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MODULATING MALE AGGRESSION AND COURTSHIP: DETECTING EXTERNAL PHEROMONAL AND NUTRITIONAL INFORMATION

Jonathan C. Andrews
University of Montana

MODULATING MALE AGGRESSION AND COURTSHIP: DETECTING EXTERNAL PHEROMONAL AND NUTRITIONAL

BY

JONATHAN ANDREWS

B.A. in Human Biology, University of Montana, Missoula 2006

B.A. in Psychology, University of Montana, Missoula (2009)

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Approved by:

Sandy Ross, Dean of The Graduate School
Graduate School

Sarah Certel, Advisor
Department of Biological Sciences

J. Stephen Lodmell, Chair
Department of Biological Sciences

Darrell Jackson
Department of Biomedical and Pharmaceutical Sciences

Fernando Cardozo-Pelaez
Department of Biomedical and Pharmaceutical Sciences

Chris Comer
Dean of the College of Humanities and Sciences

Jonathan Andrews

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Abstract

Survival and reproduction in the natural world requires an organism to identify and react to the presence of environmental stimuli in a time and cue dependent manner. Such temporal specificity requires the development and use of specialized sensory organs that receive this external sensory information. Neurons within the specialized sensory organs respond to touch, taste, pheromones, chemicals, and light, and transduce this information to the central brain. In many systems, gustatory and olfactory chemosensation in particular, provides critical information regarding sex and species identification as well as the status of food resources. The output of neurons which receive chemical information is regulated by the action of biogenic amines, including serotonin, dopamine, and norepinephrine. In this dissertation I examined the role of octopamine (the invertebrate structural homologue of norepinephrine) signaling in the regulation of two behaviors required for survival and reproduction; aggression and courtship.

In chapter II, I, along with my colleagues, demonstrate that neurons bearing the taste receptor Gr32a form putative synapses with octopamine neurons within the subesophageal zone, and that octopamine neurons promote male aggression and courtship behavior. These findings help to explain how an organism selects appropriate behavioral responses when confronted with the pheromonal signals of a rival male.

In chapter III, I examined the effects of octopamine signaling on taste sensitization. In this section, I examined the distribution and function of neurons that express the Oaβ1R receptor, and found that these neurons are sugar sensitive. As the presence of a food source is known to be a major contributor to the generation of aggressive and courtship behavior, these findings imply a mechanism by which exposure to an environmental stimulus or changes in internal octopamine signaling may sensitize a particular form of sensory input.

Acknowledgements

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Chapter I

Introduction

Survival and reproduction in a complex environment requires that an organism be able to identify signals that correspond with the presence of rivals, mates and food sources. This dissertation will investigate the activity and function of two different populations of peripheral neurons that are critical for the identification and processing of important environmental stimuli. Both neuronal populations interact with octopaminergic neurons that express the neurotransmitter octopamine (OA), either through direct contact or long distance signaling, underscoring the importance of OA neuromodulation in the regulation of goal-directed behavior. This introductory chapter will provide a brief history of *Drosophila* as a model organism in neuroscience, review insect chemosensation as it pertains to behavioral regulation, and discuss the role OA plays in the initiation and maintenance of social behavior.

Major contributions of *Drosophila* to neuroscience research

From the first appearance of the fruit fly as a model organism in the early 1900's, *Drosophila* research has lead to many landmark discoveries, a few which will be summarized here (Kohler 1993). Early studies in genetics were greatly assisted by the use of *Drosophila* as a model organism, and some of the central tenants of genetics, including sex linkage and the mutagenic effects of ionizing radiation, were identified by Thomas Hunt Morgan and Hermann Muller using a fly model (Morgan 1910, Crow 2005, Muller 1927). In 1915, fruit flies formally entered into the field of neuroscience with the identification of the *Notch* gene, an important player in neurogenesis and neuronal differentiation (Poulson 1950, Bellen 2010, Gazave 2009). By the late 1960's, advancements in mutagenic tools allowed for researchers to combine genetic and behavioral approaches for the first time to tackle questions regarding the molecular basis of behavior (Lewis 1968). This renaissance of tools and methods allowed for the identification of multiple genes, including *Period, Dunce, Rutabaga,* and others, which are important for the maintenance of circadian rhythm, learning, and memory (Benzer 1967, Konopka 1971, Bellen 2010).

Furthermore, analysis of the growing body of genes and their products in *Drosophila* revealed a number of proteins that affect the function of the nervous system. *Transient receptor potential*, or TRP, was one such gene/protein pair identified during this period, and the TRPs have been found to respond to multiple stimuli, including mechanical stretch, heat, touch, nerve growth factor, and pheromones (Minke 1975). TRP channels are key in *Drosophila* proprioception, touch sensation, hearing and olfaction, and mutations in TRP-related proteins are thought to be responsible for several different neurodegenerative disorders (Zuker 1996, Levix 1982, Zipursky 1994, Montell 1985, Montell 1999, Venkatachalam 2007).

The fruit fly has also directly contributed to our understanding of neuronal function at the molecular level through the *Shaker* and *Eag* mutants. Flies with mutations in the *Shaker* gene display aberrant patterns of movement, and convulse when anaesthetized (Kaplan 1969, Jan 1977). This unusual pattern of behavior is the direct cause of change in a voltage-gated potassium channel, which fails to repolarize neurons following an action potential in *Shaker* mutants (Wu 1983, Baumann 1987, Kamb 1987). Likewise, *Eag* mutants, who were also identified on the basis of a leg-shaking phenotype, possess a defective potassium channel (Ganetzky 1982, Wu 1983). However, the fact that *Shaker* and *Eag* double mutants display a more severe phenotype paved the way to the understanding how different types of potassium channels, with different physical properties, contribute to the repolarization process (Ganetzky 1982).

More modern techniques and genetic tools have perpetuated the use of *Drosophila* in neuroscience, including the development of the UAS/Gal4 system and its analogues, the sequencing of the *Drosophila* genome, and the creation of robust RNAi libraries, have all contributed to the current success of the fruit fly in laboratories worldwide. Currently, of the estimated 17,651 genes currently mapped to the *Drosophila* genome, 548 genes have been identified as having a direct analog to human diseases, and 74 of these are known to be involved in neurological disorders (Ashburner 2005, Reiter 2001). Given the sequential and functional

conservation, the genetic tools available to researchers using this model system, and ease of behavioral analysis possible in the fly model, the humble fruit fly will undoubtedly continue to make contributions to neuroscience. In this dissertation, I will capitalize on the genetic and behavioral tools found within this model system as a means by which to explore the neurological origins of several behaviors that are common in both vertebrates and invertebrates.

Drosophila Behavior

From the perspective of early psychology, goal directed actions/behavior is performed when the acting agent desires a goal, and believes that some form of activity will achieve this goal (Thorndike 1911, De Wit, 2009). Modern neuroscience has expanded on this definition to include a more mechanistic evaluation of behavior, including the role of reflexes, learning, memory, internal state, external stimuli and a more thorough understanding of how genetic and molecular factors can influence final behavioral choices (Fernandez 2013, Zwarts 2012). Nevertheless, some of the earliest observations of animal behavior are still relevant to a neuroscience-based approach when studying social behavior. First, performing any behavior is fundamentally an expenditure of energy in the form of ATP, and depending on the behavior performed it can be energetically expensive or hazardous to the performer (Pool 2014). Therefore, behavior must be tightly controlled, both in the initiation and execution of the intended activity. Second, all patterns of behavior must be performed in a context dependent manner. This requires the rapid integration of information supplied by multiple environmental stimuli, which in turn necessitates accurate input from multiple body systems.

Our lab has focused on two specific types of *Drosophila* behavior- courtship and aggression. As a model organism, the fruit fly is uniquely suited to study these behaviors for two major reasons. Foremost, the fly brain contains 100,000-135,000 neurons, making it structurally complex enough to generate complicated responses to social and environmental stimuli (Powers 1943, Alivisatos, 2012). Additionally, many social behaviors performed by fruit flies are highly stereotyped, and adult flies

are capable of performing these behaviors shortly after emerging as adults (Mundiyanapurath 2007, Chen 2002, Spieth 1968, Hoffmann 1990, Schilcher 1975). This implies that the neural pathways responsible for the generation of aggression and courtship are innately wired into the *Drosophila* brain. Therefore, it is possible to study the generation of these social behaviors as a consequence of gene expression or using genetic tools to alter the function of neuronal pathways and observe the results. In the next few paragraphs, I will delve into the commonalities present in all fruit fly behavior, and examine the two behaviors relevant to this dissertation: courtship and aggression.

