IDENTIFICATION OF A COMMAND NEURON DIRECTING THE EXPRESSION OF FEEDING BEHAVIOR IN *Drosophila melanogaster*

A Dissertation Presented

By Thomas F. Flood

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Neurobiology

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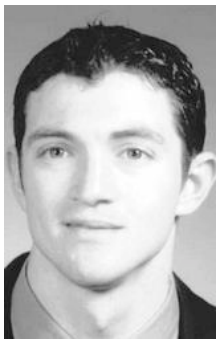
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May 12, 2011

Dedication



In Loving Memory of

Niall Dennis Murphy

November 1, 1979 – February 16, 2011

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Abstract

Feeding is one of the most important behaviors for an animal's survival. At a gross level, it is known that the nervous system plays a major role in the expression of this complex behavior, yet a detailed understanding of the neural circuits directing feeding behavior remains unknown. Here we identify a command neuron in *Drosophila melanogaster* whose artificial activation, using dTrpA1, a heat-activated cation channel, induces the appearance of complete feeding behavior. We use behavioral, genetic, cellular and optical imaging techniques to show that the induced behavior is composed of multiple motor programs and can function to uptake exogenous, even noxious, material. Furthermore, we resolve the neuron's location to the subesophageal ganglion, characterize its pre and post-synaptic sites, and determine its responsiveness to sucrose stimulation. Interestingly, the neuron's dendritic field is proximal to sweet sensing axon terminals and its baseline activity corresponds to the fly's satiation state, suggesting a potential point of integration between sensory, motor and motivational systems. The identification of a command neuron for feeding in a genetically tractable organism provides a useful model to develop a deeper understanding of the neural control of this ubiquitous and evolutionarily ancient behavior.

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CHAPTER I: UNDERSTANDING THE NEURAL CONTROL OF BEHAVIOR

IA. Introduction

> Behaviour is a mirror in which everyone shows his image.
>
> *Johann Wolfgang von Goethe* [1]

Imagine the movement of a cheetah sprinting across the rugged savannah terrain as it closes in on a gazelle at seventy miles per hour. One deft motion of the cheetah's nimble forepaw and the gazelle is grounded, pounced upon, and suffocated by the cat's crushing jaws. Or picture the remarkable flight of the dragonfly as it continually adjusts the beat pattern and frequency of its wings to effectively take off, soar, and land with the utmost precision on the smallest of perch. Lastly, consider the propulsive movements of an earthworm as it sends peristaltic waves from tip to tail while traversing the damp sidewalk and the agile split-second accommodation in your stride to clear the puddle while simultaneously saving the worm from being squashed beneath your sole. At first glance, it may be hard to see what the cheetah, dragonfly, earthworm, and human have in common. However, upon further contemplation, it becomes apparent that each is an animal expressing a unique behavior.

Animals display many different behaviors for many different reasons. Behaviors range from being relatively simple and stereotyped, such as worm locomotion, to highly complex and variable such as the elegant aerial display used to keep one's

sneakers dry. At a gross level, it is known that the nervous system is essential for coordinating the expression of behavior. However, the identification of specific, centrally located neurons regulating the majority of behaviors remains unknown. The general goal of this dissertation is to identify and characterize centrally located neurons that are competent components of behavioral circuits. Specifically, the genetically tractable organism, *Drosophila melanogaster*, will be utilized to elucidate the functional organization of the fruit fly's brain, by searching for, identifying, and characterizing command neurons governing instinctive behavior.

IB. History & Background

> If you would understand anything, observe its beginning and its development.
>
> *Aristotle* [2]

Animal Behavior

Strong selective pressure, in the form of the gain or loss of a meal, has necessitated *Homo sapiens'* desire for understanding animal behavior for hundreds of thousands of years. Even today accurate prediction is as salient as ever, as humans rely heavily on comprehending the behavioral patterns of insects, fish, and livestock to secure food for the masses [3]. Irrespective of the essential life-sustaining need for nourishment, animals have also been looked upon to meet the metaphysical need of illuminating the human condition. Dating back to ancient Greece, numerous philosophers have speculated on the nature of the animal mind with respect to humans, including Aristotle, Descartes, Hobbes, Nietzsche and Dennett [4-6]. However, it was not until the twentieth century that the analysis of animal behavior as a scientific pursuit, rather than as a speculative art, began to flourish. This change was precipitated by the nineteenth century release of Charles Darwin's *On the Origin of Species, The Descent of Man* and *The Expression of Emotions in Man and Animals,* which provided compelling evidence that the mind and behavior of animals and man were intimately related and bonded by common ancestry [7]. Ever since Darwin, animal behavior has been

rigorously analyzed by scores of scientific disciplines consisting of, but not limited to, psychology, behavioral ecology, ethology, neuroethology, neurobiology, sociobiology, zoology and evolutionary biology, with each field interpreting an organism's behavioral patterns from a unique perspective.

A primary goal of the current dissertation is to better understand the neural control of instinctive behavior. Instinctive behavior can be defined as a largely inheritable and unalterable tendency of an organism to make a complex and specific response to environmental stimuli without involving reason, which may only occur during specific developmental periods or physiological states [8]. Instinctive behavior comes in three main types; the first concerns highly stereotyped behavioral patterns that automatically occur in response to specific environmental stimuli. For example, if you place an object in a human infant's hand or stroke their palm, the fingers will automatically close in a grasping motion, which is a behavioral remnant from the days when we clung to the hair of our ancestral mother's back [9]. The second type refers to certain motivational states, such as that associated with maternity. Although maternal instinct can induce the occurrence of stereotyped behavior such as a rodent dam's characteristic nursing posture, it can also facilitate less rigid behavioral programs such as the highly variable search strategy used to locate displaced young. Maternal instinct results, in part, from hormonally induced changes in a rodent dam's reward pathways, which increase the attractive properties of her pups and

leads to approach behavior [10]. The third type of instinctive behavior is innate preference, which refers to an inherited predisposition to find a certain stimulus attractive or aversive. For example, fruit flies have an innate preference and aversion to sugar and bitter substances, respectively [11]. Further, dung beetles are genetically hardwired to prefer dung volatiles and some mosquitoes are built to favor human-like odors [12, 13]. Importantly, innate preferences allow an organism to respond to biologically relevant stimuli without the need to assess its value through mechanisms of learning. This strategy is advantageous when time is limited, competition fierce, and environmental constants, which an organism can genetically anticipate, exist. Innate preferences are also crucial for creating memory based associations, which help an organism make accurate predictions and quickly adapt to an inconstant environment [9, 14]. Hereinafter, instinctive behavior, or related terms, will refer to automatic, stereotyped, and rigid forms of inherited behavior, excluding instinctive motivational states and innate preferences. Going forward, ethology studies the control of instinctive behavior occurring in natural habitats, whereas, neuroethology aims to explain the nervous system's role in regulating these behaviors. These branches of science will be the focus of the next two sections.

Ethology

Elaborate behavioral patterns have been painstakingly catalogued since the 1930s when a branch of science, termed ethology, arose out of the pioneering work of

Niko Tinbergen, Konrad Lorenz, and Karl von Frisch. These scientists thoroughly observed and described animals in their natural environment and manipulated them to understand better how animals behave. In a classic experiment, Tinbergen and Lorenz collaborated to study the greylag goose's egg retrieval behavior. They observed that each time an egg rolled away from a nest, a goose would invariably lean forward, stretch out its neck, contact the egg with its bill, and gently roll the egg back into its nest. They then designed a set of ingeniously simple experiments to determine how reliably the goose performed the response. First, an egg was repeatedly removed from under the goose and placed a short distance in front of it. They noticed that each time an egg was moved in this way the goose would consistently retrieve the egg. Next, the researchers placed various egg-like objects in front of the goose and watched for the occurrence of the retrieval response. The goose responded to the egg-like objects as if they were real eggs and collected them back into the nest. Interestingly, if the researchers removed the egg-like object in the middle of the goose's retrieval response, the complete behavioral sequence would continue to completion, as if an invisible egg was being carefully shuttled back to its nest. To explain these results, Tinbergen and Lorenz postulated the existence of a neuronal program in the greylag goose, which when activated by an egg-like cue, coordinates a complex pattern of muscle contractions that produce the entire stereotyped egg retrieval response [15]. Similar behavioral processes, triggered in full by specific sensory stimuli, were found in additional animals such as the

stickleback fish and herring gull. These automatic, invariant and inheritable motor programs were labeled as instinctive behavior. The work of the early ethologists spurred tremendous interest in the field of animal behavior. Nevertheless, in the midst of all this progress, the nervous system's role in mediating the expression of complex behavior remained enigmatic [3, 9].

Neuroethology & The Command Neuron

To unravel the mystery of the neural control of instinctive behavior, a group of innovative researchers began to probe the nervous system of invertebrate organisms. In 1964, K. Ikeda and A.G. Wiersma used the crayfish, a freshwater crustacean whose relatively large neurons and simple nervous system makes it an experimentally amenable animal. The crayfish has four pairs of swimmerets, leg-like appendages that aid in swimming, stabilizing posture, and aerating eggs. Each pair is associated with one abdominal segment and one abdominal ganglion. A pair of swimmerets beat simultaneously whereas adjacent swimmerets beat in succession, such that all four pairs sequentially contract from back to front. Through a series of experiments, Ikeda and Wiersma demonstrated that an isolated abdominal nerve cord, devoid of all sensory input, was sufficient to coordinate the simultaneous and sequential contraction of the swimmerets to produce the full behavior [16].

Although an isolated abdominal nerve cord is sufficient to coordinate the expression of the full behavior, certain environmental contexts such as the need to aerate eggs, stabilize posture, or swim effectively were also known to elicit the swimmeret response. Therefore, Ikeda and Wiersma performed an additional set of experiments to determine if neuronal input into the abdominal nerve cord could regulate the response. By randomly stimulating the nerve cord at the anterior tip of the severed abdominal segment five distinct areas were identified that when stimulated could induce the full response. Furthermore, it was determined that the neural tracts of the descending inputs were distinct from the neuronal connectivity of the abdominal ganglia. It was concluded that activity in the descending inputs triggers activation of a neuronal program, located within the abdominal ganglia's circuitry, that once set in motion autonomously coordinates the expression of the full behavior [17].

Prior to this work, A.G. Wiersma also independently studied the escape response of the crayfish. He observed that a threatening touch on the crayfish's anterior end resulted in a rapid backward movement whereas a touch placed in a posterior location resulted in a rapid upward movement. By studying the neurophysiology of this behavior, Wiersma found that a single action potential in an interneuron within the ventral nerve cord could elicit the complete escape response. This finding, in conjunction with the work done on swimmerets, led to the development of Ikeda and Wiersma's concept of a command neuron. They

hypothesized that an environmental stimulus triggers activity in a sensory neuron that in turn triggers activity in an interneuron (the command neuron). It is activity in the command neuron that then regulates the complete expression of a complex behavior [17, 18]. Following the seminal work on the crayfish, command neurons mediating instinctive behaviors have been identified in numerous invertebrate organisms such as worms, crustaceans, mollusks and insects [19-23]. However, the concept of the command neuron was not rigorously defined in the initial studies and created much ambiguity within the field. Therefore, for the purposes of this dissertation, a command neuron will be defined as an interneuron whose activity can mediate the full expression of a complex and naturally occurring instinctive behavior.

IC. Why Use *Drosophila*?

> All animals are equal, but some animals are more equal than others.
>
> *George Orwell* [24]

Introduction

Drosophila melanogaster has been used as a research tool for more than a century to answer questions of biological importance dating back to 1910 when T.H. Morgan began using the organism to study the role of chromosomes in heredity [25]. Since this time, the use of *Drosophila* in the laboratory has expanded and has lead to many advances in the fields of development, immunology and neurogenetics [26-29]. For instance, in the 1960s Seymour Benzer began using the fruit fly to study the genetic basis of behavior. In his initial studies Benzer mutated genes and screened the mutated flies for behavioral defects. Benzer's work lead to the identification of genes involved in circadian rhythms, courtship and memory and provided strong empirical evidence that genes affect behavior [29-32].

Today, *Drosophila* continues to be employed as an experimental system in many fields, including neurobiology, in which it is used to answer fundamental questions concerning the neural control of behavior. For instance, the fruit fly has been successfully utilized to better understand synapse formation, control and plasticity, biological rhythms, processes of memory formation, consolidation and

retrieval, the influence of motivational signals on neural circuits, the effect of glia on neural processes, and sensory system organization and function [14, 33-40]. *Drosophila*'s impressive merit for decoding the mysteries of neural life arises, in part, from the organism's variegated and complex behavioral patterns, genetic tractability, and relatively simple nervous system. As will be discussed below, these qualities also make the fruit fly an ideal system to search for, identify, and characterize command neurons governing instinctive behavior.

Fruit fly & command neurons

Analogous to the crayfish, a command neuron pathway has been described in *Drosophila* that is essential for mediating an escape response [21,41]. The neural circuit consists of two giant fiber (GF) neurons that synapse on motor neurons, which innervate jump muscles, and on interneurons that innervate flight muscles. One action potential in a GF neuron can trigger the sequential firing of an action potential in the jump and then flight muscles resulting in the complete expression of the instinctive behavior [42]. The GF pathway mediating the escape response is one of only two command neuron circuits, the other being for courtship song, which have been identified in *Drosophila* [22]. However, adult *Drosophila* display numerous instinctive behaviors that require complex and choreographed movement for their full expression, such as grooming, feeding, egg laying, aggressive displays, stages of courtship (other than song) and flight. Therefore, it is reasonable to predict that additional unidentified command neurons exist in the

fly.

Powerful genetics

Drosophila's potent genetic tools confer an exceptional ability to manipulate neuronal elements to better understand nervous system organization and function. The GAL4-UAS system is one such tool, which grants an experimenter impressive control of neural activity in a spatially restricted manner [43]. GAL4 is a yeast transcription factor that binds to a sequence called the upstream activation sequence (UAS), causing the expression of downstream elements. When it was originally developed, the genetic sequence for GAL4 was coupled to a weak promoter and placed within a P element, a transposon present in the fruit fly. When P elements interact with the enzyme transposase, they begin to randomly jump around the fly genome. Crossing GAL4 flies with flies expressing transposase induces the excision and random insertion of the P element. This results in novel strains of flies, each with a different P element insertion site. Since the sequence for GAL4 is fused to the sequence of a weak promoter, adequate expression of GAL4 can only exist if the P element carrying GAL4 inserts in a location that places it under the control of transcriptional regulatory elements, such as enhancers. Because transcriptional regulation of genes can be tissue and cell- specific, a GAL4 sequence inserting at a specific location can be uniquely expressed in certain tissues or cell-types. Further, any gene placed downstream of a UAS sequence can be expressed in the same tissue or cell-type

specific pattern. For example, if the genetic sequence for a reporter gene, such as green fluorescent protein (GFP), is placed after the UAS sequence GFP will be expressed in the same cells as GAL4 [44, 45]. Importantly, this technique can also be used to restrict expression of molecules that can inhibit, stimulate, and monitor neural activity [46-48]. Further, GAL4 lines created by P element transposition are called enhancer trap strains. A large number of enhancer trap strains, each of which express GAL4 in a unique pattern, have been created and are available for use [49, 50]. In addition to P element transposition, GAL4 lines can also be created by placing GAL4 under the control of endogenous promoter elements known to drive expression of genes in a restricted manner [51].

Additionally, the GAL4/UAS system can be combined with GAL80 to increase spatial and temporal control of transgene expression [52, 53]. GAL80 blocks GAL4 activity by binding to its transcriptional activation domain when co-expressed within a cell. By combining GAL4 and GAL80 strains with partially overlapping expression patterns GAL4's activity can be limited. A temperature sensitive form of GAL80 is also available, which allows temporal control over GAL4 activity and can be used to avoid developmental lethality or compensatory changes that occur in mutant flies [54].

The Flipase (FLP)/Flipase recognition target (FRT) system is another powerful genetic technique that can be combined with the GAL4/UAS system and/or

GAL80 to further increase spatial and temporal control of transgene expression. FLP mediates the mitotic recombination between two FRT sites. The FLP/FRT system can be used to activate the expression of a transgene by causing the excision of an inhibitory element located between two FRT sites [53]. When under the control of a heat shock promoter, FLP-induced recombination events can be temporally influenced by the duration and intensity of the heat shock. FLP can also be expressed spatially if under the control of an endogenous promoter. The FLP-out technique has been used to elucidate the neural circuitry of *Drosophila* male courtship, feeding and egg laying behavior [55-57]. In summary, the GAL4, GAL80, and FLP-out techniques provide powerful tools to genetically dissect the fly brain and better understand the organization and function of neural circuits underlying the expression of complex behavior.

Simple nervous system

The fruit fly's relatively simple and highly reproducible nervous system facilitates the dissection of neural circuits underlying behavior. For instance, the fruit fly's central nervous system (CNS) has approximately one hundred thousand neurons, whereas humans have an estimated one hundred billion [28]. The fly's decreased complexity aids neural circuit analysis as identifying a neuron out of a hundred thousand is much more feasible than identifying one out of a hundred billion. Additionally, the fly CNS is composed of identifiable neurons. An identifiable neuron is characterized by relatively constant morphological and physiological

properties among all member of a species, at least of one sex. Importantly, the reproducibility of identifiable neurons allow for accurate comparisons of distinct neurons between con-specifics [58]. A command neuron in *Drosophila* can be thought of as one type of identifiable neuron [41]. However, although elementary, the fruit fly's nervous system is a sophisticated mass of the neural tissue capable of orchestrating a complex behavioral repertoire. The fly nervous system's combination of relative simplicity, reproducibility, and rich behavior make *Drosophila* a valuable tool to unravel the neural circuits underlying behavior.

Conclusion

Drosophila is an invertebrate, displays a variety of instinctive behaviors, and contains command neuron circuitry. Further, the fruit fly's nervous system can be extensively manipulated, is relatively simple, and is highly reproducible. Combined, these attributes make *Drosophila* an ideal system to elucidate the neural control of instinctive behavior.

ID. Significance

> …sometimes these dollars they go to projects having little or nothing to do with the public good. Things like fruit fly research in Paris, France. I kid you not.
>
> *Sarah Palin* [59]

Given the tremendous amount of knowledge that has been gleaned from *Drosophila* research over the past hundred years it is hard to believe why anyone would doubt the value of the fly. For instance, four Nobel Prizes in Physiology or Medicine have been granted since the awards inception in 1901 that have relied upon fruit fly research, including work on the role of chromosomes in hereditary, generation of mutations by x-ray, the genetic control of development, and odor receptors and the organization of the olfactory system [60]. Importantly, these discoveries clearly demonstrate that examining *Drosophila* can elucidate conserved biological processes common to many forms of life, including humans. Thus, understanding how the fruit fly's nervous system is organized and operates to produce complex behavior allows us to better understand ourselves.

Preface to CHAPTER II

The contents of this chapter will appear, in part, in the following publication:

Thomas Flood, Michael Gorczyca, Shinya Iguchi, Benjamin White, Kei Ito, and Motojiro Yoshihara. Decision-making neurons for feeding behavior revealed by genetic activation in *Drosophila*. ***Submitted***

Regarding contents of CHAPTER II

T. Flood, K. Ito and M. Yoshihara designed experiments

B. White provided an essential transgenic fly

K. Ito supervised T. Flood's behavioral screening of 'NP Lines'. M. Yoshihara supervised all other research contained within this chapter

T. Flood executed and analyzed the behavioral screening of 'NP lines'

T. Flood designed, executed and analyzed behavioral quantification experiments and performed image analysis

T. Flood, assisted by S. Iguchi, performed mosaic screening and image analysis

T. Flood produced (Figures 2.1-2.9); (Movies 2.1-2.30)

T. Flood wrote contents of this chapter

CHAPTER II: THE SEARCH FOR COMMAND NEURONS

IIA. Introduction

> The mosquito is an automaton. It can afford to be nothing else. There are only about one hundred thousand nerve cells in its tiny head, and each one has to pull its weight. The only way to run accurately and successfully through a life cycle in a matter of days is by instinct, a sequence of rigid behaviors programmed by the genes to unfold swiftly and unerringly from birth to the final act of oviposition.
>
> *Edward O. Wilson* [61]

For the insect continuation of a genetic legacy is a numbers game. To deal with the challenges of an unpredictable environment an insect will ration its resources to produce an abundance of inexpensive progeny ensuring at least some survive and reproduce. An insect's small size, early maturity, fast reproduction, short generation time, and low levels of parental investment support this strategy's success [62]. As opposed to mammals, which are born immature, dependent on parental care, and require substantial environmental learning to develop, the adult insect emerges into the world, almost, if not completely formed and ready to fight, flee, feed and mate. The insect's precarious existence necessitates this ready built nature, created by genes and driven by instinct, as there is no time for lengthy trial and error learning to meet essential needs [61].

Although some insects are exceptions to the former generalizations, *Drosophila* is not one. Upon eclosion from its pupal case, the fruit fly is able to perform many complex and stereotyped behaviors without an apparent need for prior

environmental training, such as grooming, feeding and flight. Thus, just as the mosquito, *Drosophila* relies heavily on instinctive behavior to efficiently navigate through life's complexities during its ephemeral existence.

Given the prevalence of instinctive behaviors that operate in the fly it is not surprising that they are well studied. For instance, the behavioral characteristics and neural regulation of feeding, fighting, courtship, egg laying and flight have been investigated [11, 22, 31, 39, 55-57, 63-68]. However, despite robust interest, a scarcity of knowledge exists concerning the precise CNS mechanisms that allow for efficient execution of complex behavior. In an attempt to remedy this situation, we designed experiments to identify novel command neurons that direct the expression of instinctive behavior. A command neuron is an interneuron whose activity can mediate the full expression of a complex and naturally occurring instinctive behavior (see Chapter IB, *Neuroethology & The Command Neuron*, for a detailed explanation). Further, command neurons that direct the expression of instinctive behavior have been found in various invertebrate organisms [19, 20, 23]. Importantly, *Drosophila* is an invertebrate organism, displays numerous forms of instinctive behavior, and contains command neuron circuitry [21, 22]. Thus, we reasoned novel command neurons would be present in the fly CNS that directs the expression of instinctive behavior.

IIB. Results

> "Moths, and all sorts of ugly creatures," replied Estella, with a glance towards him, "hover about a lighted candle. Can the candle help it?"
>
> *Charles Dickens* [69]

To test the prediction that command neurons direct the expression of instinctive behavior we performed a genetic screen in which we activated random neurons and examined the effect on behavior. To induce neuronal activity, flies carrying the cold-activated cation channel, Trpm8 or heat-activated cation channel, dTrpA1, were crossed to specific GAL4 enhancer trap lines and their progeny were observed at the activating temperature (see experimental set-up, Figure 3.1; see chapter IC, *Powerful genetics*, for a detailed explanation of enhancer trap) [47, 49, 50, 70]. Importantly, both Trpm8 and dTrpA1 can produce neuronal depolarization and behavioral induction when placed at the appropriate temperature [22, 47, 65, 70].

Genetic Screen with Trpm8

A primary screen of 835 GAL4 lines using Trpm8 identified 130 candidate lines showing an induced behavior when tested at 15°C, a temperature known to activate the Trpm8 channel [70]. Following the 15°C assay, the same flies were immediately retested at 25°C, at which the Trpm8 channel is inactive [70]. No flies

displayed the 15°C induced behavior when retested at 25°C. Further, the induced behavior quickly appeared and disappeared (within approximately 30 seconds, although there was less delay for turning the behavior off) when tested at 15°C and 25°C, respectively (personal observation, data not shown). Additionally, wild-type flies did not show an induced behavior when tested at 15°C (Movie 2.1). Together, these results strongly suggest that the behavioral induction is dependent on continuous Trpm8 channel activation.

Flies exhibiting Trpm8-induced behaviors were labeled and characterized based on observer preference, as opposed to established criteria, as a majority of the induced behaviors had not been described elsewhere. However, if a behavior were previously described, care was taken to accurately label the behavior according to its wild-type counterpart. Lastly, although the Trpm8-induced behaviors are relatively robust, highly penetrable, and all replicated at least once, the Trpm8 screening data is qualitative and preliminary.

Paralysis

Trpm8-induced behaviors identified via the screen consisted of paralysis, changes in locomotion, wing movements and various other behavior resembling wild-type behavioral acts (Figure 2.1). Trpm8-induced paralysis manifested in three dominant forms, labeled as Full Paralysis (n=20), Wing Beat Paralysis (n=27), and Frozen Still Paralysis (n=37) (Table 2.1). Full Paralysis is characterized by

extreme postural instability and complete immobilization (Movie 2.2; Figure 2.2a). Wing Beat Paralysis is characterized by continual wing beating with simultaneous postural instability and/or immobilization (Movie 2.3). Frozen Still Paralysis consists of an upright immobilized fly without postural instability (Movie 2.4). Preliminary data suggests the three types of paralysis may lie on an activation continuum with the relative level of Trpm8-induced activation, from least to most, being Frozen Still Paralysis, to Wing Beat Paralysis to Full Paralysis. For instance, the Frozen Still phenotype can be changed into Wing Beat Paralysis by decreasing the temperature (personal observation). Similarly, Wing Beat Paralysis can be induced into Full Paralysis by decreasing the temperature (personal observation). However, further verification is needed before a conclusive statement can be made.

Changes in locomotion

Locomotor effects induced by Trpm8 activation consisted of two groups, which are labeled, Short Spasm (n=2) and Tipsy (n=4) (Table 2.2). Short Spasm is characterized by short intermittent seizures, which result in the tumbling of the fly (Movie 2.5). Tipsy is characterized by slow uncoordinated locomotion (Movie 2.6).

Wing movements

Trpm8-induced 'Wing Movements' were categorized into three groups. Wing Raise (n=2), Wing Clip (n=2), and Wing Beat (n=3) (Table 2.3). The Wing Raise phenotype is characterized by bilateral wing elevation. The wings are raised at a forty-five degree angle (approximate) perpendicular to the length of the body accompanied by slight bilateral medial rotation (Movie 2.7; Figure 2.2b). This behavior closely resembles the wing raising phenotype that occurs during initiation of voluntary flight and also during aggressive displays [67, 71]. Interestingly, the neural circuit underlying the Trpm8-induced Wing Raise phenotype may be common to both wild-type behavioral acts and may represent the cooptation of a previously evolved behavior, such as a component of flight initiation, to serve as an aggressive display, analogous to the cooptation of molecules to serve divergent cellular roles [72]. Wing Clip consists of a quick scissoring of the wings, which remain parallel to the length of the body (Movie 2.8). Wing Beat refers to continual bilateral beating of the wings without prominent postural instability, as seen in 'Wing Beat Paralysis' (Movie 2.9).

Other behaviors

Lastly, some Trpm8-induced behaviors resembled wild-type behavioral acts such as aggression (n=1), courtship song (n=1), grooming (n=2), exploration (n=2), and jumping (n=5) (Table 2.4). *Drosophila* aggression manifests in violent and intimidating acts against con-specifics such as wing threat, fencing, boxing, and

chasing. Further, a wing threat can be combined with a forward thrust to chase a rival away [63, 71]. Resemblance to this latter act was identified in the screen (Movie 2.10). It is important to note that although chasing is a sex specific act, occurring only in wild-type males, the video demonstrates induction of the behavior in both sexes. The appropriate neural circuitry for aggressive behavior may be present in both genders, with circuit functioning regulated in a sex-specific way, as previously postulated for male-specific courtship song [73]. An additional possibility is that we are not activating neural circuits related to aggression.

Drosophila courtship behavior consists of a sequence of reciprocal behaviors between male and female flies. Male specific behaviors include orienting to a female, tapping, licking, singing, and mounting. Males sing to entice female flies into copulation and singing is referred to as courtship song. To sing males vibrate one wing, which they extend horizontally and perpendicular to the length of their body [73, 74]. Interestingly, we identified a Trpm8-induced behavior resembling courtship song (Movie 2.11, Figure 2.2c).

