and VNC (Fig. 2.5e). The SOG contains axons from gustatory sensory neurons and dendrites of motor neurons that drive feeding (Stocker, 1994), suggesting that DSOG1 neurons are well-positioned to modulate feeding.

To examine whether DSOG1 cells selectively inhibit water consumption or generally regulate intake, we tested whether the overconsumption behavior depended on category of the taste stimulus or satiety state. Unlike wild-type flies which terminate feeding after a brief meal, flies with DSOG1 neurons expressing *Kir2.1* exhibited insatiable ingestion culminating in regurgitation, at which point measurements were terminated (Fig. 2.7a). They consumed appetitive compounds (sucrose and water) as well as aversive compounds (denatonium, 6M sodium chloride) for extended time (~150s) and increased volume (~1μl) in both food-deprived and non-deprived conditions (Fig. 2.7b, c). Surprisingly DSOG1 inactivation also led to consumption of physiologically and ecologically irrelevant substance such as 100% ethanol that was consumed despite mortality at the end of the

meal (Fig 2.7c). In contrast, flies without *Kir2.1* induction and controls did not consume under fed conditions, but consumed water and sucrose for approximately 17 seconds (150 nl volume) after 24 hour deprivation and never consumed aversive compounds. Gustatory responses to sugar and bitter cues were unaffected in *98-Gal4, UAS-Kir2.1* flies as measured by GCaMP calcium imaging (Gordon and Scott, 2009), arguing that overconsumption of bitter compounds occurs despite normal sensory detection (Fig. 2.8).

Rapid inactivation of DSOG1 neurons using a temperature-sensitive dominant-negative dynamin (*UAS-shibire*^{ts}) (Kitamoto, 2001), which acts on the time course of minutes to inhibit neurotransmission, also led to overconsumption (Fig. 2.7d). In addition, flies lacking DSOG1 activity showed increased feeding initiation (proboscis extension) to nutrients in fed states as well as bitter compounds but no changes in starvation-induced locomotor increases (Fig. 2.9). Similar results were obtained with the *98-Gal4* line (Fig. 2.10 and 2.11). These experiments argue that DSOG1 activity is required for rejection of aversive substances and rejection of appetitive substances upon satiation, affecting both feeding initiation and consumption.

As inactivation of DSOG1 neurons promoted consumption, we tested whether inducing activity in these cells would inhibit feeding. The temperature-sensitive cation channel dTRPA1 was expressed in DSOG1 cells and flies were monitored for water and sucrose consumption at temperatures at which dTRPA1 was not active (22°C) or active (30°C). dTRPA1-induced activation of DSOG1 cells reduced sucrose consumption by a third and water consumption by half as compared to control under food/water-deprived states respectively (Fig. 2.12), demonstrating that acute activation of DSOG1 neurons directly inhibits consumption.

Our behavioral studies demonstrate that DSOG1 activity is essential to inhibit feeding. In the absence of DSOG1 activity, flies consume substances that are normally rejected (bitter compounds, high salt) as well as compounds that are rejected in sated conditions (sugar and water), suggesting that DSOG1 acts downstream of bitter cues and satiety signals to inhibit consumption. To test whether the activity of DSOG1 neurons is altered by gustatory detection or satiety state, we performed cell-attached recordings of DSOG1 in live flies (Marella et al., 2012). DSOG1 neurons showed an average baseline firing rate of ~16 Hz, with a standard deviation of 7Hz (Fig. 2.14a). DSOG1 neurons did not respond to gustatory stimulation with 1M sucrose, 1mM denatonium or water, nor was the baseline activity significantly different upon food- or food- and water-deprivation (Fig. 2.13 and 2.14). Similarly, we monitored the steady state activity of DSOG1 neurons in flies with over distended or non-distended abdomens. *98-Gal4, UAS-TNT* flies were fed water until bloated and activity in DSOG1 cells was monitored. TNT was used to block activity rather than Kir2.1 as it selectively inhibits synaptic vesicle release without altering action potential firing. No significant difference were found in animals with distended or non-distended abdomens arguing that gut distention does not activate DSOG1 neurons. These studies demonstrate that DSOG1 cells are not directly activated by gustatory stimuli nor is their tonic activity regulated by satiety signals or gut distention. This argues that DSOG1 cells, gustatory cues and physiological state signals likely impinge on a common feeding pathway, with DSOG1 activity required to gate the response to gustatory cues and satiety cues.

To investigate molecular mechanisms within DSOG1 cells that might influence consumption, we carried out an RNAi screen of candidate receptors, neuropeptides and neurotransmitter synthesis and trafficking genes in DSOG1 neurons and examined effects on consumption (Fig. 2.15). Inhibiting GABA-ergic signaling by RNAi against glutamate decarboxylase 1 or vesicular GABA transporter dramatically increased consumption, as did inhibiting synaptic transmission with nSynaptobrevin RNAi. RNAi against neuropeptide receptors implicated in feeding regulation did not elicit overconsumption (Fig. 2.16), consistent with the model that DSOG1 is not directly regulated by internal state cues. Thus, DSOG1 is a GABA-ergic interneuron that inhibits consumption.

How does DSOG1 interact with other neuronal structures implicated in consumption regulation? Three neural classes have previously been implicated directly in consumption control in insects: activating neurons labeled by the genetic driver line *NP883-Gal4* that also targets a putative feeding command neuron (FDG-neuron) in *Drosophila* was previously shown to promote feeding (Flood et al., 2013). Similarly severing the recurrent nerve or the medial abdominal nerve connecting the digestive tract to the brain in blowflies elicited hyperphagia (Dethier and Bodenstein, 1958; Dethier and Gelperin, 1967). We found that activating NP883-Gal4 neurons (Fig. 2.17) produced a robust rate of spontaneous meal initiations, however only modest and inconsistent short consumption bouts of appetitive substances were observed and either no or short drinks of bitter tastants (Fig 2.17a and b). Although it is currently unclear whether it is the purported feeding command neuron that is mediating the consumption in the genetically labeled cell population, some consumption regulatory neurons appear to be present in NP883-Gal4 line. Severing the medial abdominal nerve (Fig. 2.18b, d) surprisingly did not elicit any abnormal consumption (Fig 2.18b and d). Interestingly however severing the recurrent nerve produced an elevated consumption of sucrose but not water or bitter solutions. Previous work in related insect species suggests that the recurrent nerve relays a post-ingestive food intake inhibiting signal from the hypocerebral ganglion to the SOG (Dethier and Gelperin, 1967). Our data suggests that a similar function is conserved in *Drosophila* and that it plays an important role in determining nutrient intake (Fig. 2.18a and c).

We explored the interaction between DSOG1 neurons, the recurrent nerve and the putative feeding command neuron labeling NP883-Gal4 line. We suspected that recurrent nerve lesioning derived nutrient hyperphagia could partially be mediated through DSOG1 neurons. To explore this scenario, we tested whether acute activation of DSOG1 neurons could rescue the RN lesioning derived sucrose overconsumption. We did observe a partial suppression of sucrose intake, however, if DSOG1 cells were downstream of the RN a full rescue would have been expected (Fig 2.18e). Our results suggest that RN and DSOG1 neurons converge on a common downstream target but are likely to be independent of each other.

The recently identified putative feeding command neuron is predicted to have a diametrically opposite effect on feeding as compared to DSOG1 neurons (Flood et al., 2013). This raised the intriguing possibility that the FDG-neurons in the NP883-Gal4 line could be targets for DSOG1 inhibition. However functional epistasis experiments where we silenced both DSOG1 and NP883-Gal4 positive neurons showed that activity in FDG-neurons is not required for DSOG1 silencing derived insatiable phenotype (Fig. 2.17d)

suggesting that the consumption regulating neurons in NP883-Gal4 line are either upstream or in a parallel pathway with DSOG1 cells. Therefore the neural epistasis experiments argue that neither recurrent nerve inputs nor FDG are in a linear pathway with DSOG1. Instead, these experiments suggest that recurrent nerve inputs, FDG-neurons and DSOG1 impinge on a common pathway for feeding, with DSOG1 providing essential inhibitory control.

To identify neurons that are downstream of DSOG1 cells we carried out a phenotype suppression screen where we inactivated DSOG1 neurons with different subsets of brain cells targeted by a large collection of Gal4 driver lines. Genetic drivers labeling downstream neurons were expected to suppress the DSOG1 silencing derived overconsumption phenotype (Fig 2.19a). We screened 242 Gal4 driver lines crossing them to the *98-Gal4; UAS-Kir2.1, tub-Gal80^{ts}* reporter. The progeny from these crosses were temperature shifted to conditionally inactivate DSOG1 neurons with candidate downstream cells. The flies were subsequently quantified for water and 1mM denatonium consumption. 12 lines showed partial or full suppression of consumption of either one or both tastants suggesting that these lines label neurons that are required to express the insatiable phenotype and are thus likely downstream of DSOG1 neurons (Fig 2.19b). Four lines showing most prominent phenotype suppression were dissected and analyzed for anatomy with all of them showing labeled neurons in the SOG (Fig 2.19c). Future work will determine whether these lines contain postsynaptic neurons to DSOG1 or cell populations further downstream in the consumption regulatory network.

4. Conclusions

These studies examined the neural basis for inhibition in the *Drosophila* feeding circuit. To identify neurons that are required for regulation of consumption we carried out an unbiased neural inactivation screen looking for behavioral defects in consumption regulation. The screen yielded 6 transgenic lines that were insatiable for water as a result of perturbed activity in a subset of cells in the central nervous system. I followed up on one of the lines from the screen and used clonal analysis and intersectional approaches to identify 4 interneurons in the suboesophageal ganglion that were causal for this phenotype. Based on their morphology we named these cells descending SOG cell type 1 (DSOG1) neurons. Behavioral analysis showed that inactivation of these cells resulted in a complete loss of taste and satiety dependent consumption regulation. Acute activation of DSOG1 neurons resulted in reduced consumption of appetitive substances suggesting that activity in these cells directly suppresses ingestion. Physiological analysis demonstrated that these cells are not regulated by taste nor canonical satiety signals. I used RNAi based approaches to show that these interneurons are GABA-ergic. Furthermore analysis of other consumption regulatory neural systems identified the recurrent nerve as a source of post-ingestive inhibition of nutrient intake and demonstrated that the DSOG1 neurons comprise a distinct feeding regulatory mechanism. Finally I identified a number of candidate transgenic lines that are likely to label neurons downstream of the DSOG1 cell cluster.

Taken together, our studies reveal a novel layer of feeding regulation in *Drosophila*, in which four GABA-ergic interneurons act as an essential brake to suppress a latent state of ubiquitous consumption. Whereas dedicated inhibitory circuit nodes that suppress feeding have been identified in multiple model systems, in most cases this inhibition stems selectively from metabolic homeostasis, taste processing or acute post-ingestive signals (Morton et al., 2006; Sternson, 2013), DSOG1 neurons provide an inhibitory tone without which feeding behavior is not regulated by satiety signals or taste quality. Our studies are most consistent with the model where DSOG1 decreases the activity of the ingestive circuitry to a range where it may be subjected to inhibition by aversive taste cues and satiety signals, which are inadequate to regulate consumption on their own (Fig. 2.20). Identifying a 4 neuron substrate to such inhibition will pave the way to understanding how the taste and satiety signaling circuits are coupled to the expression of feeding motor action patterns. Whether other animals employ similar feeding inhibitory mechanisms remains an open question.

5. Materials and Methods

Transgenic flies

The Gal4 enhancer trap collection used for the behavior screen was the InSite collection from the Clandinin lab. The Gal4 enhancer trap collection used for the behavior screen was the InSite collection from the Clandinin lab. Publications for transgenic flies used are cited in the text.

Immunohistochemistry

Immunofluorescence on fly brains was performed as described earlier (Gordon and Scott, 2009) using the following primary antibodies: rabbit anti-GFP (1:500; Invitrogen, cat# A11122), mouse anti-GFP (1:1000; Invitrogen; cat# A11120), rabbit anti-DsRed (1:1000; Biovision; cat# 3993-100), and nc82 (1:500, Developmental Studies Hybridoma Bank). Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG (1:100; Invitrogen, cat # A11008), Alexa Fluor 568 goat anti-mouse IgG (1:100; Invitrogen, cat# A11004), Alexa Fluor 568 goat anti-rabbit IgG (1:100; Invitrogen; cat# A11036) and Alexa Fluor 488 goat anti-mouse IgG (1:100; Invitrogen; cat# A11029). Displayed images are collapsed confocal stacks.

Behavior

Temporal and volumetric consumption assays

Females were collected at eclosion and aged 5-6 days before behavioral testing. Fasted cohorts were kept in vials with wet kimwipes for 24 hours (food-deprived). Flies were kept in an empty vial for 24 hours for food- and water-deprivation experiments (water/food-deprived). Sated, non-deprived flies were taken directly from food vials (fed). Flies were mounted onto glass slides with nail polish, allowed to recover in a humidified chamber for two hours. Individual flies were presented with either a syringe or a capillary (Drummond 3μL Wiretrol I, 5-000-1003) filled with water, 1M sucrose or 1mM denatonium and consumption for single flies was measured by ingestion time or volume (loss of fluid in the capillary) respectively. Measurements were terminated after flies did not initiate consumption to 10 consecutive tastant exposures or regurgitated. For illustration purposes only, 0.25 mg/ml FD&C #1 (Erioglaucine, Sigma 861146) blue dye was included in solutions.

For silencing experiments, Gal4 lines were crossed to *UAS-KIR2.1*, *tub-Gal80^{ts}*. Two- to three-day-old females were collected and incubated at 30°C for 48 hours to inactivate Gal80^{ts} then mounted for consumption assays.

For neural activation experiments, Gal4 lines were crossed to *UAS-dTRPA1; UAS-dTRPA1*. One to two-day-old flies were fasted or water deprived for 24 hours and mounted on glass slides. Activity was induced by exposing flies to 120 seconds of elevated temperature (30°C) on a heat block after which consumption was monitored.

Proboscis extension response assays

Assays were carried out with tarsal stimulation as previously described[29] with the exception that flies were not water satiated prior to the assay.