Commonalities in *Drosophila* behavior

Before discussing the specifics of aggression and courtship, it is valuable to note what these forms of behavior have in common. First, courtship and aggression, like all other behaviors, are reliant on external sensory cues, such as pheromones or specific tastants, for regulation (Coen 2014, Moon 2009, Fan 2013) . Aggression, for example, relies on the presence of pheromonal signals to ensure it is not performed at the wrong time or against the wrong targets. Second, all *Drosophila* behavior relies on central processing within the brain (Zwarts 2012, Kravitz 2015). While neurons from the periphery do form synapses within the ventral ganglion, input from the brain is required to coordinate complex social behavior. Finally, courtship and aggression rely on neuromodulation for the integration and propagation of the information necessary to choose and perform the appropriate pattern of behavior (Cohn 2015). As the number of variables required to perform complex social tasks are numerous, neuromodulation is uniquely suited to transmit relevant information to and from multiple body systems and throughout the brain, allowing for behavior to be properly executed and tightly controlled. In the following sections, I will further discuss the specific patterns of action that represent *Drosophila* courtship and aggression.

Drosophila aggression

Aggressive behavior is a commonality among many different animal species, and is essential in the acquisition of territory, food, and mates or in the defense of the actor or its progeny against potential predation by conspecifics or other species (Zwarts 2012). As noted previously, aggression must be tightly regulated, and relies on the integration of both internal and external signals to be performed properly. Aggressive behavior in the fly was first recorded by Sturtevant in 1915, who recorded descriptions of fights between males vying for mating partners (Sturtevant 1915). Since that time, a more complete ethogram of *Drosophila* aggression has emerged, describing an array of agonistic behaviors performed in response to the presence of a male conspecific (Chen 2002, Spieth 1968, Hoffmann 1990, Schilcher 1975).

Like many other organisms, fruit flies display sex-specific patterns of behavior. Of particular interest to this dissertation are the behaviors used as means of quantifying male aggression in our assays, which include lunges, fencing, boxing, tussling, and wing threats. Lunges are one of the primary forms of aggressive behavior, where one male fly strikes his opponent by standing on his mid and hindlegs, then hurling himself upon his opponent. Boxing and tussling represent higher-intensity forms of the lunge, with either both flies balanced on their hindlegs repeatedly striking at their opponent, or both flies tumbling over each other while attempting to strike. Fencing represents a lower-intensity form of aggression, where both flies make jabs at each other with their extended legs. The wing threat is, as the name implies, a threat display involving prolonged extension of the wings in a near-vertical manner. Photos of these behaviors can be seen in **Figure 1**. Female fruit flies also perform aggressive behavior, but instead of the patterns reported above, females primarily rely on a “head butt” or shove to displace competitors (Chen 2002, Skrzipek 1979, Lee 2000, Jacobs 1979, Schilcher 1975, Ueda 2002).

Cues affecting aggression

The environmental cues responsible for the initiation of aggressive behavior are sex dependent. Both male and female flies are capable of fighting over food territories, especially if the territory contains yeast (Nilsen 2004, Ueda 2002, Lim 2014). However, males alone will commit to skirmishes for the opportunity to mate (Nilsen 2004). When fighting over mates, male *Drosophila* will form a hierarchy of dominance dependent on "winning" or "losing" encounters with other conspecifics. "Winners" are defined as animals that have previous victories over conspecifics, typically driving their opponent off of a shared territory, while "losers" are driven off by the action of their rival (Parker 1974, Beacham 1987, Beaugrand 1991, Trannoy 2016). Established dominance is stable over short periods of time, and can contribute significant changes in behavior, where flies that drive off their opponents are more likely to do so in the future, and flies that are driven off are more likely to leave contested territories (Yurkovic 2006, Trannoy 2015).

Observation of lab-grown and wild *Drosophila* has also identified several environmental factors that contribute to aggression. The amount of space available for movement has been shown to be a significant contributing factor to aggression in lab grown flies. Smaller arenas have been shown to lead to increased levels of arousal and subsequently more aggression (Kamyshev 2002). The presence of a food source is also aggression promoting, with smaller territories inciting the highest levels of aggressive behavior (Hoyer 2008, Chen 2002, Kamyshev 2002, Lim 2014). Body size is another important factor, and changes of as little as 8% in size are sufficient to increase the likelihood of aggression from the larger fly (Hoyer 2012). Lastly, social conditioning is important in a fly's willingness to fight. Flies housed with other flies display less aggressive behavior, and housing in isolation greatly increases aggressive tendencies (Stevenson 2013, Ueda 2009). These discoveries highlight the multifactorial nature of aggressive behavior, and how multiple cues may guide aggression.

Drosophila Courtship

Courtship in any species is a means by which sexually mature individuals identify conspecific mates who possess traits indicative of high levels of fitness (Yamamoto 2013). Like aggression, *Drosophila* courtship is highly stereotyped, and composed of several sequential steps that are primarily modulated by the presence of pheromones and courtship ritual. Upon encountering a female conspecific, male fruit flies orient towards her, and will circle around to have access to her abdominal region. The male will touch the female with a foreleg, referred to as "tapping", and will follow the female if she flees. Post-tapping, male flies will engage in "singing", a behavior where the male extends a single wing and vibrates it in a courtship song. Females typically reduce their rate of movement in response to courtship song, provided that they are receptive. Following the initial steps of courtship, male fruit flies will extend their proboscis towards the female's genitalia (referred to ethologically as licking), and attempt to copulate. The female, if receptive, will withdraw her vaginal plate, and copulation will last for 15 to 20 minutes (Bastock 1955, Greenspan 2000, Hall 1982, Ferveur 2010). Photos of these courtship behaviors can be found in **Figure 1**. Some plasticity can be observed during this sequence of events, but all actions are repetitive in nature, until copulation (Fernandez 2013). In the presence of rival conspecifics, males will also increase the duration of copulation (Kim 2012). Like the "loser" effects seen in *Drosophila* aggression, males that are unsuccessful in courtship reduce their rate of courtship for several hours following rejection (Siegel 1979). Much like aggression, *Drosophila* courtship is reliant on processing within the central nervous system and pheromonal cues. Many of the cues are gustatory, and will be discussed in more detail during the next section.

Drosophila gustation

Drosophila, like many vertebrates, utilizes a system of specialized sensory cells to detect chemical cues present within the environment, an important form of information gathering critical to finding both food and identifying conspecifics. Gustation, or the detection of non-volatile compounds, is performed by gustatory