Grooming consists of cleaning components of the head, thorax and abdominal segments with coordinated bilateral movements of the legs. We identified a Trpm8-induced behavior resembling repetitive grooming of the head (Movie 2.12).

Wild-type fruit flies are known to explore their environment to locate valuable resources, such as food, mates, and egg laying sites[66, 75]. We identified a Trpm8-induced behavior resembling fly exploration (Movie 2.13). Trpm8-induced flies continuously wandered for the duration of the assay, which was atypical compared to a wild-type control or other screened GAL4 strains that were not disrupted by an induced behavior (Movie 2.1).

Successful jumping is necessary for initiation of voluntary flight and the execution of the escape response [67]. We identified a Trpm8-induced behavior resembling jumping (Movie 2.14). Wing elevation did not precede jumping, which would occur in initiation of flight, but rather the wings remained nestled against the fly's body reminiscent of the escape response [67]. Thus, Trpm8-induced jumping may result from activation of the cellular network directing the expression of the escape response.

A surprising result

Vigorous and coordinated male courtship behavior was induced in male flies at 25°C immediately after testing at 15°C (Movie 2.15; Figure 2.3a,b). However, the occurrence of the full behavior required the presence of a female con-specific. Interestingly, courtship behavior was not seen during the initial 15°C test. However, it is possible that at 15°C flies were stimulated to court, but background

Trpm8-induced cellular activation prevented the occurrence of the full behavior by producing a seizure like state. Raising the temperature would inactivate Trpm8 and allow for the expression of the full courtship sequence. Importantly, this scenario requires that behavioral expression continue independent of Trpm8 channel activity and may represent induction of a sexually motivated state. The requirement for the presence of a female also suggests induction of a sexually motivated state. However, other possibilities exist to explain this effect. For instance it is possible, as mentioned above, that widespread Trpm8-induced cellular activation establishes a seizure like state in the fly and results in significant scrambling of the normal CNS firing patterns. At 25°C the CNS can begin to recover and re-establish its normal firing. The courtship circuitry may be the first to recover proper functioning, whereas circuits established to inhibit courtship behavior are inactive for a longer period. Interestingly, paralyzed strains take an extended time to recover from immobilization when placed at 25°C, which suggests resetting of the CNS needs to occur, although other possibilities, such as a muscle refractory period, also exist (personal observation). However, courtship behavior is not always induced upon recovery, rather grooming behavior seems to be most prevalent upon arousal after complete paralysis, which may arise due to CNS resetting or may be stimulated from flies falling on the ground and getting dirty during paralysis (personal observation). The induction of courtship behavior has another peculiar feature that deserves consideration. Induction requires fast transition of temperature from 15°C to

25°C otherwise the behavior will not be produced (e.g. within approximately 1 minute). It is plausible that the fast rate of temperature change is inducing the courtship behavior rather than Trpm8. However, this effect is not seen in all fly strains subjected to a rapid temperature change, thus confusion as to the mechanism persists and requires further experimentation. Regardless, the ability to easily and reliably induce vigorous male courtship behavior may provide a valuable tool to researchers interested in unraveling the neural control of mating.

Genetic Screen with dTrpA1

Forty-five NP strains that displayed a Trpm8-induced behavior were re-screened using the heat-activated cation channel dTrpA1. Four induced behaviors resembling components of wild-type instinctive behavior were identified such as wing raising, egg laying, initiation of flight and feeding. Interestingly, only one strain NP0377, displayed the same dTrpA1-induced phenotype as when tested with Trpm8 (Figure 2.2b; Figure 2.4a,b; Movie 2.16). This disparity may result from differences in Trp channel properties or expression levels, which could alter behavior by affecting the induction and frequency of action potentials. Further, the Trpm8 and dTrpA1 screening temperatures of 15°C and 31°C, respectively, may alter endogenous nervous system functioning and indirectly affect the induced behavioral response. In general, the dTrpA1 screening procedure was more robust at behavioral induction relative to Trpm8. dTrpA1 will be utilized for the remainder of the experiments contained within this dissertation.

Egg Laying

Egg-laying behavior in *Drosophila* consists of a fixed sequence of relatively stereotyped motor patterns. For instance, a female fruit fly will search and assess its environment by probing the surface with its proboscis, ovipositor, and legs. Once a suitable site is determined the ovipositor motor program (OMP) will commence. The OMP consists of series of stereotyped motor programs that function to properly lay an egg, such as bending of the abdomen, ovipositor substrate insertion and egg ejection. After egg deposition the fly will invariably groom its ovipositor. This final cleansing act completes the full sequence of egg laying behavior [66]. dTrpA1-induced egg-laying was identified during the rescreen in strain NP0406 (Movie 2.17; Movie 2.18). The dTrpA1-induced behavior consists of abdominal bending and egg expulsion and resembled the wild-type behavior (Figure 2.5a). The induced egg-laying behavior only occurred in the NP0406-GAL4;uas-*dTrpA1* flies at 32°C (temperature increased to facilitate egg-laying effect) and not in control animals (Figure 2.5b). No egg-laying behavior was observed when NP0406-GAL4;uas-*dTrpA1* flies were tested at 21°C (personal observation; data not shown). Thus, we conclude that dTrpA1 activity is inducing abdominal bending and egg expulsion. Additional components of egg-laying behavior, such as the search and groom sequence described above, are not obvious at 32°C. It would be interesting to determine if the induced abdominal bending and egg expulsion could trigger ovipositor grooming, which is the next behavior in the invariant sequence. This could be tested by inducing

abdominal bending and egg expulsion at 32°C, returning the temperature to 21°C, and see if ovipositor grooming commenced. Although crude, this type of analysis may provide insight concerning how the neural circuitry mediating the full behavior is organized. Additionally, we identified line, NP0120, which showed robust dTrpA1-induced abdominal bending (Movie 2.19). In NP0120 abdominal bending only occurred in female flies, as opposed to NP0406, which demonstrated abdominal bending in both sexes. This effect can be attributed to NP0120's GAL4 X chromosome insertion site as NP males were used for all crosses. Further, egg expulsion was not seen in uas-*dTrpA1*;NP0120-GAL4 flies at 31°C. Importantly, understanding the nature of this difference between the two strains may prove useful for dissecting the precise neural circuitry regulating this complex behavior.

Feeding

An animal's feeding behavior consists of a collection of diverse behaviors such as foraging, recognizing food, and food ingestion [76, 77]. This dissertation focuses solely on behaviors associated with food ingestion and refers to them throughout as feeding, feeding behavior or the feeding response at the exclusion of other components.

In response to an appropriate gustatory stimulus a starved wild-type fly will arrest locomotion, extend its proboscis, contact and taste a potential source of

nourishment, and then retract the proboscis [76, 77]. The fly will reiterate this process till sated. NP0883-GAL4/uas-*dTrpA1* flies displayed an induced behavior closely resembling the entire wild-type feeding response (Movie 2.20; Movie 2.21). Furthermore, NP0883-GAL4/uas-*dTrpA1* show robust feeding on dyed agar at room temperature following a five-minute incubation at 31°C (personal observation; data not shown). This effect at room temperature may result from dTrpa1-induced activation of a motivational circuit or plasticity of neural elements underlying the induced feeding response.

We have made significant progress in better understanding the behavioral characteristics and neural control of induced feeding behavior. This data will be presented in Chapter III.

Initiation of Flight

During initiation of voluntary flight, a fly raises its wings and then contracts its middle leg muscles, which propels the fly into the air, while simultaneously performing a wing down-stroke. Once airborne continuous wing beating commences. This coordinated and relatively stereotyped sequence ensures a smooth and stable take off [67]. We identified strain NP0761, which demonstrated a dTrpA1-induced behavior resembling initiation of voluntary flight (Movie 2.22). Interestingly, all components of the wild-type behavioral sequence appear to be present in the dTrpA1-induced behavior, such as wing elevation, jumping, and

continuous wing beating. Impressively, actual flight was induced (Movie 2.23; Figure 2.6a,b). Further, induced flight only occurred in uas-*dTrpA1*; NP0761 flies at 31°C and not in control animals or when tested at 21°C (Figure 2.6c; behavior at 21°C, personal observation; data not shown). Thus, we conclude that dTrpA1 activity is inducing the expression of the motor patterns resembling the initiation of voluntary flight.

Since the coordinated expression of multiple motor patterns resembling the initiation of voluntary flight occurred in NP0761 we hypothesized that a command neuron would be present in its CNS GAL4 expression domain (Figure 2.7). As an initial, albeit crude, strategy to resolve the responsible neuron we performed the following two experiments. The fly CNS can be divided into a brain that is housed in the head capsule and a thoracic ganglion, which is contained within the thoracic segment. The cervical connective, located in the fly's neck, connects the brain and thoracic ganglion. Importantly, decapitated flies can be artificially induced to behave [78]. Therefore, we decapitated uas-*dTrpA1*; NP0761 flies and then tested them at 31°C. Results demonstrate that decapitated flies still display the artificially induced response resembling initiation of the voluntary flight (Movie 2.24; Figure 2.8). Further, this response did not occur in control flies at 31°C or when tested at 21°C, confirming that dTrpA1 activity is inducing the behavior (behavior at 21°C, personal observation; data not shown). The decapitation results suggest that the command neuron is

located in the thoracic ganglion. However, it is possible that severed descending interneurons, which are activated by the dTrpA1 channel, are inducing the behavior. To test this, we locally heated the cut cervical connective. Interestingly, locally heating the severed cervical connective resulted in the induction of the initiation of voluntary flight-like behavior. This effect occurred only in uas-*dTrpA1*;NP0761 flies and not wild-type controls (personal observation; data not shown). Further, the application of a non-heated probe had no effect (personal observation; data not shown). These experiments suggest that a command neuron, located in the brain, sends descending projections through the cervical connective and into the thoracic ganglion to coordinate the expression of multiple motor programs that are characteristic of the initiation of voluntary flight.

Next, to better resolve the identity of the predicted command neuron we combined NP0761, dTrpA1, GFP, and GAL80 with FLP-genetic recombination technology, as previously described and successfully used for neural circuit analysis (see Chapter 1, *Powerful techniques*, for explanation of GAL80 and FLP genetic recombination technology; see Methods section for more detailed description of methodology) [56, 57]. This technique produces mosaic flies that co-express the dTrpA1 channel and GFP in a limited subset of cells within the full GAL4 expression domain. Mosaic flies were screened at an elevated temperature for the initiation of voluntary flight-like behavior (Movie 2.25). Positive flies

were isolated, dissected, and GFP expression patterns were analyzed. Results demonstrate that mosaic flies positive for the induced behavior contain cervical connective interneurons labeled with GFP (Figure 2.9a). Further these labeled cervical connective neurons have a cell body located in the brain (2.9b). The number of neurons expressing GFP within the mosaic fly is substantially reduced relative to the full GAL4 expression domain (compare to Figure 2.7). Further, since GFP is co-expressed with dTrpA1, it is believed that dTrpA1 activation in the GFP positive cervical connective interneurons drives the expression of multiple motor programs, which are characteristic of flight initiation. Further, we believe these interneurons are command neurons for the initiation of voluntary flight. Future experimentation is needed to validate these preliminary results. Although inchoate, these experiments provide an important starting point for a rigorous genetic based exploration of this fundamental and complex instinctive behavior.

Other Behaviors

Briefly, additional lines identified during the dTrpA1 rescreen that demonstrated an induced behavior worth mentioning are NP0022 (Movie 2.26; airplane), NP1118 (Movie 2.27; backstroke), NP0523 (Movie 2.28; crazy leg paralysis), NP0114 (Movie 2.29; wing raise/aggression), NP0502 (Movie 2.30; wing raise/aggression). The name found within the parentheses loosely describes the induced behavior; additional information can be found within each movie legend.

IIC. Discussion

> The argument of this book is that we, and all other animals, are machines created by our genes.
>
> *Richard Dawkins* [79]

Adult insects, including *Drosophila*, are able to perform stereotyped behavior on initial encounters with environmental stimuli without an apparent need for prior training. To account for this phenomenon, it is believed that an organism's genes encode messages that rigidly construct certain forms of behavior during development. However, a detailed mechanistic understanding of this process, from genes, to neurons, to neural circuits, to behavior, remains largely unknown. Identification of specific, centrally located components of neural circuits governing the expression of instinctive behavior in *Drosophila*, a genetically tractable organism, would provide pertinent knowledge, as well as act as a significant resource to help unravel these outstanding mysteries. Therefore, to promote the understanding of the neural control of instinctive behavior we performed preliminary experiments to identify novel command neurons within the fly CNS.

Our genetic screen identified numerous artificially induced instinctive behaviors that closely resembled full, or partial components of wild-type behavioral acts such as feeding, flight, courtship, and egg laying. Importantly, the induced

behaviors were quickly triggered, robust, complex, and coordinated, suggesting underlying command neuron control.

We hypothesize that a unique command neuron is present in the CNS GAL4 expression domain of each strain demonstrating an induced instinctive behavior. However, enhancer trap expression domains are relatively widespread making command neuron identification difficult. To overcome this challenge we took advantage of the sophisticated genetic techniques available in *Drosophila* by combining GAL4, GAL80, and FLP technology to create mosaic flies with significantly restricted expression domains. Incipient experiments performed on NP0761, which displayed an initiation of flight phenotype, confirmed the feasibility of this technique for command neuron identification. However, before accurate identification can be made, considerable work is needed to thoroughly describe and identify the induced behavior as an authentic wild-type behavioral act. Furthermore, extensive experimentation, complementary to the FLP technique, is required to conclusively resolve the cellular elements responsible for the behavioral induction. Importantly, these criteria have been satisfied for one induced behavior identified via our genetic screen. Our progress in characterizing this highly conserved and crucial instinctive behavior as well as understanding its neural control will be the subject of the next chapter.

IID. Materials & Methods

> If your experiment needs statistics, you ought to have done a better experiment.
>
> *Ernest Rutherford* [80]

Trpm8 screening procedure

uas-*Trpm8* virgin females were crossed to enhancer trap NP line male flies and reared at 25°C. 3-14 day old progeny were tested in a custom-made chamber[49, 50]. Temperature was regulated with a TS-4 SPD Controller and Stage (Physitemp) and monitored by a IT-23 probe (Physitemp) (Figure 3.1). Behavior was viewed and recorded using a dissection microscope (Stemi, 2000-c, Zeiss) with attached CCD camera. During testing, approximately 10 flies were introduced into a 15°C chamber. Flies were observed for 1 minute at 15°C, then the temperature was lowered to 14°C and the flies were observed for an additional minute. All induced behavior was recorded. For all flies showing an induced behavior, the temperature was immediately raised to 25°C following the 2 minute low temp (14-15°C) observation and behavior was observed for an additional 2 minutes. This functioned as one type of negative control, as the Trpm8 channel should be relatively inactive at 25°C.

dTrpA1 screening procedure

uas-*dTrpA1* virgin females were crossed to enhancer trap NP lines male flies and reared at 21°C. 3-6 day old progeny were tested in a custom-made chamber. The temperature was regulated with a TS-4 SPD Controller and Stage (Physitemp) and monitored by an IT-23 probe (Physitemp) (Figure 3.1). Behavior was viewed and recorded using a dissection microscope (Stemi, 2000-c, Zeiss) with attached CCD camera. During testing, approximately 10 flies were introduced into a 31°C chamber. Flies were observed for 2 minute at 31°C. All induced behavior was recorded. For all flies showing an induced behavior, the temperature was immediately lowered to 21°C following the 2 minute observation at 31°C and behavior was observed for an additional 2 minutes. This functioned as a negative control, as the dTrpA1 channel should be relatively inactive at 21°C.

Mosaic analysis of NP0761

The following transgenes were combined into individual flies: NP0761-GAL4, uas-*dTrpA1*, uas-*mCD8::GFP*, *heat shock-FLP*, *tubulin*>GAL80>. All flies were reared and kept at 23°C until testing. Baseline expression, (e.g. no heat shock), of FLP at 23°C was adequate to mediate random excision of GAL80 resulting in restricted expression of GAL4 within NP0761's expression domain. Flies tested were aged to 2-5 days after eclosion and assayed in an empty vial inserted into a 35°C water bath. 35°C was empirically determined to produce the most robust behavior in mosaic flies (data not shown). Flies positive for continuous jumping

were isolated, then re-tested at 35°C in a homemade chamber (Figure 3.1), and finally dissected, stained, and imaged with a confocal spinning disk microscope (Improvision). Only flies positive for behavior during the initial screen and re-test were used for analysis. For more information on *hs*-FLP generated mosaic flies for neural circuit analysis the interested reader can refer to the following publications[56, 57].

Figure 2.1

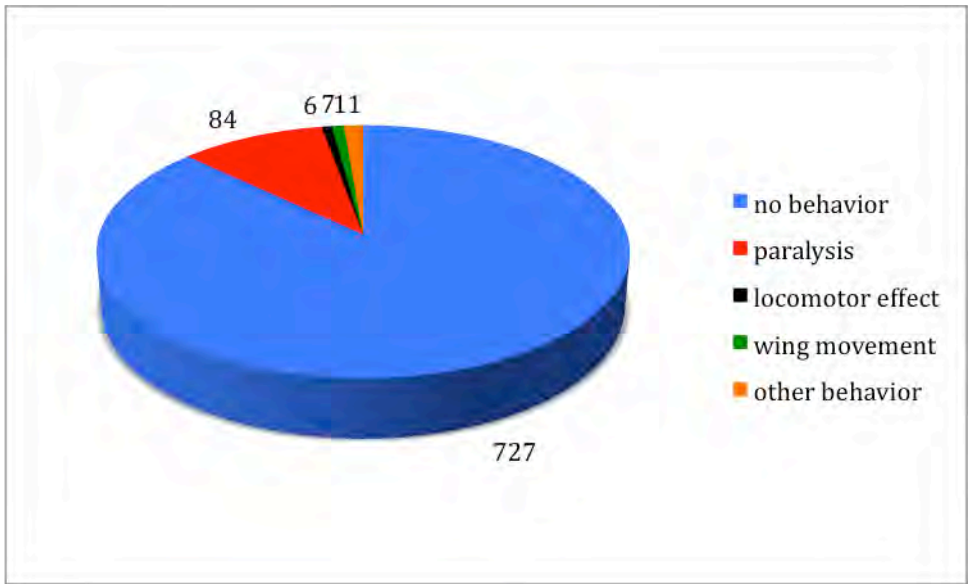


Figure 2.1| Trpm8-induced behaviors consisted of paralysis, changes in locomotion, wing movements and various other behavior. Pie chart summarizing results of the Trpm8-induced behavioral screen. Color indicates general type of induced behavior. Number of NP lines identified for each category is indicated.

Figure 2.2

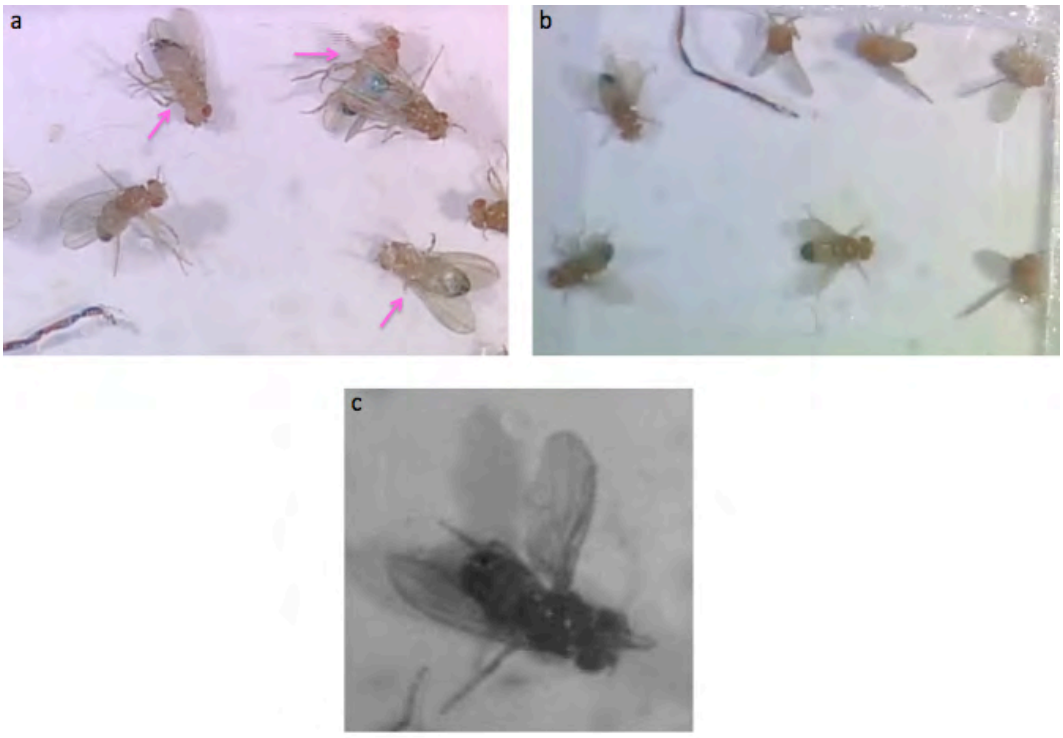


Figure 2.2| Representative Trpm8-induced behaviors. a, 'Full Paralysis' is characterized by extreme postural instability and complete immobilization. Magenta arrows mark immobile, toppled flies. Progeny of NP2106 x uas-*Trpm8* flies at 15°C (Movie 2.2). **b**, 'Wing Raising' is characterized by bilateral wing elevation with medial rotation. Progeny of NP0377 x uas-*Trpm8* flies at 15°C (Movie 2.7). **c**, Male fly demonstrating unilateral wing extension characteristic of courtship song. This image immediately precedes wing vibration. Progeny of NP0437 x uas-*Trpm8* flies at 15°C (Movie 2.11)

Figure 2.3

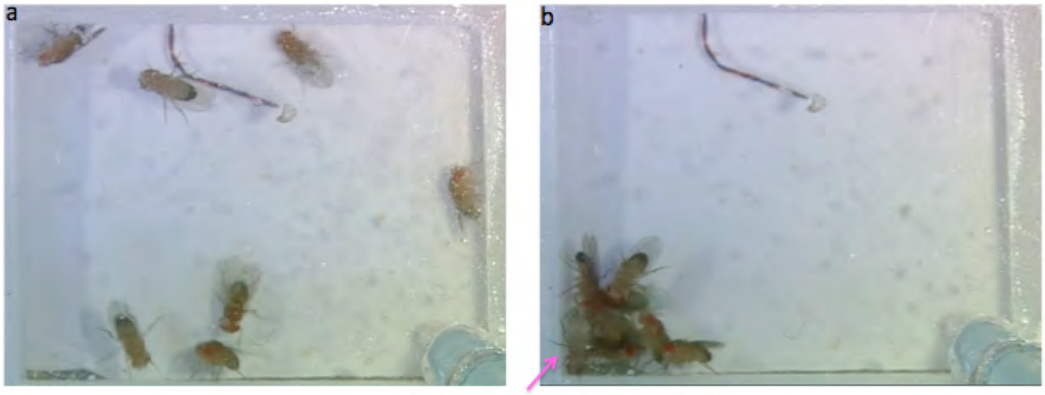


Figure 2.3| Trpm8-induced 'Courtship' at 25°C. a, Male flies at 25°C immediately after spending 2 minutes at 15°C, and immediately before a virgin female is introduced into the chamber. **b**, Male flies crowd around virgin female (magenta arrow) and repeatedly display courtship behavior. Progeny of NP0351 x uas-*Trpm8* at 25°C (Movie 2.15).

Figure 2.4

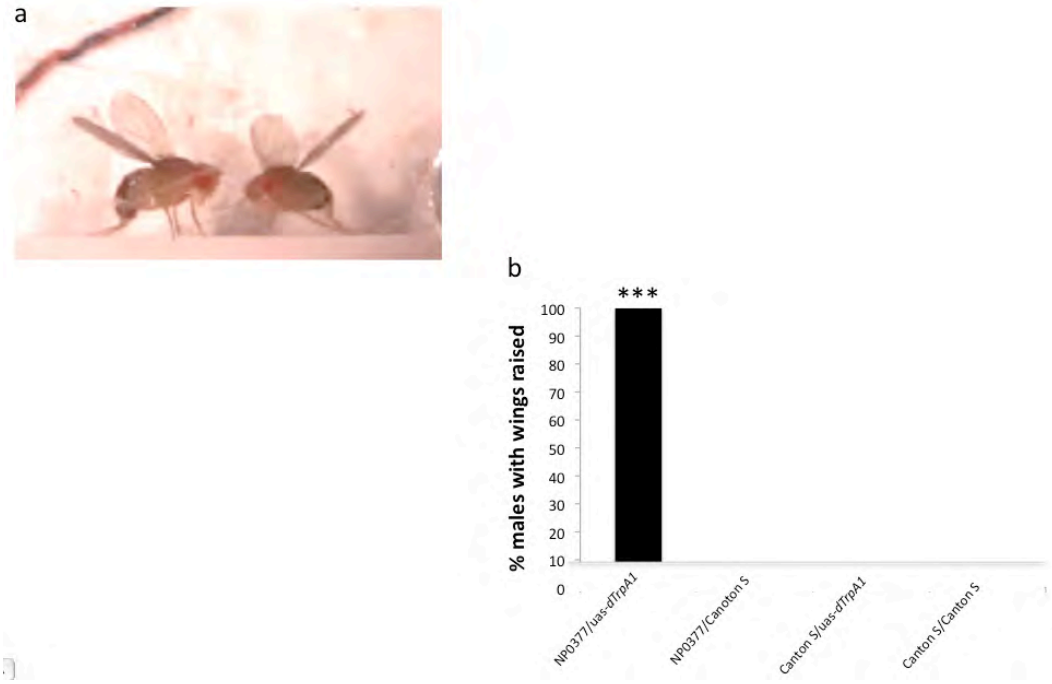


Figure 2.4| dTrpA1-induced 'Wing Raising'. 'Wing Raising' is characterized by bilateral wing elevation with medial rotation and may comprise part of the motor program for an aggressive display and/or initiation of flight. **a**, One strain NP0377, displayed the same dTrpA1-induced phenotype as when tested with Trpm8 (see Figure 2.2b). **b**, Male progeny of NP0377 x uas-*TrpA1* show a significant increase in wing elevation as compared to control animals (*** : $p < .001$; Fisher's exact test). To be scored as a positive 'wing raise' a fly's wings had to remain continuously elevated, as seen in **a**, for >5 seconds during a 5 minute observation. Multiple flies were tested together, n=20 animals for all groups. In **a,b** progeny of NP0377 x uas-*TrpA1* at 31°C (Movie 2.16).