Locomotor assay
For the locomotor activity assay, we generated flies bearing *tub>Gal80>*; *98-Gal4/276-FLP*; *UAS-Kir2.1*, *tub-Gal80^{ts}*. Flies were reared at 22°C for controls whereas flies were reared at 30°C for DSOG1 inactivation for 48 hours prior to the assay. Individual 4-6 days-old female flies were lightly anesthetized by CO_2 and introduced into polycarbonate tubes (5 mm (D) X 65 mm (L)). One end of the tubes was filled with 2% agar medium (no supplement for the starved groups, agar supplemented with 5mM sucrose for the fed groups). Tubes were then inserted and secured in *Drosophila* activity monitors (DAM2) for the duration of experiments. Multiple activity monitors were connected to a computer through a PSIU9 interface unit (Trikinetics). Experiments were started before the end of a light-on period (Day 0) and typically lasted for 3 days (Day 1-3). Midline crossing activity was sampled for every minute and pooled into 30-minute bins for analysis. The average midline crossing activity was calculated for Day 1-2. Flies that showed no midline crossing activity for a prolonged duration were considered dead and the relevant data points were removed from analysis. Starvation induced enhanced locomotion was measured by averaging the activity during the 24H starting from day 2 lights-on period (hours 36-60H).

Mosaic Analysis of 98-Gal4
Flies of the genotype *hs-FLP^{122}/ tub>Gal80>*; *98-Gal4/UAS-TNT*; *UAS-CD8:GFP* were raised at 22˚C and heat-shocked 0 - 5 min at 37˚C during pupal stages. Eclosed flies were collected and aged for 4-6 days. Flies were separated based on consumption phenotype: those consuming 120 seconds of water and 20 seconds of 1mM denatonium on two separate measurement sessions were classified as 'insatiable', those consuming no denatonium and fewer than 5 seconds of water on two sessions were classified as 'wt'. Animals exhibiting intermediate phenotypes were discarded. Mosaic approaches were used for single cell labeling of DSOG1, using flies containing the transgenes *hs-FLP^{122}*, *tub>Gal80>*, *98-Gal4, UAS-DenMark*, *UAS-synaptobrevin-GFP* to label axons and dentrites.
The expression of 98-Gal4 was restricted to DSOG1 using the transgenes *98-Gal4, 276B-FLP, tub>Gal80>, UAS-CD8:GFP* or to DSOG2 cells using *934-FLP* instead of *276B-FLP.* Flies contained *UAS-Kir2.1, tub-Gal80^{ts}* for neural inactivation experiments or *UAS-dTRPA1* for activation experiments instead of *UAS-CD8:GFP*.

Electrophysiology
Extracellular recordings in cell attached mode in live flies were performed as previously described[29]. Flies used for recording were 3-5 day-old females and were anesthetized using CO_2. Flies were then placed into a small slit on a plastic mount at the cervix such that the head was in a different compartment than the rest of the body. The head was then immobilized using nail polish and bathed in adult hemolymph-like solution (AHL)(Wang et al., 2003). The antennae and surrounding cuticle were gently removed using fine forceps, exposing the SOG. The proboscis remained intact and exposed to the environment. The perineural sheath was removed on the lateral side of the SOG.
Electrodes (5-7MOhm) containing AHL were used to carry out extracellular recording in a loose patch configuration with resistances from 50-500 MOhm. DSOG1 was identified by

the presence of GFP and location of the cell bodies in the ventral SOG. Spikes were recorded in voltage-clamp mode using a multiclamp 700B recorder at 20kHz and low-pass filtered at 5kHz. Recordings were then bandpass filtered between 100 and 3000Hz using a butterworth type filter. Spikes were identified by threshold detection, typically between 5-10pA, using a custom Python script.
For taste stimulation experiments, tastants (1M sucrose, 1mM denatonium or water) were delivered to proboscis by glass capillary. Stimulus artefact in the recordings indicated the time of stimulus onset. Prestimulus spike rates were calculated using 10 seconds of recording preceeding simulation. Stimulus spike rates were calculated using 1 second of recording post stimulation.
Steady state activity was measured in fed, food-deprived (24 hour wet starvation) and food- and water-deprived (24 hour dry starvation) conditions. Tonic gut distention was induced in flies with blocked synaptic transmission in DSOG1 neurons (genotype 98-Gal4/UAS-CD8:GFP4; UAS-TNT) by pre-feeding flies with water for 60 seconds prior to recordings. Steady state activity was estimated from recordings averaging the spike rate across an interval of 30 – 200 seconds of activity.

Labelling neurons by photoactivation
For photoactivation experiments, we generated flies expressing photoactivatable GFP in DSOG1 cells (genotype tub>Gal80>; 98-Gal4/276-FLP; UAS-C3PA-GFP). Brains from 2-3 day-old flies were carefully dissected in ice cold Ca2+ and Mg2+ free AHL and attached to a perfusion chamber by pins. Single cell was picked for photoconversion under 920 nm laser light using baseline fluorescence. Photoactivation was carried out with 760nm laser light (at 11-12 mW of laser power measured at the back apperture of the objective) with 3 intervals of photoactivation of the cell body volume (2 min) and 10 minute intervals for diffusion. A single DSOG1 was photoconverted per brain and the resulting anatomy was acquired by 2-photon confocal microscopy at 920nm (laser power ranging from 12 – 26 mW).

RNAi screen
UAS-RNAi and UAS-shRNA lines from the TRiP and VDRC collections targeting transcripts encoding neuropeptide receptors and neurotransmitter metabolism related proteins were used to knock down gene expression in DSOG1 neurons. UAS-RNAi lines were crossed to 98-Gal4; UAS-dcr2 and UAS-shRNA lines were crossed to 98-Gal4. Water and 1mM denatonium consumption was monitored as detailed above.

2-photon lesions of the recurrent and medial abdominal nerves
Recurrent nerve (RN) or the medial abdominal nerve (AN) were visualised by pan-neuronal expression of GFP (genotype nSyb-Gal4; UAS-CD8:GFP). 4-6 day old flies were taken off of fly food, mounted to custom made plastic chambers with lesion sites bathed in AHL. RN or AN was visualized at 920 nM wavelength with a 40X water immersion objective (N/A 0.8) to define the lesioning target volume. Nerve lesions were carried out at 760 nm laser light (at 42-47 mW laser power) with a 3 minute lesioning protocol for RN and a 10 minute lesioning protocol for AN. RN was lesioned between the head and the thoracic segment. AN was lesioned beneath the abdominal cuticle immediately below the thoracic segment. Mock lesions in comparable volumes were made laterally from the targeted

nerve. Following the lesioning, flies were glued on glass slides and allowed to recover for 3 hours in a humid chamber followed by consumption measurements. For DSOG1 activation experiments, UAS-dTRPA1 expression was targeted to DSOG1 neurons by 98-Gal4. The recurrent nerve lesions were guided by anatomical landmarks lesioning the tissue above the foregut and medially from the trachea in the neck. DSOG1 activity was induced by temperature shifting flies to 30°C on a heat block for two minutes followed by consumption measurements.

Electrical stimulation of Recurrent Nerve

Genetically encoded calcium sensor G-CaMP5 was targeted to DSOG1 neurons by *98-Gal4*. The whole nervous system with the intact proboscis, foregut and partial midgut was dissected in cold Ca^{2+} and Mg^{2+} free AHL. The dissected brains were transferred to AHL containing calcium and magnesium and gently fixed with pins with the ventral surface facing up. The recurrent nerve was then inserted into a stimulating suction electrode and an electrical stimulus of 10V, 300 μs was delivered at 100 Hz for 100 ms. G-CaMP responses were monitored by spinning disk confocal microscopy. Perfusing 1M KCl to the imaging chamber was used as a positive control for DSOG1 Ca^{2+} response.

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6. Figure Legends

Figure 2.1. **Neuronal screen identifies flies with insatiable behavior. a.** Water consumption time of single flies was monitored with temporal consumption assay. **b.** Flies consumed water in proportion to water deprivation. n=20-31 flies; mean±s.e.m.; one-way ANOVA, Tukey post-hoc, ***p<0.001. **c.** 363 Gal4 lines, expressing Kir2.1 in adult neurons, were assayed for water consumption under non-deprived conditions identifying water overconsuming lines (mean, n=20 flies). **d.** Neuropeptide/neurotransmitter-Gal4 lines were tested for water consumption upon neural inactivation (left) or activation (right) under non-deprived conditions. n=20 flies; mean±s.e.m.; t-test; ***p<0.001.

Figure 2.2. Expression pattern of Gal4 lines with an overconsumption phenotype. Expression in brain, ventral nerve cord (VNC) and proventriculus (PV, 98-Gal4) or hypocerebral ganglion (HG, other lines) is shown for Gal4 lines expressing UAS-CD8:GFP. Scale is 50 µm.

Figure 2.3. The water consumption phenotype of 98-Gal4, UAS-Kir2.1 requires neural expression. a. *98-Gal4, UAS-Kir2.1* flies overconsume water. Inclusion of *elav-Gal80* or *cha-Gal80* eliminated the overconsumption phenotype. n=20-22 flies; mean ± s.e.m.; t-test to *98-Gal4, UAS-Kir2.1*, ***p<0.001. b. Expression of *98-Gal4* driving *UAS-CD8:GFP* in the brain, ventral nerve cord (VNC) and peripherally in the proventriculus (PV). Inclusion of *elav-Gal80* (**c**) or *cha-Gal80* (**d**) abolished neural expression leaving the peripheral expression intact. Scale is 50 µm.

Figure 2.4. Different cell-types in the 98-Gal4 line. Single cell clones for the seven cell-types identified in mosaic animals (flies contained *hs-FLP, tub>Gal80>, 98-Gal4, UAS-GFP, UAS-Kir2.1*). N6 and N7 are located in the ventral nerve cord, other neurons are localized in the brain. Scale is 50 µm.

Figure 2.5. A subset of *98-Gal4* neurons influences consumption. a. *98-Gal4* drives GFP in the brain and ventral nerve cord (scale 100 µm). **b.** Frequency distribution of cell-types in wild-type 'wt' (grey bars; n=105) and 'insatiable' (black bars; n=44) mosaics (*hs-FLP*^{122}*/ tub>Gal80>; 98-Gal4/UAS-TNT; UAS-CD8:GFP*). mean+/-s.e.m.; Fisher's test, ***p<0.001. **c.** Number of DSOG1 cells (4 cells total) or DSOG2 cells (16 total) labeled in 'wt' (grey bars) or 'insatiable' (black bars) mosaics. **d.** Water consumption under non-deprived conditions was measured for flies with DSOG1 or DSOG2 inactivated. *98-Gal4* expression was restricted to DSOG1 (*276B-FLP*) or DSOG2 (*934-FLP*) with *tub>Gal80>* and cells were inactivated in *UAS-Kir2.1, tub-Gal80*^{ts} animals at 30°C (cell inactivation) or kept at 22°C (control). n=20; mean+/-s.e.m.; t-test, ***p<0.001. **e.** GFP expression in brain (top) and VNC (bottom) for DSOG1 (left) and DSOG2 (second). Single-cell labeling of DSOG1 with GFP (third) or dendritic (DenMark; magenta) and synaptic (SYT-GFP; green) markers (right). Scale 50µm.

Figure 2.6. The DSOG1 cell cluster is comprised of morphologically indistinguishable neurons. Anatomical diversity among DSOG1 neurons was explored by photoconverting photo-activatable GFP (pa-GFP) by 2-photon microscopy to visualize single DSOG1 cells. Flies were tub>Gal80>; 98-Gal4/276B-FLP; UASC3PA-GFP) a-f. 6 individual photoactivated DSOG1 cells. All 6 cells were characterized by a large cell body (arrow head) that sends widely arborizing projections throughout the SOG, with characteristic innervation around the oesophageal passage (asterisk), and a descending projection on the contralateral side of the cell body (arrow). Scale 20 μm.

Figure 2.7. Inactivation of DSOG1 neurons leads to indiscriminate overconsumption of appetitive and aversive substances. a. Controls and flies expressing Kir2.1 in DSOG1 before and after water consumption. The genotype for DSOG1 inactivation was *98-Gal4*, *276B-FLP*, *tub>Gal80>*, *tub-Gal80*^{ts}, *UAS-Kir2.1*, with flies at 30°C for Kir2.1 induction or 22°C for controls. **b.** Conditional inactivation of DSOG1 neurons increased consumption time in deprived (24h wet-starved for 1M sucrose and 1mM denatonium; 24h dry-starved for water) and non-deprived (fed) conditions. 100% ethanol (EtOH) and 6M sodium chloride (NaCl) were consumed by non-deprived animals. n=20; mean+/-s.e.m.; one-way ANOVA, Tukey post-hoc, ***p<0.001. † flies die after consumption. **c.** Conditional inactivation of DSOG1 neurons increased consumption volume in deprived and non-deprived conditions. n=20; mean +/- s.e.m.; one-way ANOVA, Tukey post-hoc, ***p<0.001. **d.** DSOG1 inactivation with shibire^{ts} (Shi^{ts}) caused 1mM denatonium consumption in non-deprived animals. n=20; mean+/-s.e.m.; one-way ANOVA, Tukey post-hoc, ***p<0.001.

Figure 2.8. Gustatory sensory activity is not affected by 98-Gal4 neural inactivation. The response of Gr66a, bitter-sensing cells to 1, 10, 100 mM caffeine was monitored in flies with (RT) or without *98-Gal4* neural activity (30°C) (left) using GCaMP calcium imaging. The response of Gr5a, sugar-sensing cells to 10, 100 and 1000 mM sucrose was also monitored (right). Responses with or without *98-Gal4* neural silencing were not statistically different. n=3-5; mean+/-s.e.m.; t-test.