Figure 1

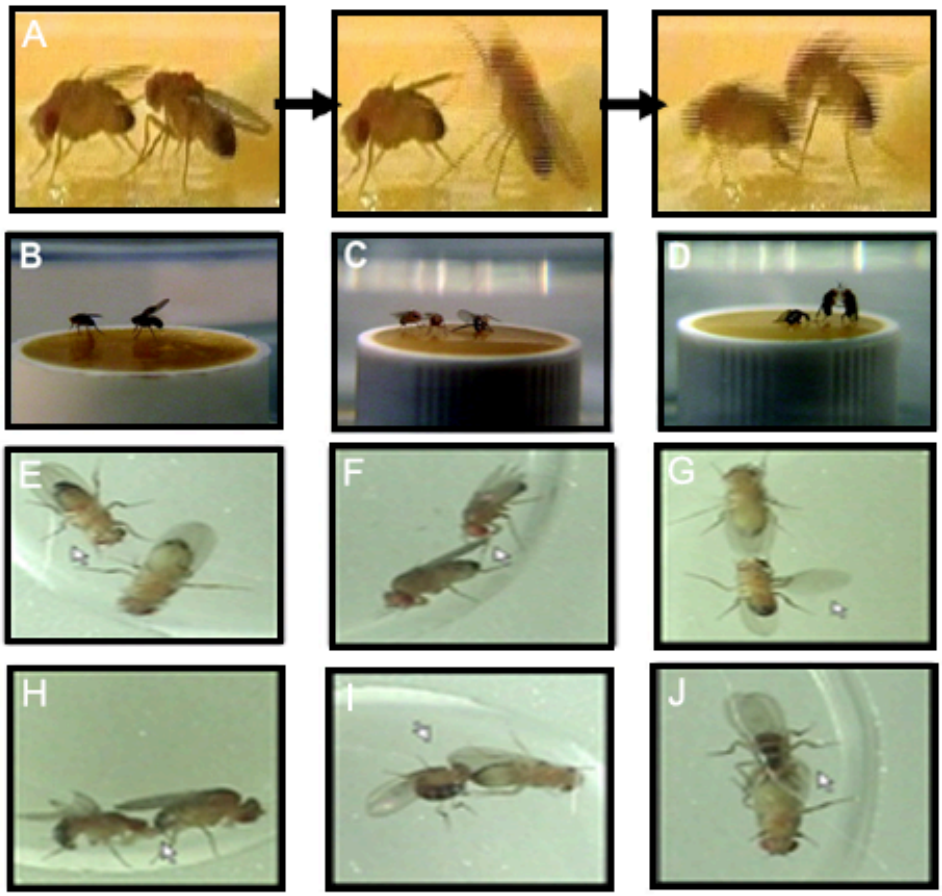

Figure 1: Stereotyped aggressive and courtship behaviors in *Drosophila*

(**A**) Example of a lunge, the predominant form of aggressive behavior measured in our assays. (**B**) Aggressive behavior: a wing threat. (**C**) Aggressive behavior: Fencing. (**D**) Aggressive behavior: Boxing/Tussling. (**E**) Courtship behavior: Orientation. (**F**) Courtship behavior: Tapping (**G**) Courtship behavior: Singing/Wing Extension. (**F**) Courtship behavior: Licking. (**I**) Courtship behavior: Attempted Copulation. (**J**) Copulation.

Images Adapted From: (**A**) Kravitz lab. (**B-D**) Chen, 2002. (**E-J**) Dai, 2008.

sensilla. These hairlike projections that can be found projecting from labellar palps (mouthparts), leg tarsi, wing margins, within the pharynx, and from the ovipositor in females (**Fig. 2A**). Each sensilla is structurally similar, containing a pore which allows for the entry of tastants, between two and four gustatory receptor neurons, a single mechanosensory neuron and a number of support cells (**Fig. 2B**). Gustatory receptor neurons themselves are bipolar neurons. Each neurons sends a single dendrite into the shaft of the sensillum, and projects an axon centrally towards the subesophageal zone (Montell 2009, Falk 1976, Stocker 1994, Singh 1997). Each hair fiber can be further classified by its location. Sensilla present on the labellum fall into three categories, based on the length of the hair that composes the central shaft of the sense organ. L-type (long) and S-type (short) share a similar chemosensory profile and the gustatory receptor neurons present in these hairs respond to the presence of environmental sugars, water, and salt (Hiroi 2002, Fujishiro 1984,). I-type (intermediate) sensilla have a more diverse detection profile, with two different subpopulations within the labellum. The first is stimulated by the presence of sugars, salts and other attractive agents, while bitter compounds and other aversive chemicals excite the second (Meunier 2003, Hiroi 2004).

Tarsal sensilla, unlike their labellar counterparts, are highly sexually dimorphic (Ling 2014). Males have thirteen more sensilla on their forelegs than their female counterparts, although the number of hair fibers remains identical on both the mid- and hindlegs (Nayak 1983). In both males and females, most tarsal sensilla are present in bilaterally symmetric pairs on either side of the leg, and many of the sensilla identified in the forlegs have analogues on both the rear legs as well Miyamoto 2013). Despite these similarities, sensilla on *Drosophila* forelegs provide a more robust response to both sugars and bitter compounds than the equivalent sensilla on either the mid- or hindlegs (Ling 2014, Dahanukar 2001, 2007, Weiss 2011, Rodrigues 1978). Tarsal gustatory receptor neurons are also more broadly tuned than their labellar equivalents. In a study by Ling et al (2014), a single tarsal neuron was identified that detected 19 separate bitter compounds. These differences have lead to the speculation that the chemosensory sensilla present on

Figure 2

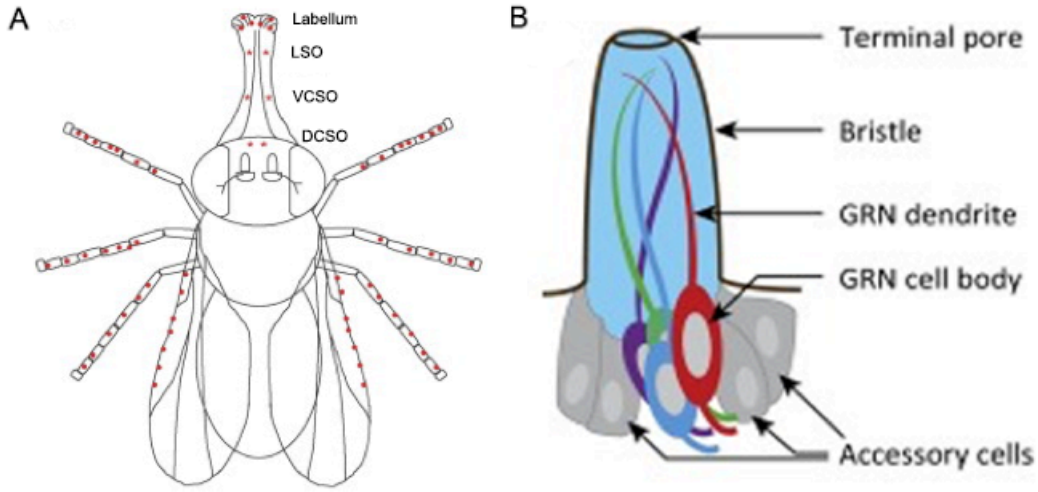

Figure 2: Location and Structure of Gustatory Sensilla

(**A**) Red dots indicate the location of gustatory sensilla upon the labellum, pharynx, tarsi, and wing margin. (**B**) Structure of a gustatory sensillum, highlighting the pore and GRN cells housed within. GRN dendrites project into the shaft of the bristle.

Image Adapted From: (**A**) Amrein, 2005 (**B**) Joseph, 2015

the tarsal segments may act as an early warning system, with the gustatory receptors on the legs serving to detect important environmental features before initiating patterns of behavior associated with feeding or aggression.

Pheromone sensation

Drosophila relies on both olfactory and gustatory senses to detect and evaluate pheromonal signals, which are critical to gender and species identification. As with other chemical senses in the fruit fly, olfaction is reliant on the action of odorant sensitive neurons housed in sensilla. Olfactory neurons present within the antenna provide the majority of olfactory sensation, and relay chemical signals to the mushroom body and lateral horn of the *Drosophila* brain (Jefferis 2007). While flies are known to respond to a number of volatile stimuli, only one olfactory pheromone have been confirmed to alter behavior. 11-*cis*-vaccenyl acetate (cVA) is a male pheromone that is transferred to female flies during copulation, and is detected by the olfactory receptors Or67d and Or65a (Naters 2007, Ha 2006, Kurtovic 2007). cVA has been extensively studied, and found to act as a repellant to male flies and as an attractive agent to females (Ha 2006, Kurtovic 2007).