Figure 2.5

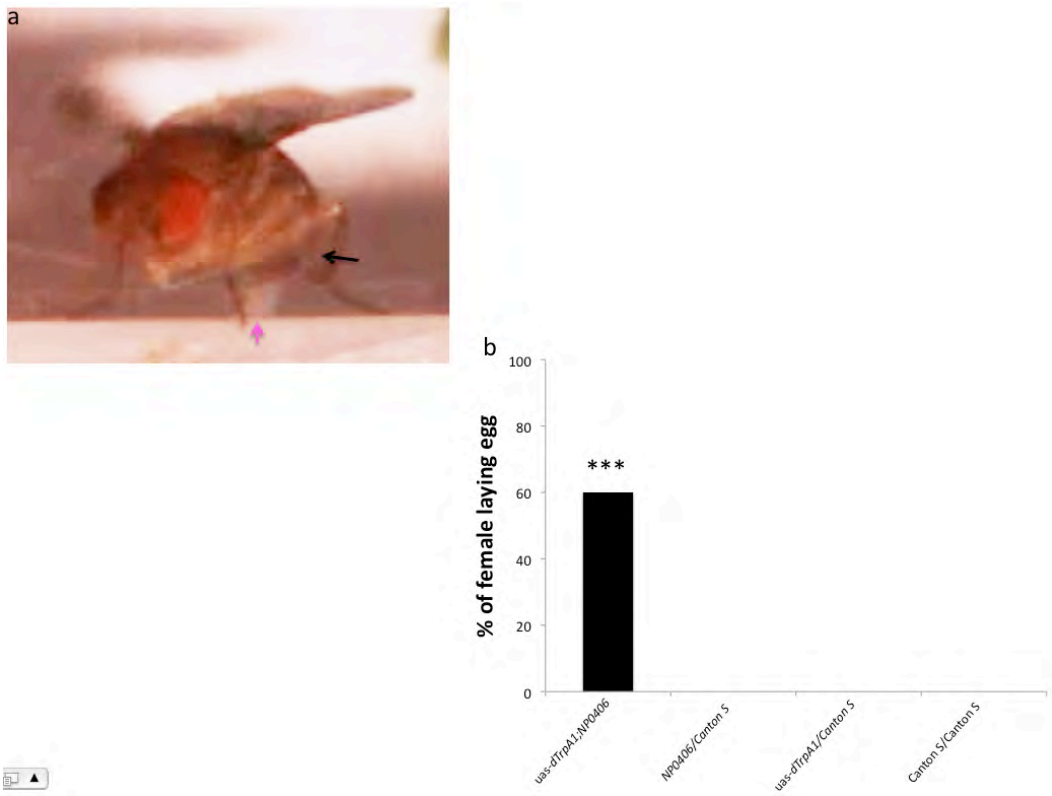


Figure 2.5| The dTrpA1-induced behavior consists of abdominal bending and egg expulsion and resembles wild-type egg laying behavior. a, Female fly displaying artificially induced abdominal bending (black arrow) and egg expulsion (magenta arrow). Progeny of NP0406 x uas-*dTrpA1* at 32°C **b**, uas-*dTrpA1*; NP0406 female flies lay eggs significantly greater than control animals at 32°C (*** : $p < .001$, Fisher's exact test). To be scored as a positive egg lay the egg must protrude ≥ halfway out of ovipositor during a 2 minute observation. Single fly tested per trial, n=20 animals for all groups. (Movie 2.17, Movie 2.18).

Figure 2.6

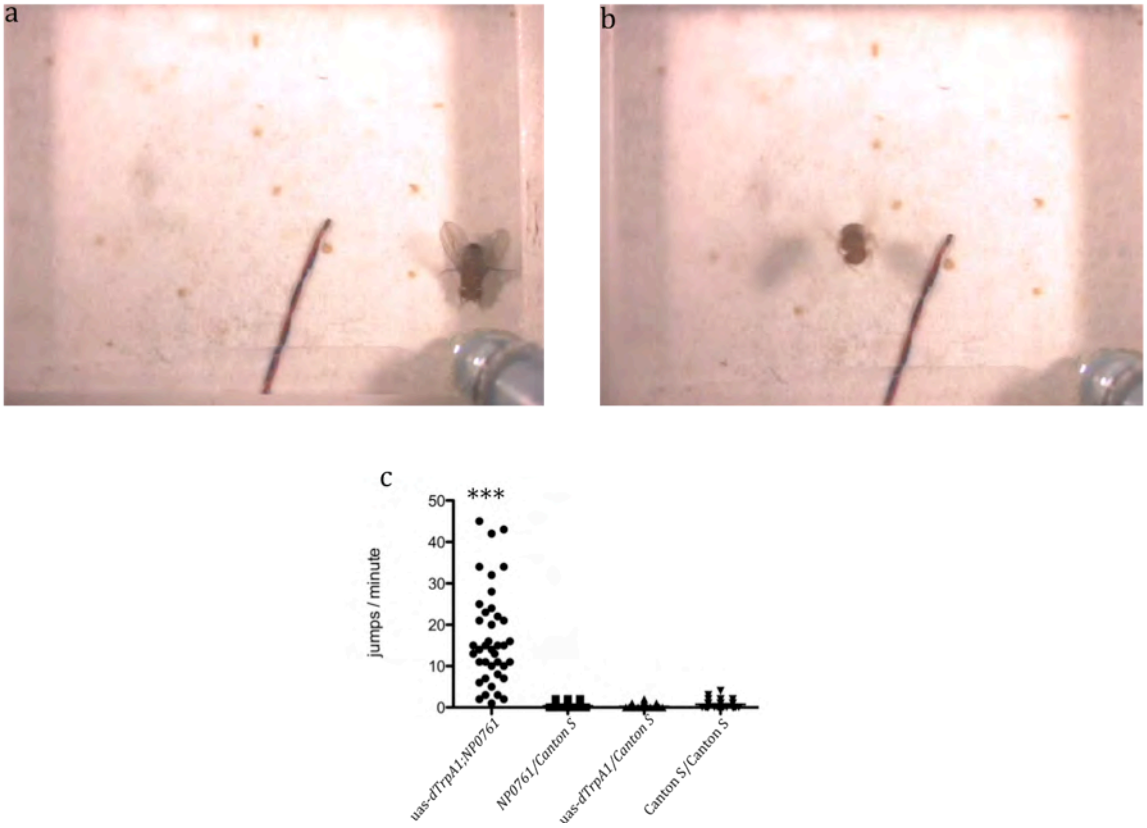


Figure 2.6| dTrpA1-induced behavior resembling initiation of voluntary flight. During initiation of voluntary flight a fly raises its wings and then contracts its middle leg muscles, which propels the fly into the air, while simultaneously performing a wing down-stroke. Once airborne continuous wing beating commences. We identified strain NP0761, which demonstrated dTrpA1-induced wing raising, jumping, and wing beating that together resemble initiation of voluntary flight. **a**, Wing elevation preceding a jump. **b,** Fly in actual flight within the 4 mm high observation chamber. **a,b** Progeny of NP0761 x uas-*dTrpA1* at 31°C. **c**, uas-*dTrpA1*;NP0761 flies display a significantly elevated rate of jumping relative to control animals (*** : $p < .001$, anova, Tukey's) To be scored a positive jump a fly must make a sudden translocation of > 5 mm (approx.) excluding walking. Jumps were scored for a 1-minute observation at 31°C. Single flies were tested per trial, n= 40 animals for all (Movie 2.22, Movie 2.23).

Figure 2.7

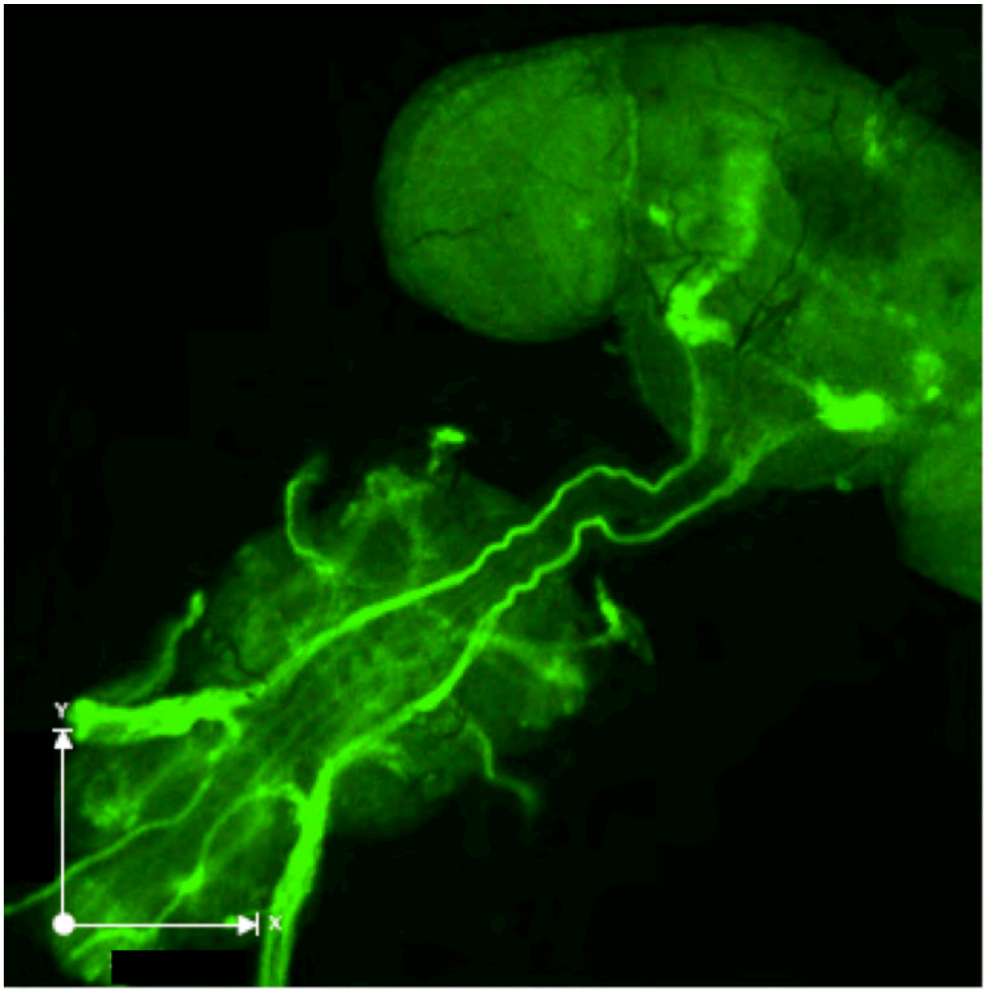


Figure 2.7| Full CNS GAL4 expression pattern of NP0761 visualized with uas-*mCD8-GPF*. Interestingly this neuroarchitecture is reminiscent of the giant fiber pathway, which is used for the escape response.

Figure 2.8

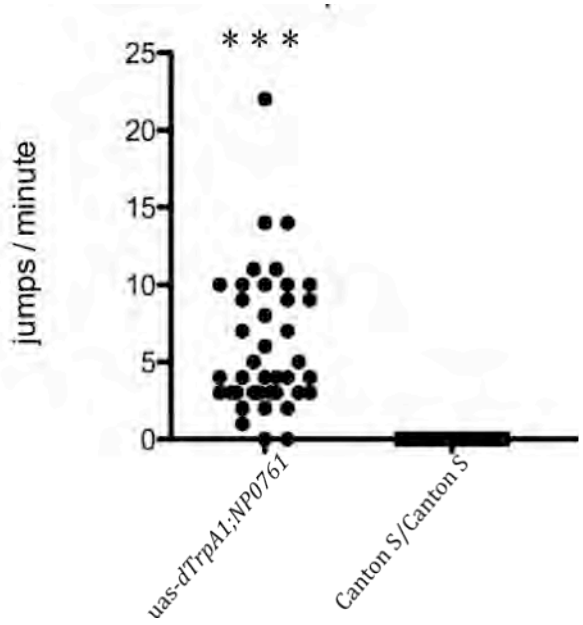


Figure 2.8| Decapitated flies demonstrate artificially induced motor programs resembling initiation of voluntary flight. Decapitated progeny of NP0761 x uas-*dTrpA1* display a significantly elevated rate of jumping relative to control animals (*** : $p < .001$, student's t-test) To be scored a positive jump a fly must make a sudden translocation of > 5 mm (approx.) excluding walking. Jumps were scored for a 1-minute observation at 32°C. Single flies tested per trial, n= 40 animals for all. Flies were anesthetized on ice, decapitated, allowed to recover at room temperature for 20 minutes and then tested (Movie 2.24).

Figure 2.9

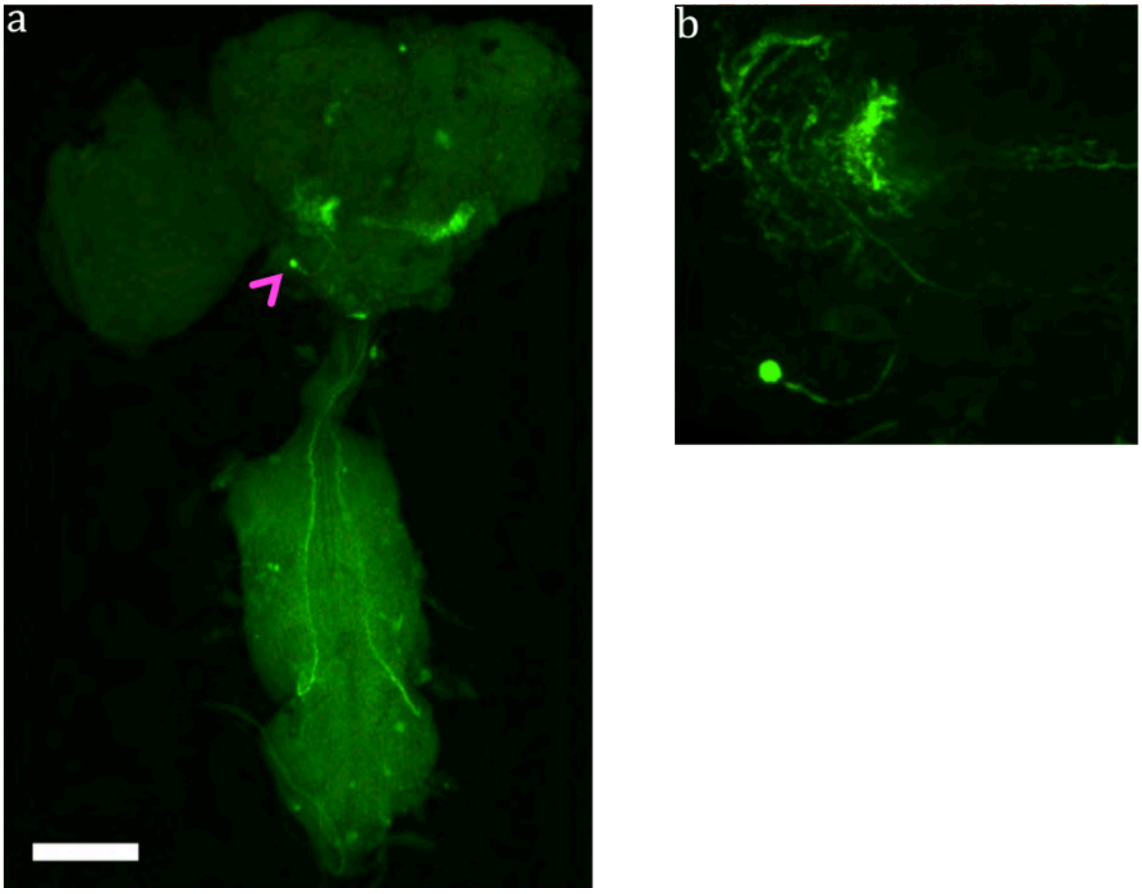


Figure 2.9| Restricted CNS expression pattern of mosaic fly positive for 'initiation of flight' behavior. Mosaic flies were screened at an elevated temperature for initiation of voluntary flight. A positive mosaic fly displaying coordinated initiation of flight behavior was dissected. **a**, GAL4 CNS expression pattern of mosaic fly visualized with uas-*mCD8-GFP* showing a nicely restricted pattern relative to the full GAL4 expression pattern (compare with Figure 2.7). Magenta arrowhead indicates cell body of putative command neuron, which sends projections through the cervical connective into the thoracic ganglion. **b**, same as **a**, high magnification image of putative command neuron cell body and brain projections. Mosaic fly contains transgenes: NP0761, uas-*dTrpA1*, uas-*mCD8-GFP*, *tubulin*->GAL80>, *hs*-FLP. Screened at 35°C. Scale bar 100 μm in **a.**

Table 2.1

	Full Paralysis	Wing Beat & Paralysis	Frozen Still
1	NP0018	NP0430	NP0242
2	NP0101	NP0514	NP0280
3	NP0120	NP0527	NP0281
4	NP0187	NP0625	NP0294
5	NP0282	NP0638	NP0323
6	NP0347	NP0644	NP0393
7	NP0368	NP0648	NP0431
8	NP0523	NP0688	NP0432
9	NP0552	NP0706	NP0507
10	NP0712	NP0708	NP0513
11	NP0906	NP0745	NP0615
12	NP1128	NP0777	NP0681
13	NP1168	NP0795	NP0685
14	NP1183	NP0808	NP0696
15	NP1297	NP0822	NP0704
16	NP1542	NP0830	NP0740
17	NP1557	NP0857	NP0753
18	NP2106	NP0887	NP0812
19	NP2213	NP0891	NP0813
20	NP2355	NP0894	NP0829
21		NP0902	NP0855
22		NP0974	NP0864
23		NP1137	NP0903
24		NP1164	NP0912
25		NP1198	NP0933
26		NP1201	NP1090
27		NP2064	NP1106
28			NP1221
29			NP1284
30			NP2045
31			NP2147
32			NP2311
33			NP2358
34			NP2360
35			NP2366
36			NP2376
37			NP2411

Table 2.1| Trpm8-induced paralysis manifested in three dominant forms. From left to right, Full Paralysis (n=20), Wing Beat Paralysis (n=27), and Frozen Still Paralysis (n=37). See text for description of each. NP lines identified displaying each form of paralysis are listed in columns.

Table 2.2

	Short Spasm	Tipsy
1	NP0035	NP0115
2	NP2309	NP0206
3		NP0212
4		NP1208

Table 2.2| Locomotor effects induced by Trpm8 activation consisted of two groups. From left to right, Short Spasm (n=2) and Tipsy (n=4). See text for description of each. NP lines identified displaying each form of locomotor effect are listed in columns.

Table 2.3

	Wing Raise	Wing Clip	Wing Beat
1	NP0210	NP0502	NP0271
2	NP0377	NP0635	NP1241
3			NP1609

Table 2.3| Trpm8-induced 'Wing Movements' were categorized into three groups. From left to right, Wing Raise (n=2), Wing Clip (n=2), and Wing Beat (n=3). See text for description of each. NP lines identified displaying each form of wing movement are listed in columns.

Table 2.4

	Aggression	Song	Grooming	Exploration	Jumping
1	NP0022	NP0437	NP0895	NP0939	NP0510
2			NP1245	NP1144	NP0957
3					NP1603
4					NP1629
5					NP2486

Table 2.4| Some Trpm8-induced behaviors resembled wild-type behavioral acts. From left to right, aggression (n=1), courtship song (n=1), grooming (n=2), exploration (n=2), and jumping (n=5). See text for description of each. NP lines identified displaying each form of induced behavior are listed in columns.

Movie 2.1

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.1| Wild-type behavior at 15°C. Canton S flies at 15°C demonstrate normal behavior including environmental exploration, con-specific inspection and grooming. This video begins after the flies were exposed to 15°C for 30 seconds.

Movie 2.2

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.2| Trpm8-induced 'Full Paralysis'. Full paralysis is characterized by extreme postural instability and complete immobilization, as demonstrated in this video by flies toppling over and not moving. In video: progeny of NP2106 x uas-*Trpm8* flies at 15°C.

Movie 2.3

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.3| Trpm8-induced 'Wing Beat Paralysis'. Wing beat paralysis is characterized by continual wing beating with simultaneous postural instability and/or immobilization. In video: progeny of NP0648 x uas-*Trpm8* flies at 15°C.

Movie 2.4

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.4| Trpm8-induced 'Frozen Still Paralysis'. Frozen Still Paralysis consists of an upright immobilized fly without postural instability. In video: progeny of NP2376 x uas-*Trpm8* flies at 15°C.

Movie 2.5

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.5| Trpm8-induced 'Short Spasm'. Short Spasm is characterized by short intermittent seizures, which result in the tumbling of the fly. In video: progeny of NP2309 x uas-*Trpm8* flies at 15°C.

Movie 2.6

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.6| Trpm8-induced 'Tipsy'. Tipsy is characterized by slow uncoordinated locomotion. This strain also appears to have intermittent seizures similar to the 'Short Spasm' line. In video: progeny of NP1208 x uas-*Trpm8* flies at 15°C.

Movie 2.7

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.7| Trpm8-induced 'Wing Raising'. The Wing Raise phenotype is characterized by bilateral wing elevation. As seen in the video the wings are raised at a forty-five degree angle (approximate) perpendicular to the length of the body and medially rotated. Further, this video is unedited and can be used to visualize the delayed behavioral induction. In general all positive strains produce an induced behavior within 30 seconds of being at 15°C. In video: progeny of NP0377 x uas-*Trpm8* flies at 15°C.

Movie 2.8

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.8| Trpm8-induced 'Wing Clip'. Wing Clip consists of a quick scissoring of the wings, which remain parallel to the length of the body. Interestingly, this behavior is induced much quicker than the 'Wing Raising' (Movie 2.7). In video: progeny of NP1280 x uas-*Trpm8* flies at 15°C.

Movie 2.9

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.9| Trpm8-induced 'Wing Beat'. Wing Beat refers to continual bilateral beating of the wings without prominent postural instability. In video: progeny of NP1241 x uas-*Trpm8* flies at 15°C.

Movie 2.10

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.10| Trpm8-induced 'Aggression'. A wing threat can be combined with a forward thrust to chase a rival away. In this video flies raise their wings and run throughout the chamber. Interestingly, there seems to be a lot of interaction between flies. Further, this phenotype is similar to "Wing Raise", although noticeably different. In video: progeny of NP0022 x uas-*Trpm8* flies at 15°C.

Movie 2.15

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.11| Trpm8-induced 'Courtship Song'. To sing males vibrate one wing, which they extend horizontally and perpendicular to the length of their body. An induced behavior resembling courtship song is identified in this video. In video: progeny of NP0437 x uas-*Trpm8* flies at 15°C.

Movie 2.12

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.12| Trpm8-induced 'Grooming'. Grooming consists of cleaning components of the head, thorax and abdominal segments with coordinated bilateral movements of the legs. We identified Trpm8-induced repetitive grooming of the head. In video: progeny of NP1245 x uas-*Trpm8* flies at 15°C.

Movie 2.13

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.13| Trpm8-induced 'Exploration'. Wild-type fruit flies explore their environment to locate valuable resources, such as food, mates, and egg laying sites. In this video Trpm8-induced flies continuously wandered for the duration of the assay, resembling exploration. In video: progeny of NP1144 x uas-*Trpm8* at 15°C.

Movie 2.14

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.14| Trpm8-induced 'Jumping'. Successful jumping is necessary for initiation of voluntary flight and the execution of the escape response. We identified Trpm8-induced jumping. Wing elevation does not precede jumping, which would occur in initiation of flight, but rather the wings remained nestled against the fly's body reminiscent of the escape response. In video: progeny of NP0957 x uas-*Trpm8* at 15°C.

Movie 2.15

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.15| Trpm8-induced 'Courtship' at 25°C. *Drosophila* courtship behavior consists of a sequence of reciprocal behaviors between male and female flies. Male specific behaviors include orienting to a female, tapping, licking, singing, and mounting. In this video, vigorous and coordinated male courtship behavior was induced in male flies at 25°C immediately after testing at 15°C. Vigorous courtship was dependent on the presence of a female, although an agitated state occurred with males alone (first 45 s of video). A virgin female is introduced into the chamber at approximately 45 seconds, immediately after you see a finger on the left of the screen. In video: progeny of NP0351 x uas-*Trpm8* at 25°C.

Movie 2.16

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.16| dTrpA1-induced 'Wing Raising'. One strain NP0377, displayed the same dTrpA1-induced phenotype as when tested with Trpm8. The increased movement relative to the Trpm8 induced behavior is most likely due to the elevated temperature. In video: progeny of NP0377 x uas-*TrpA1* at 31°C.

Movie 2.17

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.17| dTrpA1-induced 'Egg Laying'. Once a suitable egg laying site is determined the ovipositor motor program (OMP) will commence. The OMP consists of series of stereotyped motor programs that function to lay an egg, such as bending of the abdomen, ovipositor substrate insertion and egg ejection. The dTrpA1-induced behavior consists of abdominal bending and egg expulsion and resembled the wild-type behavior. The fly in the bottom right corner of the video demonstrates this well. In video: progeny of NP0406 x uas-*TrpA1* at 32°C.

Movie 2.18

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.18| dTrpA1-induced 'Egg Laying' part 2. Higher magnification video image of dTrpA1-induced abdominal bending and egg expulsion. Fly is upside down. In video: progeny of NP0406 x uas-*TrpA1* at 32°C.

Movie 2.19

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.19| dTrpA1-induced 'Abdominal Bending'. Robust dTrpA1-induced abdominal bending. GAL4 is X-linked. In video: progeny of NP0120 x uas-*TrpA1* at 31°C.

Movie 2.20

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.20| dTrpA1-induced 'Feeding'. In response to an appropriate gustatory stimulus a starved wild-type fly will arrest locomotion, extend its proboscis, contact and taste a potential source of nourishment, and then retract the proboscis. The fly will reiterate this process until sated. NP0883/uas-*dTrpA1* flies displayed repetitive proboscis extension/retraction and decreased locomotion resembling the wild-type feeding response. In video: progeny of NP0883 x uas-d*TrpA1* at 31°C.

Movie 2.21

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.21| dTrpA1-induced 'Feeding' part 2. High magnification video image of NP0883-GAL4/uas-*dTrpA1* fly displaying repetitive proboscis extension/retraction and decreased locomotion. In video: progeny of NP0883 x uas-d*TrpA1* at 31°C.

Movie 2.22

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.22| dTrpA1-induced 'Initiation of Voluntary Flight'. During initiation of voluntary flight a fly first raises its wings and then contracts its middle leg muscles, which propels the fly into the air, while simultaneously performing a down-stroke. Once airborne continuous wing beating commences. This coordinated and relatively stereotyped sequence ensures a smooth and stable take off. We identified strain NP0761, which demonstrated dTrpA1-induced wing raising, jumping, and wing beating that together resemble initiation of voluntary flight. In video: progeny of NP0761 x uas-*dTrpA1* at 31°C.

Movie 2.23

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.23| dTrpA1-induced 'Initiation of Voluntary Flight' part 2. During initiation of voluntary flight a fly first raises its wings and then contracts its middle leg muscles, which propels the fly into the air, while simultaneously performing a down-stroke. Once airborne continuous wing beating commences. This coordinated and relatively stereotyped sequence ensures a smooth and stable take off. All components of the wild-type behavioral sequence appear to be present in the dTrpA1-induced behavior such as wing elevation, jumping, and continuous wing beating. Impressively, in this video actual flight is induced. In video: progeny of NP0761 x uas-*dTrpA1* at 31°C.

Movie 2.24

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.24| dTrpa1-induced 'Initiation of Voluntary Flight' part 3. Decapitated flies still demonstrate the induced response resembling 'initiation of voluntary flight'. In video: decapitated progeny of NP0761 x uas-*dTrpA1* at 32°C.

Movie 2.25

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.25| dTrpA1-induced 'Initiation of Voluntary Flight' part 4. Mosaic flies were screened at an elevated temperature for initiation of voluntary flight. The positive mosaic fly displayed coordinated behavior consisting of wing elevation and jumping resembling initiation of voluntary flight. In video: mosaic fly contains transgenes: NP0761, uas-*dTrpA1*, uas-*mCD8-GFP*, *tubulin*->GAL80>, *hs*-FLP at screened 35°C.

Movie 2.26

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.26| dTrpA1-induced 'airplane'. 'Airplane' is characterized by bilateral wing extension perpendicular to the length of the body. Further the wings are rotated 90 degrees. In video: progeny of NP0022 x uas-*dTrpA1* at 31°C.

Movie 2.27

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.27| dTrpA1-induced 'backstroke'. In 'Backstroke' flies move around the chamber on their back with wings slightly spread and legs flailing. Interestingly, this phenotype may result from disruption of the fly's proprioceptive or gravity sensing faculties. In video: progeny of NP1118 x uas-*dTrpA1* at 31°C.

Movie 2.28

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.28| dTrpA1-induced 'Crazy Leg Paralysis'. 'Crazy Leg Paralysis**'** is characterized by full paralysis with continuously and vigorously flailing legs. Studying this induced behavior may lead a better understanding of the neural control of leg movement and/or paralysis. In video: progeny of NP0523 x uas-*dTrpA1* at 31°C.