Figure 2.9. Additional behavioral phenotypes of DSOG1 activity modulation. a. Flies with DSOG1 neurons expressing Kir2.1 showed increased proboscis extension to sugar under fed conditions. All flies with *Kir2.1* also contain *tub>Gal80> and tub-Gal80*^{ts} and were incubated at 30°C for 2 days for *Kir2.1* induction prior to behavioral testing or remained at 22°C for same genotype controls. n=60 flies per data point; mean +/- s.e.m; t-test to same genotype control; ***p<0.001. **b.** Extension was significantly different in flies with DSOG neurons expressing Kir2.1 upon 24 hr food-deprivation. n=60 flies per data point; mean +/- s.e.m; t-test to same genotype control; ***p<0.001. **c.** Flies with DSOG1 neurons expressing Kir2.1 showed increased proboscis extension to water in non-deprived states. n=60 flies per line; t-test to same genotype control; mean +/- s.e.m; ***p<0.001. d. Flies with silenced DSOG1 neurons showed increased proboscis extension to bitter compounds in fed and deprived states. n=60 flies per line; mean +/- s.e.m; t-test to same genotype control; ***p<0.001. **e.** Locomotor activity measurements in a single fly beam-crossing assay. Starvation-induced locomotor changes were unaffected in flies with DSOG1 neurons inactivated. Flies in the deprived groups were food deprived throughout the assay but had

access to water. Fed groups had access to sucrose and water. **f.** Average locomotor activity of starved and unstarved flies during the second day (36-60H) activity peak. n=19-32 flies per genotype and condition; mean +/- s.e.m; t-test to same genotype control; ***p<0.001.

Figure 2.10. Activity in 98-Gal4 neurons controls consumption. a. Conditional inactivation of 98-Gal4 neurons with Kir2.1 increased feeding times in deprived (24h no food for 1M sucrose and 1mM denatonium; 24h no food or water for water stimulation) and non-deprived conditions. n=22; mean +/- s.e.m.; one-way ANOVA, Tukey post-hoc, ***p<0.001. **b.** Conditional inactivation of 98-Gal4 neurons with Kir2.1 increased volume consumed in deprived and non-deprived conditions, as in b. n=22; mean +/- s.e.m.; one-way ANOVA, Tukey post-hoc, ***p<0.001. **c.** Inactivation of 98-Gal4 neurons with shibire^{ts} (Shi^{ts}) caused overconsumption. The percentage of flies that drank denatonium for >1s and >120s was recorded. n=20; mean; Fisher's test, ***p<0.001. **d.** Heat induced dTRPA1 activation of *98-Gal4* neurons reduced consumption of sucrose and water. n=24-60; mean +/- s.e.m.; one-way ANOVA, square root transformation, Tukey post-hoc, ***p<0.001.

Figure 2.11. Additional behavioral phenotypes of 98-Gal4 flies. a. Flies with *98-Gal4* neurons silenced showed increased proboscis extension to sugar under fed conditions. n=60 flies per data point; mean +/- s.e.m; t-test to same genotype control; **p<0.01, ***p<0.001. **b.** Extension was not significantly different in flies with silenced *98-Gal4* neurons upon 24 hr food-deprivation. n=60 flies per data point; mean +/- s.e.m; t-test to same genotype control; ***p<0.001. **c.** Flies with silenced *98-Gal4* showed increased proboscis extension to water in non-deprived states. n=60 flies per line; t-test to same genotype control; mean +/- s.e.m; ***p<0.001. **d.** Flies with silenced 98-Gal4 neurons showed increased proboscis extension to bitter compounds in fed and deprived states. n=60 flies per line; mean +/- s.e.m; t-test to same genotype control; ***p<0.001.

Figure 2.12. Activation of DSOG1 neurons suppresses consumption of appetitive substances in deprived states. a. Heat-induced dTRPA1 activation of DSOG1 (*98-Gal4*, *276B-FLP*, *tub>Gal80>*, *UAS-dTRPA1*) reduced sucrose and water consumption times in deprived flies food (wet starved) and water (dry starved) deprived flies respectively. n=61-71; mean+/- s.e.m.; one-way ANOVA, square root transformation, Tukey post-hoc, ***p<0.001. **b.** Heat-induced dTRPA1 activation of DSOG1 neurons (*98-Gal4, 276-FLP, tub>Gal80>, UAS-dTRPA1*) reduced consumption volume for sucrose and water in food and water deprived flies respectively. n=30; mean +/- s.e.m.; one-way ANOVA, Tukey posthoc, ***p<0.001.

Figure 2.13. Taste detection does not modulate activity in DSOG1 neurons. DSOG1 firing rate before (Baseline) and during 1s stimulation (Stimulus) with respective raster plots (1s pre-stimulus in white and 1s after stimulus onset in blue) for stimulation of proboscis gustatory receptor neurons with 1M sucrose (**a**), water (**b**) or 1mM denatonium (**c**) in non-deprived conditions. Baseline rate was averaged 10s pre-stimulus. n=5 flies per taste compound, different flies for each stimulation. Wilcoxon matched pairs test.

Figure 2.14. DSOG1 neurons display a regular and stable firing rate regardless of physiological state or gut distention. a. DSOG1 activity in wt animals that are water-deprived(dry starved), food-deprived (wet starved) or non-deprived and flies expressing TNT in 98-Gal4 under non-deprived conditions or after water overconsumption. TNT may influence DSOG1 activity by inhibiting postsynaptic feedback. Activity was averaged for 30-200s. n=7-12; mean +/- s.e.m.; Kruskal Wallis. **b-f.** Representative DSOG1 cell attached extracellular recording traces and spike rate histograms from animals that are 24 hours dry starved (**b**), 24 hours wet starved (**c**), non-deprived (**d**), non-deprived with synaptic output from DSOG1 neurons blocked by TNT (**e**), tonic gut distention by allowing flies to overconsume water for 60 seconds prior to recording with synaptic output from DSOG1 neurons blocked by TNT (**f**).

Figure 2.15. DSOG1 neurons are GABA-ergic interneurons. A screen with RNAi and shRNA based knockdown of gene expression targeting neurotransmitter synthesis and trafficking genes was carried out to identify the neurotransmitter profile of DSOG1 cells. Increased consumption of water and 1mM denatonium after gene knockdown indicates functional role in DSOG1 physiology. 14 UAS-RNAi lines were crossed to *98-Gal4; UAS-dcr2* and *UAS-shRNA* lines were crossed to *98-Gal4*. n=20; mean+/-s.e.m.; t-test to control (no RNAi); ***p<0.001.

Figure 2.16. RNAi/shRNA based knock down of neuropeptide receptors implicated in feeding in DSOG1 neurons does not elicit overconsumption. *UAS-RNAi* and *UAS-shRNA* lines targeting transcripts encoding neuropeptide receptors implicated in food intake regulation were used to knock down gene expression in DSOG1 neurons in *98-Gal4; UAS-dcr2* and *98-Gal4* genetic background respectively. Water and 1mM denatonium consumption was monitored. n=20 per tastant per genotype; mean+/- s.e.m.; t-test to control (no RNAi).

Figure 2.17. The feeding phenotype of a putative feeding command neuron (FDG) is different from DSOG1 inactivation. a. Flies with NP883-Gal4 neurons activated with dTRPA1 (32°C, green) showed mild consumption under fed conditions. Deprived controls are shown as a reference. In contrast, 98-Gal4, UAS-Kir2.1 flies consumed ~150s. n=20 flies/genotype, each data point is one fly; mean +/- s.e.m; t-test to NP883-dTRPA1 (22°C); **p<0.01. **b.** Spontaneous proboscis extensions were observed in NP883-dTRPA1 flies upon dTRPA1 activation (32°C). n=20 flies/genotype; mean +/- s.e.m; t-test to NP883-dTRPA1 (22°C); ***p<0.001. **c.** The consumption of NP883-dTRPA1 flies was not significantly different when 98-Gal4 neurons were also activated (green bars), with the exception of the water response. n=20-22 flies; mean +/- s.e.m; ttest to NP883-dTRPA1 (22°C); *p<0.05. **d.** Consumption of 98-Kir2.1 flies was not different when NP883 neurons were also inactivated (red bars), demonstrating that the putative feeding command neuron is dispensable for the DSOG1 overconsumption phenotype and is not linearly downstream in the feeding circuit. n=10-22 flies; mean +/- s.e.m; t-test to NP883-Kir2.1 (22°C); ***p<0.001.

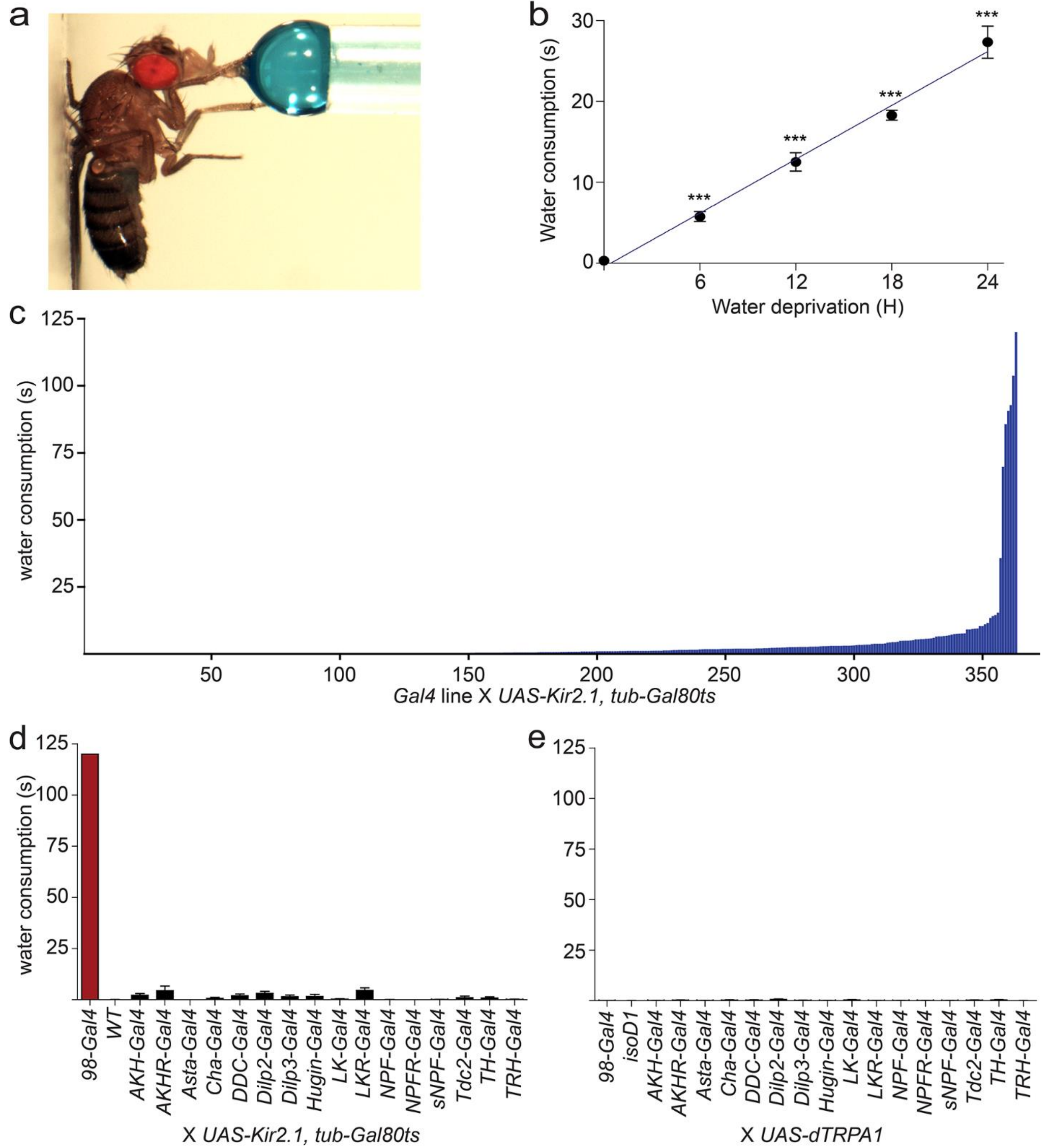
Figure 2.18. The feeding phenotype of recurrent nerve lesions is different from DSOG1 inactivation. a-b. Flies with pan-neuronal GFP (nSyb-Gal4; UAS-CD8:GFP) were used to target lesions to the recurrent nerve (RN) or the medial abdominal nerve (AN). A 2-photon laser was used for lesioning (760 nm light, X power, 3 minute lesions for RN and 10 minute lesions for AN) and the disappearance of GFP was used to indicate successful lesioning. Arrowheads mark the lesioned nerves. Scale is 50 µm. **c.** Flies with RN lesions showed increased 1M sucrose consumption but no consumption of water or 1mM denatonium. Consumption of 24H food deprived flies is shown as a reference. **d.** AN lesion did not affect consumption. For mock lesions, the laser was directed to tissue adjacent to the nerve. n=10; mean +/- s.e.m; ttest to mock lesion; ***p<0.001. **e.** DSOG1 activation partially suppressed the 0.5M sucrose consumption induced by severing the RN. n=20; mean +/- s.e.m; t-test to 22°C same genotype; ***p<0.001.

Figure 2.19. Overconsumption phenotype suppression screen identifies Gal4 lines labeling candidate neurons downstream of DSOG1. a. Genetic logic underlying the 98-Gal4 overconsumption phenotype suppression screen. Silencing 98-Gal4 neurons with downstream components in the ingestion regulatory circuit should abolish the observed hyperphagic phenotype. **b.** Water and denatonium consumption profile of identified 12 Gal4 driver lines either partially or fully suppressing 98-Gal4 silencing derived overconsumption phenotype (n=5–12 per tastant condition). **c.** Expression of 4 Gal4 lines yielding most reliable phenotype suppression as visualized by driving *UAS-CD8:GFP* expression. Scale 50µm.

Figure 2.20. Model for the role of DSOG1 neurons in food intake regulation. DSOG1 inhibits ingestion probability to enable regulation by satiety and bitter cues (left). In the absence of DSOG1, bitter and satiety cues are not sufficient to inhibit ingestion (right). Food indicates a positive sensory drive.