In contrast to olfactory signaling, six gustatory (non-volatile) pheromones have been identified. All of these pheromones, save one, are classified as cuticular hydrocarbons, and are produced by oenocytes, a layer of specialized cells found on the inner surface of the abdominal cuticle (Ferveur, 2005). Three cuticular hydrocarbons ((z)-7-tricosene, (z)-7-pentacosene, and (z)-11-pentacosene) have an inhibitory effect on courtship behavior, while two ((z,z)-7-11-heptacosadiene and (z,z)-7-11-nonacosadiene) act as aphrodisiacs (Billeter 2009, Miyamoto 2008, Fan 2013, Moon 2009). The only non-cuticular hydrocarbon pheromone is (3*R*,11*Z*,19*Z*)-3-acetoxy-11,19-octacosadien-1-ol (CH503), which is produced in the male ejaculatory bulb, and acts as an anti-aphrodisiac (Yew 2009). The structure of these compounds can be found in **Figure 3**. In *Drosophila melanogaster*, aphrodisiac and anti-aphrodisiac compounds are detected by two different sets of gustatory receptor neurons. Compounds that inhibit courtship behavior are detected by neurons

Figure 3

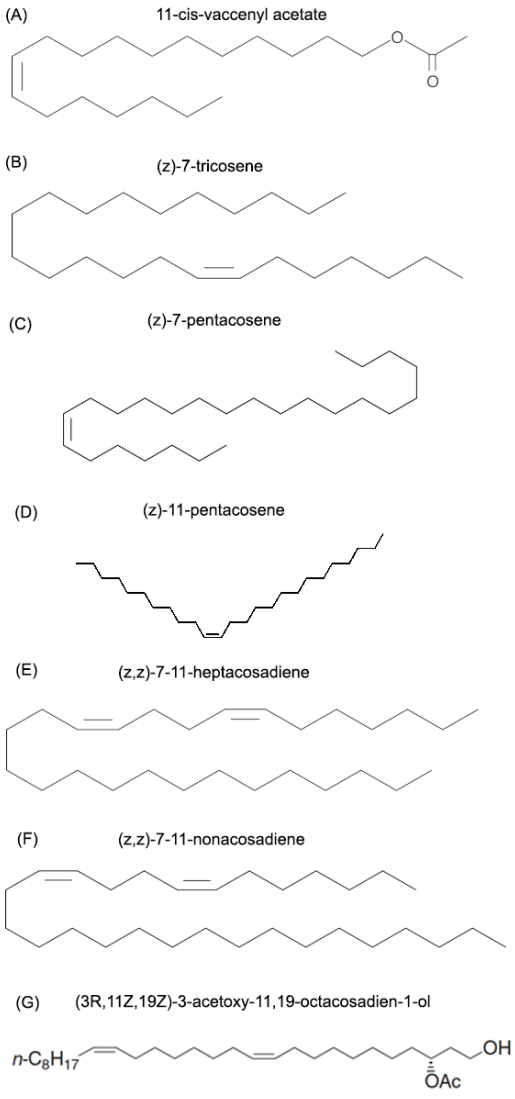

Figure 3: Structure of *Drosophila* Pheromones

(**A**) is a volatile/olfactory pheromone, while (**B-G**) are nonvolatile/gustatory pheromones. (**B-D and G**) act as anti-aphrodisiacs, while (**E-F**) promote sexual activity.

Image Adapted From: (**A-C, E, F**) Cayman Chemical, (**D**) The Pherobase, 2016. (**G**) Shikichi, 2013.

bearing the Gr32a, Gr33a, Gr68a or ppk23 receptors (Moon 2009, Lacaille 2007, Tetsuya 2008. Conversely, courtship-promoting signals are processed via neurons expressing ppk23 and ppk25 (Bray 2003, Ejima 2007, Toda 2012). Several other receptors, including, Gr39a and IR52c/IR52d, have also been suggested as important players in the sensation of pheromones, but no ligand has been found that interacts with these receptors (Ferver 2005).

Sensation of dietary sugars and bitter compounds

In order to maintain metabolic homeostasis, each step involved in feeding behavior requires input from internal and external sensors that detect the presence of sugars and amino acids. Internally, the Gr43a receptor acts as a fructose sensor. Neurons expressing this receptor in the superior protocerebrum promote feeding in starving flies (Miyamoto 2012). Conversely, cessation of feeding is largely the result of the activity of the kinase GCN2. When triggered in dopaminergic neurons, GCN2 signaling results in food rejection (Hao 2005, Domingos 2013, Gonzalez 2008). Externally, a suite of receptors are responsible for the sensation of sugar, including Gr5a, Gr64a. Gr64b, Gr64c, Gr64d, Gr64e, and Gr64f. These receptors are crucial for two different appetitive behaviors: the proboscis extension response and locomotion suppression (Wang 2004, Thorne 2004, Jiao 2007, Dahanukar 2007, Ledue 2015, Knapek 2016). The activity of these sugar receptors is important to the third chapter of this dissertation, where I will discuss the link between OA neuromodulation and feeding behavior.

The detection of bitter compounds is handled by a separate set of receptors on the proboscis and tarsi. Gr66, Gr32a, Gr33a, Gr93a, Gr89a are each capable of detecting bitter tastants that are considered aversive, and therefore deter feeding (Thorne 2004, Wang 2004, Lee 2009, Moon 2009). It is worth noting that some of the receptors responsible for the detection of bitter compounds are also involved in the regulation of courtship and aggression as well. The activity of one of these receptors, Gr32a, is the topic of the second chapter of this dissertation. As neurons expressing both bitter and sweet-sensing GRN's send information from the periphery into the

subesophageal zone, I will provide a brief overview of the relevant portions of *Drosophila* neuroanatomy.

The Subesophageal zone of the *Drosophila* brain

Before discussing how connections in the fruit fly brain and periphery contribute to courtship and aggression in the subsequent chapters, it is necessary to be familiar with the general anatomy of the *Drosophila* brain. Originally described by Maxwell Powers in 1943, the *Drosophila* central nervous system contains an estimated 100,000-135,000 neurons within the supraesophageal, subesophageal, and thoracico-abdominal ganglion (Powers 1943, Alivisatos, 2012). At the time of its first description, the central brain was characterized as containing an external cellular cortex surrounding a dense fibrous core, which could be organized into aggregates of fibers or glomeruli (Powers 1943).

Using the imaging methods available at the time, these "bodies" were sub-divided into seven different regions: the central complex, the corpora peduncilata, the protocerebral bridge, the antennal glomeruli, the optic lobes, the important fiber bundles, and the cellular cortex (Powers 1943). As modern imagining techniques have increased the spatial resolution and accuracy of brain imagining significantly since the initial morphological study of the fruit fly, contemporary studies of the fly brain use a more refined system of nomenclature that divides the insect brain into twelve supercatagories and five landmark fiber bundles that can be identified in both *Drosophila* and other common insect models (Rein 2002, Ito et al 2014).
Of these supercatagories, two regions, the Gnathal ganglia (GNG) and peroesophageal neuropils (PENP) are of particular interest to the following chapters of this dissertation (**Fig. 4**). Together, they compose the primary supercatagories that make up the subesophageal zone, and are therefore involved in generation of feeding behavior, locomotion, courtship, and aggression. During development, the gnathal ganglia are comprised of three clear divisions: the mandibular, maxillary and labial neuromeres (Ito et al 2014). By the time the fly reaches its adult stage, the boundaries separating these regions within the gnathal ganglia are less distinct, and