Movie 2.29

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.29| dTrpA1-induced 'Wing Raise/Aggression'. A wing threat can be combined with a forward thrust to chase a rival away. In this video flies raise their wings and run throughout the chamber. This behavior is similar to that identified in Movies 2.7, 2.10, 2.16, 2.30. Examining these strains may lead to a better understanding of the neural control of aggression. In video: progeny of NP0114 x uas-*dTrpA1* at 31°C.

Movie 2.30

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 2.30| dTrpA1-induced 'Wing Raise/Aggression' version 2. A wing threat can be combined with a forward thrust to chase a rival away. In this video flies raise their wings and run throughout the chamber. This behavior is similar to that identified in Movies 2.7, 2.10, 2.16, 2.29. Examining these strains may lead to a better understanding of the neural control of aggression. In this video flies raise their wings and run throughout the chamber. In video: progeny of NP0502 x uas-*dTrpA1* at 31°C.

Preface to CHAPTER III

The contents of this chapter will appear, in part, in the following publication:

Thomas Flood, Michael Gorczyca, Shinya Iguchi, Benjamin White, Kei Ito, and Motojiro Yoshihara. Decision-making neurons for feeding behavior revealed by genetic activation in *Drosophila*. ***Submitted***

Regarding contents of CHAPTER III

T. Flood, M. Gorczyca, S. Iguchi, and M. Yoshihara designed research

T. Flood designed and executed experiments and analyzed data contained within (Figures 3.2-3.12, 3.14, 3.15, 3.16c, 3.25); (Movies 3.1-3.7, 3.10-3.14); Table 3.1.

M. Gorczyca, S. Iguchi, and M. Yoshihara performed neuroanatomy and associated data analysis found in (Figures 3.13, 3.16a,b, 3.17 - 3.22); Movie 3.8

M. Yoshihara performed calcium imaging (Figures 3.23 - 3.24); Movie 3.9

M. Yoshihara produced Figure 3.1

Figure 3.26 contains work produced by T. Flood, M. Gorczyca, S. Iguchi, and M. Yoshihara. Figure 3.26 designed by M. Gorczyca and M. Yoshihara

Contents of CHAPTER III written by T. Flood,

Immunohistochemistry, Mosaic flies, and Calcium imaging sections of Material & Methods (written by M. Gorczyca and M. Yoshihara).

M. Gorczyca and M. Yoshihara assisted in writing the figure legends for neuroanatomy and calcium imaging sections.

M. Yoshihara supervised all research contained within this chapter

CHAPTER III: IDENTIFICATION OF A COMMAND NEURON DIRECTING THE EXPRESSION OF FEEDING BEHAVIOR

IIIA. Introduction

> Time flies like an arrow. Fruit flies like a banana.
>
> *Anonymous* [81]

An animal must ingest nutritious substances in order to survive. To fulfill this requirement the animal performs feeding behavior. Due to its importance, both in understanding basic biological processes and in promoting health and curing disease, the neurobiological control of feeding behavior has been very well studied. For example, sensory neuron input, motor neuron output, major brain regions and numerous hormones, peptides and neurotransmitters have been identified that regulate the expression of feeding behavior [76, 77, 82-84]. However, a detailed understanding of the neural circuits within the CNS that control feeding behavior remain poorly understood. Herein we identify a novel command neuron within the CNS of *Drosophila melanogaster*, which regulates the expression of complete feeding behavior.

IIIB. Results

> "Boy, the food at this place is really terrible." The other one says, "Yeah, I know; and such small portions."
>
> *Woody Allen* [85]

The command neuron concept was developed from electrophysiological experiments that studied the crayfish's swimmeret and escape responses, and can be defined as an interneuron whose activity can mediate the full expression of a complex and natural instinctive behavior [16-18]. Two command neurons, one for the escape response and one courtship song, have been indentified in *Drosophila* [22, 41]. To uncover novel command neurons we performed a genetic screen in which we activated random neurons and examined the effect on behavior. To induce neuronal activity, flies carrying Trpm8, a cold-activated cation channel, or dTrpA1, a heat-activated cation channel, were crossed to specific GAL4 enhancer trap lines, and their progeny were placed at the activating temperature (see experimental set-up; Figure 3.1) [47, 49, 50, 70]. A primary screen of 835 GAL4 lines using TRM8, followed by a secondary screen of 45 candidate lines using dTrpA1 identified one line, NP0883, which showed an induced behavior closely resembling wild-type feeding behavior (Figure 3.2a,b). An additional GAL4-line, NP5137, which displayed a nearly identical dTrpA1-induced behavior, was identified based on its GAL4 chromosomal insertion site being close to the insertion of NP0883 (Movie 3.3). In both lines, the dTrpA1-induced behavior is

quickly triggered, robust, and steady for an extended period of time and rapidly terminates upon removal from the elevated temperature (Figure 3.3a-c; see Chapter IIB: *Results* for description of behavioral termination). Wild-type feeding behavior consists of the co-expression of multiple motor programs such as locomotion arrest, head lowering, foreleg bending, proboscis extension, opening and closing of the labellar lobes and substrate sucking (Figure 3.2a; Movie 3.1) [76, 77]. Importantly, these motor programs were induced in the NP-GAL4/uas-*dTrpA1* flies at the activating temperature (Figure 3.2b; Figure 3.4a,b; Figure 3.5a-d). Furthermore, these motor programs occurred in a simultaneous and coordinated manner that appeared nearly identical to natural feeding behavior (Movie 3.2). Interestingly, females displayed enhanced characteristics of feeding behavior as compared to males in two of the three assays tested, although males did manifest all of the same induced behaviors (Figure 3.6a-c; Movie 3.4). Importantly, none of the induced behaviors were seen at 21°C when the dTrpA1 channel is inactive (Figure 3.3b; Figure 3.7a,b). In summary, these results demonstrate the coordinated expression of multiple motor programs typical of wild-type feeding behavior in the NP-GAL4/uas-*dTrpA1* flies and support the notion that activation of a command neuron is the underlying cause.

Upon appropriate sensory stimulation a hungry wild-type fly will extend its proboscis to a surface, spread its labellar lobes and initiate sucking via activation of the cibarial pump resulting in the ingestion of food (Movie 3.1; Movie 3.12) [76,

[77]. As shown above, dTrpA1 activation in our NP-GAL4 lines accurately replicates this behavior (Movie 3.2). Therefore, to test if real feeding behavior was being induced we attempted to force-feed the NP-GAL4/uas-*dTrpA1* flies an exogenous substance, dyed agar. The results demonstrate that NP-GAL4/uas-*dTrpA1* flies can be forced to ingest (Figure 3.8b). However, only the mouthparts, and not the crop, were stained with dye. This result may stem from inadequate pump muscle relaxation or asynchrony at the pump-oesophageal junction, both of which are necessary to pass ingested material to the crop [77, 86]. Support for this claim, that lack of coordination, and not lack of pump induction is the reason for impaired dye motility is found by the results demonstrating that the sucking pump is activated and functioning (Figure 3.5c,d; Figure 3.9; Movie 3.4; Movie 3.5). Further, the artificially induced cibarial activity resembles a functioning wild-type pump (Figure 3.10; Movie 3.12). Taken together, these results confirm that the behavioral induction is functional, at least partially, and provide additional evidence that the induced behavior is the natural feeding response. Next, to further characterize the ingestive effect we tested flies on dyed agar containing quinine, an aversive substance known to inhibit wild-type feeding behavior [82]. Results demonstrate that quinine inhibits dye ingestion at 31°C, but interestingly, not when tested at 33°C (Figure 3.8c,d). Additionally, a lower concentration of quinine did not inhibit ingestion, no difference was found between NP0883-GAL4/uas-*dTrpA1* and control flies at 21°C and NP0883-GAL4/uas-*dTrpA1* flies sense quinine normally (Figure 3.11b,c; Figure 3.7c;

Figure 3.12a-c). Importantly, these results demonstrate that aversive gustatory signals compete with dTrpA1-induced activation to inhibit or stimulate behavioral expression, respectively, and, by providing a link to gustatory sensation, add additional evidence that the induced behavior is indeed feeding. Furthermore, although it is possible that Tpra1 activation could make the flies motivated to ingest, we believe that ingestion of dyed agar, especially with an aversive element, combined with the findings that the induced behavior can occur in the absence of food and under highly artificially conditions, provide convincing evidence against this possibility (Movie 3.6). Rather, these results strongly suggest that the induced behavior is occurring via direct activation of a feeding motor circuit.

Since the behavioral data demonstrate the simultaneous, coordinated, and functional induction of multiple motor programs characteristic of the natural feeding response, we hypothesized that a command neuron would be present in the CNS expression domains of our NP-GAL4 lines (Figure 3.13a-c). However, before identifying a command neuron, we first needed to establish that the induced behavior was due to neuronal activation. Therefore, we generated flies carrying NP0883-GAL4, uas-*dTrpA1* and uas-GAL80, which is a negative regulator of GAL4, and tested the effect on the induced behavior. Results demonstrate expressing GAL80 pan-neuronally or in cholinergic neurons completely abolishes the induced behavior, whereas expression in muscle or

dopaminergic neurons does not (Figure 3.14) [57, 87-89]. These results confirm that activation of a neuron is responsible for the induced response and more specifically they suggest it is by activation of a cholinergic neuron, although we cannot rule out the possibility that GAL80 may be expressed in non-cholinergic neurons [90]. Next, to further resolve the responsible neuron(s), we combined NP0883, dTrpA1, GFP, and GAL80 with FLP genetic recombination technology as previously described and successfully used for neural circuit analysis [56]. Mosaic flies were individually screened and their induced behavior was quantified (Figure 3.15 and Table 3.1). After screening, positive and negative flies were dissected, stained and GFP expression patterns were examined. This analysis revealed a putative command neuron, which we term the FC-neuron (**F**eeding **C**ommand-neuron) (Figure 3.16a,b). Further, the FC-neuron's neuritic processes are stereotyped and identifiable and it exists as a bilaterally symmetric command neuron pair (Figure 3.17). In positive flies, the FC-neuron was enriched as compared to control neurons and was also found to increase in frequency with increasing strength of the induced behavior (Figure 3.18a-d, Figure 3.19a-c). However, the FC-neuron was not present in all positive flies. Thus, neurons other than the FC-neuron may be present within NP0883's GAL4 expression domain that also influence the feeding circuit (see LtCl; Figure 3.19a-c). Another possibility is that the threshold for behavioral induction may be lower than that for GFP detection. These two explanations are neither mutually exclusive nor exhaustive. Additionally, the FC-neuron was not present in the expression pattern

when combined with GAL80 strains that inhibited induced feeding, however it remained present when combined with a GAL80 stain that had no effect (Figure 3.20). Importantly, the induced behavior of mosaic FC-neuron positive flies demonstrate the coordinated induction of multiple motor programs closely resembling wild-type feeding behavior (Figure 3.16c; Movie 3.7). This data, in conjunction with the FC-neuron image analysis, strongly suggest that the FC-neuron is a command neuron that can direct the expression of multiple motor patterns characteristic of the natural feeding response.

If, as postulated, the FC-neuron is regulating the motor circuit for feeding behavior then it is reasonable to predict that it will be neuroanatomically situated in a position conducive to performing this function. In line with this prediction the FC-neuron's neuritic processes are located within the subesophageal ganglion (SOG) of the fly brain, an area of known importance for the initiation and execution of feeding behavior [56, 76, 82]. Its cell body is situated lateral of the SOG and its neuritic processes extend and ramify centrally into the SOG's ventromedial and medial portions, while also sending one minor branch to the contralateral hemisphere (Figure 3.16a) [76]. Based on FC-neuron morphology we predicted that the dense, highly branched, and tuft processes were of dendritic origin, whereas the sparse, less convoluted areas containing bulbous structures, were axonal (Figure 3.21a,b, Movie 3.8). Further, we predicted the bulbous structures to be varicosities characteristic of axon terminals. To confirm these

predictions we combined pre- and post-synaptic markers fused to GFP and the FLP-out GAL80 technique, as previously used to resolve the FC-neuron identity [91, 92]. Results demonstrate that the pre and post-synaptic markers were localized to distinct and separate neuronal compartments and their presence correlated with the morphological features characteristic of dendrites and axon terminals, respectively (Figure 3.22c-h). Importantly, these results confirm our morphological predictions (Figure 3.21). Next, since the SOG contains axon terminals of sensory neurons important for the initiation of feeding behavior, we hypothesized that these terminals may be intimately connected with the FC-neuron's dendritic region [82]. To test this possibility we combined the FLP-out GAL80 technique with GFP, driven by a sweet sensory neuron promoter element [82]. Our results demonstrate that sweet sensing neurons contain axon terminals that overlap with FC-neuron dendritic regions (Figure 3.22i). However, on close inspection these terminals do not appear to directly connect to the FC-neuron's dendritic processes (Figure 3.22j).

Although a direct connection was not identified, the degree and nature of the intermingling still suggested the possibility of information transfer from gustatory sensory neurons to the FC-neuron. Therefore, to determine a functional relationship between sweet sensation and the FC-neuron we performed *in vivo* calcium imaging of the fly brain using GCamp3, a genetically encoded calcium indicator [48]. We simultaneously monitored sucrose induced neuronal activity and

behavioral output in starved and sated flies (Movie 3.9). In starved flies the FC-neuron responded to sucrose stimulation with increased neuronal activity and behavioral output, whereas, in sated flies, this effect was not seen (Figure 3.23a,b). Importantly, an identical trend was demonstrated in a motor neuron for proboscis extension, and also replicated in our study (Figure 3.23a,b) [56]. Interestingly, sweet sensing neurons responded to sucrose stimulation regardless of satiation state, whereas proboscis extension occurred only in starved flies (Figure 3.23a,b). Surprisingly, the FC-neuron's baseline activity was found to vary depending on the animal's satiation state (Figure 3.24a-c). For example, in the starved state, the FC-neuron's baseline activity was found to fluctuate, which was not observed in sated animals or control cells from starved flies (Figure 3.24a-c). Taken together, these results implicate the FC-neuron as part of the neural circuit directing the expression of natural feeding behavior and demonstrate that its activity is influenced by satiety and/or hunger signals.

Next, to further verify the FC-neuron's role in natural feeding behavior we inhibited its function and tested starved flies' responsiveness to sucrose stimulation. Results demonstrate that adult NP0883 flies that transiently express the inward rectifying channel kir2.1, which can hyperpolarize neurons, show decreased responsiveness to sucrose stimulation (Figure 3.25a,b) [54, 93]. Flies expressing the kir2.1 channel appeared vigorous and healthy before testing, suggesting that the defect was specific to the feeding circuit (Movie 3.10).

Interestingly, decreased responsiveness occurred when sucrose was presented to either the proboscis or the tarsi and suggests that the FC-neuron is common to both sensory pathways. Further, as previously demonstrated, the induced feeding behavior and FC-neuron are responsive to bitter and sugar stimulation, respectively, and the FC-neuron is influenced by hunger-associated signals (Figure 3.8; Figure 3.23; Figure 3.24). Thus, the FC-neuron may be integrating, both internal and external, spatial and qualitative gustatory-related information and acting as a decision maker for the expression of the natural feeding response.

IIIC. Discussion

Man lives for science as well as bread.

William James [94]

Overall, these studies have demonstrated the experimental induction, via neuronal activation, of multiple motor programs that together resemble natural feeding behavior. Further, we have shown that the induced behavior is partially functional and likely results from direct activation of the feeding motor circuit. Also, we have identified the FC-neuron, which regulates the expression of this behavior and have shown that it is positioned within the SOG, among sensory axon terminals and motor neuron dendrites, of the feeding neural circuit [56, 76, 82]. The FC-neuron was also found to respond to food-related sensory cues and motivational signals and its inhibition resulted in decreased responsiveness to sucrose. Taken together, we believe the FC-neuron is a command neuron directly regulating the expression of feeding behavior via activation of the feeding motor circuit. Further, we believe the FC-neuron may integrate sensory and motivational signals and acts as a decision maker for initiating the feeding response (Figure 3.26). Future experiments will help elucidate the precise neural circuitry, as well as the internal and external environmental regulation, of the FC-neuron in relation to the feeding response.

It is well established that gustatory related sensory cues and satiation/hunger signals regulate the expression of feeding behavior [76, 77, 82-84]. However, given the limited knowledge of the underlying neural circuitry, where and how these signals are precisely functioning to generate a purposeful feeding response remains unknown. The present study's identification of a command neuron in *Drosophila*, which directly regulates feeding behavior, creates the opportunity to more thoroughly ask and answer these exciting questions. Furthermore, in addition to increasing our understanding of sensory and motivational integration, these results confer the ability to assess the integration of behavioral circuits and can shed light on the decision-making processes occurring within an animal's brain. Lastly, animals share a common evolutionary history and the process of natural selection can robustly converge adaptations into a limited number of economically effective forms. Therefore, understanding how the fruit fly's nervous system operates to control complex behavior, such as food intake, will illuminate general mechanisms of how the nervous systems of animals across all phyla are set up to do the same.

IIID. Materials & Methods

> Thanks to our present surgical methods in physiology we can demonstrate at any time almost all phenomena of digestion without the loss of even a single drop of blood, without a single scream from the animal undergoing the experiment.
>
> *Ivan Pavlov* [95]

Fly Strains

Fly stocks were raised on standard cornmeal food at 23°C and 60% relative humidity. Canton S was used as the wild-type control. The following fly strains were used: uas-*Trpm8*[70], uas-d*TrpA1*[47], uas-*mCD8-GFP*[96], *hs*-FLP[97], *tubulin*->GAL80>[98], *elav*-GAL80[57], *Mhc*-GAL80[88], *Cha-3.3kb*-GAL80[87], uas-*Brp-GFP*[91], uas-AChR-*GFP*[92], *Gr5a-GFP-IRIS-GFP-IRIS-GFP*[99], *E49*-GAL4[56], *Gr5a*-GAL4[100], uas-*GCamp3.0*[48], uas-*Kir2.1*[93], *tubP*-GAL80[ts54], NP0883 & NP5137[49, 50], uas-*mCD8*-mCherry was made by A. Sheehan and generously provided by M. Freeman before publication.

Behavioral Assays

Unless otherwise indicated the following criteria apply to all behavioral experiments: flies tested were reared at 23°C on standard fly food and aged to 3-6 days after eclosion. Approximately, 20-40 flies were tested for each assay. Both male and female flies were tested for each experiment. Temperature for behavioral assays was regulated with a TS-4 SPD Controller and Stage

(Physitemp) and monitored by a IT-23 probe (Physitemp) (Figure 3.1). Behavior was viewed using a dissection microscope (Stemi, 2000-c, Zeiss).

Proboscis Extension Assay

A positive proboscis extension (PE) was scored as follows: the tip the fly's proboscis must touch the surface of the arena (Movie 3.11). Each time the tip touches the surface counts as one proboscis extension.

Single flies were blown into a rectangular arena that was pre-warmed to 31°C (see set up; Figure 3.1b). After an incubation period of 30 seconds (s) the number of proboscis extensions (PE) were counted for 10s, immediately followed by another 30s and then another PE count for 10s. The two PE counts for each fly were averaged. During the 10s observation the fly's mouthparts must remain in view for the total duration. If out of view, the 10s observation would be immediately re-done and repeated as necessary, but for no more than 30s of elapsed time.

For the Duration Assay the above description was modified as follows (Figure 3.3c). Single flies were blown into a rectangular arena that was pre-warmed to 31°C. After an incubation period of one minute the number of PEs were counted for 10s. The 10s count was repeated once per minute for 10 minutes.

For Figure 3.3a the above description was modified as follows. Single flies were blown into a rectangular arena that was pre-warmed to 31°C. The time elapsed until observation of the first PE was recorded. A 30s incubation period was not utilized.

For mosaic analysis (Figure 3.15 and Table 3.1) the above description was modified as follows. Single mosaic flies aged 2-5 days after eclosion were blown into a rectangular arena that was pre-warmed to 37°C. After an incubation period of 30s the number of PEs were counted for 1 minute. Flies displaying for ≥ 6 PEs per minute were treated as positive for analysis, whereas flies displaying 0 PEs per minute were labeled as negative. Flies displaying 1-5 PEs per minute were not used for analysis (see Table 3.1).

For Figure 3.21 and Figure 3.22 the above description was modified as follows. Approximately 8-10 mosaic flies, ages 2-5 days after eclosion, were blown into a larger rectangular arena that was pre-warmed to 37°C (Figure 3.1a). After an incubation period of 30s, flies displaying repetitive proboscis extension behavior were isolated, dissected, stained and imaged.

Locomotion Assay

Single flies were blown into a rectangular arena that was pre-warmed to 31°C (Figure 3.1b). A line was drawn to bisect the rectangle. After an incubation

period of 30s the number of times the fly crossed the mid-line during one minute was counted. To count as a line cross the fly's entire body must cross the line.

Suction Assay

Single flies were blown into a rectangular arena that was pre-warmed to 31°C (Figure 3.1b). After an incubation period of 30s the amount of time a fly was stuck to the surface of the arena during a five-minute observation was recorded. To count as being stuck the tip of the proboscis must remain in contact with the surface of the arena for ≥ 5s.

Dye Assay

Flies were tested in a rectangular arena (12mm x 4mm x 4mm) made out of 1% agar supplemented with 5mg/mL Brilliant Blue FCF (Acros Organics) (Figure 3.11a). The arena was pre-heated to 31°C and covered with a wire screen to regulate humidity. For each experiment 4-6 flies were blown into the arena, given a 30s incubation and then a five-minute observation. After the trial, flies were aspirated from the arena (proboscis tips stain with dye if anesthetized in arena) and then anesthetized and immediately placed at -80C for a lethal duration. Next, the fly mouthparts, including the labellar lobes and pump, were scored for the presence or absence of dye. After scoring the mouthparts each fly's crop was dissected and scored for the presence or absence of dye (Figure 3.8a; Movie 3.13).

Preference Assay

Methodology was adapted from a published protocol [101]. Briefly, 50-60 flies were starved 18hrs on a wet kim wipe at room temperature. Starve flies were tested on a 96 well plate. Alternating wells were filled with either 5mM sucrose + red dye or 1mM sucrose + blue dye. The color paired with each sugar was switched between experiments to rule out color-induced effects. Flies were left on the plates for 90 minutes and then placed at -80°C for a lethal duration. Each fly's abdomen was examined for the presence of red, blue or purple (mix of each color) and a preference index (PI) was calculated: PI = (# with blue belly) + ½(# with purple belly)/ (# with blue belly + # with red belly + # with purple belly). This formula corresponds to experiments with 5mM sucrose paired with red dye and is adjusted accordingly for experiments that differ in the sucrose/color pairing.

Video Imaging

Videos were recorded using a CCD color camera (ImagingSource) mounted to a dissection microscope (Zeiss). Videos were acquired at 15 or 30 frames per second.

Immunohistochemistry

We performed immunostaining according to a protocol described previously[102] with a modification for adult brains. Paraformaldehyde (4% in PBS) was used for fixation throughout. For the original flipping screening, we used anti-GFP

antibody (rabbit polyclonal, Invitrogen) at 1:1500 and a monoclonal antibody, nc82[91] (Developmental Studies Hybridoma Bank at Iowa) at 1:10. Secondary antibodies used were FITC-conjugated donkey anti-rabbit IgG (Chemicon) at 1:500 and Cy-3 conjugated donkey anti-mouse IgG (Chemicon) at 1:100. For double staining GFP and mCherry, we used anti-GFP (mouse monoclonal, Roche Diagnostics) at 1:300 and anti-DsRed (rabbit polyclonal, Clonetech) at 1:2000 (mCherry is a derivative of DsRed). We used secondary antibodies Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) at 1:250 and DyLight 594-conjugated anti-rabbit IgG (Jackson ImmunoResearch) at 1:100. Confocal micrographs of samples were acquired with an Olympus microscope connected to an Improvision Inc. spinning disk system using Volocity software, ver. 4.3.

Mosaic flies

We used flies with the following genotype for flipping experiments for dTrpA1 and GFP;

hs-FLP (X-chromosome) ; *tubulin*->GAL80> uas-*dTrpA1*/ NP883 uas-*mCD8-GFP* (2nd Chromosomes).

We used flies with the following genotype for flipping experiments for dTrpA1, mCherry and BRP-GFP;

hs-FLP (X-chromosome) ; *tubulin*->GAL80> uas-*TrpA1*/ NP883 (2nd Chromosomes); uas- *mCD8-mCherry* uas-*BRP-GFP* / + (3rd Chromosomes).

We used flies with the following genotype for flipping experiments for dTrpA1, mCherry and AChR-GFP;

hs-FLP (X-chromosome) ; *tubulin*->GAL80> uas-*dTrpA1*/ NP883 (2^{nd} Chromosomes); uas- *mCD8-mCherry* uas-*AChR-GFP* / + (3^{rd} Chromosomes).

We used flies with the following genotype for flipping experiments for dTrpA1, mCherry and Gr5a-GFP;

Gr5a-GFP-IRIS-GFP-IRIS-GFP /*hs*-FLP (X-chromosome) ; *tubulin*->GAL80> uas-*dTrpA1*/ NP883 (2^{nd} Chromosomes); uas-*mCD8-mCherry* / + (3^{rd} Chromosomes).

These flies were aged for 2-5 days after eclosion, and tested at 37°C to observe feeding behavior. Behavior was quantified by counting PEs for 1min after an incubation period of 30s. Heat shock was not necessary as flipping was active at normal temperature.

Calcium imaging

An adult fly was anesthetized in a 15 ml plastic tube standing on ice and set in a tube attached to a custom made chamber made from a 35mm Falcon dish. One end of the dish was deformed by melting and carving to make an appropriate angle, and a hole was bored to accept the fly head while keeping mouth parts freely exposed at the outside of the chamber. Light-curing glue was used to seal the proximally adjacent part of the rostrum to the inner edge of the chamber's

hole. A sugar-free saline used previously for *Drosophila* embryonic electrophysiology was also employed here[103]. The saline contained (in mM): NaCl, 140; KCl, 2; $MgCl_2$, 4.5; $CaCl_2$, 1.5; and HEPES-NaOH, 5, pH 7.1. The head capsule was opened by a tungsten "sword", and forceps were modified to act as scissors to better clip the cuticle and trachea and expose the SOG. The esophagus, Muscle 16[86], and some air sacks were removed, and tracheas connecting the brain and head capsule were detached to avoid movements which could make the Ca^{2+} signal noisy. Ca^{2+} imaging was performed following a previous report[104]. We scanned terminals of Gr5a neurons, or the soma of an FC-neuron or rostral protractor motoneuron through a 40X water immersion lens, using a spinning disk confocal microscope (Improvision) with a BX51WI microscope (Olympus, Japan) for detecting GCaMP3.0 signal at a rate of 4 Hz with and exposure time of 122 ms. GCaMP fluorescence at the cell body of an FC-neuron or motoneuron or the terminals of Gr5a neurons was quantified as a region of interest using the Velocity software (Improvision). Image J 1.40g (NIH) was used for subtraction of a fluorescence image before stimulation from an image after stimulation to calculate the difference in fluorescence (DF). The average of fluorescence before simulation in a region of interest was calculated as "F" using Image J, and pseudocolor images of % DF/F were made by comparing DF with the F, using Adobe Photoshop. To expose Gr5a neuronal terminals, which tend to be covered by inner mouth parts, the proboscis was partly lifted by a thread. Identification of an FC-neuron by its location was confirmed by

immunostaining with anti-GFP antibody recognizing GCaMP 3.0 after Ca^{2+} imaging experiments. The proboscis was stimulated by a 100 mM aqueous sucrose solution on a wick inserted into a 1ml syringe. A special ultrathin and smooth, traditional Japanese Washi paper (Haibara, Japan) was used as a wick. This was sturdy and held solution well and was transparent when wet, all improvements for reducing experimental variation when compared to KimWipes[105]. After making a very small droplet of sugar solution at the wick, the piston of the syringe was pulled, and at the moment when the droplet was sucked, the wet surface of the Washi wick was applied to the tip of proboscis. These manipulations were done quickly to prevent the animal from drinking sucrose solution and mitigating its starved state (Movie 3.9). Labellar bristles sensed the sucrose and the proboscis extended reproducibly if flies were starved for 24 hours immediately before (Movie 3.9). Proboscis extension response (PER) behavior was monitored and recorded through a CCD camera attached to a dissection microscope at the same time as GCaMP3.0 was being imaged by the spinning disk confocal microscope. For starved experiments, flies were placed in a vial with only a wet paper towel for approximately 24 hours. We checked PER before dissection, and only flies exhibiting PER behavior were dissected. For satiated experiments, flies were placed in a grape juice/yeast pasted food vial for more than1 hour, and only flies that did not show a PER to sucrose stimulation were dissected. Each fly was given four or five presentations of sucrose solution while GCaMP and PER responses were monitored, and the percentage of positive

responses was calculated as: the number of cases where GCamp signal increases after stimulation / total number of stimulations. The preparation was stable for approximately 1 hour. Thus, we took data within one hour after starting dissection. Details of these methods will be published elsewhere (M.Y., manuscript in preparation).