7. Figures

Figure 2.1



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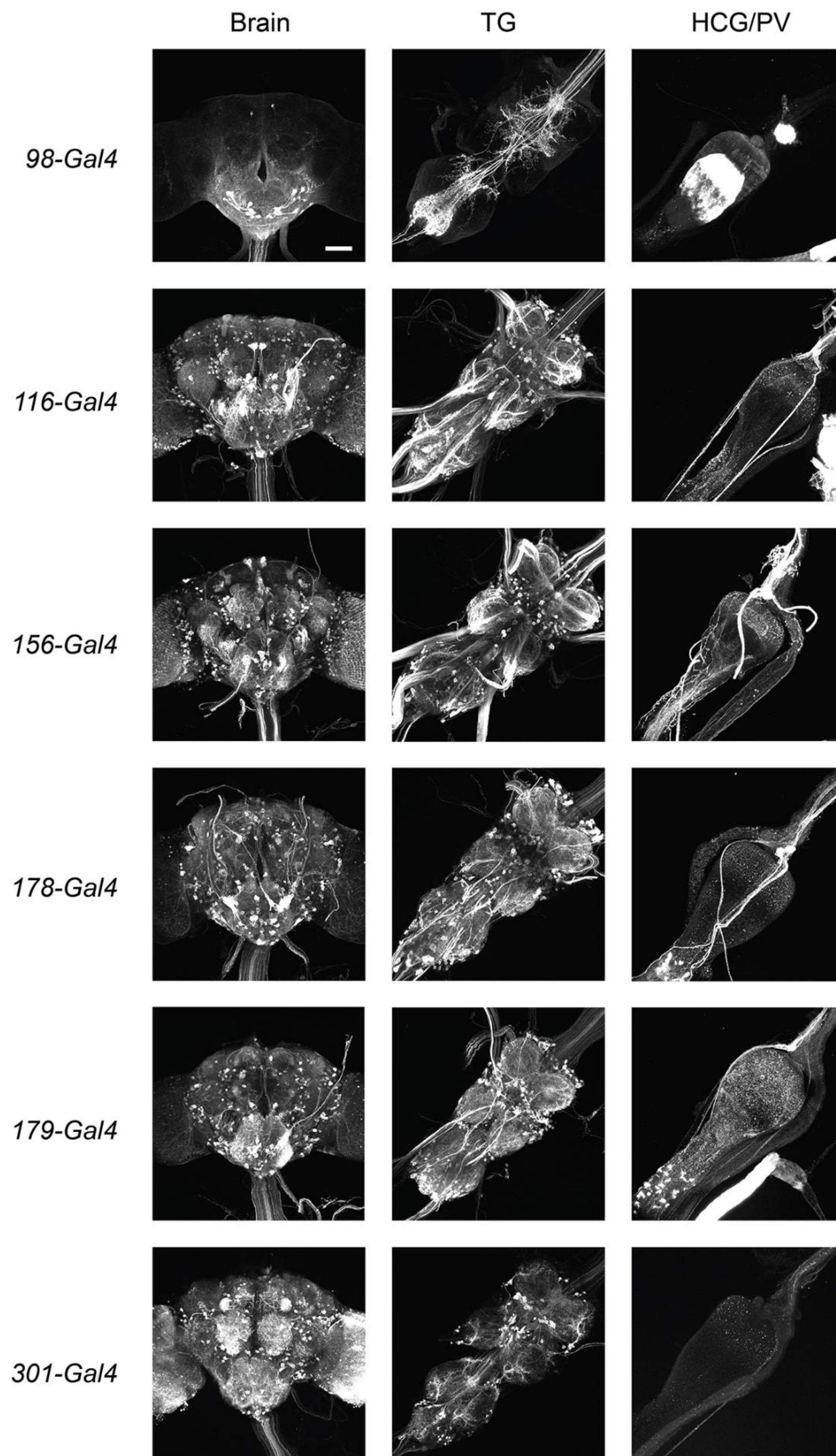

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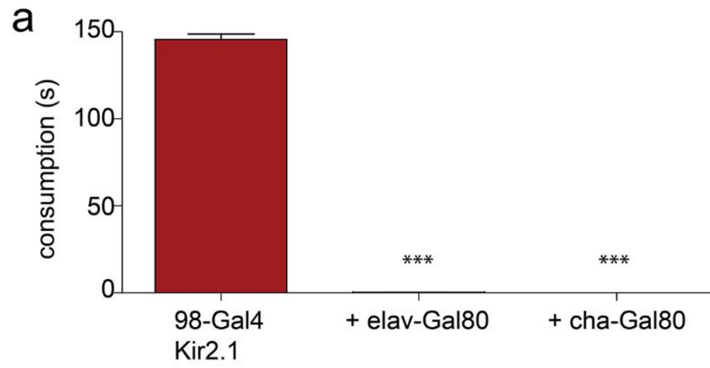
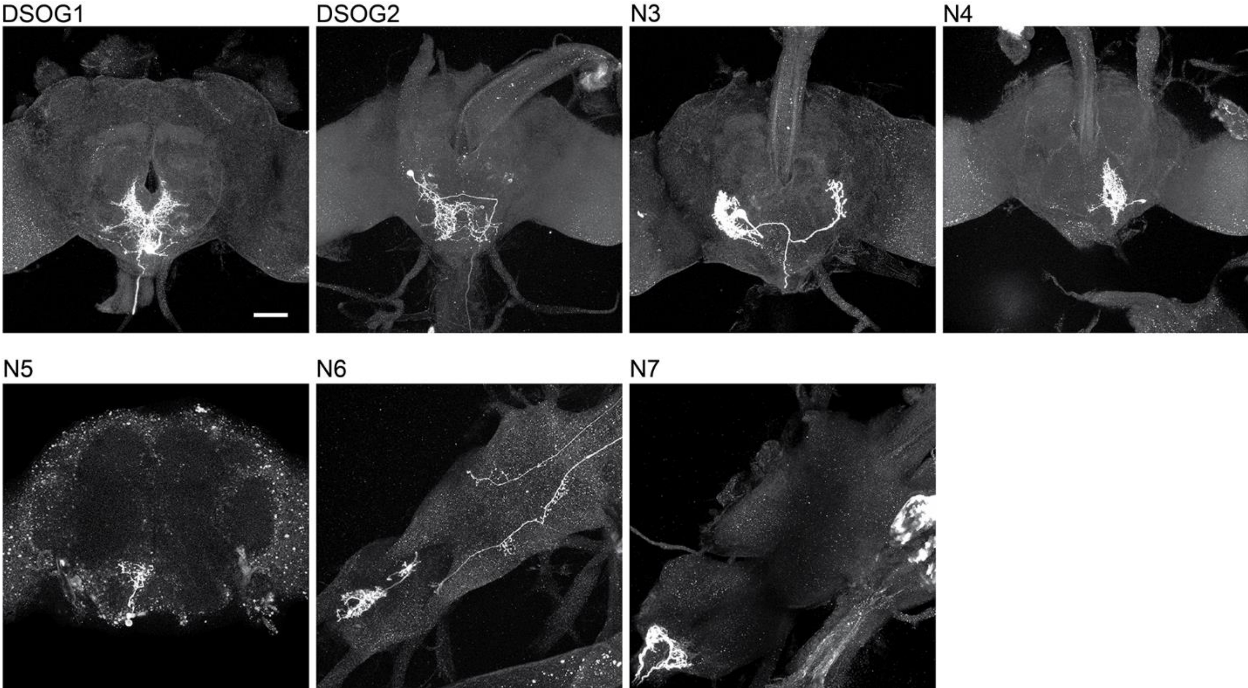


b
Brain
VNC
HCG
98-Gal4

c
98-Gal4
elav-Gal80

d
98-Gal4
cha-Gal80

Figure 2.4



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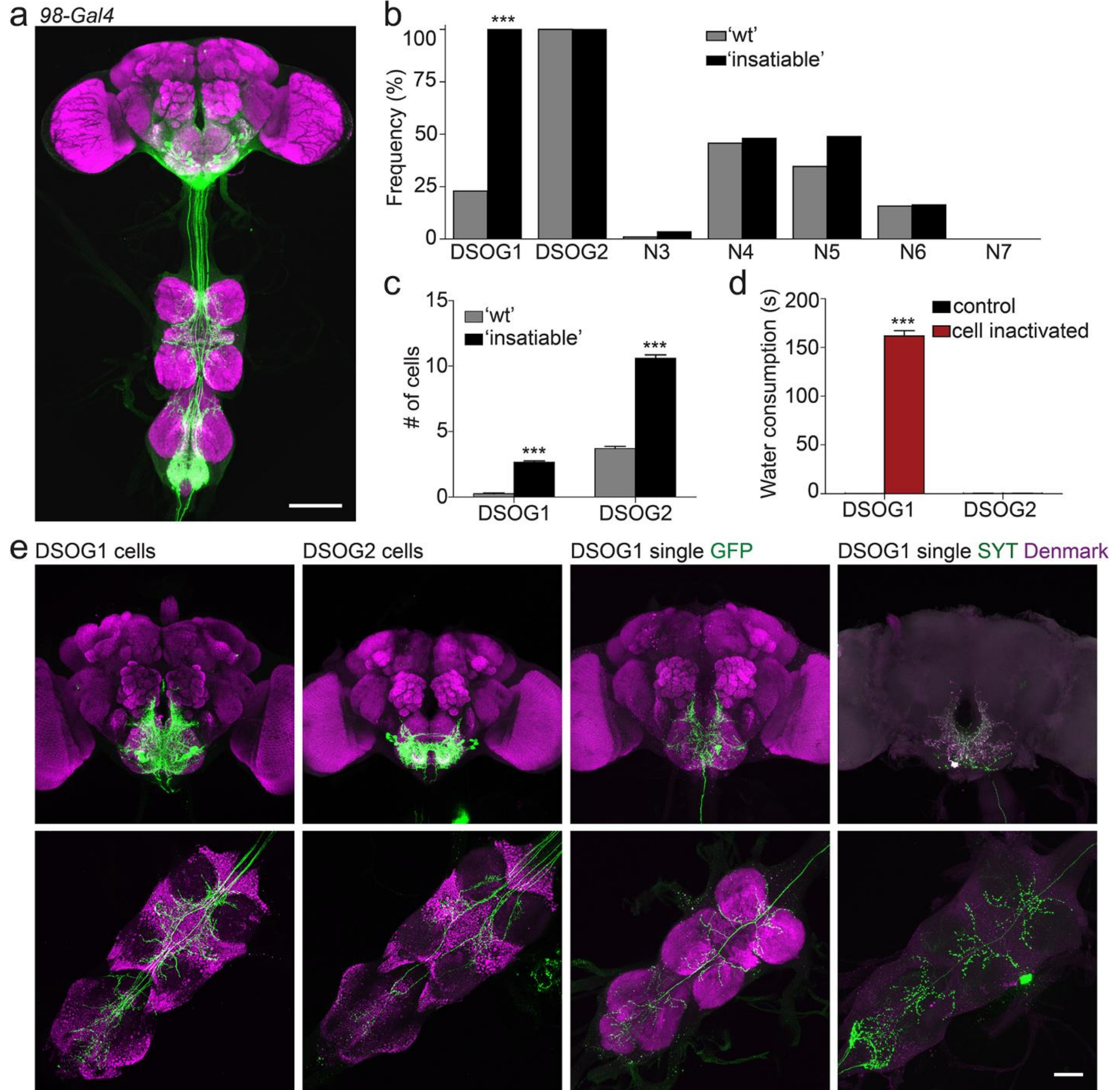

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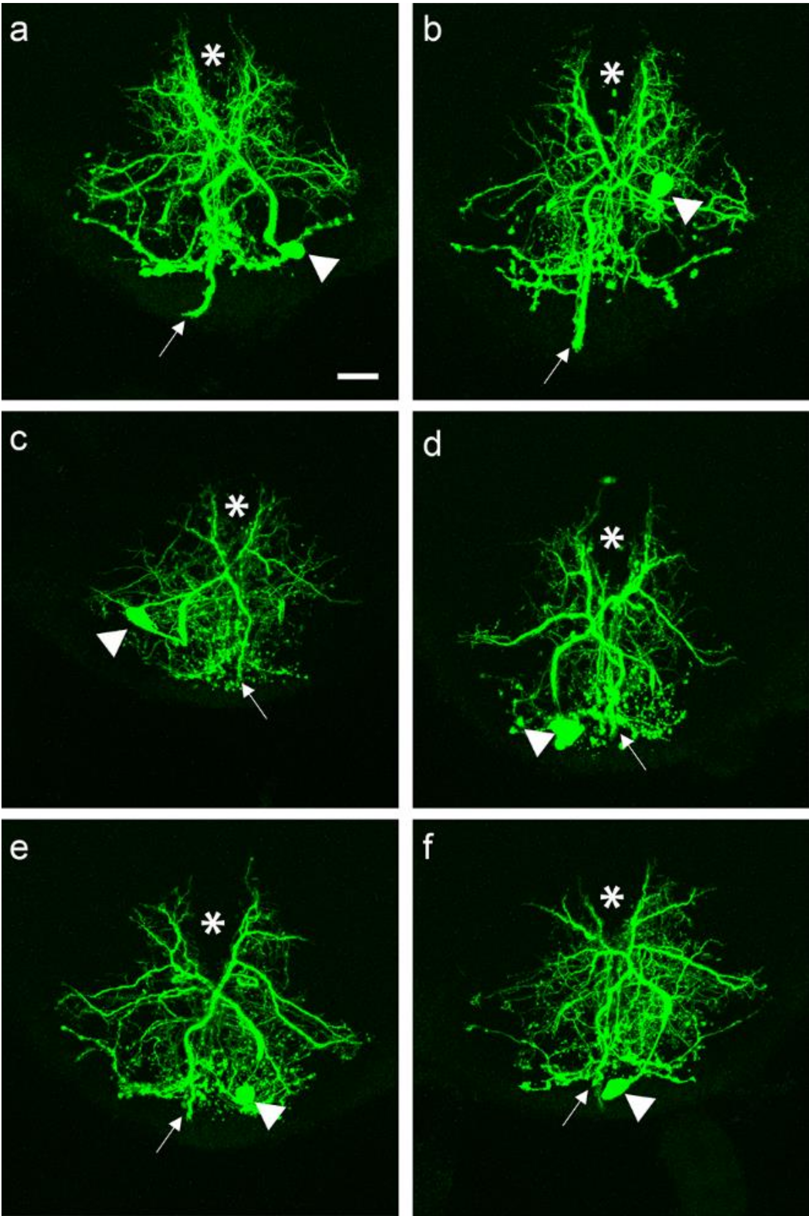