Figure 4

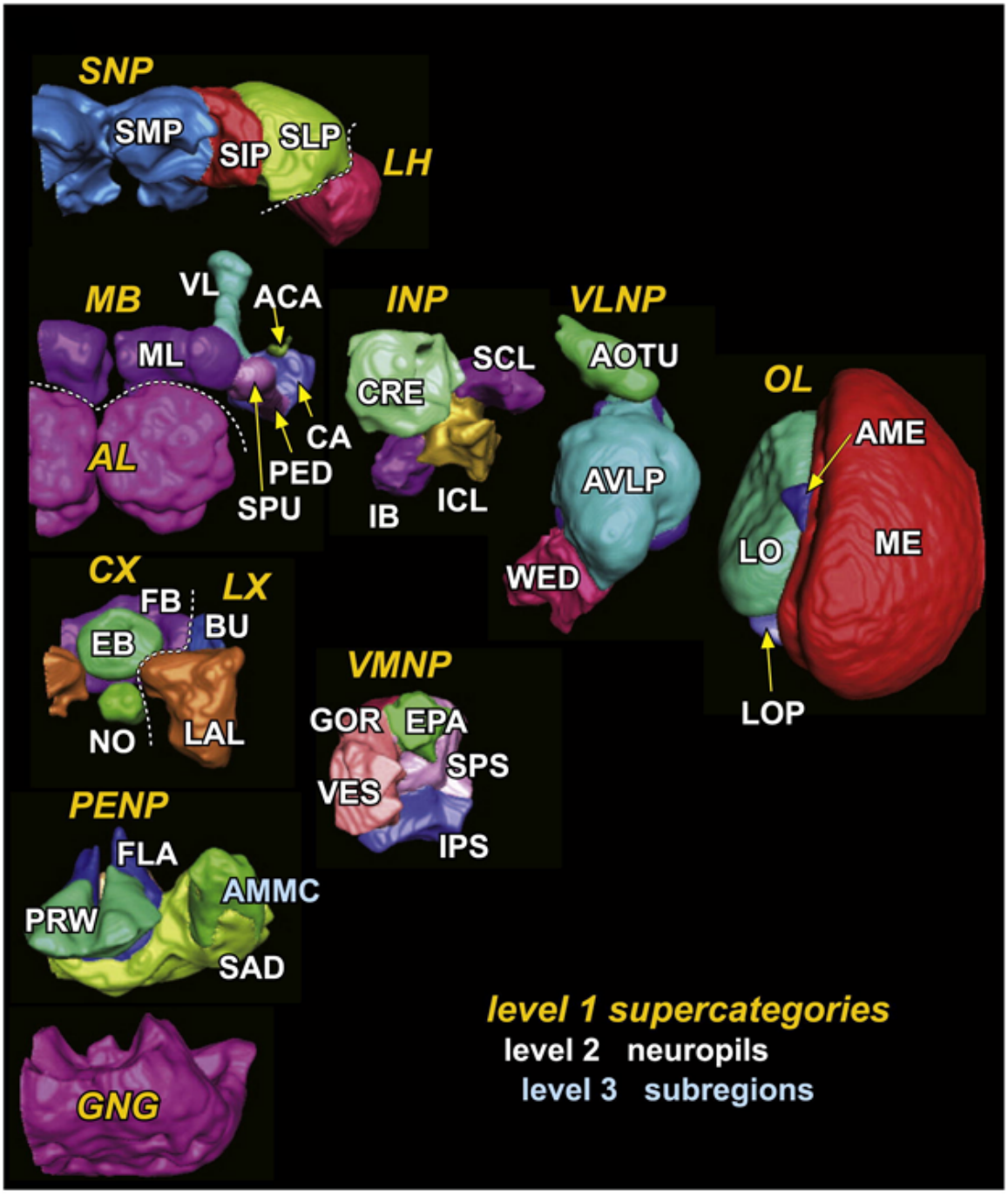

Figure 4: The *Drosophila* brain

3D model of regions of the *Drosophila* brain, expanded for viewing. Areas of interest to this dissertation include the gnathal ganglia (GNG) and peroesophageal neuropils (PENP), including the saddle (SAD), and prow (PRW). Image Source: Ito, 2014.

are defined only by the presence of fiber bundles extending from the ventral unpaired cluster neurons and by synaptic labeling of the pharyngeal nerve (for the mandibular neuropil) and the maxillary-labial nerve (for the maxillary and labial neuropils). These neuropils can therefore be more accurately segregated into the inferior pharyngeal sensory center, the anterior maxillary sensory center, the posterior maxillary sensory center, and the labial sensory center based upon the terminal regions of the pharyngeal and accessory pharyngeal nerve and the maxillary-labial nerve (Ito K et al 2014).

Immediately superior to the gnathal ganglion, the periesophageal neuropils occupy the space directly adjacent to the esophagus and inferior to the antennal lobe and ventromedial neuropils. While this region is composed of five different neuropils, only two are traditionally considered to be part of the subesophageal zone: the prow and the saddle. The prow is the most superior region of the subesophageal zone, consisting of the brain tissue just inferior and anterior to the esophageal foramen. As part of the tritocerebrum, the prow acts to integrate sensory information from the proto- and deutocerebrum, which receive sensory input from the eyes and antennae respectively. Conversely, the saddle is a more complex structure that combines two separate regions. The first region runs laterally and encapsulates the axons of the antennal nerve until their termination within antennal mechanosensory and motor center, while the second forms the boundary between the gnathal ganglia and the esophagus. Encapsulated within the saddle lies the antennal mechanosensory and motor center, which receives input from the Johnstons organ neurons of the antenna. These features are highlighted in **Figure 4** (Ito K et al 2014).

Octopaminergic signaling and Aggression

Like many vertebrates, *Drosophila* also utilizes a number of amines to regulate aggressive behavior. OA is of particular relevance to this dissertation, and is possibly the most thoroughly studied amine in the context of *Drosophila* aggression, and an important player throughout the remainder of this document. While OA was

initially discovered in the salivary glands of *octopus vulgaris*, it is now primarily known for its role as a neurotransmitter, neuromodulator and neurohormone in many different insects, including *Drosophila* (Erspamer 1951, Axelrod 1977, Roeder 1999, Orchard 1982). In the absence of OA, fruit flies lunge at significantly lower rates and take much longer to initiate aggressive behavior (Baier 2002, Hoyer 2008, Zhou 2008, Certel 2007, 2010). This pattern of behavior is not atypical for animals that have difficulty recognizing salient features of other conspecifics, and will be discussed in greater detail during chapter two. Overexpression of Tβh, use of an OA agonist, or forced activation of OA neurons via genetic means all resulted in increased aggression, even in flies housed together (Rou 2008, Hoyer 2008). In addition to the direct effects of OA on aggression, OA signaling acts to increase starvation-induced locomotion and enhance food intake, activity that is mutually exclusive with aggressive behavior and may therefore tangentially play a role in limiting aggression (Zhe 2015, Koon 2012).

Much like vertebrates, *Drosophila* are also influenced by their gut microbes, and members of the *Wolbachia* family are known to influence fruit fly aggression by influencing the synthesis of OA. *Drosophila* infected by *Wolbachia* display reduced expression levels of both Tdc2 and Tβh, and subsequently lower levels of OA within the head (Rohrscheib 2015). All of these factors, when combined, make OA one of the most potent influences on fruit fly aggression, and an excellent candidate for further study. This dissertation will further expand our knowledge of OA signaling by evaluating signaling partners associated with OA neurons or expressing OA receptors within the periphery of *Drosophila*.

Octopaminergic Signaling and Courtship

As with aggression, OA is known to play a significant role in *Drosophila* courtship. OA is known for its role in courtship conditioning, where males who fail to copulate post courtship display reduced levels of courtship several hours. A loss of OA is also known to have an effect on male-female courtship specificity. Male flies that lack OA display higher levels of male-male courtship than do their control

counterparts, even when presented with a female fly as an alternate target for courtship. Stimulation of OA-expressing neurons via the heat activated *UAS-dTrpA1* line also results in increased male-male courtship, a phenotype that is attributed to the loss of proper attenuation within the courtship circuit (Certel 2007, 2010). This phenotype is similar to those exhibited by flies who lack the Gr32a receptor, a fact which served as the foundation for the investigation documented in chapter two of this dissertation (Wang 2011, Miaymoto 2008, Fan 2013).

OA in the *Drosophila* brain

As OA is one of the principle players in fruit fly behavior, understanding where it is found in the brain is vital. By using the TDC2 promoter as part of the UAS-Gal4 binary expression system, along with more traditional immunohistochemical methods, a map of OA neurons within the *Drosophila* brain was completed in 2009 (Cole 2005). This study revealed a total of 137 Gal4-positive neurons distributed across 8 regions of the fruit fly brain (Busch 2009). Of particular interest are the 27 neurons present along the ventral midline of the subesophageal zone (**Fig. 5**), which can be divided into three clusters along the anteroposterior axis that roughly correspond to the mandibular, maxillary and labial neuromeres (Busch 2009). Other regions containing OA neurons include the ventromedial margin of the antennal lobes (6 neurons), the ventrolateral protocerebrum (2 neurons), the anterior medial protocerebrum (8 neurons), the protocerebral bridge (65 neurons), the posterior superior medial protocerebrum (5 neurons), the anterior margin of the antennal lobe (1 neuron) and the region ventral to the protocerebral bridge (2 neurons) (Busch 2009).