Suppression by Kir channel

Assay methodology was adapted from a published protocol [106]. Briefly, all flies were reared at 19°C. Flies were collected within one day after eclosion and then placed back at 19°C overnight. Next, flies were either placed at 30°C for 18hrs or kept at 19°C. All flies were then starved on wet kim wipes for approximately 24hrs at room temperature (23°C).

Tarsus stimulation

Starved flies were anesthetized on ice, affixed to a 4mm high acrylic block, and allowed to recover in a humidified chamber for a minimum of 2hrs. During the assay, a solution containing 100 mM sucrose was touched to the forelegs as a liquid ball on a pipette tip for a three second count. Each fly was given 6 presentations of sucrose per trial. Before and after each presentation the fly was given water to satiation. Each experiment consisted of testing 10-15 flies per genotype, for a total of 6 experiments.

Proboscis stimulation

Starved flies were introduced into a cut pipette tip [105]. The fly's head was exposed to air while the body remained enclosed within the tip. This set-up allowed for proboscis stimulation without concurrent tarsus stimulation. During the assay, a solution containing 100 mM sucrose was touched to the proboscis, using the 'washi' wick method, as described in Ca imaging methods. Each fly was given 6 presentations of sucrose per trial. Before and after each presentation the fly was given water to satiation. Each experiment consisted of testing 3-5 flies per genotype, for a total of 5 experiments.

Figure 3.1

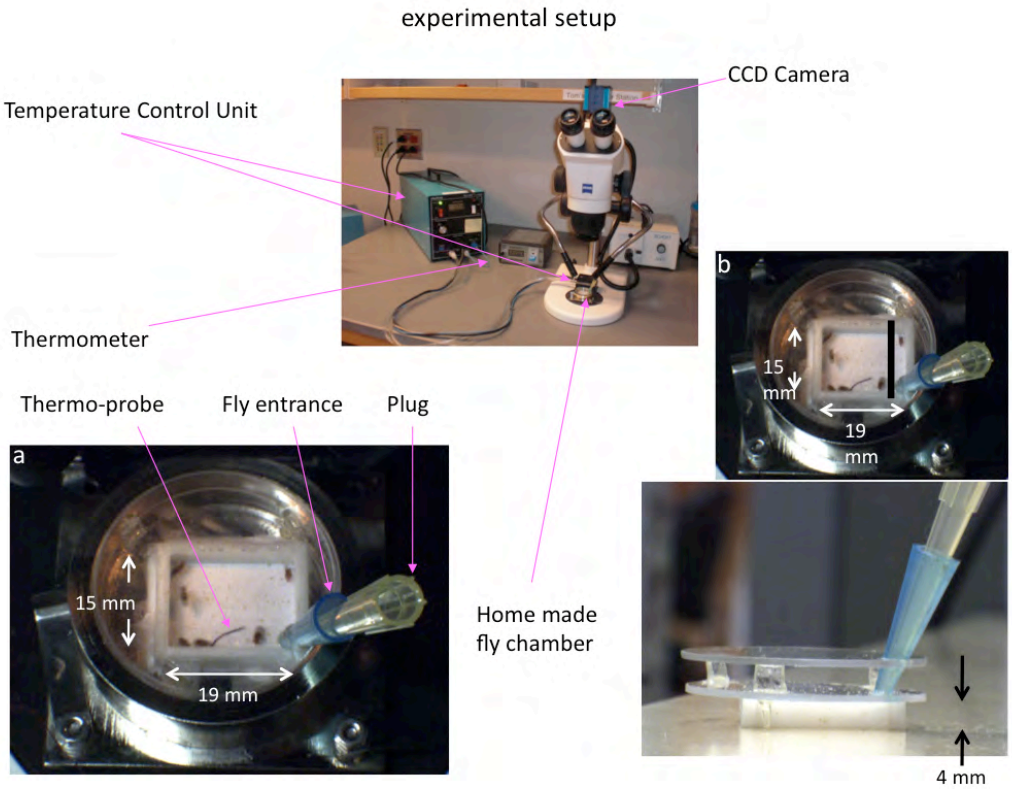


Figure 3.1| Experimental set-up used to analyze behavior. Major elements of the set-up are identified with arrows. **a**, arena dimensions used for initial screening **b**, arena dimensions used for single fly behavioral analysis.

Figure 3.2

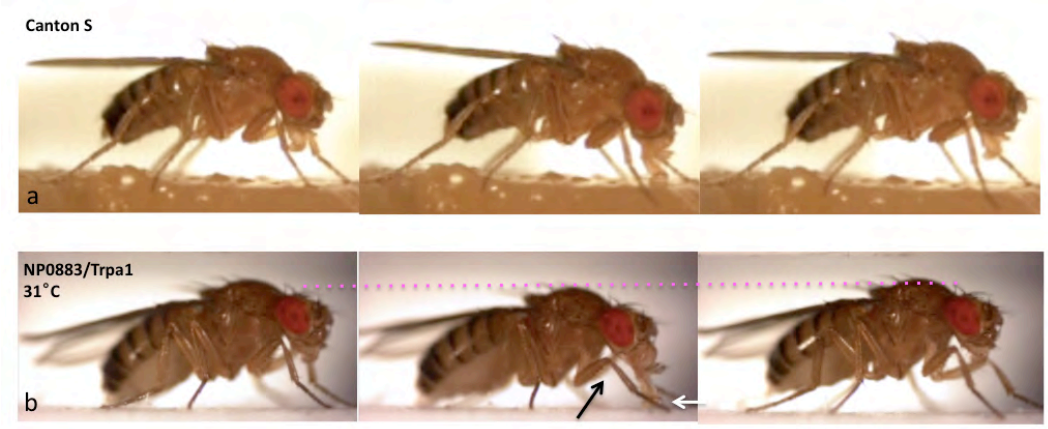


Figure 3.2| Genetically-induced behavior closely resembles wild-type feeding. **a,** Natural feeding behavior of a starved wild-type fly on normal food: from left to right; before, during, and after the feeding response. **b**, dTrpA1-induced behavior of a satiated unrestrained NP0883-GAL4/uas-*dTrpA1* fly at 31°C. No food is present. Left to right; before, during, and after the dTrpA1-induced response. Characteristics of natural feeding, such as head lowering, foreleg bending and proboscis extension are marked by the dashed magenta line, black arrow, and white arrow, respectively.

Figure 3.3

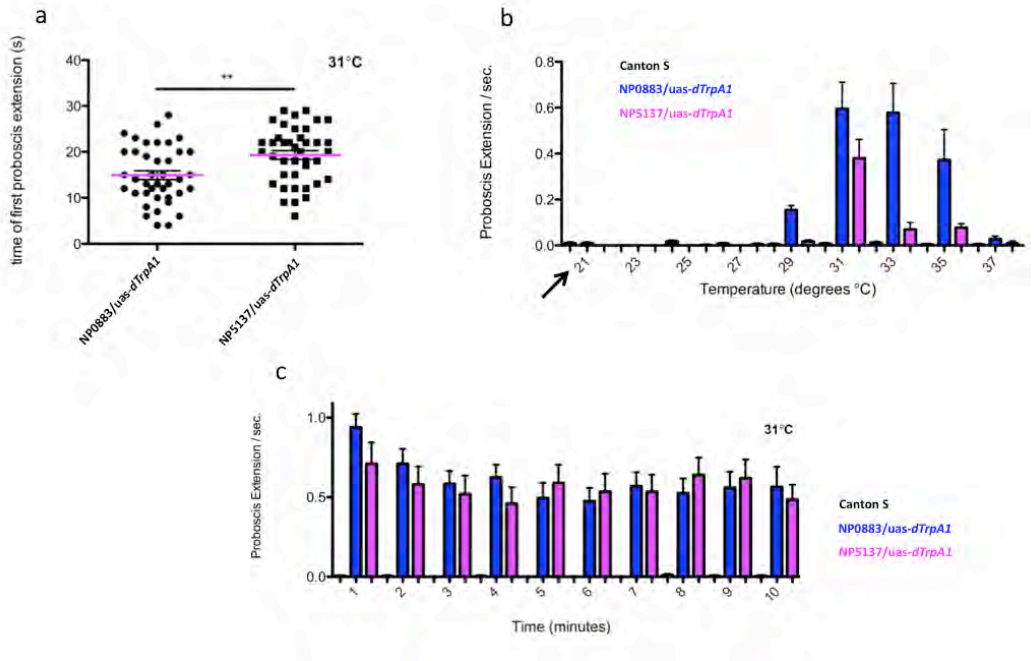


Figure 3.3| Proboscis extension behavior initiates quickly, is robust, and is steady for an extended period of time. a, Time of first proboscis extension, 31°C. All flies extended their proboscis within 30 seconds of being placed at 31°C, (*** : < .001, student's t-test). **b,** Temperature dependence of proboscis extension rate for each genotype. No induced behavior at 21°C (black arrow). **c,** Duration of proboscis extension response, 31°C. Rate of proboscis extension was steady and continued for at least 10 minutes (see Methods for description of assays).

Figure 3.4

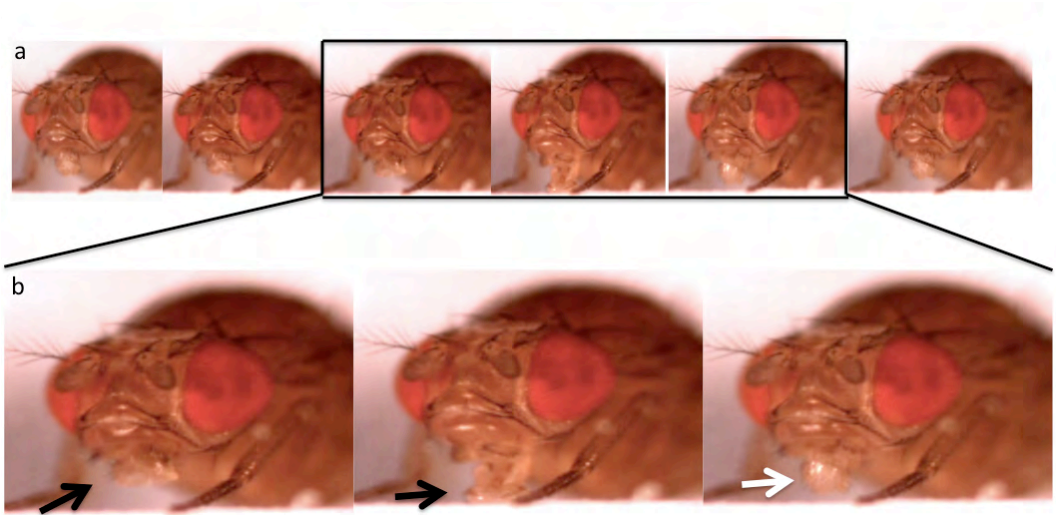


Figure 3.4| Genetically-induced labellar lobe opening and closing. a, Time lapse image of dTrpA1-induced proboscis extension in NP0883-GAL4/uas-*dTrpA1* flies at 31°C; anterior view. **b**, Higher magnification images of boxed area in **a**, black arrows mark spreading of labellar lobes on extension, white arrow marks closure on retraction.

Figure 3.5

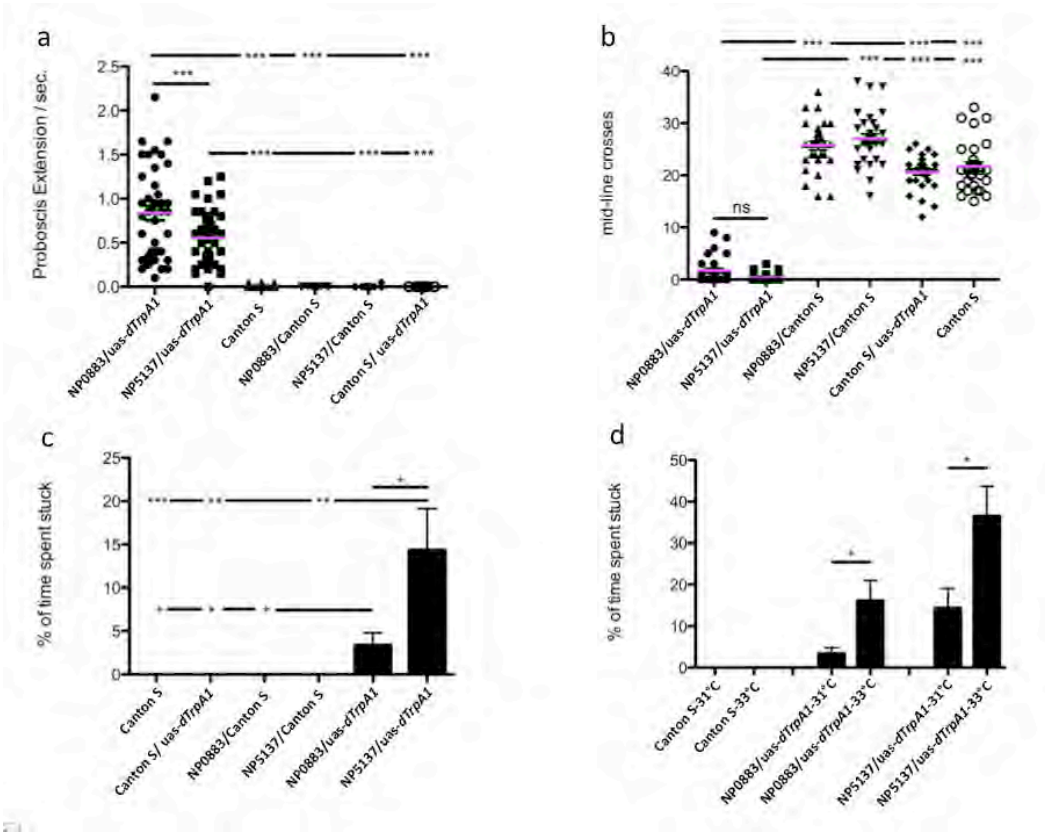


Figure 3.5| Genetically-induced motor programs characteristic of the wild-type feeding response. a, Proboscis Extension Assay at 31°C. NP-GAL4/uas-*dTrpA1* flies repetitively extend/retract proboscis in response to elevated temperature (see Movie 3.11). **b**, Locomotion Assay at 31°C. NP-GAL4/uas-*dTrpA1* flies decrease locomotion in response to elevated temperature. **c**, Suction Assay at 31°C. The proboscis tip of NP-GAL4/uas-*dTrpA1* flies sticks to surface of the behavioral arena in response to elevated temperature. **d**, Suction Assay at 31°C and 33°C. The percentage of time NP-GAL4/uas-*dTrpA1* flies stick to surface of the behavioral arena increases with increasing temperature. (for a,b,c,d (* : $p < .05$, *** : $p < .001$), anova, Tukey, n= 30-40 flies per genotype). (See methods for explanation of each assay).

Figure 3.6

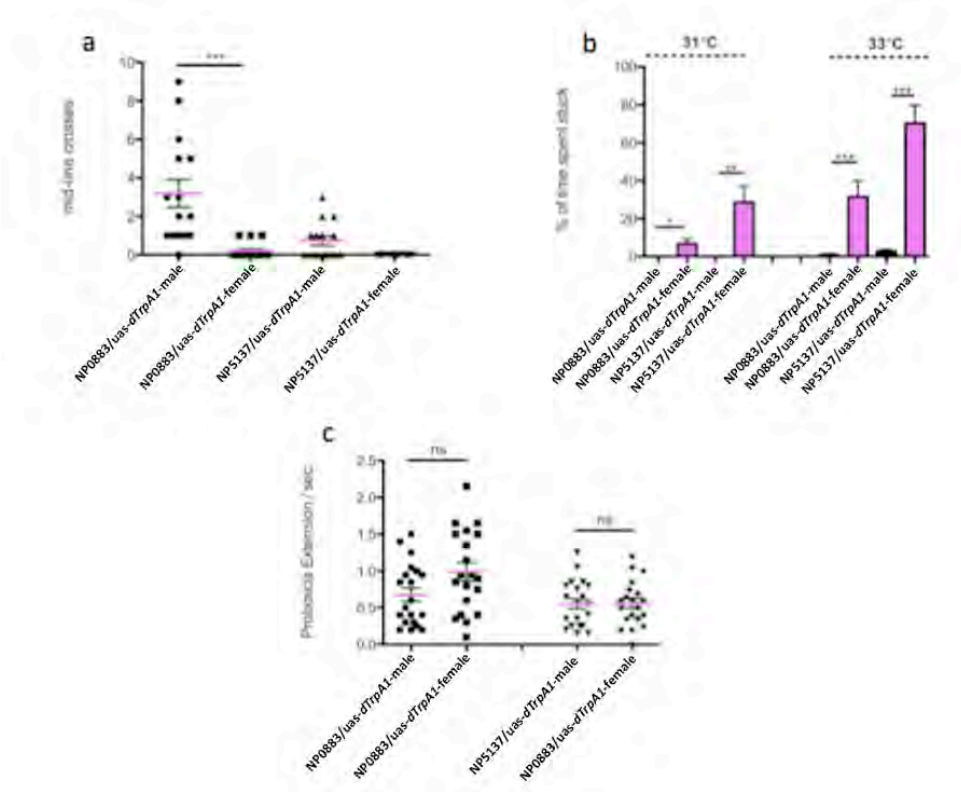


Figure 3.6| Females show enhanced behavior in 2 of 3 behavioral assays. a, Locomotion Assay, male vs. female comparison, 31°C. NP0883-GAL4/uas-*dTrpA1* females show enhanced behavior (*** : < .001, student's t-test). **b,** Suction Assay, male vs. female comparison, 31°C and 33°C. Degree of suction is enhanced in females and increases with increasing temperature (* : p < .01, *** : < .001, student's t-test). **c,** Proboscis Extension Assay, male vs. female comparison, 31°C. No statistically significant gender difference was found.

Figure 3.7

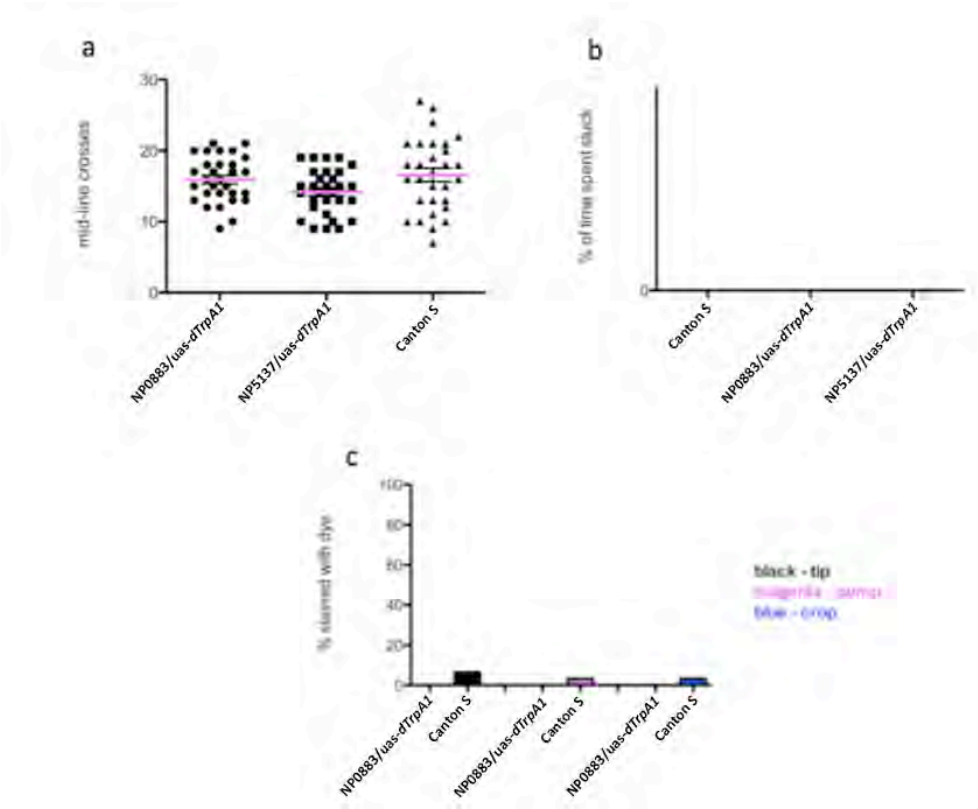


Figure 3.7| Behavioral induction was not seen at 21°C. a, Locomotion Assay at 21°C. No difference was found between NP-GAL4/uas-*dTrpA1* flies and wild-type control in degree of locomotion. **b,** Suction Assay at 21°C. No difference was found between NP-GAL4/uas-*dTrpA1* flies and wild-type control in percentage of time spent stuck to surface of arena. **c**, Dye Assay with 15 mM quinine at 21°C. No difference was found between NP-GAL4/uas-*dTrpA1* flies and control in amount of dyed agar ingested.

Figure 3.8

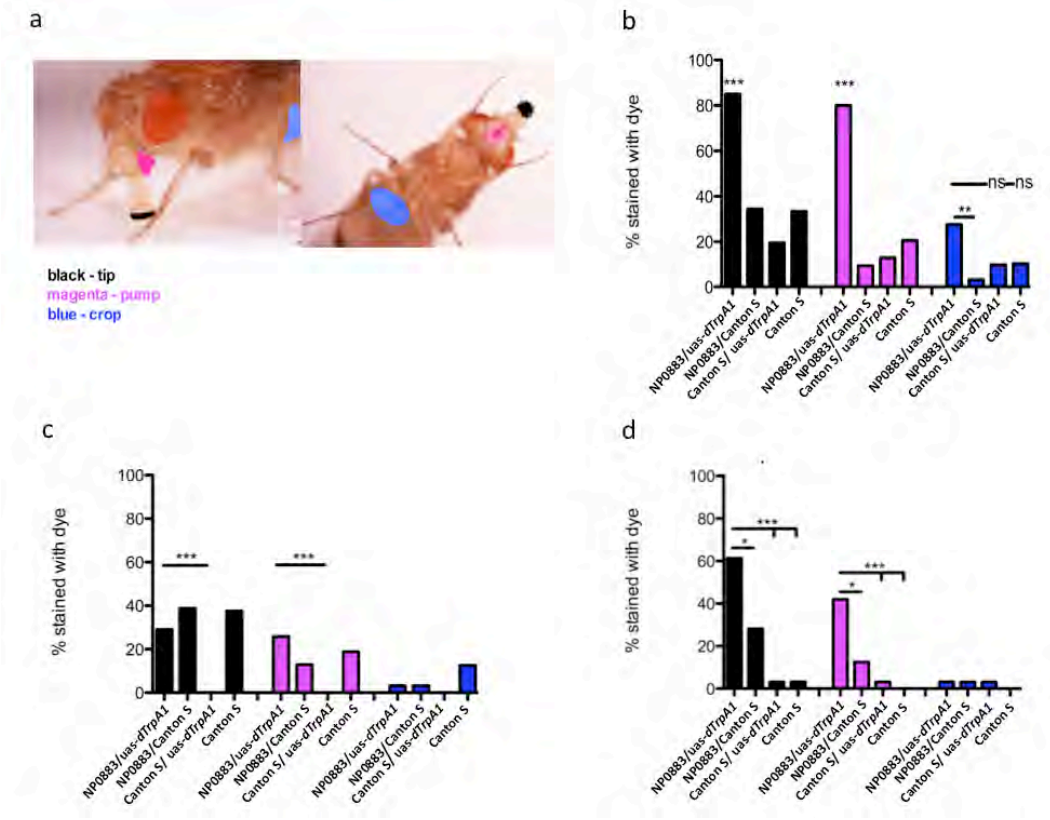


Figure 3.8| NP0883-GAL4/uas-*dTrpA1* flies can be forced to ingest. a, Illustrated schematic outlining three anatomical regions along the gastrointestinal tract analyzed in the Dye Assay (See methods, Figure 3.11a, Movie 3.13 for additional details). **b**, Dye Assay 0 mM quinine at 31°C, NP0883-GAL4/uas-*dTrpA1* flies can be forced to ingest dyed agar into the labellar lobes and pump region, but not the crop. **c**, same as **b**, but with 100 mM quinine. 100 mM quinine inhibits NP0883-GAL4/uas-*dTrpA1* dye ingestion relative to the 0 mM condition in **b**. NP0883-GAL4/uas-*dTrpA1* flies no longer display an increase in dye ingestion at any anatomical level relative to control animals. **d**, same as **c**, but at 33°C, Increased temperature results in an increase in dye ingestion in NP0883-GAL4/uas-*dTrpA1* flies at the level of the labellar lobes and pump but not the crop, relative to control animals (for b,c,d, * : $p < .05$, ** : $p < .01$, *** : $p < .001$, Fisher's exact test, n=30-40 flies per genotype.

Figure 3.9

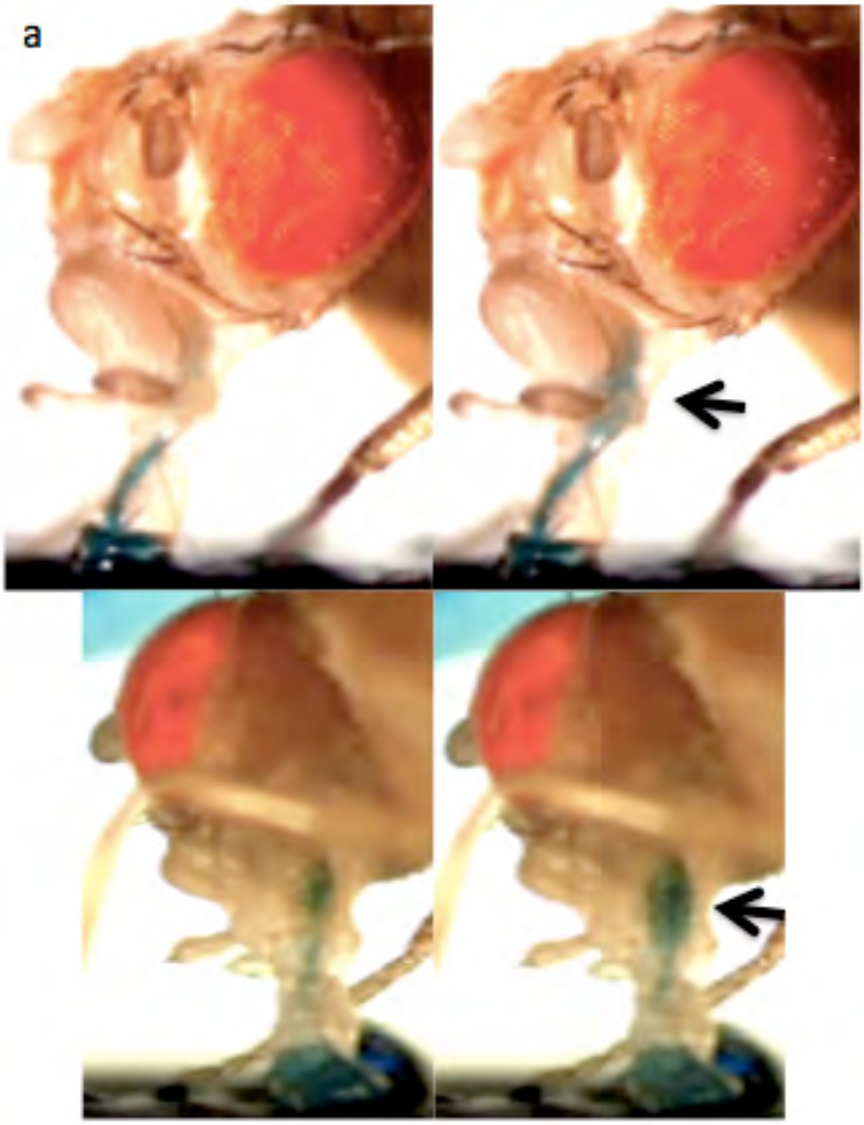


Figure 3.9| Cibarial pump is activated and functioning. a, Time lapse image of tethered NP0883-GAL4/uas-*dTrpA1* flies on 3% dyed agar at 35°C showing functional activation of the cibarial pump; anterior/lateral view. Lower image, same as upper, posterior view. Arrow marks suction of dye into the rostrum, which houses the pump.