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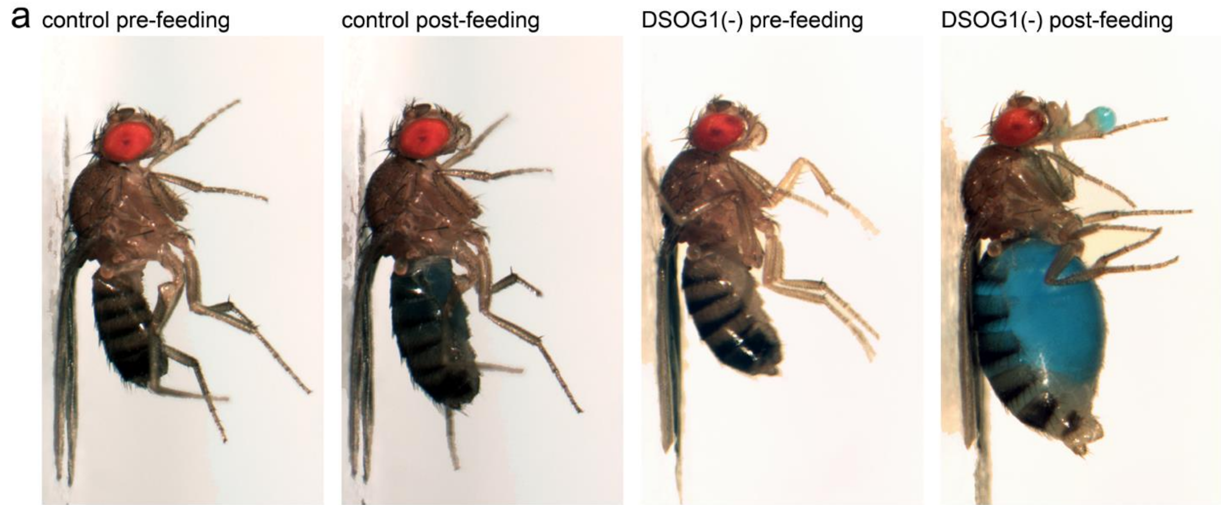

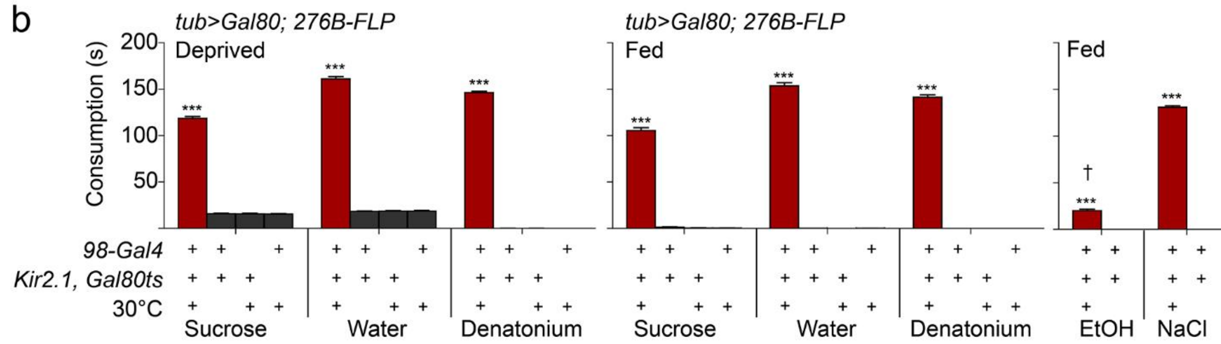

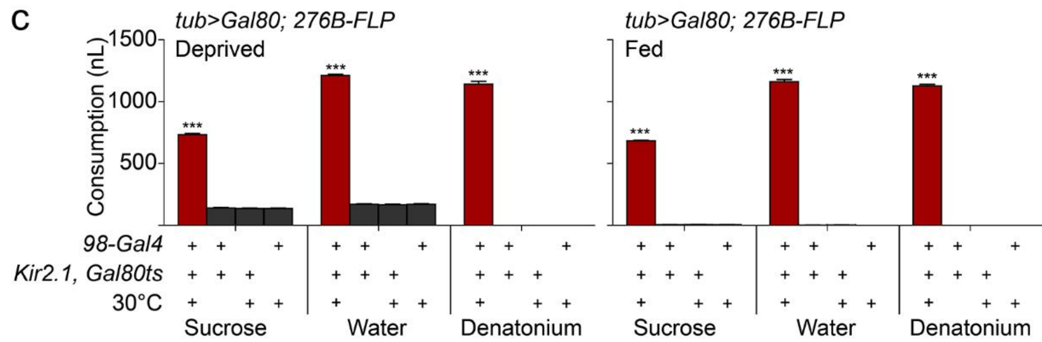

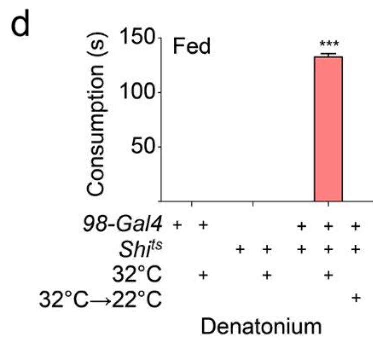

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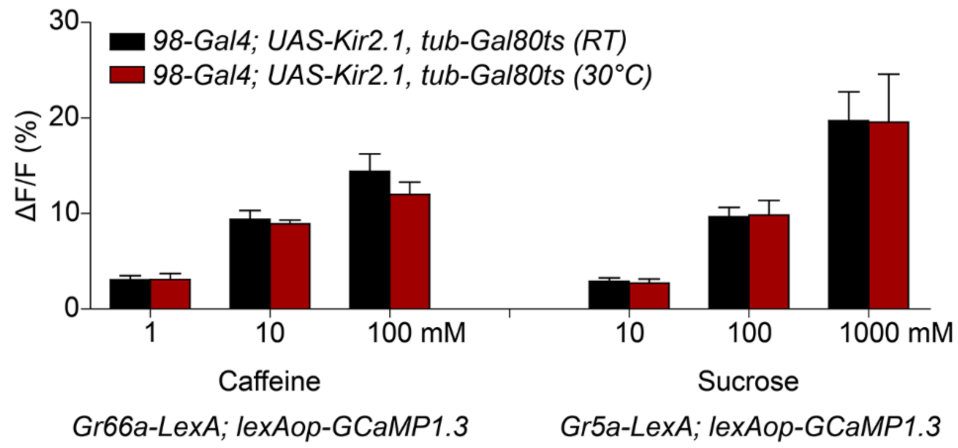

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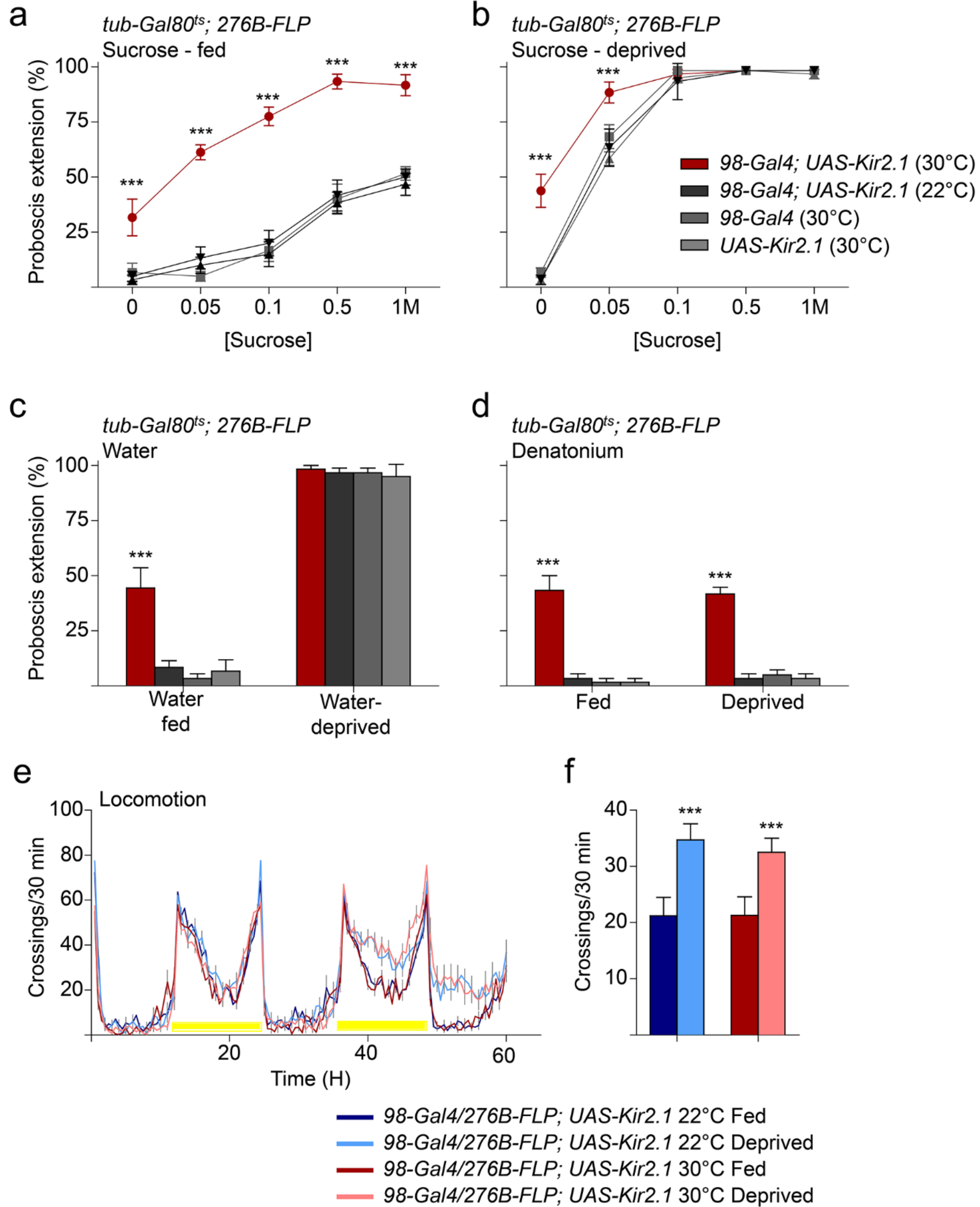

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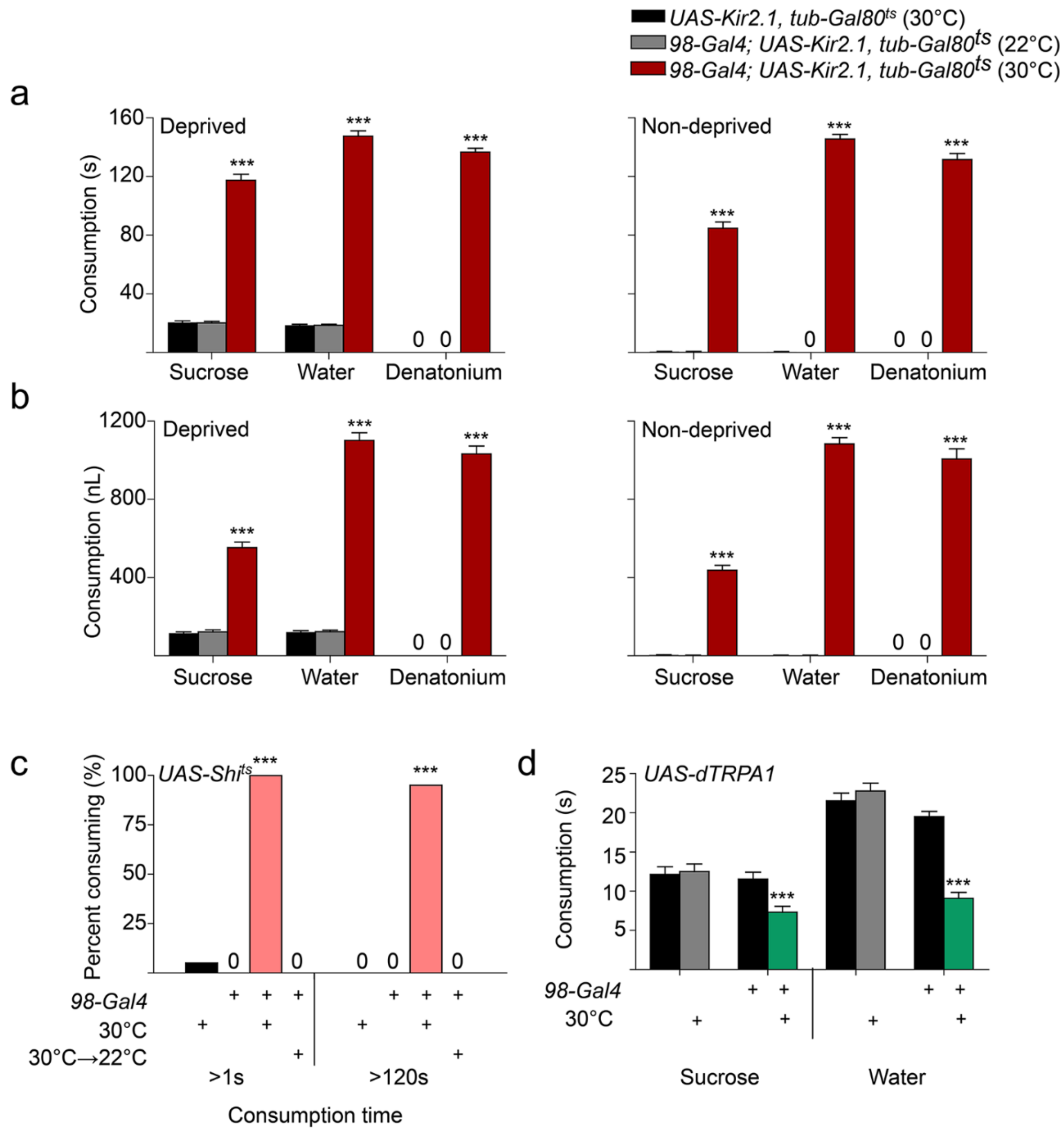