This widespread array of neurons sends projections to nearly all structures within the brain, but of particular interest to this dissertation are the innervations sent into the primary gustatory center of the subesophageal zone (Busch 2009, Tanaka 2008, Sinakevitch 2006). Primary sensory regions also receive extensive OA innervation, with ramifications present in the antennal lobes, antennal nerves, and medulla, lobula, and lobula plate of the optic lobes (Busch 2009). Several regions are also

Figure 5

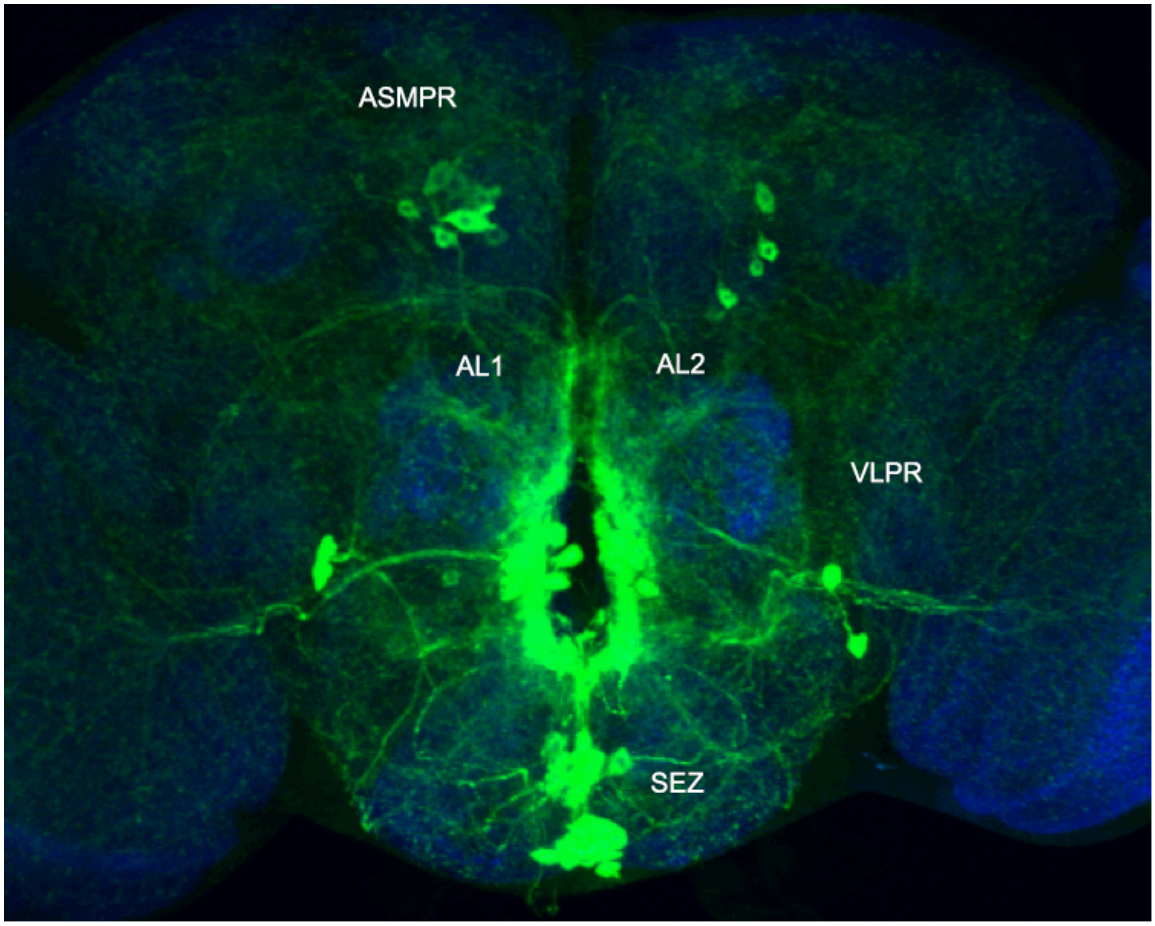

Figure 5: OA in the *Drosophila* brain.

Image of the OA expression pattern within the brain, as marked by *Tdc2-Gal4 ; UAS-CD8::GFP*. Cell bodies can be clearly seen within the subesophageal zone (SEZ). Areas identified are the anterior superior medial protocerebrum (ASMPR), the antennal lobes (AL1/AL2), the ventrolateral protocerebrum (VLPR) and the subesophageal zone (SEZ).

Image Source: Sarah Certel, 2014.

conspicuously lacking in innervation, including the pedunculus and α/β lobes of the mushroom body and the ellipsoid body (Busch 2009). An image of OA neurons and their projections can be found in **Figure 5.** Due to its widespread innervation of sensory structures, OA-expressing neurons are an excellent potential candidate for the modulation of sensory information, which will be further discussed during the third chapter of this dissertation.

OA Structure and Synthesis

As a biogenic amine, OA shares a great deal of structural similarity with its vertebrate analog, norepinephrine. Although there are three different structural isomers of OA, each existing as a D(-) or L(+) enantiomer, only para-OA has been identified in naturally occurring sources (Danielson 1977, Williams 1978, Ibrahim 1985, Brown 1988)(**Fig 6B,C,D**). In insects, the highest concentrations of OA can be found in the centeral and peripheral nervous systems and the hemolymph (Starratt 1981, Erspamer 1951). OA biosynthesis from L-tyrosine requires a two-step process. First, L-tyrosine is decarboxylated into tyramine by tyrosine decarboxylase (TDC1 or TDC2). Following this conversion, tyramine is hydroxylated by tyramine β-hydroxylase (Tβh) on its β-carbon side chain to become OA. A diagram detailing this process can be found in **Figure 6A.** The enzymes involved in the synthesis of OA can be found in both neuronal and non-neuronal tissues, with TDC2 and Tβh present in neuronal tissues, and TDC1 being found outside the nervous system (Monastirioti 1996, Lehman 2000, Cole 2005).

OA receptors

Modern classification schemes of OA sensitive receptors are composed of three different groups based on OA sensitivity and intracellular response to OA administration (Evans 1981, Farooqui 2007). All known OA receptors belong to the superfamily of G-protein coupled receptors, and share a common structural motif of seven transmembrane domains and serve to transduce a signal in response to the presence of an agonist (Evans 1993, 1993, 2005). Two of the groups, dmOCTα-R and dmOCTβ-R, respond with a higher affinity to OA than other neurotransmitters, while