Figure 3.10

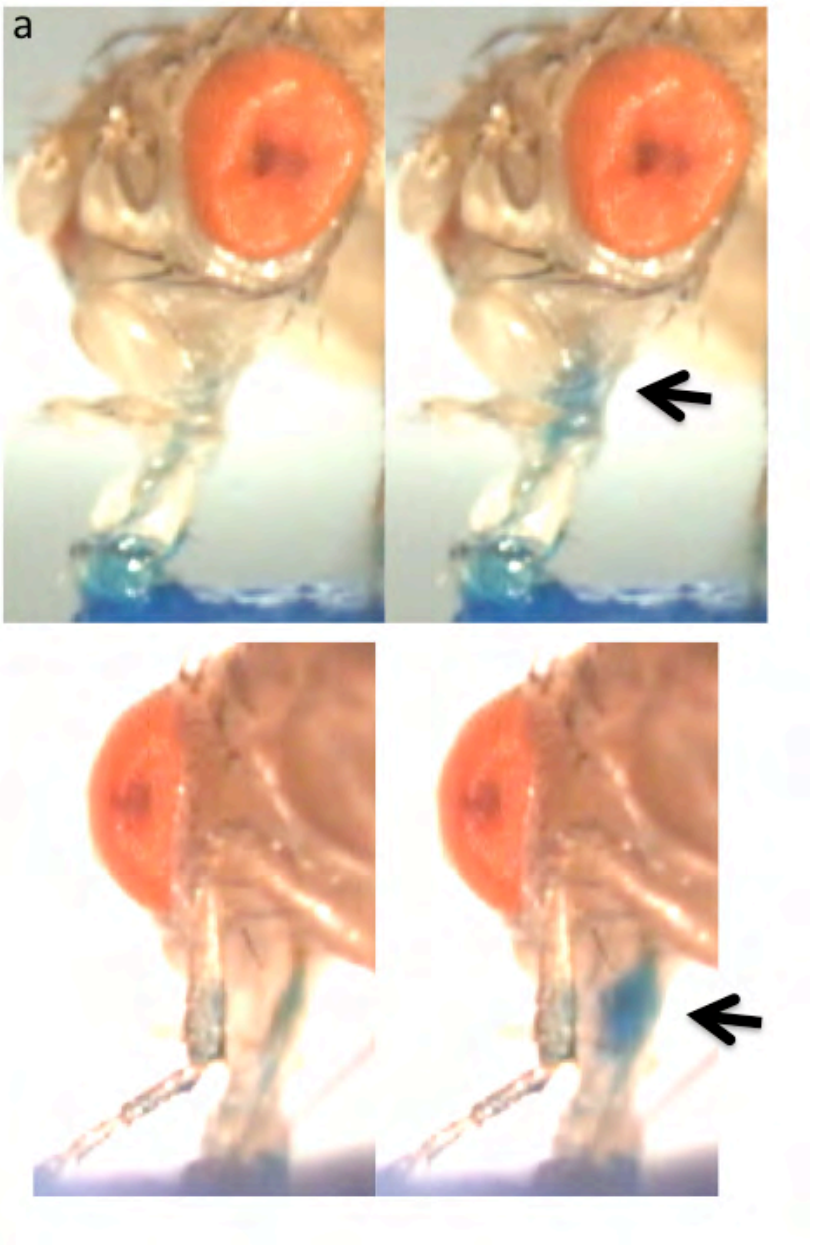


Figure 3.10| Wild-type cibarial pump functioning resembles artificially induced behavior. a, Time lapse images of a starved Canton S fly on filter paper saturated with 100 mM dyed (5mg/mL Brilliant Blue FCF) sucrose solution showing food uptake via activation of the cibarial pump; anterior/lateral view. Lower image, same as upper, posterior view. Arrowhead marks suction of dye into the rostrum, which houses the pump. Analysis performed at room temperature; unrestrained fly.

Figure 3.11

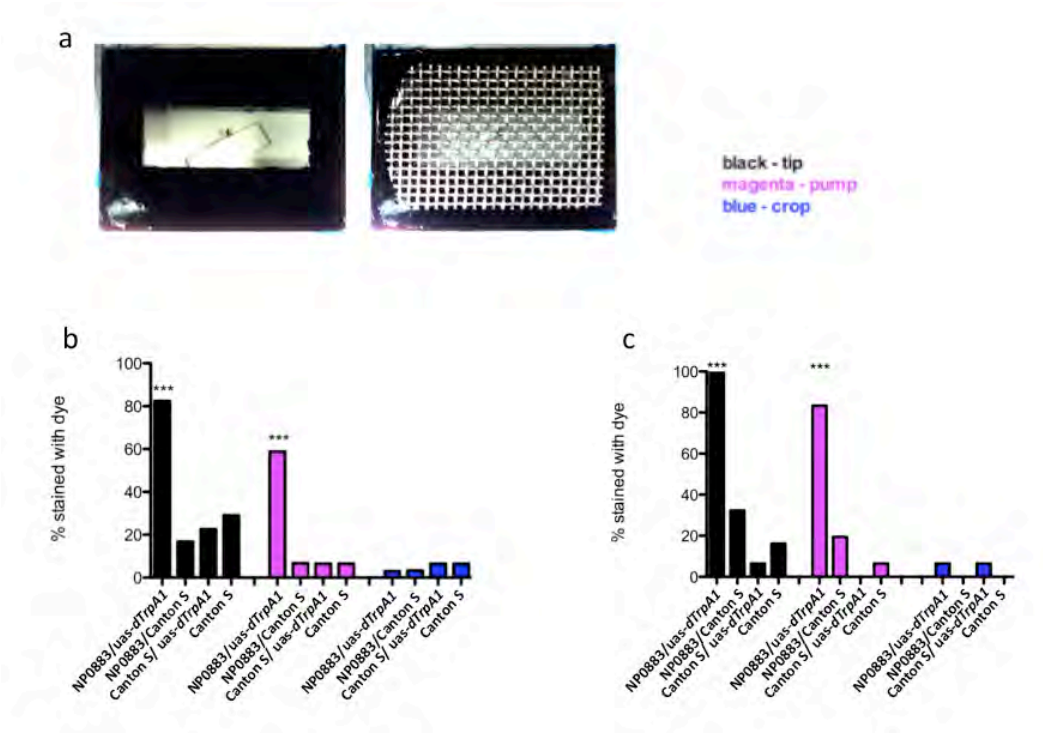


Figure 3.11| 15 mM quinine does not inhibit dye ingestion. a, Dye Assay experimental set-up, left, rectangular arena made of 1% dyed agar; right, arena covered with metal screen to regulate humidity (see Methods). **b**, Dye assay with 15 mM quinine at 31°C. 15 mM quinine does not inhibit ingestion of dye to the labellar lobes and pump. (*** : $p < .001$, Fisher's exact test). **c**, Same as **b**, but at 33°C.

Figure 3.12

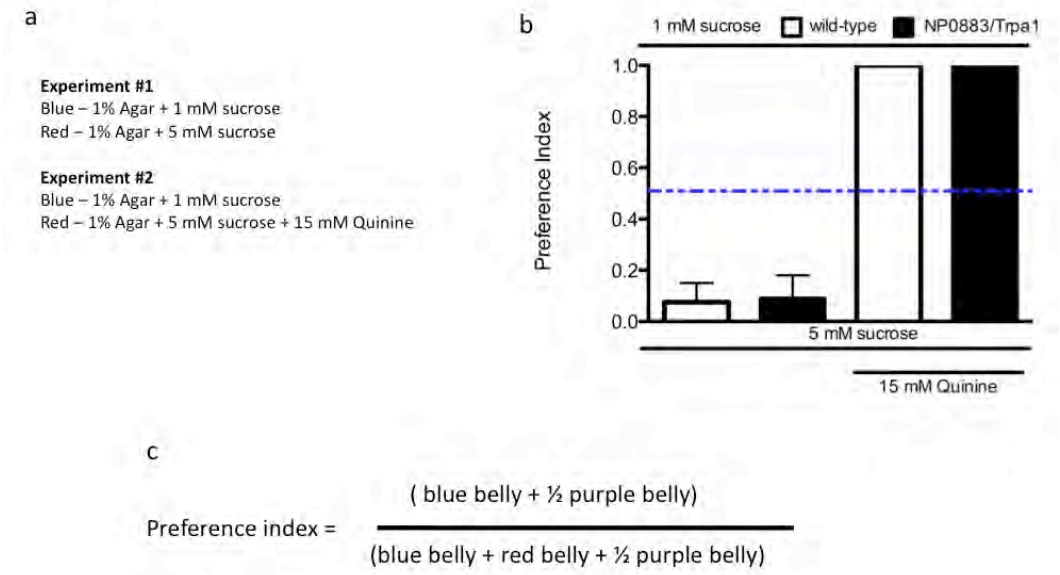


Figure 3.12| NP883-GAL4/uas-*TrpA1* flies sense quinine normally. a, Experimental set-up of Preference Assay experiments 1 and 2. **b**, Preference Assay results; no difference was found between NP0883-GAL4/uas-*dTrpA1* and control flies tested with and without quinine **c**, Formula to calculate the preference index.

Figure 3.13

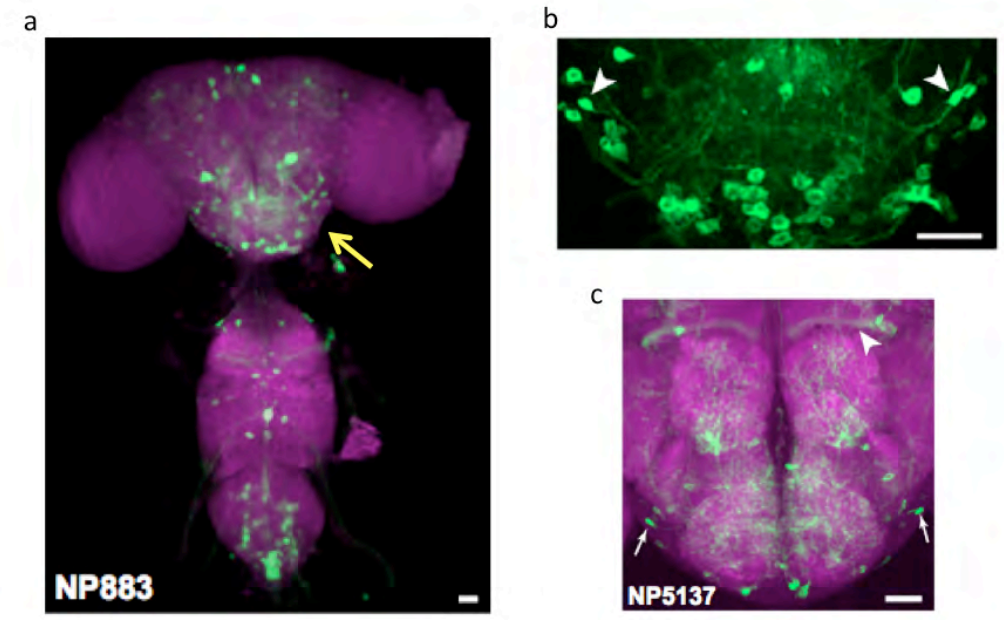


Figure 3.13| GAL4 expression patterns of artificially induced feeding NP lines. a, Full NP0883-GAL4 CNS expression domain visualized with uas-*mCD8::GFP* (green), co-stained with nc82 (magenta) to mark neuropil. The expression was limited to no fewer than 120 cells throughout the entire CNS. Scale bar, 100 μm. Yellow arrow marks suboesophageal ganglion (SOG). **b**, Full NP0883-GAL4 expression domain within the SOG, marked in **a,** (yellow arrow), visualized with uas-*mCD8::GFP*, white arrowheads mark cell body location of FC-neuron. Scale bar, 30 μm **c**, Full NP5137-GAL4 expression pattern within the SOG and central brain visualized by uas-*mCD8-GFP* (green), co-stained with nc82 (magenta). White arrowhead marks mushroom body detection, white arrows mark cell body location of FC-neuron. Functional neuroanatomical experiments focused on NP0883, since NP5137's mushroom body's GAL4 expression may have complicated analysis. Scale bar, 30 μm.

Figure 3.14

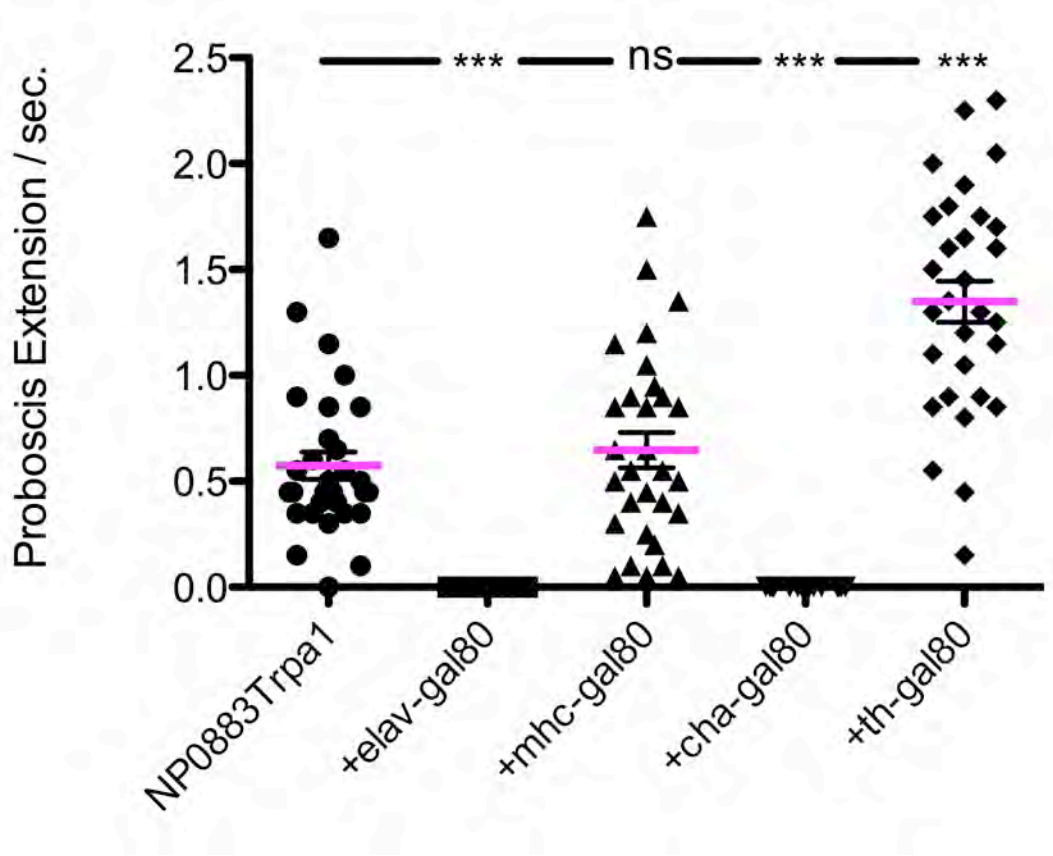


Figure 3.14| The cellular element inducing feeding behavior is most likely a cholinergic neuron. Proboscis Extension Assay with various GAL80 constructs *elav*-GAL80 (pan-neuronal), *Mhc*-GAL80 (muscle), *Cha3.3kb*-GAL80 (cholinergic), *TH*-GAL80 (dopaminergic). Expressing GAL80 pan-neuronally or in cholinergic neurons completely abolishes the induced behavior, whereas expression in muscle or dopaminergic neurons does not. Interestingly blocking dTrpA1 channel activity in dopamine producing cells increases the rate of proboscis extension, which suggests a role for dopamine in the expression of *Drosophila* feeding behavior. Future experiments are needed to better understand this effect (*** : $p < .001$, anova, Tukey, n=30 per genotype).

Figure 3.15

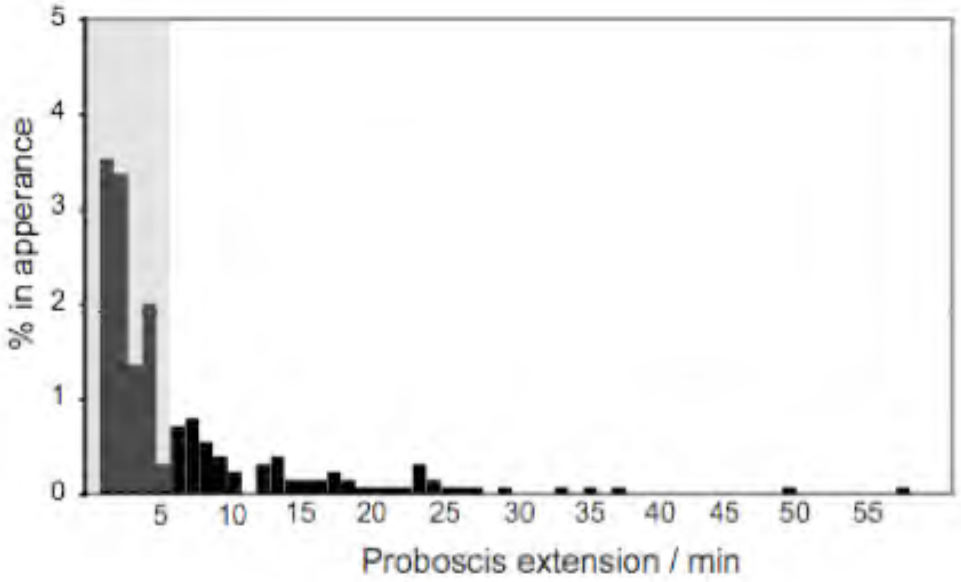


Figure 3.15| Behavioral screening results from mosaic flies. Mosaic flies displaying ≥6 proboscis extension per minute were considered positive for behavior, whereas mosaic flies displaying 0 proboscis extensions per minute were negative. Positive and negative flies were used for all analysis. Mosaic flies displaying between 1-5 proboscis extensions per minute were not used for analysis due to overlap with control flies (Table 3.1). (genotype: *hs*-FLP ; *tubulin*->GAL80>, uas-*dTrpA1*/ NP0883-GAL4 uas-*mCD8-GFP*).

Figure 3.16



Figure 3.16| Identification of FC-neuron as a command neuron directing feeding behavior. a, Representative image of FC-neuron in central brain from mosaic fly (genotype: *hs*-FLP ; *tubulin*>GAL80>, uas-*dTrpA1*/ NP0883-GAL4 uas-*mCD8-GFP*). A confocal montage of the SOG (lower half) and antennal lobes (upper half), neuron visualized with uas-*mCD8::GFP* (green), brain co-stained with nc82 (magenta) to mark neuropil. Importantly, the FC-neuron is located within the SOG of the fly brain, an area of known importance for the initiation and execution of feeding behavior. FC-neuron cell body is situated lateral of the SOG and its neuritic processes extend and ramify within the SOG proper. A minor branch is sent to the contralateral hemisphere. Success of mosaic fly GAL4 expression refinement is evident when compared to Figure 3.13a,b. Scale bar, 30 μm. **b**, same prep as **a**, but low mag of entire CNS. White arrow indicates location of FC-neuron cell body in brain. Compare to full expression pattern of Figure 3.13a. Scale bar, 30 μm. **c**, Coordinated feeding behavior closely resembling wild-type behavior is induced by a very restricted GAL4 expression pattern. dTrpA1-induced behavior of mosaic FC-neuron positive fly at 37°C. Same fly dissected for **a,b** above. Left to right; before, during, and after dTrpA1-induced response. Characteristics of natural feeding such as head lowering and

full proboscis extension are indicated by dashed line (magenta) and black arrow, respectively.

Figure 3.17

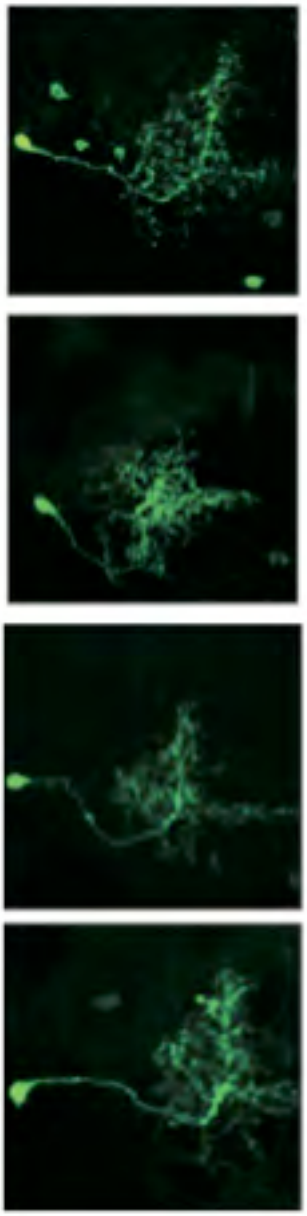


Figure 3.17| The FC-neuron is an identifiable command neuron pair. Four representative FC-neuron images from mosaic flies demonstrating its relatively stereotyped branching pattern. Further, FC-neurons with cell bodies located in both the right and left hemispheres were isolated via the mosaic screen, although they were aligned to one side above to ease viewing (genotype: *hs*-FLP ; *tubulin*>GAL80>, uas-*dTrpA1*/ NP0883-GAL4 uas-*mCD8-GFP*).

Figure 3.18

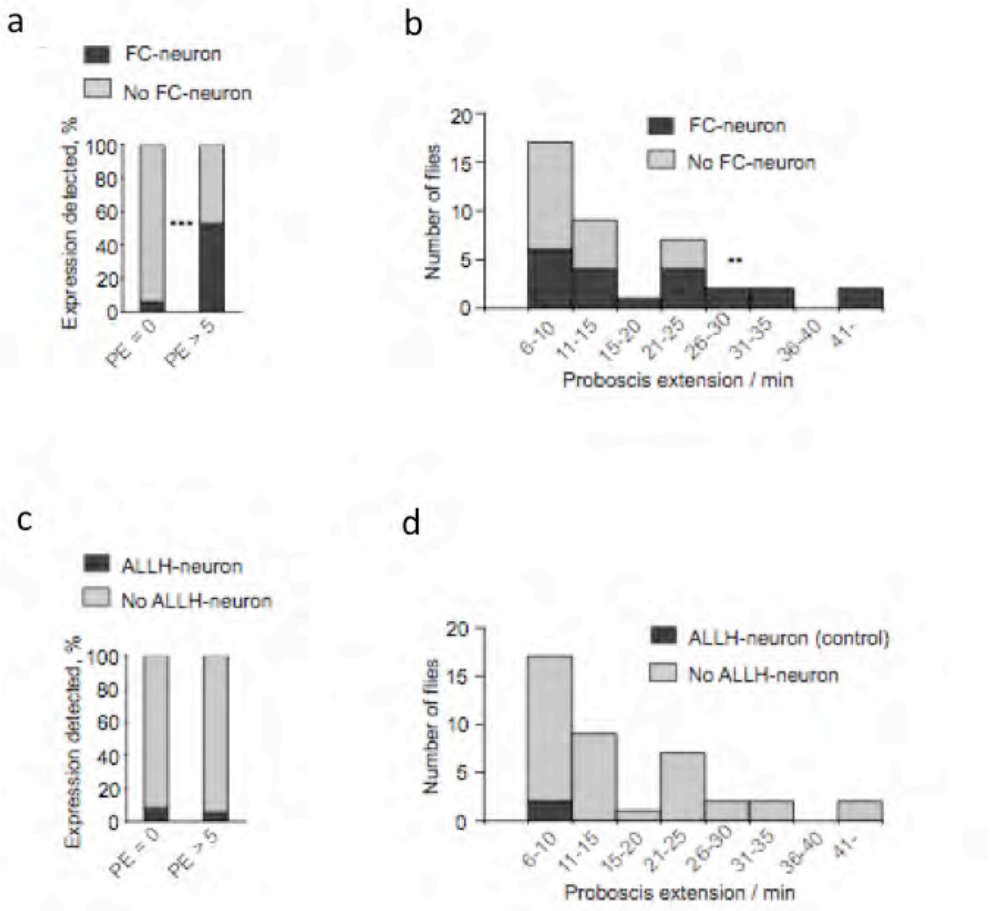


Figure 3.18| The FC-neuron increases in frequency with increasing strength of the induced behavior. a, Confocal image analysis of the FC-neuron from mosaic brains of flies positive (PE > 5/min, see Table 3.1 for explanation of cut off) and negative (PE = 0/min) for proboscis extension behavior. Left column, brains of negative flies (n=195), right column, brains of positive flies (n=40). Black area represents percentage that each specific neuron was identified. Behavioral induction correlates with the presence of an FC-neuron in mosaic flies (*** : $p < .001$, student's t test). **b**, Same analysis as in **a**, but data is separated according to degree of behavioral response and only brains from positive flies are analyzed. The FC-neuron increases in frequency with increasing strength of the induced behavior. (Comparison of PE distributions of flies with and without FC-neuron expression, Mann-Whitney's U-test, ** : $P < 0.01$). **c,d**, same analysis using the same flies and brains as in **a,b,** respectively, but for the ALLH neuron, which was used as a control neuron (see Figure 3.19a,b for ALLH neuron identity). Behavioral induction did not correlate with the presence of the ALLH

neuron. (genotype: *hs*-FLP ; *tubulin*>GAL80>, uas-*dTrpA1*/ NP0883-GAL4 uas-*mCD8-GFP*).