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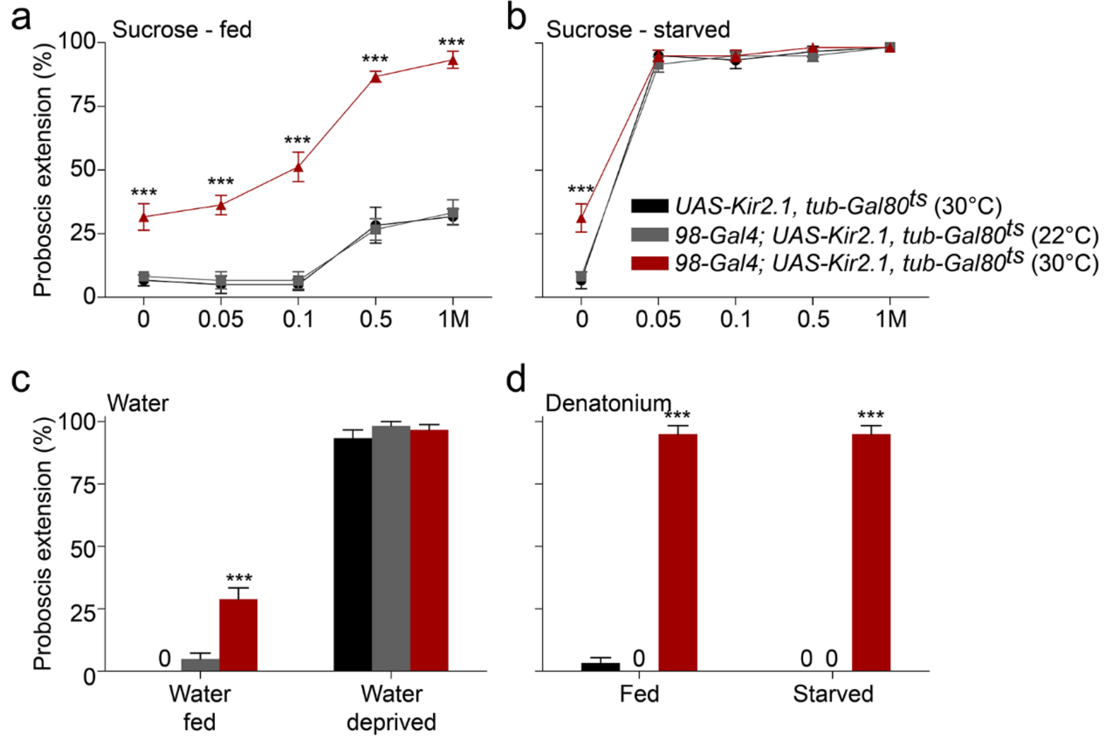

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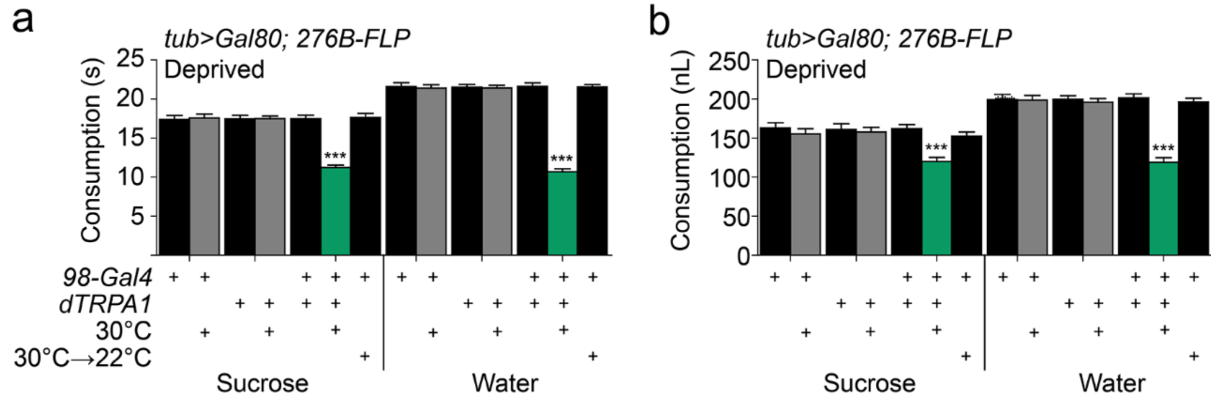

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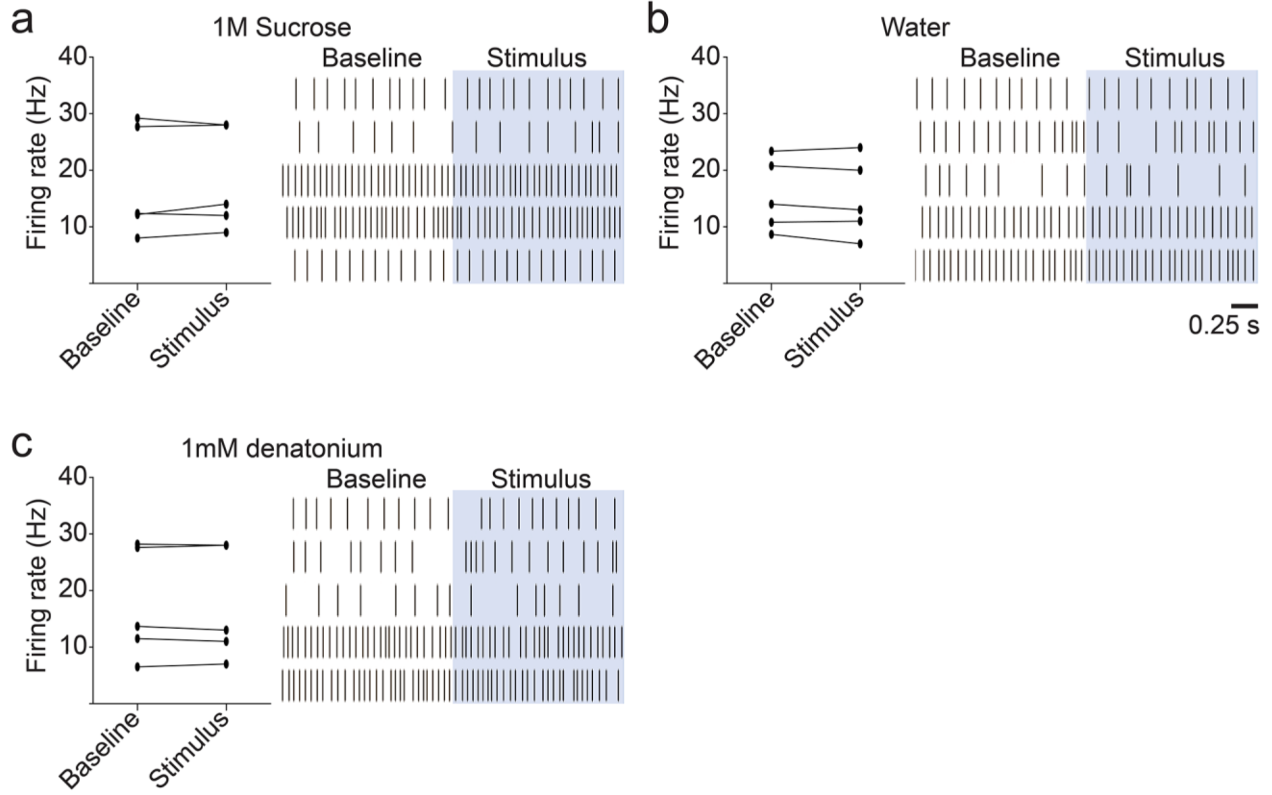

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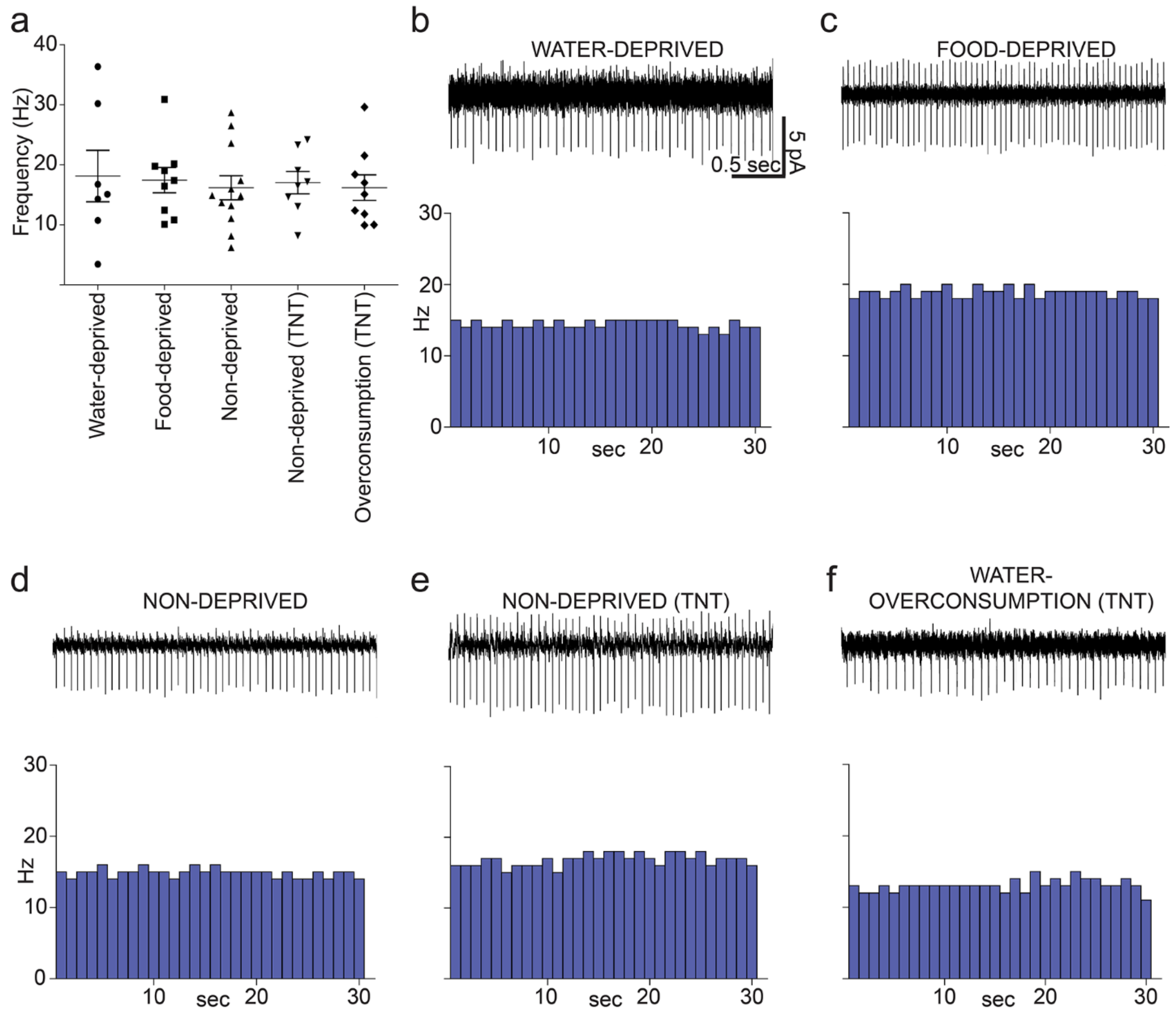

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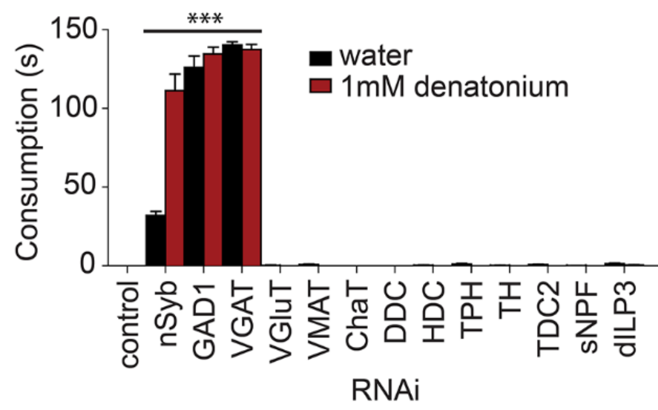

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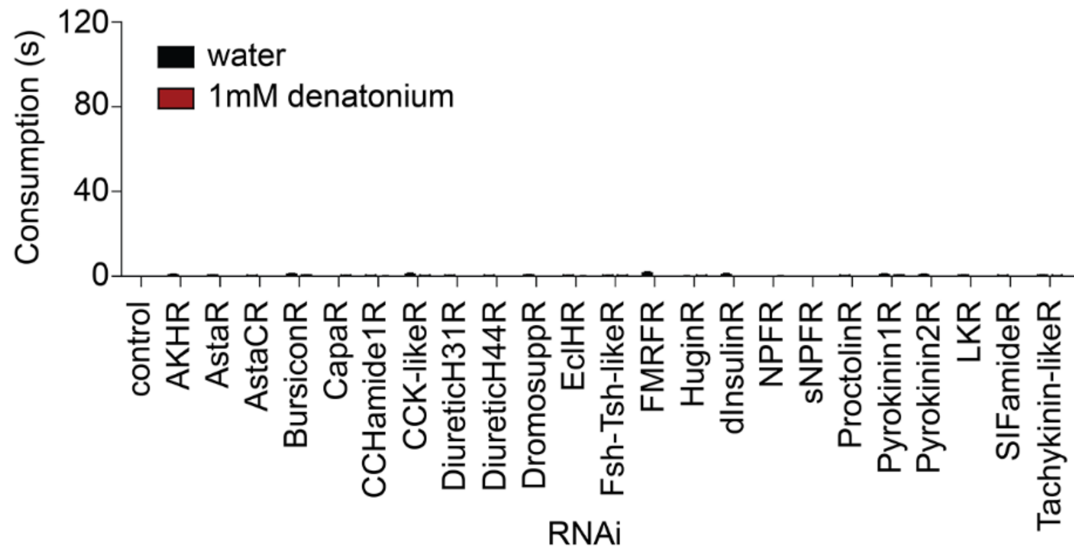