Figure 6

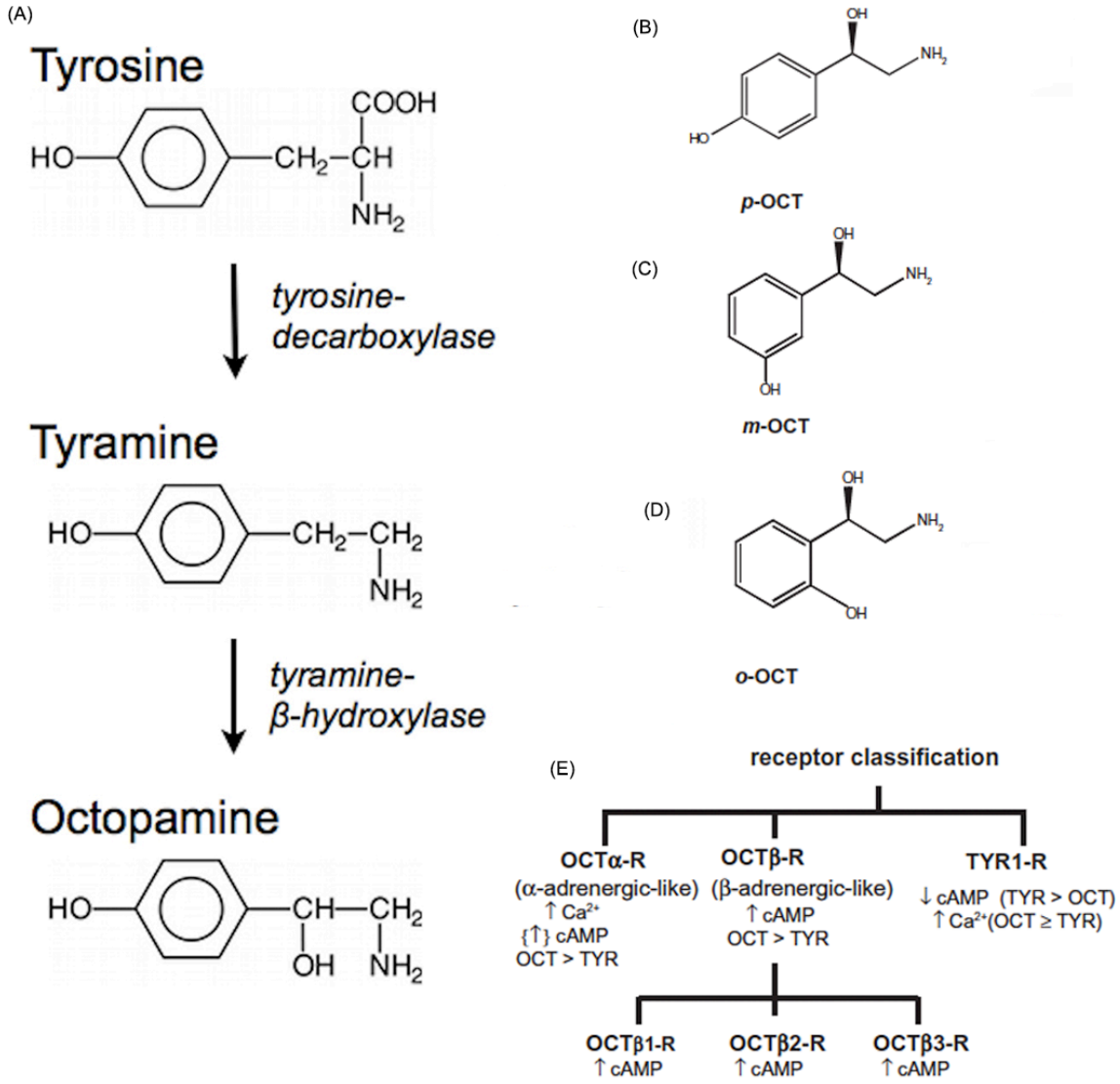

Figure 6: Octopamine structure, synthesis, and receptors.

(**A**) Octopamine synthesis pathway. (**B-D**) Para-, Meta-, and Ortho- variations of octopamine. (**E**) Classification of OA receptors, based on ligand response.

Image Sources: (**A**) Barron, 2010. (**B-E**) Farooqui, 2012.

dmTYR1-R can be stimulated with either OA or tyramine. dmOCTα-R displays a high degree of sequence homology with vertebrate α1-adrenergic receptors, and activation of these receptors is responsible for an increase in intracellular Ca^{2+} levels, as well as rise in cAMP (Han 1998). Of particular interest to this dissertation is the dmOCTβ1-R receptor. dmOCTβ-R has been subdivided into three different receptor categories based on the unique pharmacological profiles of dmOCTβ1-R, dmOCTβ2-R, and dmOCTβ3-R. While each of the subtypes display structural similarities to vertebrate β-adrenergic receptors and cause an increase in intracellular cAMP levels when activated by OA, they can be differentiated pharmacologically by their response to administration of phentolamine, a non-selective α-adrinergic antagonist (Evans 2005, Han 1998, Hrohmann 2003, Duportes 2010, Balfanz 2005, Ohani 2006). The final group of *Drosophila* OA receptors, dmTYR1-R, is also structurally similar to α2-adrenergic receptors but displays differential responses to OA and tyramine. In response to tyramine, dmTYR1-R inhibits adenylyl cyclase, resulting in a reduction of cAMP levels. However, when exposed to OA, activation of dmTYR1-R results in increased intracellular Ca^{2+} levels (Evans 2005, Saudou 1990, Broeck 1995, Poels 2001, Ohta 2003, Blenau 2000). A diagram of these receptors and their responses to OA can be found in **Figure 6E**.

OA in vertebrates

Only trace amounts of OA have been reported in the nervous systems of vertebrates. Because of this, very little is known about what effects OA may have on vertebrate neurobiology. It has been confirmed that OA can displace other endogenous amines in storage vesicles, which has lead to some speculation as to if OA can act as a "false transmitter" in the brain (Roeder 1999, Orchard 1982, Evans 1985, Berry 2004, Borowsky 2001). OA has also been suspected, along with other trace amines, as either a contributor to or biomarker for a number of neurological disorders, including schizophrenia, depression, parkinson's disease and migraine headaches (D'andrea 2010, 2010, 2013, Branchek 2003). To date, two receptors have been identified in vertebrate systems that respond to OA. The first, the β_3-

adrenoceptor is found on lipocytes, and is known to induce lipolysis (Broadley 2010). The second is the trace amine-associated receptor (TAAR). TAAR's have been identified in mice, rats, chimpanzees, and humans, but the effect of TAAR signaling on neurons is not yet understood (Frascarelli 2008). While these findings are far from conclusive, it highlights the possibility of a role for OA in vertebrate nervous systems as well as in invertebrates.

Summary

In this chapter, we have laid the foundation necessary to further explore the interplay between OA and *Drosophila* behavior. Notably absent from this summary, and from our knowledge as a whole, is the formal structure of the neural networks that govern fruit fly behavior. While the individual neurotransmitters, neurohormones, receptors, and some genes have been well characterized, the specific neuronal connections that underlie aggression, courtship and feeding behaviors are still being discovered. It is this gap in knowledge that I will address in this dissertation. To this end, in the second chapter, I will further elaborate on the interactions between Gr32a-bearing neurons and OA neurons within the subesophageal zone, and how manipulation of these neurons exposes their role in *Drosophila* behavior. The third chapter will contain a report on my current project, which is being prepared for publication. This material will expand upon our understanding of how OA signaling modulates the sensitivity of sugar detecting neurons in the periphery, and how this may influence energy intensive behavior, such as courtship and aggression. These two chapters will contribute to our understanding of how OA signaling contributes to *Drosophila* behavior.

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Foreword to Chapter II

The research contained within this chapter was conducted as a portion of my PhD work at the University of Montana. This work has been published, and contains the relevant background information for the study. Of the presented material, my direct contribution to the work includes the material presented in Figures 3, 4, 5, supplemental figure S7, Supplemental Table 1, and the analysis of the data pertinent to those figures. The citation for the work is as follows:

Andrews JC, Fernández MP, Yu Q, Leary GP, Leung AKW, Kavanaugh MP, et al. (2014) Octopamine Neuromodulation Regulates Gr32a-Linked Aggression and Courtship Pathways in *Drosophila* Males. PLoS Genet 10(5): e1004356. doi:10.1371/journal.pgen.1004356

Chapter II

Abstract

Chemosensory pheromonal information regulates aggression and reproduction in many species, but how pheromonal signals are transduced to reliably produce behavior is not well understood. Here we demonstrate that the pheromonal signals detected by Gr32a-expressing chemosensory neurons to enhance male aggression are filtered through octopamine (OA, invertebrate equivalent of norepinephrine) neurons. Using behavioral assays, we find males lacking both octopamine and *Gr32a* gustatory receptors exhibit parallel delays in the onset of aggression and reductions in aggression. Physiological and anatomical experiments identify Gr32a to octopamine neuron synaptic and functional connections in the suboesophageal ganglion. Refining the Gr32a-expressing population indicates that mouth Gr32a neurons promote male aggression and form synaptic contacts with OA neurons. By restricting the monoamine neuron target population, we show that three previously identified OA-Fru^{M} neurons involved in behavioral choice are among the Gr32a-OA connections. Our findings demonstrate that octopaminergic neuromodulatory neurons function as early as a second-order step in this chemosensory-driven male social behavior pathway.