Figure 3.19

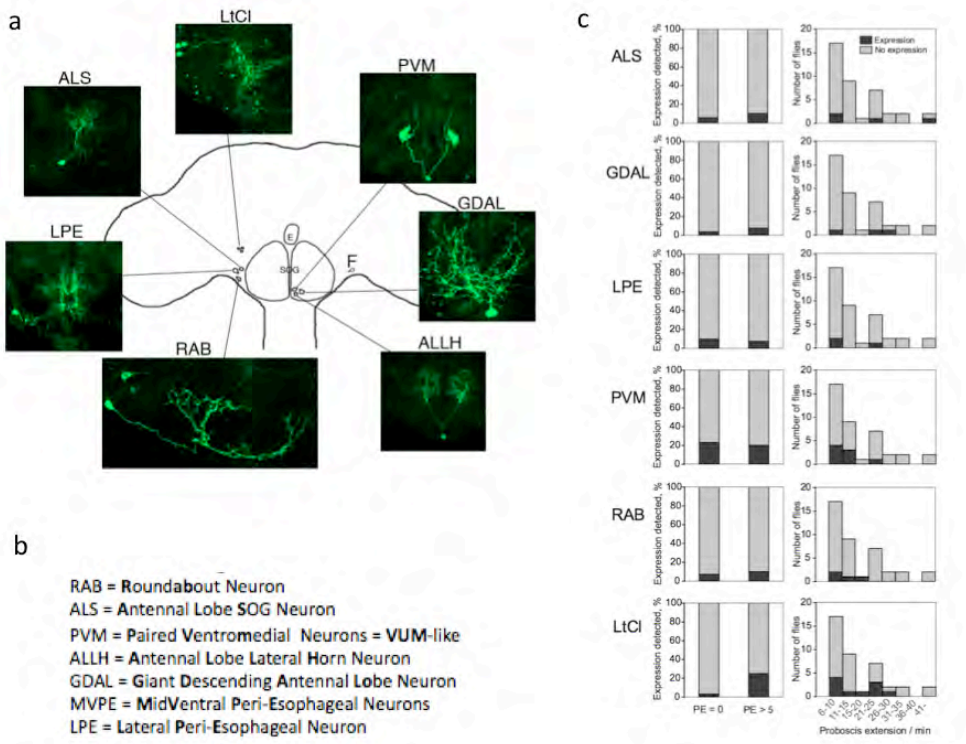


Figure 3.19| Behavioral induction does not correlate with control neurons in mosaic flies. a, brain schematic depicting the cell body location of ALLH neuron (Figure 3.18c,d) and additional control neurons that are found in **c**. representative confocal images of each neuron are connected to cell body location via a line. Images from mosaic flies and visualized with uas-*mCD8::GFP*. All cell bodies are located within the brain area delineated in Figure 3.13b, **b**, Abbreviations for each of the identified neurons. Names were assigned in the present study. **c**, Confocal image analysis of the 6 control neurons from mosaic brains of flies positive (PE > 5/min, see Table 3.1 for explanation of cut off) and negative (PE = 0/min) for proboscis extension behavior. Left column, brains of negative flies (n=195), right column, brains of positive flies (n=40) (same flies and brains as used in Figure 3.18a-d). Black area represents percentage that each specific neuron was identified. **b**, Same analysis as in **a**, but data is separated according to degree of behavioral response and only brains from positive flies are analyzed. Behavioral induction did not correlate with the presence of control neurons. (genotype: *hs*-FLP ; *tubulin*>GAL80>, uas-*dTrpA1*/ NP0883-GAL4 uas-*mCD8-GFP*).

Figure 3.20

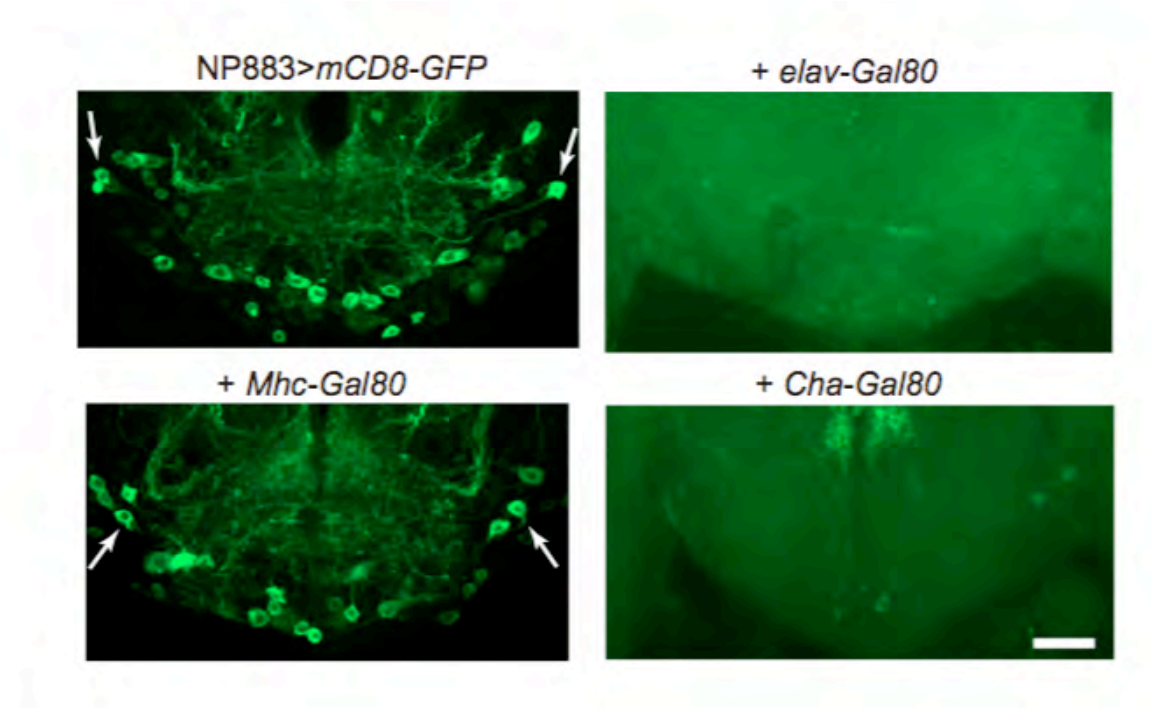


Figure 3.20| The FC-neuron was not present when combined with GAL80 strains that abolished dTrpA1-induced feeding. The FC-neuron was not present in the GAL4 expression pattern when GAL80 was co-expressed pan-neuronally (*elav*-GAL80, top right*)* or in cholinergic cells (*Cha*-GAL80, bottom right*)*. Arrows indicate cell bodies of FC-neuron pair that were present in the full expression pattern of NP0883-GAL4 (top left) and when GAL80 was co-expressed in muscle (*Mhc*-GAL80, bottom left). The presence of the FC-neuron positively correlated with the presence of behavioral induction (See Figure 3.14). All flies contained uas-*mCD8::GFP*, which was used to visualize the GAL4 expression patterns. Scale bar, 30 μm

Figure 3.21

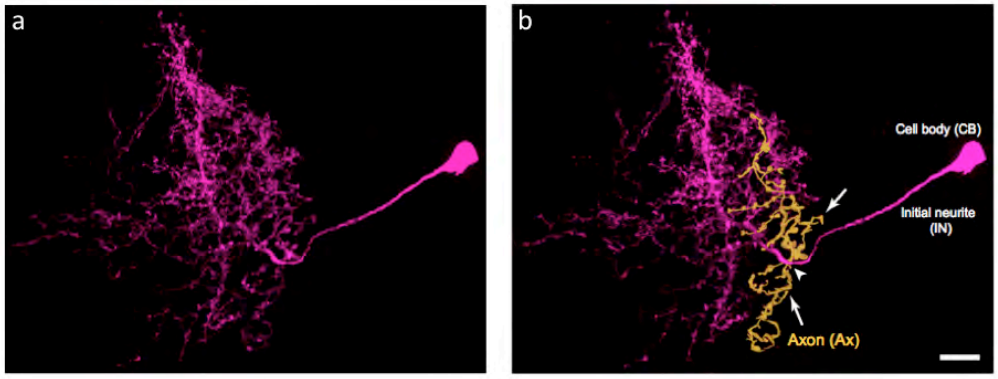


Figure 3.21| Predicted dendritic and axonal regions of the FC-neuron. a, Confocal Image of FC neuron visualized with uas-*mCD8-mCherry* (magenta). **b**, Same sample as **a**, with predicted axonal region digitally painted orange. Arrows indicate terminals. Arrowhead marks initial neurite branch point. A predicted dendritic region is the dense, highly branched area located at the FC-neuron apex. Scale bar, 10 μm. (genotype: *hs*-FLP; >*tubulin*-GAL80> uas-*dTrpA1*/ NP0883-GAL4; uas- *mCD8-mCherry* / +).

Figure 3.22

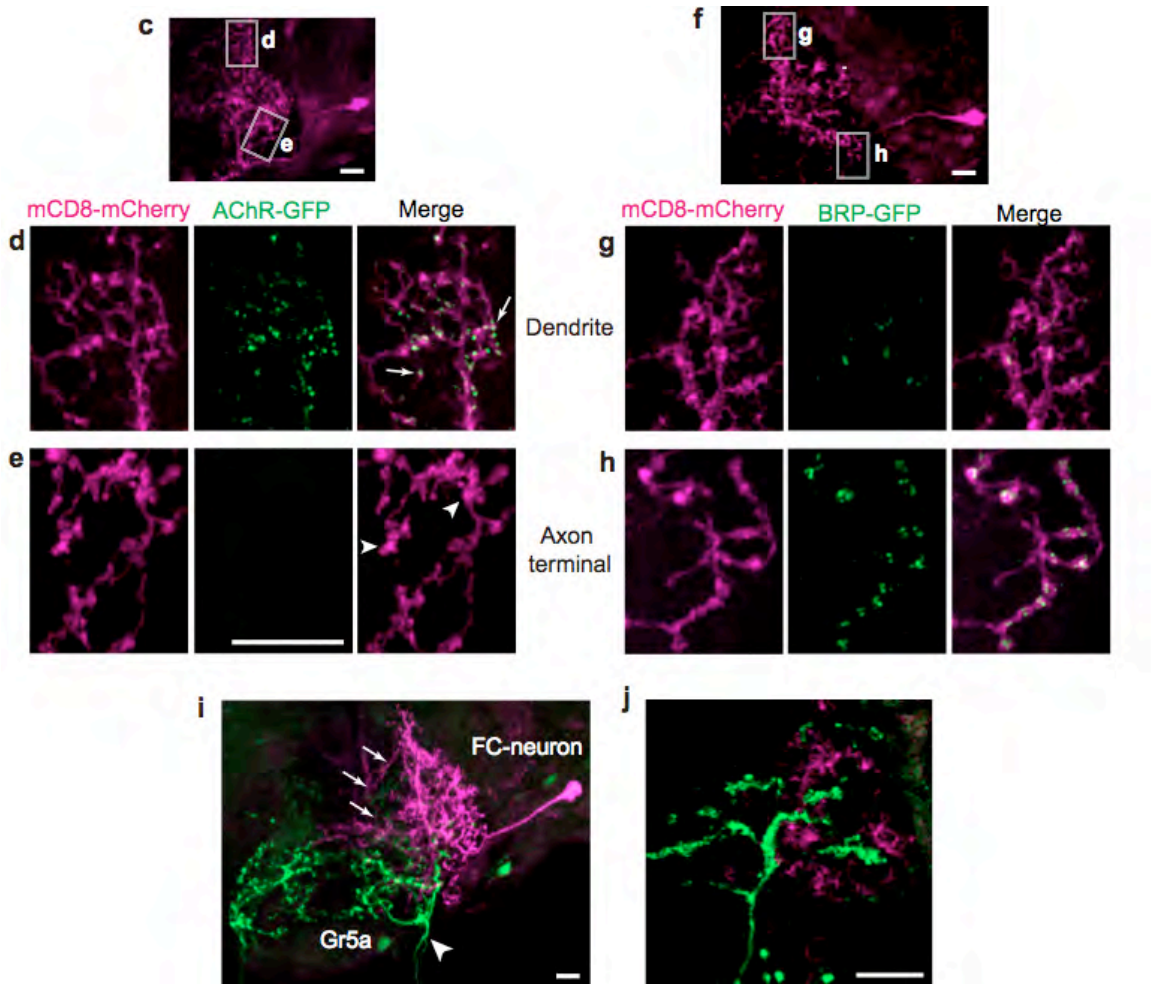


Figure 3.22| The FC-neuron's pre- and post-synaptic compartments are located to respond to gustatory related sensory cues and execute feeding behavior. c, Ach-GFP localizes to predicted dendritic region. FC-neuron (low mag) from mosaic fly, visualized with uas-*mCD8-mCherry* (magenta) and *Aceytlcholine receptor*-GFP (*AchR*-GFP) (green). **d**, High mag of image **c** corresponding to box marked d. Right, middle, left are mCherry, GFP and merged signal, respectively. Predicted dendritic region boxed by d displays multiple AchR-GFP puncta (white arrows). **e**, High mag of image **c** corresponding to box marked e. Right, middle, left same as **d**. Predicted axonal region boxed by e does not display AchR-GFP puncta. White arrowheads mark AchR-GFP puncta negative large varicosities. **f**, Brp-GFP localizes to predicted axonal region. FC-neuron (low mag) from mosaic fly, visualized with uas-*mCD8-mCherry* (magenta) and *Bruch pilot*-GFP (*Brp*-GFP) (green). **g**, High mag of image **f**

corresponding to box marked g. Right, middle, left are mCherry, GFP and merged signal, respectively. Predicted dendritic region boxed by g display only a few small Brp-GFP puncta, visible. **h**, High mag of image **f** corresponding to box marked h . Right, middle, left, same as **g**. Predicted axonal region boxed by h display Brp-GFP positive puncta localized to large axonal varicosities. **i,** Axon terminals of sweet sensing neurons visualized with GFP overlap with the FC-neuron's dendritic region (magenta; mCherry) (mosaic fly) **j**, Same as **i**, except at higher magnification and constructed with thinner confocal sections (7 um thick in **j**, 25 um in **i**). Thinner higher mag stack show the connections are juxtaposed but do not contact. All scale bars in figure 10 μm. All fly genotypes can be found in Methods section.

Figure 3.23

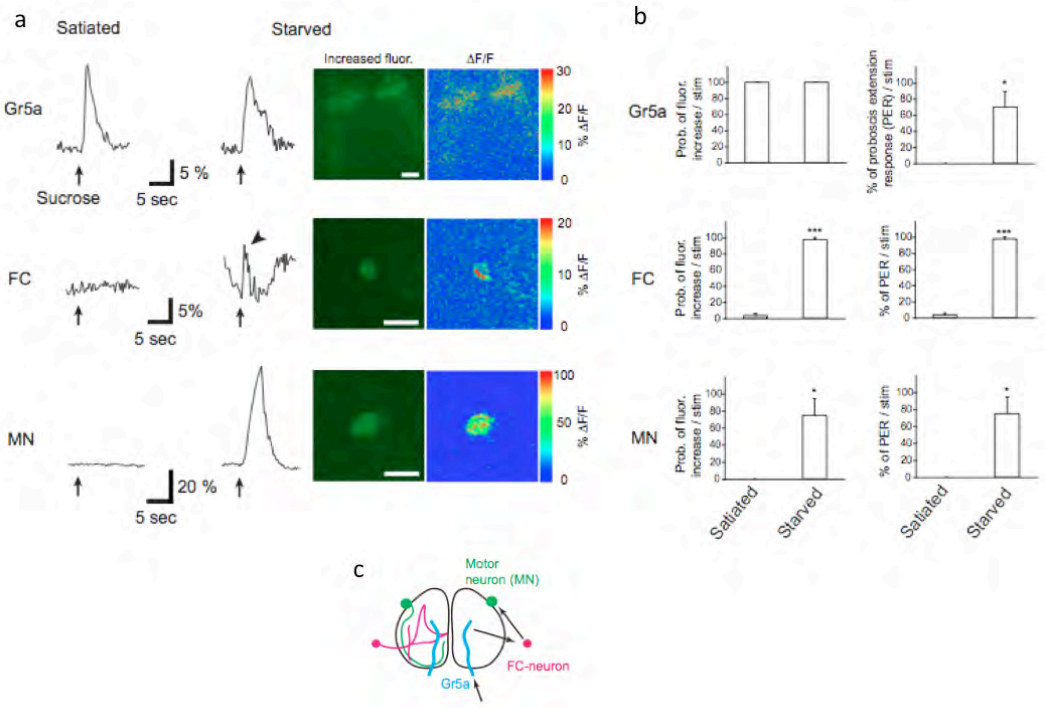


Figure 3.23| The FC-neuron responds to sucrose stimulation and is part of the natural feeding circuit. FC-neuron responds to sucrose stimulation in a state dependent manner, similar to a motor neuron for proboscis extension, but different than sweet sensing neurons. **a**, Representative calcium imaging traces in response to 100mM sucrose stimulation, in sated, (left trace) and starved flies (right trace). Gr5a: *Gr5a*-GAL4 (expresses GCamp3 in sweet sensing neurons), F: NP0883-GAL4 (expresses GCamp3 in FC-neuron), MN: *E49*-GAL4 (expresses GCamp3 in motor neuron for rostrum extension). In a starved state, an FC-neuron responds to a sucrose stimulus by a sharp increase in fluorescence (arrowhead) followed by a restoration phase of several seconds to the base line**.** Right panels show GCaMP fluorescence increase by sucrose stimuli in starved animals, and pseudocolor images of the percent increase in fluorescence. Image subtraction (see Methods) was used to calculate final values for each genotype. All scale bars, 10 μm. **b**, Left, quantification of calcium response from sucrose stimulation in sated and starved flies. Right, quantification of behavioral response from sucrose stimulation in sated and starved flies. (n=6 satiated and starved Gr5a neurons), (n=11 satiated and starved FC-neurons), and (n=5 satiated and starved motoneurons). (* : $p < .05$, *** : $p < .001$, student's t test). Error bars in all figures are SEM. (genotype: NP883-GAL4 and two doses of uas-*GCamp3.0* to increase the signal; for the motoneuron, a fly with *E49*-GAL4 and a single dose of

uas-*GCamp3.0;* for the sweet sensing neuron, a fly with *Gr5a*-GAL4 and a single dose of uas-*GCamp3.0*). **c**, Schematic drawing of SOG showing cell body location and neuritic projections of sugar sensing (Gr5a) neurons (blue), FC-neuron (magenta), and a motoneuron (MN) for the rostral protractor (green). Gr5a cell body is localized outside of SOG, only axon terminals shown.

Figure 3.24

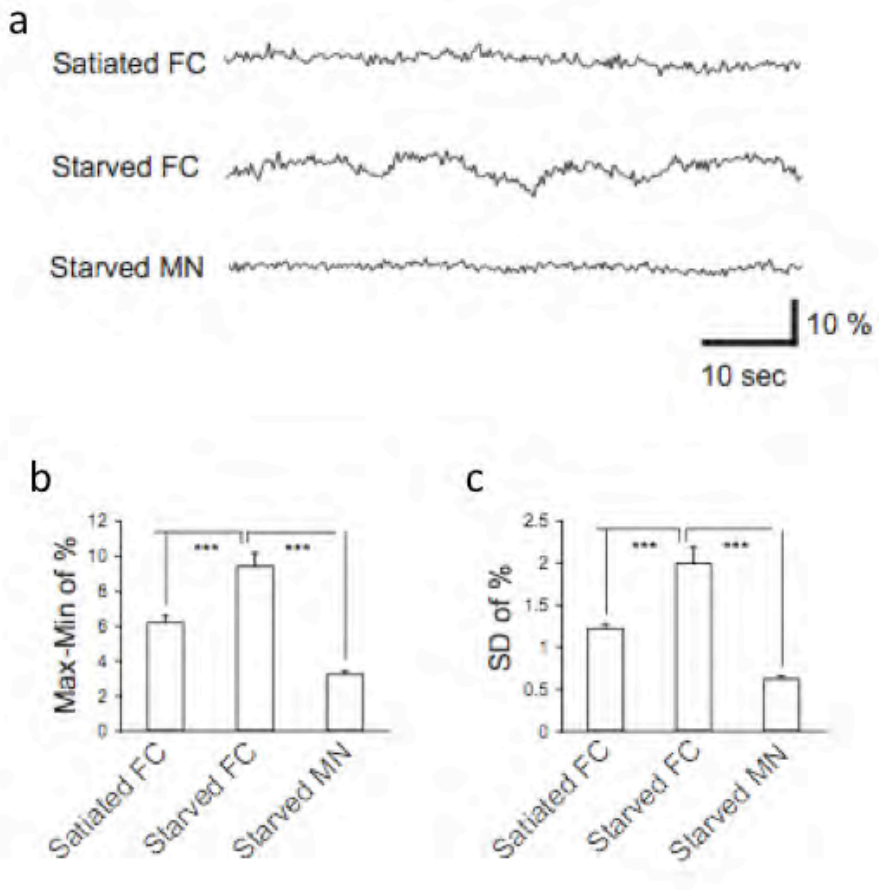


Figure 3.24| The FC-neuron is influenced by hunger related signals. a, Representative calcium imaging traces, no sucrose stimulation. Starvation induces baseline fluctuation of FC-neuron calcium imaging signal **b,c**, Quantification of baseline calcium signal (no sucrose stimulation) demonstrating significant fluctuation of starved FC-neuron trace. Fluctuation quantified by measuring maximum – minimum (**b**) and standard deviation (**c**) of percentage values in a given 30s interval in each animal. (n=6,11,10 for satiated FC-neuron, starved FC-neuron, and starved MN (motoneurons), respectively (*** : $p < .001$, anova, Tukey's). Error bars in all figures are SEM. (genotype: NP883-GAL4 and two doses of uas-*GCamp3.0* to increase the signal; for the motoneuron, a fly with *E49*-GAL4 and a single dose of uas-*GCamp3.0*.; for the sweet sensing neuron, a fly with *Gr5a*-GAL4 and a single dose of uas-*GCamp3.0*)

Figure 3.25

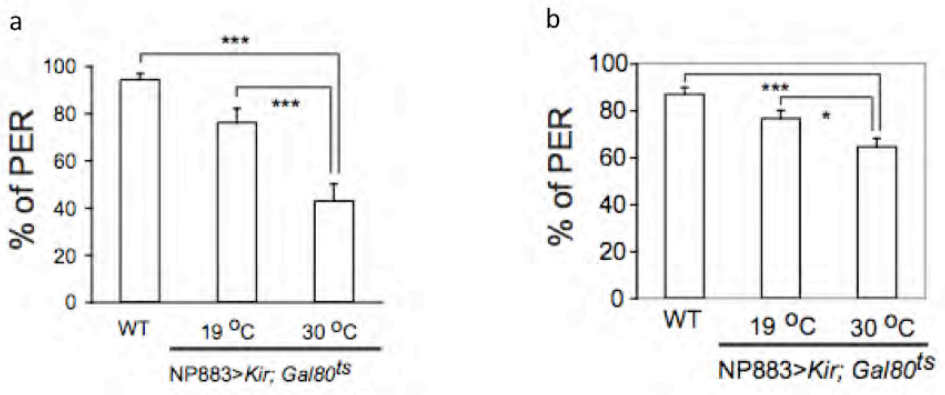


Figure 3.25| FC-neuron inhibition results in decreased responsiveness to sucrose stimulation within multiple sensory pathways. a, Inhibition of FC-neuron decreased a starved fly's responsiveness to 100 mM sucrose presented to the proboscis, as compared to control animals. 5 experiments, 3-5 flies per experiment, the mean Proboscis Extension Response (PER) of 6 sucrose presentations from each fly was compared. (*** : $p < .001$, anova, Tukey). **b,** same as **a**, except sucrose presented to tarsi. 6 experiments, 10-15 flies per experiment, the mean PER of 6 sucrose presentations from each fly was compared. (** : $p < .01$, *** : $p < .001$, anova, Tukey).

Figure 3.26

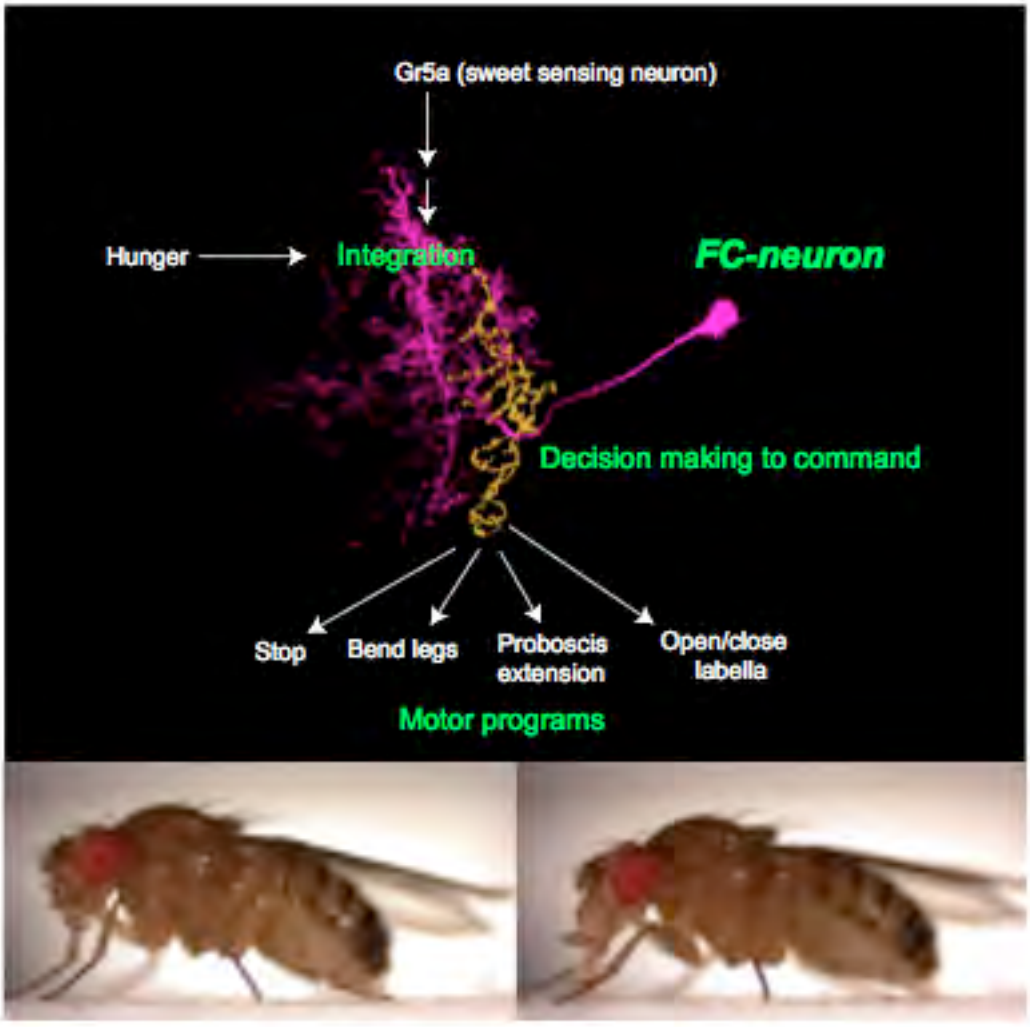


Figure 3.26| Proposed model of FC-neuron's role in feeding behavior. The schematic depicts the FC-neuron as a command neuron directly regulating the expression of feeding behavior via activation of multiple motor programs. The FC-neuron may also integrate sensory and motivational signals and acts as a decision maker for initiating the feeding response, as pictured.

Table 3.1

PE/minute	0	1	2	3	4	5	6 & above	% with PE
Canton S (n=100)	89	6	1	2	2	0	0	0.11
No FLP (n=100)	94	4	1	0	1	0	0	0.06

Table 3.1| Control flies show a low level of proboscis extension behavior. Wild-type flies display 0-4 proboscis extensions per minute (n=100). NP883-GAL4/uas-*dTrpA1-tubulin*->GAL80>;uas-mCD8::GFP (no *hs*-FLP present) flies display 0-4 proboscis extensions per minute (n=100).

Movie 3.1

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.1| Wild-type feeding behavior of starved fly on food. Wild-type feeding behavior consists of the co-expression of multiple motor programs such as locomotion arrest, head lowering, foreleg bending, proboscis extension, opening and closing of the labellar lobes and substrate sucking. Sucking occurs at end of video when 100 mM sucrose solution is applied to filter paper. The rest of the video is taken on normal fly food. Unrestrained flies.

Movie 3.2

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.2| dTrpA1-induced behavior closely resembles wild-type feeding behavior. Wild-type feeding behavior consists of the co-expression of multiple motor programs such as locomotion arrest, head lowering, foreleg bending, proboscis extension, opening and closing of the labellar lobes and substrate sucking. These characteristics of wild-type behavior are replicated in NP0883-GAL4/uas-*dTrpA1* and NP5157-GAL4/uas-*dTrpA1* flies at 31℃ with no food present. Unrestrained flies.