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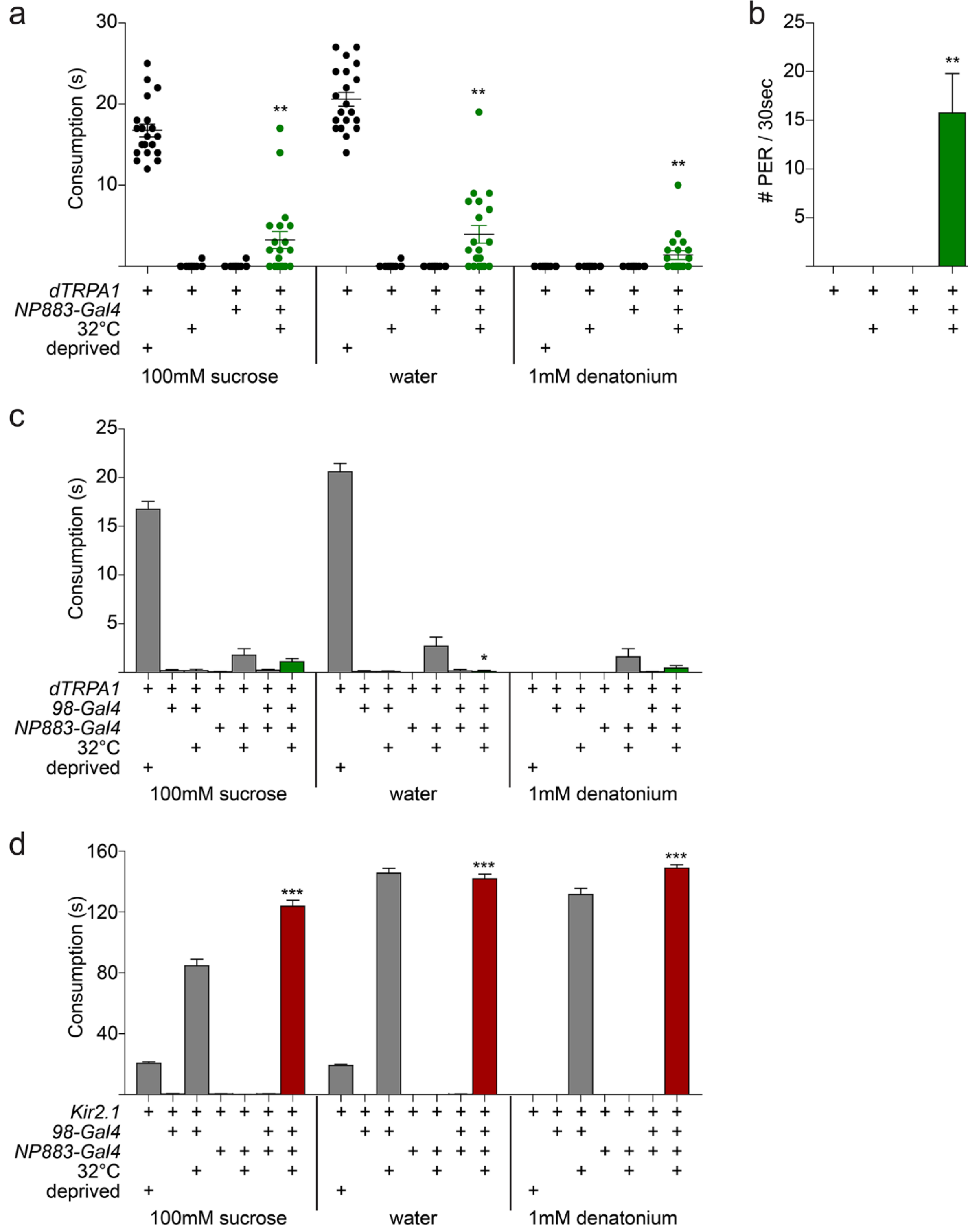

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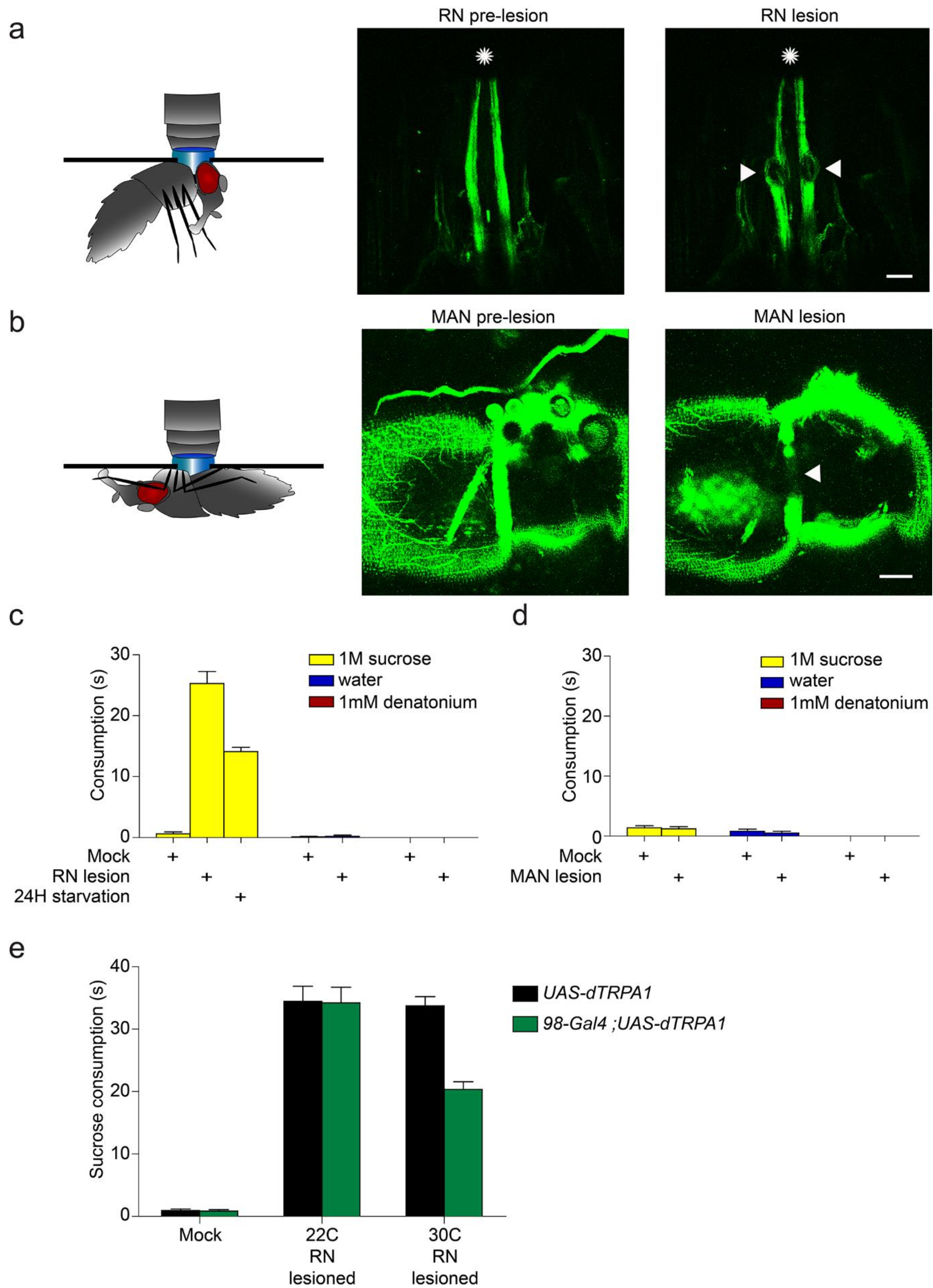

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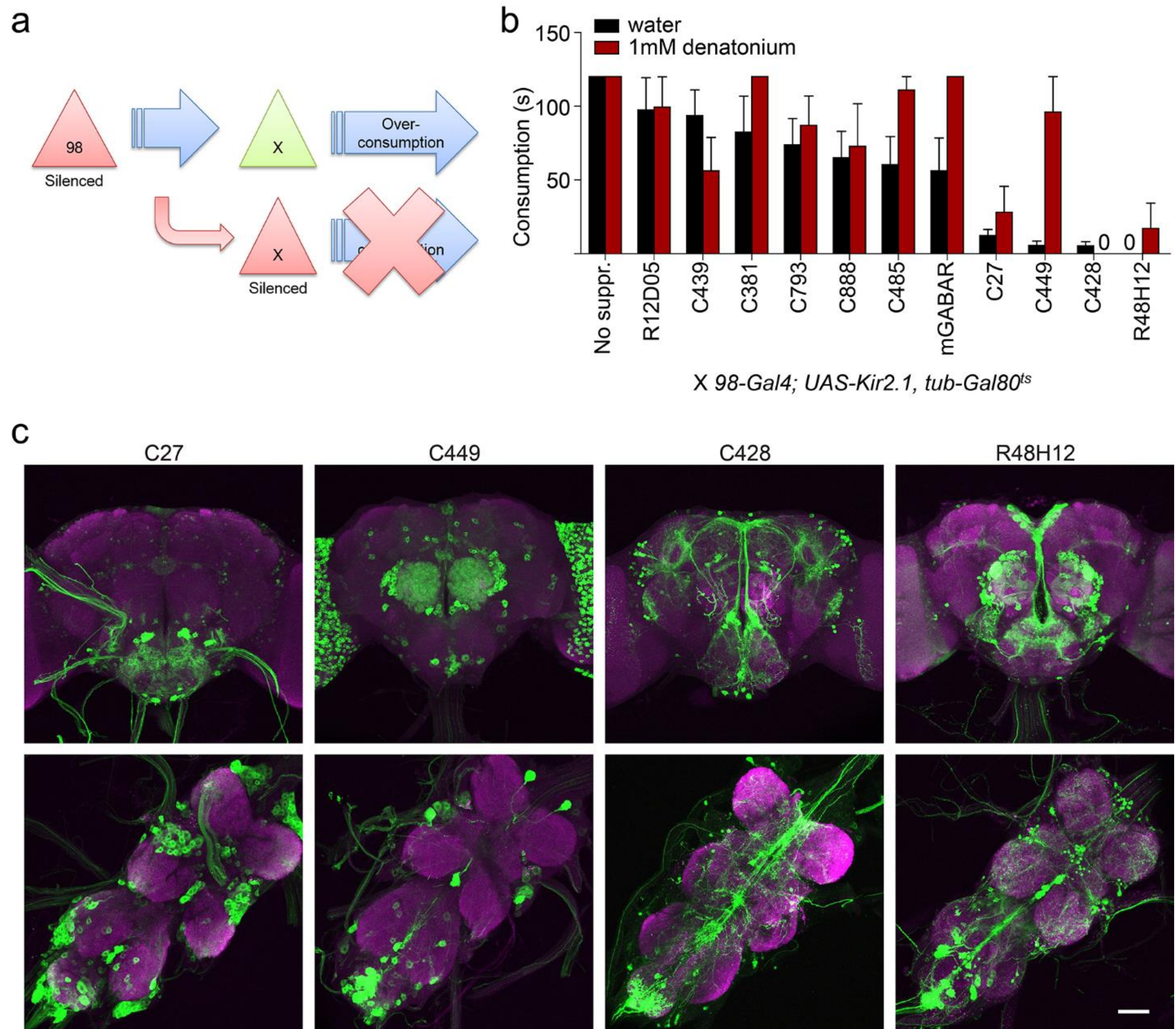

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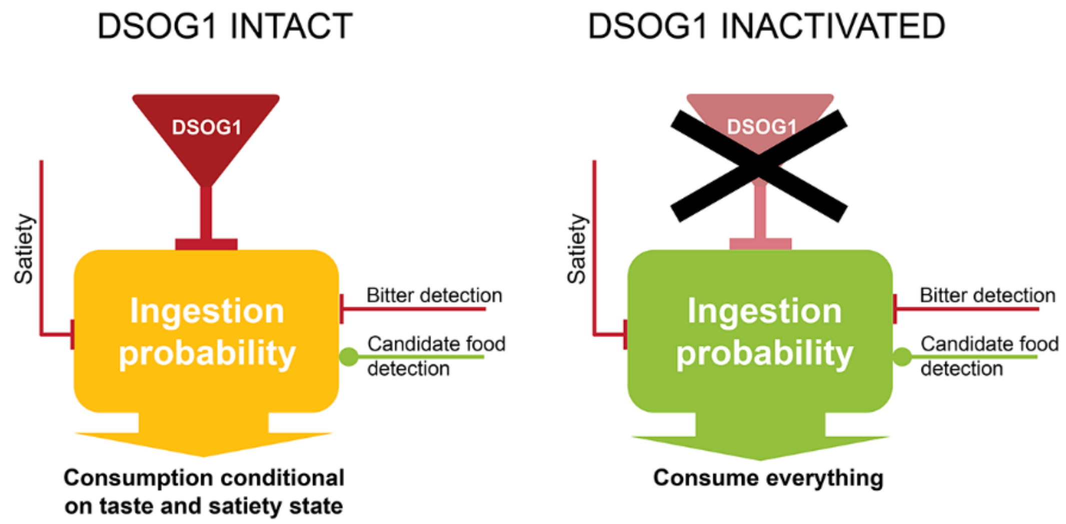


Chapter 3:
Discussion

Inhibitory control in the *Drosophila* feeding circuit

The pioneering studies in the blowfly led to the proposal of a simple model for food intake regulation in insects. It was suggested that the decision to feed in insects is a linear summation of excitatory signals stemming from the taste system and inhibitory post-ingestive signals carried by the recurrent nerve and the medial abdominal nerve that downregulate feeding based on foregut stretch and crop pressure (Dethier and Gelperin, 1967). Although the possibility of central mechanisms for establishing the feeding threshold was entertained, the lack of tools precluded any detailed analysis (Dethier, 1976).

Genetic and functional studies in *Drosophila* have significantly refined this early model by enabling targeted functional dissection of circuits in the central nervous system. To date more than a dozen central circuit nodes and molecular signaling systems have been implicated in some aspect of feeding threshold modulation (Fig. 3.1). Central feeding thresholds are inhibited by several neuropeptide systems like hugin and leucokinin as well as distinct interneurons like drosulfakinergic medial neurosecretory cells and PERin interneurons in the thoracic ganglion (Al-Anzi et al., 2010; Mann et al., 2013; Melcher and Pankratz, 2005; Söderberg et al., 2012). However, the effect of these feeding inhibitory systems is much more nuanced and distinct from the robust peripheral hyperphagic phenotypes described in the blowfly. The majority of central inhibitory mechanisms appears to fine tune the expression of a single or a subset of feeding motor action patterns – for example the PERin neurons selectively suppress meal initiations in response to locomotor activity whereas leucokinin signaling selectively adjusts the meal duration. Alternatively, the central inhibitory mechanisms adjust the feeding threshold for a distinct category of substances – for example allatostatin and hugin modify the nutrient intake threshold by adjusting the tolerance for noxious taste cues (Hergarden et al., 2012; Melcher and Pankratz, 2005).

DSOG1 interneurons as a novel layer of inhibitory control

In this thesis, I identified a collection of 4 GABA-ergic cells – DSOG1 interneurons located in the suboesophageal ganglion of the fly brain - that play an important role in establishing and maintaining a central feeding threshold. Inhibitory mechanisms controlling food intake are not uncommon in either vertebrate nor invertebrate systems (Carter et al., 2013; Hergarden et al., 2012; Jennings et al., 2013), yet there are several aspects that distinguish the mode of inhibition mediated by DSOG1 neurons from previous reports. DSOG1 ablated animals lack essentially any feeding threshold, excessively overconsuming and failing to reject any substance. They are centrally taste blind although their peripheral taste detection is intact. Furthermore, perturbing output from DSOG1 neurons also precludes the influence of specific satiety mechanisms on feeding behavior. This is notably different from the hyperphagic phenotypes resulting from recurrent nerve and abdominal nerve lesions, where blowflies display a clear taste preference as well as

avoid consuming noxious substances (Belzer, 1978; Dethier, 1976). This also differs substantially from inhibitory mechanisms described in mammalian feeding circuits where suppression arises from acute post-ingestive signals, taste processing pathways or central energetic state sensing mechanisms and is specific for a particular homeostatic category like osmoregulation or caloric intake (Bourque, 2008; Morton et al., 2006; Murphy and Bloom, 2006; Sternson, 2013).

DSOG1 inactivation derived insatiable phenotype raises a number of interesting questions about the logic of how insect feeding systems are organized. If taste quality of food is not important to trigger ingestion in the insatiable flies, what is driving feeding in these animals? Feeding appears to be contingent on some sort of sensory stimulation as the insatiable flies are not spontaneously pumping or initiating meals. Curiously even organic solvents like ethanol, for which no taste detection mechanism has been described, also trigger consumption upon DSOG1 inactivation. However, a simple dry mechanosensory stimulus on the proboscis does not appear to lead to feeding attempts. This seems to suggest that in the absence of central feeding threshold setting mechanism consumption behavior in insects can be driven by any liquid stimulus.

Another question that we addressed was the issue of regulation of DSOG1 neurons. Our experimental data suggests that DSOG1 neurons impose an unchanging inhibitory tone to the feeding circuit. We explored all feeding relevant variables including taste stimulation, food and water deprivation as well as inducing gut stretch and found that none of the external or internal cues modulate DSOG1 activity. It is feasible however that some internal cue that we did not test does pattern the activity of DSOG1 neurons. Nevertheless baseline activity in DSOG1 neurons seems to be necessary in its own right to maintain any feeding threshold. This raises the question of what happens downstream of DSOG1 neurons. Does DSOG1 inactivation relieve some continuous inhibition on feeding command neurons? Alternatively, output from DSOG1 neurons could prevent coupling between taste detection circuits and the feeding motor output circuits. More of the feeding circuit will have to be mapped to definitively answer these questions.

DSOG1 upstream regulators and downstream targets

How do DSOG1 neurons fit into a wider circuit? In my current work, multiple efforts were made to identify putative upstream signaling systems and neural pathways. Through 2-photon lesioning and functional epistasis studies, I ruled out the contribution of neural pathways mediating post-ingestive feedback from the gut (Fig 2.18). RNAi based gene knockdown approaches targeting neurotransmitter and neuropeptide receptor genes demonstrated that activity in DSOG1 neurons is not driven by a single neuropeptide (Fig 2.16) nor neurotransmitter system. This screen targeted an exhaustive selection of neuropeptide receptors as well as all ionotropic and metabotropic glutamate and acetylcholine receptor subunit genes. There are several reasons as to why this did not yield any candidate upstream signaling systems: it is feasible, that knocking down any single receptor subunit is not sufficient to eliminate sensitivity to a particular neurotransmitter. Alternatively, DSOG1 activity might be driven by as of yet untested signaling mechanism or be a function of multiple systems converging on the cell. The dendritic arborizations of

DSOG1 neurons is extensive in the SOG, which makes photoactivatable-GFP based mapping of candidate upstream neuron implausible. Alternatively, profiling the gene expression pattern in DSOG1 neurons could prove fruitful. Cell type specific transcriptome profiling in *Drosophila* has become available in the recent years and could delineate the input to DSOG1 neurons (Costa-Mattioli et al., 2012).

Excitingly, the phenotype suppressor screen yielded multiple genetic driver lines that label candidate downstream neurons from DSOG1 cells. Preliminary data seems to suggest that DSOG1 presynaptic terminals relevant for consumption regulation are located in the SOG. Intriguingly, all of the suppressor lines label subpopulations of neurons in this brain structure. The phenotype suppressors may label neurons either directly or indirectly downstream of DSOG1 neurons. For example, line *C27-Gal4* labels neurons that look identical to ingestion motorneurons and are thus obligatory members of the downstream network (Manzo and Scott, 2012; Tissot et al., 1998). Genetic approaches should be telling in distinguishing direct from indirect downstream neurons.

Gut derived post-ingestive feedback

Despite profound effects on feeding in other species, the medial abdominal nerve and the recurrent nerve systems had not been previously examined in *Drosophila*. Using two-photon lesioning, I showed that severing the recurrent nerve has profound effects on nutrient intake (Fig. 2.18). No effect on water consumption nor on ingestion of aversive bitter substances was observed following the RN lesion. This is consistent with previous findings in the blowfly, where RN lesioning derived hyperphagia was specific for appetitive substances such as carbohydrate and protenacious solutions and had no effect on ingestion of aversive substances (Belzer, 1978).

Which subpopulation of neurons projecting through the recurrent nerve carry the nutrient intake inhibiting signal? Anatomic evidence from *Phormia regina* ruled out the contribution of neuroendocrine cells that project through the RN nerve and identified the projections from the hypocerebral ganglion to the SOG as causal for the hyperphagic phenotype (Dethier and Gelperin, 1967). Previous anatomic studies have also demonstrated the presence of a similar projection in the fruit fly (Rajashekhar and Singh, 1994b).

The molecular basis for RN derived inhibition is unknown. Whether the recurrent nerve carries information about foregut stretch as predicted by earlier work in the blowfly or a chemosensory signal remains to be determined and is addressable in *Drosophila*. The cellular diversity in the *Drosophila* hypocerebral ganglion has not been studied in detail. Interestingly, however Miyamoto and colleagues recently showed that *Gr43a* – a fructose responsive gustatory receptor and internal nutrient sensor - is also expressed by 4 cells in in the hypocerebral ganglion that heavily innervate the foregut region in front of the proventriculus and send direct projection to the SOG (Miyamoto et al., 2012).

Surprisingly, the medial abdominal nerve lesions resulted in no abnormal consumption phenotypes in *Drosophila*. There might be several reasons as to why that is the case. In initial reports, the hyperphagic phenotype in blowflies was observed in pre-starved animals feeding on 2M glucose (Dethier and Gelperin, 1967), whereas I report data

for 1M sucrose, water and denatonium ingestion in unstarved conditions. Based on initial studies in the blowfly showing a 4 fold increase in carbohydrate ingestion time, a nutrient ingestion phenotype would have been expected in *Drosophila*. It is conceivable however that the role of the mAN system, if it has any effect on feeding in the fruit fly, is specific for meal termination and has very little to no effect on meal initiation rates, which might be suppressed by another mechanism such as input from the recurrent nerve.

DSOG1 and plasticity in the feeding circuit

How is plasticity achieved in a tonically inhibited feeding circuit? Although DSOG1 neurons appear to impart constant inhibition on circuits mediating meal initiation and ingestion, these behaviors are clearly modulated by nutritional state and feeding history (Edgecomb et al., 1994). That implies that DSOG1 derived inhibition is somehow temporally overcome or bypassed in deprivation states. The lack of feeding behaviors in sated states might reflect a general level of higher inhibition on feeding circuits stemming from systems detecting nutritional status as well as activity in the foregut. The source of this inhibition may be mediated by already described inhibitory systems such as the recurrent nerve pathway described in this thesis, allatostatinergic and drosulfakinergic signaling (Hergarden et al., 2012; Söderberg et al., 2012) as well as possibly other uncharacterized sources of inhibition. Importantly, none of these supplementary feeding inhibitory systems are sufficient on their own to suppress feeding behaviors in the absence of DSOG1 neurons. Conversely in a state of deprivation feeding behaviors may emerge by either a decline in these supplementary inhibitory signals or by dedicated pro-feeding signals imparted possibly by orexigenic signaling such as dNPF, sNPF or CRZ (Hergarden et al., 2012; Lee et al., 2004). Mapping out the feeding inhibitory circuit from DSOG1 neurons as well as efforts to dissect the taste processing pathways will prove to be informative in providing a mechanistic understanding of plasticity in the feeding circuit.

Future directions

The immediate future challenges entail identifying the upstream and downstream neurons to provide the circuit context for DSOG1 function. The phenotype suppressor screen reported in Fig. 2.19 is likely to yield downstream neurons from DSOG1 cells and clarify many of the questions raised by this study in terms of gating mechanisms governing feeding behavior. Genetic drivers labeling cells expressing different GABA-ergic receptor subunits might also provide an inroad to identifying downstream neurons. Finally recent advances in mapping the taste circuits might reveal targets innervated by the DSOG1 cluster.

Beyond mapping connectivity in this inhibitory circuit node it will be informative to observe how taste and satiety related information processing is altered upon modulating activity in DSOG1 neurons. Recent advances in global Ca^{2+} imaging that afford cellular resolution with almost full brain coverage are likely to provide important insight into the global circuit level mechanisms underlying the insatiable condition.

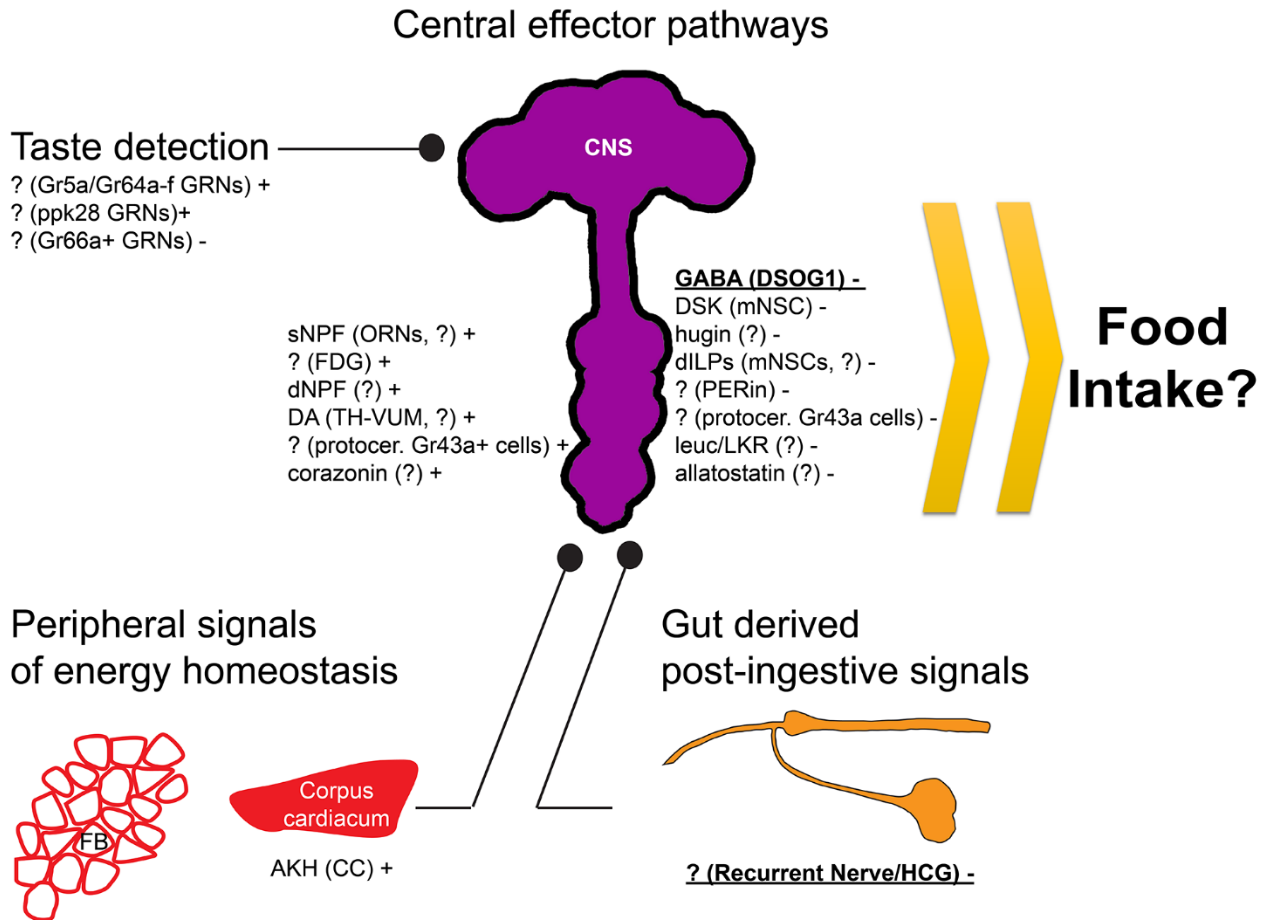
Finally more in depth investigation of the *Drosophila* recurrent nerve system is warranted. Given the prominent control of nutrient intake exerted by this pathway in *Drosophila* and other insects, tracing this pathway is likely to be an informative inroad to understanding the neural basis of energy metabolism.

Figure legends

Fig 3.1. Currently known food intake regulatory systems in *Drosophila melanogaster.* Molecular signaling systems are presented with their anatomic source in parenthesis and with a '+' or '-' indicator do denote whether they promote or inhibit feeding respectively based on currently published reports and the present thesis. The mechanisms identified in this thesis are underlined. FB – fat body, GRN – gustatory receptor neuron, HCG – hypocerebral ganglion, ORN – olfactory receptor neuron.

Figures

Figure 3.1



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