Movie 3.3

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.3| NP5137-GAL4/uas-*dTrpA1* induced behavior resembles natural feeding. NP5137-GAL4/uas-*dTrpA1* fly at 31°C; no food present. Unrestrained fly.

Movie 3.4

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.4| Male NP-GAL4/uas-*dTrpA1* flies display suction. NP5137-GAL4/uas-*dTrpA1* male fly at 31°C; no food present. Unrestrained fly.

Movie 3.5

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.5| Cibarial pump is activated and functioning. NP0883-GAL4/uas-*dTrpA1* fly on 3% blue dyed agar at 35°C. Fly is tethered. Dye can be seen being sucked into pump. The video playback is ½ normal speed for ease of viewing. A 1 second clip is continuously looped. Anterior view.

Movie 3.6

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.6| Behavior can be induced in unnatural contexts. Tethered NP0883-GAL4/uas-*dTrpA1* fly at 31°C showing robust proboscis extension in the absence of appropriate sensory stimulus and under unnatural conditions.

Movie 3.7

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.7| Mosaic FC-neuron positive flies demonstrate the induction of multiple motor programs characteristic of wild-type feeding behavior. Wild-type feeding behavior consists of the co-expression of multiple motor programs such as locomotion arrest, head lowering and proboscis extension. In the video, a mosaic FC-neuron positive fly at 37°C demonstrates the co-expression of these motor programs. This fly displays a very restricted GAL4 expression pattern (Figure 3.16a,b). (genotype: *hs*-FLP ; *tubulin*->GAL80>, uas-*dTrpA1*/ NP0883-GAL4 uas-*mCD8-GFP*). Unrestrained fly.

Movie 3.8

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.8| 3-D movie of FC-neuron from mosaic fly showing predicted pre and post-synaptic compartments. First half, made from 20x confocal image. Second half, confocal 60x image. It is predicted that the dense, highly branched, and tuft processes represent dendrites, whereas the sparse, less convoluted areas containing bulbous structures, represent axons. Visualized by mCherry. (genotype: *hs*-FLP; *tubulin*->GAL80> uas-*dTrpA1*/ NP0883; uas-*mCD8-mCherry* / +).

Movie 3.9

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.9| Proboscis extension behavior during simultaneous calcium imaging. NP0883-GAL4;uas-*GCamp3* fly is stimulated with 100 mM sucrose to tip of proboscis.

Movie 3.10

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.10| Flies expressing the kir2.1 channel appeared vigorous and healthy before testing. NP883-GAL4/uas-*kir2.1*;*tubulin*-GAL80ts flies reared at 19C, placed at 30C for 18hrs, then starved for 24 hrs at room temperature. Appearance of flies approximately 2 hrs before testing. Unrestrained flies.

Movie 3.11

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.11| Positive proboscis extension (PE). A positive PE was scored as follows: the tip the fly's proboscis must touch the surface of the arena. NP0883-GAL4/uas-*dTrpA1* fly at 31°C. Unrestrained fly.

Movie 3.12

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.12| Visualization of cibarial pump activity in wild-type flies. Starved Canton S fly on filter paper saturated with 100 mM dyed (5mg/mL Brilliant Blue FCF) sucrose solution showing food uptake via activation of the cibarial pump. Unrestrained fly.

Movie 3.13

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.13| Dye Assay analysis at three points along the gastrointestinal tract. First fly demonstrates dye staining of the labellar lobes and pump. Second fly is negative for dye staining on lobes and pump. Third fly demonstrates a crop dissection. In video, the crop is negative for dye staining. All flies in Dye Assay experiments were analyzed in this way (see Methods).

Movie 3.14

(see accompanying disc or go to
http://escholarship.umassmed.edu/gsbs_diss/523/)

Movie 3.14| Fly love. Male and female fly forced to love one another via a wet kiss. Note suction. In Video: NP0883/uas-*dTrpA1* male and female, at 31°C.

CHAPTER IV: DISCUSSION

IVA. Summary & Conclusions

> But then arises the doubt – can the mind of man, which has, as I fully believe, been developed from a mind as low as that possessed by the lowest animal, be trusted when it draws such grand conclusions?
>
> *Charles Darwin* [107]

We set out to elucidate the functional organization of the fruit fly brain, by searching for, identifying, and characterizing command neurons governing instinctive behavior. To achieve this objective we established an artificially induced cellular activation-based genetic screen for functional neural circuit analysis in the fruit fly, *Drosophila melanogaster*. By performing this screen we identified complex motor patterns resembling full, or partial, wild-type behavioral acts such as courtship, egg laying, feeding, initiation of flight, and aggressive displays.

Of these artificial instinct-like behaviors we focused our research efforts on understanding the feeding response. A series of behavioral experiments confirmed that this induced behavior was composed of multiple motor programs, which occurred in a simultaneous, coordinated, and functional manner nearly indistinguishable from wild-type feeding. Further, aversive gustatory signals were found to compete with dTrpA1-induced activation to inhibit or stimulate behavioral expression, respectively, suggesting the induced behavior is arising

from activation of gustatory-related neural circuitry. Based on this evidence, we hypothesized that command neuron activation was directly stimulating the feeding motor circuit resulting in the full expression of the natural feeding response.

To test this hypothesis we relied upon *Drosophila*'s powerful and sophisticated genetic techniques to resolve the neural elements responsible for induced feeding. Through our analysis we identified the FC-neuron as a neuron directing the expression of the multiple motor programs characteristic of feeding behavior. Further, we determined the FC-neuron to be positioned within the SOG, among sensory axon terminals and motor neuron dendrites of the natural feeding neural circuit [56, 76, 82]. Additionally, *in vivo* calcium imaging revealed that the FC-neuron responded to food-related sensory cues and motivational signals in a state-dependent manner. Lastly, FC-neuron inhibition decreased a starved fly's responsiveness to sucrose stimulation. Taken together, we conclude that the FC-neuron is a command neuron directing the expression of feeding behavior in *Drosophila*.

IVB. Significance & Future Directions

> What you can imagine depends on what you know.
>
> *Daniel C. Dennett* [108]

Over time, the processes of natural selection shape an organism's anatomy, physiology, and behavior to meet unique requirements for survival within a distinct ecological niche. Out of this powerful process arose the great diversity of feeding behavior found on earth, a microcosm of which can be appreciated at a family picnic. Imagine yourself sitting in a park eating a sandwich. You tear into the ham, lettuce and bread with your front incisors, tongue the nutritious lump over the molars, and grind away, eventually swallowing the morsel. Next, over by the pond, you witness a frog adroitly cast its long tongue to catch and consume an aerial prey. Suddenly, the buzz of a hummingbird catches your ear, you turn and catch a glimpse of its skilled stationary flight and long thin beak as it obtains a meal of nectar. Lastly, you vigorously swat away a housefly remembering that it feeds on dead decaying matter, which it spits on, liquefies, and ingests through its straw-like proboscis. Having experienced a picnic, it should be easy to appreciate that animals consume many different things in many different ways. Importantly, the apparent richness of consumptive behaviors may stem from evolved differences in sensory and motor system design and operation, while preserving the organization and function of centrally located neural elements

across species. If valid, then central nervous system mechanisms of behavioral control discovered in the fruit fly can be generalized to better understand the neural control of feeding behavior throughout the animal kingdom. However, before such generalizations can be made, additional centrally located neural elements controlling feeding behavior need to be identified. Thus, the identification of the FC-neuron, which is a centrally located neuron that directs the expression of feeding behavior in *Drosophila*, is an important step in the right direction.

An animal must ingest nutritious substances in order to survive. To fulfill this requirement the animal performs feeding behavior. Due to its importance, both to basic and applied scientific endeavors, the neurobiological control of feeding behavior has been well studied. For example, sensory neuron input, motor neuron output, major brain regions and numerous hormones, peptides and neurotransmitters have been identified that regulate the expression of feeding behavior [76, 77, 82-84]. However, a detailed knowledge of the neural circuits within the CNS that control feeding behavior remain poorly understood. Herein we identified a novel command neuron within the CNS of *Drosophila melanogaster*, which directs the expression of complete feeding behavior. This discovery provides important insight into how the fly's brain is organized and operates to produce a complex behavior and constitutes a significant contribution to the field of behavioral sciences. However, this finding's real significance may lie in its

potential to be used as a tool to answer many exciting and unsettled questions concerning the neural control of behavior.

Identification of the FC-neuron and the suite of motor programs it controls provides a unique experimental system, fully equipped with the power of *Drosophila* genetics to develop a rigorous and unprecedented understanding of nervous system mechanisms controlling feeding behavior. For example, this new tool will allow for an examination of the formation, control, and adaptability of a specific, centrally located neuron that constitutes an important part of the feeding behavioral neural circuit. Further, by determining inputs and outputs it will be possible to piece together sensory-command-motor neuron networks and elucidate principles of nervous system design that enable efficient execution of complex behavior. Further, this discovery will allow for the assessment, both morphologically and functionally, of the integration of various stimuli from distinct sensory systems or various behavioral circuits onto the command neuron and will help to better understand an animal's decision-making and behavioral selection process. Additionally, a rigorous examination of the influence of internal factors, such as homeostatic signals, hormones, biological rhythms, and cytokines on feeding neural circuit function and behavioral output can be performed. For instance, one intriguing experiment would be to assess the effect of hunger related signals on neural circuit connectivity. In Figure 2.22i,j, the sweet sensing axon terminals were determined to closely approach the FC-neuron

dendritic processes but failed to connect. However, interestingly, the axon terminals and FC-neuron processes apparently interdigitate (Figure 2.22j). Therefore, it is possible that hunger related signals directly affect synaptic structure and lead to increased synaptic connectivity between juxtaposed and functionally related circuits, such as sweet sensing neurons and the FC-neuron. Further, increased connectivity would directly regulate circuit functioning by allowing sensory stimuli the ability to transmit their signal to downstream neural elements, which direct behavior. This connectivity could be dynamically controlled to quickly and efficiently adjust to changing nutritional needs. Importantly, the discovery of the FC-neuron, presented herein, allows for these types of exciting analyses. Lastly, the effect of external factors, such as the presence of con-specifics, environmental conditions, and life experience, on various elements of FC-neuron function, circuitry and behavior can be examined.

In addition to the discovery of the FC-neuron, our genetic screen identified numerous candidate lines that can be used to better understand the neural control of behavior. One line in particular, resembling the ‘initiation of voluntary flight’, seems like it would be amenable to a similar sort of analysis as performed to resolve the FC-neuron. However, examining the other lines displaying an instinctive-like induced behavior may also prove fruitful. Furthermore, studying the unnatural induced behaviors, including the different forms of paralysis, would

lead to a better understanding of how the nervous system works, as well as shed light on what happens when things go wrong.

Final Summary

In summary, the discovery of the FC-neuron provides important insight into how the fly's brain is organized and operates to produce feeding behavior. Further, this discovery also provides a novel system, equipped with a myriad of potent and sophisticated genetic tools, that enable a mechanistic analysis of how genes, molecules, and neurons are organized, controlled and influenced to produce complex behavior. Furthermore, numerous candidate lines have been identified that can be used for additional neural circuit analyses.

Further, animals are bonded by developmental constraints of common ancestry or independently shaped by natural selection to display similar design principles. Therefore, understanding how the fruit fly nervous system's organization and structure, and its integration and processing of internal and external signals, influence the expression of complex behavior will help elucidate fundamentals of nervous system form and function occurring across all phyla, including in humans. Lastly, it is hoped that this research will lead to better understanding and treatment of human afflictions that manifest in the disruption of behavior such as autism, stroke, spinal cord injury, eating disorders, and neurodegenerative disease and maybe, just maybe, even world peace (Movie 3.14).

References

1. Goethe, J. W. v., Stopp, E. & Hutchinson, P.,. in *Maxims and reflections* (Penguin Books, London; New York, 1998).
2. Szasz, F. M. The Many Meanings of History, Part I. *The History Teacher* 7, 552-563 (1974).
3. Alcock, J. in *Animal behavior : an evolutionary approach* (Sinauer, Sunderland, Mass., 2001).
4. Page, G. in *Inside the animal mind* (Doubleday, New York, 1999).
5. Lemm, V. in *Nietzsche's animal philosophy : culture, politics, and the animality of the human being* (Fordham University Press, New York, 2009).
6. Dennett, D. C. in *Darwin's dangerous idea : evolution and the meanings of life* (Simon & Schuster, New York, 1995).
7. Cziko, G. in *The things we do : using the lessons of Bernard and Darwin to understand the what, how, and why of our behavior* (MIT Press, Cambridge, Mass., 2000).
8. Gove, P. B., & Merriam-Webster, I. in *Webster's third new international dictionary of the English language, unabridged* (Merriam-Webster, Springfield, Mass., 1993).
9. Gray, P. in *Psychology* (Worth Publishers, New York, 1999).
10. Numan, M., & Insel, T. R.,. in *The neurobiology of parental behavior* (Springer, New York, 2003).
11. Masek, P. & Scott, K. Limited taste discrimination in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 14833-14838 (2010).
12. Dormont, L., Jay-Robert, P., Bessiere, J. M., Rapior, S. & Lumaret, J. P. Innate olfactory preferences in dung beetles. *J. Exp. Biol.* **213**, 3177-3186 (2010).
13. Dekker, T., Takken, W. & Braks, M. A. Innate preference for host-odor blends modulates degree of anthropophagy of Anopheles gambiae sensu lato (Diptera: Culicidae). *J. Med. Entomol.* **38**, 868-871 (2001).
14. Krashes, M. J. & Waddell, S. Rapid consolidation to a radish and protein synthesis-dependent long-term memory after single-session appetitive olfactory conditioning in Drosophila. *J. Neurosci.* **28**, 3103-3113 (2008).
15. Tinbergen, N.,. in *The study of instinct.* (Clarendon Press, Oxford, 1951).
16. IKEDA, K. & WIERSMA, C. A. Autogenic Rhythmicity in the Abdominal Ganglia of the Crayfish: the Control of Swimmeret Movements. *Comp. Biochem. Physiol.* **12**, 107-115 (1964).
17. WIERSMA, C. A. & IKEDA, K. Interneurons Commanding Swimmeret Movements in the Crayfish, Procambarus Clarki (Girard). *Comp. Biochem. Physiol.* **12**, 509-525 (1964).

18. Cattaert, D. & Le Ray, D. Adaptive motor control in crayfish. *Prog. Neurobiol.* **63**, 199-240 (2001).
19. Pirri, J. K., McPherson, A. D., Donnelly, J. L., Francis, M. M. & Alkema, M. J. A tyramine-gated chloride channel coordinates distinct motor programs of a Caenorhabditis elegans escape response. *Neuron* **62**, 526-538 (2009).
20. Hedwig, B. Control of cricket stridulation by a command neuron: efficacy depends on the behavioral state. *J. Neurophysiol.* **83**, 712-722 (2000).
21. King, D. G. & Wyman, R. J. Anatomy of the giant fibre pathway in Drosophila. I. Three thoracic components of the pathway. *J. Neurocytol.* **9**, 753-770 (1980).
22. von Philipsborn, A. C. *et al*. Neuronal control of Drosophila courtship song. *Neuron* **69**, 509-522 (2011).
23. Kupfermann, I. & Weiss, K. R. The command neuron concept. *Behavioral and Brain Sciences* **1**, 3-39 (1978).
24. Orwell, G.,. in *Animal farm;* (Harcourt, Brace, New York, 1954).
25. Keros, T. *et al*. The centenary progress of molecular genetics. A 100th anniversary of T. H. Morgan's discoveries. *Coll. Antropol.* **34**, 1167-1174 (2010).
26. Lewis, E. B. Developmental genetics of Drosophila. *Ann. N. Y. Acad. Sci.* **1038**, 94-97 (2004).
27. Tanji, T. & Ip, Y. T. Regulators of the Toll and Imd pathways in the Drosophila innate immune response. *Trends Immunol.* **26**, 193-198 (2005).
28. Weiner, J. in *Time, love, memory : a great biologist and his quest for the origins of behavior* (Knopf, New York, 1999).
29. Benzer, S. From the gene to behavior. *JAMA* **218**, 1015-1022 (1971).
30. Konopka, R. J. & Benzer, S. Clock mutants of Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.* **68**, 2112-2116 (1971).
31. Hotta, Y. & Benzer, S. Courtship in Drosophila mosaics: sex-specific foci for sequential action patterns. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 4154-4158 (1976).
32. Dudai, Y., Jan, Y. N., Byers, D., Quinn, W. G. & Benzer, S. dunce, a mutant of Drosophila deficient in learning. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 1684-1688 (1976).
33. Yoshihara, M., Adolfsen, B., Galle, K. T. & Littleton, J. T. Retrograde signaling by Syt 4 induces presynaptic release and synapse-specific growth. *Science* **310**, 858-863 (2005).
34. Yoshihara, M. & Littleton, J. T. Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. *Neuron* **36**, 897-908 (2002).
35. Budnik, V. & Salinas, P. C. Wnt signaling during synaptic development and plasticity. *Curr. Opin. Neurobiol.* **21**, 151-159 (2011).

36. Reppert, S. M. & Weaver, D. R. Comparing clockworks: mouse versus fly. *J. Biol. Rhythms* **15**, 357-364 (2000).
37. Krashes, M. J. *et al*. A neural circuit mechanism integrating motivational state with memory expression in Drosophila. *Cell* **139**, 416-427 (2009).
38. Fuentes-Medel, Y. *et al*. Glia and muscle sculpt neuromuscular arbors by engulfing destabilized synaptic boutons and shed presynaptic debris. *PLoS Biol.* **7**, e1000184 (2009).
39. Scott, K. *et al*. A chemosensory gene family encoding candidate gustatory and olfactory receptors in Drosophila. *Cell* **104**, 661-673 (2001).
40. Kang, K. *et al*. Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception. *Nature* **464**, 597-600 (2010).
41. Thomas, J. B. & Wyman, R. J. Mutations altering synaptic connectivity between identified neurons in Drosophila. *J. Neurosci.* **4**, 530-538 (1984).
42. Tanouye, M. A. & Wyman, R. J. Motor outputs of giant nerve fiber in Drosophila. *J. Neurophysiol.* **44**, 405-421 (1980).
43. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415 (1993).
44. Chalfie, M. GFP: Lighting up life. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 10073-10080 (2009).
45. Yeh, E., Gustafson, K. & Boulianne, G. L. Green fluorescent protein as a vital marker and reporter of gene expression in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7036-7040 (1995).
46. Hodge, J. J. Ion channels to inactivate neurons in Drosophila. *Front. Mol. Neurosci.* **2**, 13 (2009).
47. Hamada, F. N. *et al*. An internal thermal sensor controlling temperature preference in Drosophila. *Nature* **454**, 217-220 (2008).
48. Tian, L. *et al*. Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Methods* **6**, 875-881 (2009).
49. Yoshihara, M. & Ito, K. Improved Gal4 screening kit for large-scale generation of enhancer-trap strains. *Dros. Inf. Serv.*, 199-202 (2000).
50. Hayashi, S. *et al*. GETDB, a database compiling expression patterns and molecular locations of a collection of Gal4 enhancer traps. *Genesis* **34**, 58-61 (2002).
51. Friggi-Grelin, F. *et al*. Targeted gene expression in Drosophila dopaminergic cells using regulatory sequences from tyrosine hydroxylase. *J. Neurobiol.* **54**, 618-627 (2003).
52. Suster, M. L., Seugnet, L., Bate, M. & Sokolowski, M. B. Refining GAL4-driven transgene expression in Drosophila with a GAL80 enhancer-trap. *Genesis* **39**, 240-245 (2004).
53. Theodosiou, N. A. & Xu, T. Use of FLP/FRT system to study Drosophila development. *Methods* **14**, 355-365 (1998).

54. McGuire, S. E., Mao, Z. & Davis, R. L. Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila. *Sci. STKE* **2004**, pl6 (2004).
55. Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirian, L. & Dickson, B. J. Neural circuitry that governs Drosophila male courtship behavior. *Cell* **121**, 795-807 (2005).
56. Gordon, M. D. & Scott, K. Motor control in a Drosophila taste circuit. *Neuron* **61**, 373-384 (2009).
57. Yang, C. H. *et al*. Control of the postmating behavioral switch in Drosophila females by internal sensory neurons. *Neuron* **61**, 519-526 (2009).
58. Bullock, T. H. Revisiting the concept of identifiable neurons. *Brain Behav. Evol.* **55**, 236-240 (2000).
59. http://tech.mit.edu/V128/N51/katz.html.
60. http://www.animalresearch.info/en/medical/nobelprize.
61. Wilson, E. O. in *On human nature* (Harvard University Press, Cambridge, 1978).
62. Wilson, E. O. in *Sociobiology : the new synthesis* (Belknap Press of Harvard University Press, Cambridge, Mass., 1975).
63. Mundiyanapurath, S., Certel, S. & Kravitz, E. A. Studying aggression in Drosophila (fruit flies). *J. Vis. Exp***. (2**), 155 (2007).
64. Penn, J. K., Zito, M. F. & Kravitz, E. A. A single social defeat reduces aggression in a highly aggressive strain of Drosophila. *Proc. Natl. Acad. Sci. U. S. A***. 10**7, 12682-12686 (2010).
65. Kohatsu, S., Koganezawa, M. & Yamamoto, D. Female contact activates male-specific interneurons that trigger stereotypic courtship behavior in Drosophila. *Neuro***n 6**9, 498-508 (2011).
66. Yang, C. H., Belawat, P., Hafen, E., Jan, L. Y. & Jan, Y. N. Drosophila egg-laying site selection as a system to study simple decision-making processes. *Scienc***e 31**9, 1679-1683 (2008).
67. Card, G. & Dickinson, M. Performance trade-offs in the flight initiation of Drosophila. *J. Exp. Biol***. 21**1, 341-353 (2008).
68. Straw, A. D., Lee, S. & Dickinson, M. H. Visual control of altitude in flying Drosophila. *Curr. Biol***. 2**0, 1550-1556 (2010).
69. Dickens, C.,. in *Great expectations*. (Dodd, Mead, New York, 1942).
70. Peabody, N. C. *et a*l. Characterization of the decision network for wing expansion in Drosophila using targeted expression of the TRPM8 channel. *J. Neurosci***. 2**9, 3343-3353 (2009).
71. Kravitz, E. A. & Huber, R. Aggression in invertebrates. *Curr. Opin. Neurobiol***. 1**3, 736-743 (2003).
72. Shen, K. & Cowan, C. W. Guidance molecules in synapse formation and plasticity. *Cold Spring Harb Perspect. Biol***.** 2, a001842 (2010).

73. Clyne, J. D. & Miesenbock, G. Sex-specific control and tuning of the pattern generator for courtship song in Drosophila. *Cel***l 13**3, 354-363 (2008).
74. Manoli, D. S., Meissner, G. W. & Baker, B. S. Blueprints for behavior: genetic specification of neural circuitry for innate behaviors. *Trends Neurosci***. 2**9, 444-451 (2006).
75. Tinette, S. *et a*l. Exploratory behaviour in NO-dependent cyclase mutants of Drosophila shows defects in coincident neuronal signalling. *BMC Neurosci.* **8**, 65 (2007).
76. Singh, R. N. Neurobiology of the gustatory systems of Drosophila and some terrestrial insects. *Microsc. Res. Tech.* **39**, 547-563 (1997).
77. Dethier, V. G. in *The hungry fly : a physiological study of the behavior associated with feeding* (Harvard University Press, Cambridge, Mass., 1976).
78. Lima, S. Q. & Miesenbock, G. Remote control of behavior through genetically targeted photostimulation of neurons. *Cell* **121**, 141-152 (2005).
79. Dawkins, R.,. in *The selfish gene* (Oxford University Press, Oxford; New York, 1989).
80. Bailey, N. T. J. in *The mathematical approach to biology and medicine* (Wiley, London; New York, 1967).
81. Shapiro, F. R. in *The Yale book of quotations* (Yale University Press, New Haven, 2006).
82. Wang, Z., Singhvi, A., Kong, P. & Scott, K. Taste representations in the Drosophila brain. *Cell* **117**, 981-991 (2004).
83. GLLFERIN, A. Neural Control Systems Underlying Insect Feeding Behavior. *Amer. Zool.* **12**, 489-496 (1972).
84. Coll, A. P., Farooqi, I. S. & O'Rahilly, S. The hormonal control of food intake. *Cell* **129**, 251-262 (2007).
85. Allen, W. *et al*. Annie Hall. (1998).
86. Demerec, M. in *The biology of Drosophila* (Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1994).
87. Kitamoto, T. Conditional disruption of synaptic transmission induces male-male courtship behavior in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 13232-13237 (2002).
88. Pauli, A. *et al*. Cell-type-specific TEV protease cleavage reveals cohesin functions in Drosophila neurons. *Dev. Cell.* **14**, 239-251 (2008).
89. Sitaraman, D. *et al*. Serotonin is necessary for place memory in Drosophila. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 5579-5584 (2008).
90. Liu, X. & Davis, R. L. The GABAergic anterior paired lateral neuron suppresses and is suppressed by olfactory learning. *Nat. Neurosci.* **12**, 53-59 (2009).
91. Fouquet, W. *et al*. Maturation of active zone assembly by Drosophila Bruchpilot. *J. Cell Biol.* **186**, 129-145 (2009).

92. Leiss, F. *et al*. Characterization of dendritic spines in the Drosophila central nervous system. *Dev. Neurobiol.* **69**, 221-234 (2009).
93. Baines, R. A., Uhler, J. P., Thompson, A., Sweeney, S. T. & Bate, M. Altered electrical properties in Drosophila neurons developing without synaptic transmission. *J. Neurosci.* **21**, 1523-1531 (2001).
94. http://www.famousquotes.com/show/1867032/.
95. *Physiology or medicine*. (World Scientific, Singapore; River Edge, N.J., 1992).
96. Lee, T. & Luo, L. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuro***n 2**2, 451-461 (1999).
97. Struhl, G. & Basler, K. Organizing activity of wingless protein in Drosophila. *Cel***l 7**2, 527-540 (1993).
98. Shang, Y., Griffith, L. C. & Rosbash, M. Light-arousal and circadian photoreception circuits intersect at the large PDF cells of the Drosophila brain. *Proc. Natl. Acad. Sci. U. S. A***. 10**5, 19587-19594 (2008).
99. Fischler, W., Kong, P., Marella, S. & Scott, K. The detection of carbonation by the Drosophila gustatory system. *Natur***e 44**8, 1054-1057 (2007).
100. Thorne, N., Chromey, C., Bray, S. & Amrein, H. Taste perception and coding in Drosophila. *Curr. Biol***. 1**4, 1065-1079 (2004).
101. Kim, S. H. *et a*l. Drosophila TRPA1 channel mediates chemical avoidance in gustatory receptor neurons. *Proc. Natl. Acad. Sci. U. S. A***. 10**7, 8440-8445 (2010).
102. Yoshihara, M., Rheuben, M. B. & Kidokoro, Y. Transition from growth cone to functional motor nerve terminal in Drosophila embryos. *J. Neurosci***. 1**7, 8408-8426 (1997).
103. Yoshihara, M., Suzuki, K. & Kidokoro, Y. Two independent pathways mediated by cAMP and protein kinase A enhance spontaneous transmitter release at Drosophila neuromuscular junctions. *J. Neurosci***. 2**0, 8315-8322 (2000).
104. Marella, S. *et a*l. Imaging taste responses in the fly brain reveals a functional map of taste category and behavior. *Neuro***n 4**9, 285-295 (2006).
105. Shiraiwa, T. & Carlson, J. R. Proboscis extension response (PER) assay in Drosophila. *J. Vis. Exp***. (3)**, 193 (2007).
106. Claridge-Chang, A. *et a*l. Writing memories with light-addressable reinforcement circuitry. *Cel***l 13**9, 405-415 (2009).
107. Darwin, Charles; Carrol, Joseph (editor) in *On the origin of species by means of natural selection.* (Broadview Press, Peterborough, Ont; 2003)
108. http://www.goodreads.com/quotes/show/352638.