Summary

To mate or fight? When meeting other members of their species, male fruit flies must determine whether a second fly is male or female and proceed with the appropriate behavioral patterns. The taste receptor, Gr32a, has been reported to respond to chemical messages (pheromones) that are important for gender recognition, as eliminating Gr32a function increases male courtship and decreases male aggressive behavior. Here we demonstrate that different subsets of Gr32a-expressing neuron populations mediate these mutually exclusive behaviors and the male Gr32a-mediated behavioral response is amplified through neurons that contain the neuromodulator octopamine (OA, an invertebrate equivalent of

norepinephrine). Gr32a-expressing neurons connect functionally and synaptically with distinct OA neurons indicating these amine neurons may function as early as a second-order step in a chemosensory-driven circuit. Our results contribute to understanding how an organism selects an appropriate behavioral response upon receiving external sensory signals.

Introduction

Organisms live in complicated environments requiring successful interaction with their surroundings for reproduction and survival. Information about the environment is transformed into neural activity by specialized sensory organs that detect signals via touch-, taste-, vibration-, odor- and image-sensitive neurons. Pheromones commonly used as olfactory or contact signals in social behavior like courtship and aggression provide information about gender, receptivity, or conspecificity (Dahanukar 2010, Ferrero 2010, Matsunami 2003). In many systems, chemosensory signal-detecting systems are regulated by biogenic amines including dopamine, serotonin, and norepinephrine (or octopamine, its invertebrate analog) acting as neuromodulators (Birmingham 2003, Farooqui 2007, Mowrey 2012). Despite extensive investigation in a wide variety of organisms, it has proven difficult to assign specific roles to individual amines in the circuitry concerned with social behavior (Harris-Warrick 2011, Marder 2012, Stevenson 2012, Yanowitch 2011). In this study, we directly connect amine regulation to pheromonal communication by identifying specific chemosensory to octopamine neuron contacts and then investigating their tissue-specific functional roles in male aggression and courtship selection.

In *Drosophila*, pheromonal signals are communicated primarily via cuticular hydrocarbons (CHC) and long carbon chain esters that trigger olfactory (volatile) or gustatory (contact) receiving pathways in conspecifics (Fernandez 2013, Ferveur 2005, Ferveur 1996). Contact pheromones are detected by gustatory receptor-expressing sensory neurons (GRNs) found in taste sensilla in mouth, leg,

and wing segments. Despite the importance of this non-volatile sensory information, only a small number of gustatory receptors (GRs) have been reported to be involved in the perception of pheromones that regulate social behavior. In one well-studied example, the behavior of males lacking the gustatory receptor Gr32a is altered in at least three ways; levels of male courtship towards females are reduced, levels of male courtship towards second males are elevated, and aggression as measured by the numbers of lunges (a key higher level behavioral pattern) is reduced (Koganezawa 2010, Miyamoto 2008, Want 2011). In addition, a recent study describes a role of tarsal/leg Gr32a-expressing neurons in the inhibition of interspecies courtship between *Drosophila* species (Fan 2013). To transduce pheromonal stimuli, axons of Gr32a-expressing neurons project to distinct zones in the suboesophageal ganglion (SOG) (Miyamoto 2008, Stocker 1994), and other sites within the central nervous system (Park 2011). The SOG is a central brain region that in addition to axons of gustatory neurons contains extensive neuronal processes of octopamine neurons (Busch 2009, Certel 2010, Chiang 2010).

Reduced levels of the amine octopamine (OA) yield phenotypes similar to those seen in flies lacking Gr32a function (Certel 2007, Hoyer 2008, Zhou 2008). Males without OA exhibit increased male-male courtship (Certel 2007) and a delay in the initiation of male aggressive behavior (Zhou 2008), as do Gr32a loss-of-function flies (Wang 2011). OA function is also necessary for males to make correct choices between courtship and aggression (Certel 2010, Certel 2007) and OA has been suggested to be essential for the display of higher-level aggression (Hoyer 2008, Zhou 2008). As studies in multiple systems reveal that the context of sensory information and internal states are often shaped molecularly by neuromodulators, we tested the hypothesis that the structural composition of the Gr32a pheromonal network includes synaptic connections to OA neuromodulatory neurons.

We used behavioral assays, Ca^{2+} imaging, and the GRASP (GFP Reconstitution Across Synaptic Partners) method (Feinberg 2008, Gordon 2009) to demonstrate the existence of functional and putative synaptic connections between Gr32a neurons and octopaminergic SOG neurons. Removing Gr32a-expressing neurons, eliminating OA, and altering both simultaneously confirmed essential roles for these chemosensory and OA neuronal groups on male aggression initiation and courtship selection. A role for the labellar Gr32a subpopulation in male aggression was revealed by functionally and anatomically separating Gr32a-expressing neurons into mouth and leg populations. Ca^{2+} imaging experiments demonstrate that OA-expressing neurons in the SOG respond to male cuticular hydrocarbon extracts and this response is eliminated in the absence of Gr32a neurons. Finally, GRASP connectivity between Gr32a neurons and three OA neurons that co-express the male forms of Fruitless (Fru^M), link anatomical characterization with previous functional data [21] and indicate that this small subset of aminergic neurons is important to provide male selective modulation of behavior. The results presented here begin to decipher social behavior at the level of small subsets of sensory and neuromodulatory neurons and provide insight into how amine-expressing neurons anatomically contribute to circuitry directing sex-specific behavior.

Results

Gr32a neurons contact OA neurons in the suboesophageal ganglion

To test the hypothesis that OA neurons might anatomically function in the Gr32a pheromonal input pathway, we generated a *Tdc2-LexA:VP16* line and utilized this tool with the split-GFP system developed in *C. elegans* (Feinberg 2008) and adapted for *Drosophila* (Gordon 2009). In invertebrates, OA is synthesized from the amino acid tyrosine via the action of tyrosine decarboxylase (TDC) and tyramine β-hydroxylase (Tβh). The *Tdc2* gene encodes the neuronal TDC (Cole 2005) and the *Tdc2-LexA* line can be used to label and manipulate OA neurons ((Burke 2012), Figure S1 and possibly a small population of tyramine (TA)-expressing neurons (Busch 2009). The Gr32a receptor is expressed in sensory neurons in the mouth

(labellum - a gustatory organ of the proboscis and pharynx) and in tarsal segments of all three legs (Koganezawa 2010, Miyamoto 2008, Dunipace 2001). Axons of Gr32a receptor-expressing neurons project through three peripheral nerves to the SOG (**Fig. 1A, B**) (Stocker 1994, Miyazaki 2010, Stocker 1981, Wang 2004). Peripheral chemosensory neuron expression of OA has not been detected in this study or previously (Cole 2005). However, within the central brain, individual OA neurons project extensive arborizations targeting multiple neuropil regions including the SOG, which functions at least in part, to receive key contact pheromone information (**Fig. 1B,C, S1**) (Busch 2009, Certel 2010, Cole 2005).

To determine if Gr32a-expressing neurons directly contact OA neurons, we used the GFP Reconstitution Across Synaptic Partners (GRASP) method, which detects putative synaptic connections based on the reconstitution of two fragments of a split-GFP protein on the outer membrane of targeted neuronal populations (Feinberg 2008, Gordon 2009). We observed GFP reconstitution in a reproducible, distinct pattern within the central SOG (**Fig. 1E-J**) in flies containing one fragment of split-GFP under *Tdc2* (OA/Tyramine) control (*Tdc2-lexA; lexAop-CD4::spGFP11*) and the second fragment driven by the promoter of *Gr32a* (*Gr32a-Gal4; UAS-CD4::spGFP1-10*). Little or no fluorescence was observed upon expression of either split-GFP fragment alone (**Fig. S2**). To confirm that at least a portion of the fluorescence seen is in contact zones that are likely synaptic, we added the *UAS-syt:HA* reporter (Robinson 2002) (**Fig. 1E-G**, displayed as red puncta). The overall syt:HA pattern shows clear preferential localization of terminal regions of Gr32a neurons and an extensive overlap is seen between syt:HA localization and split-GFP reconstitution at both low and higher magnification (**Fig. 1E-H**). In the merged channels (**Fig. 1E, F**), regions of syt:HA expression where no GFP reconstitution is observed indicating that only specific neurons amongst the populations of Gr32a and OA neurons contact each other. In particular, the synaptic endings derived from Gr32a neurons that project directly to the ventrolateral protocerebrum region (Miyamoto 2008) do not express reconstituted GFP (**Fig. 1E**, arrow)

Figure 1

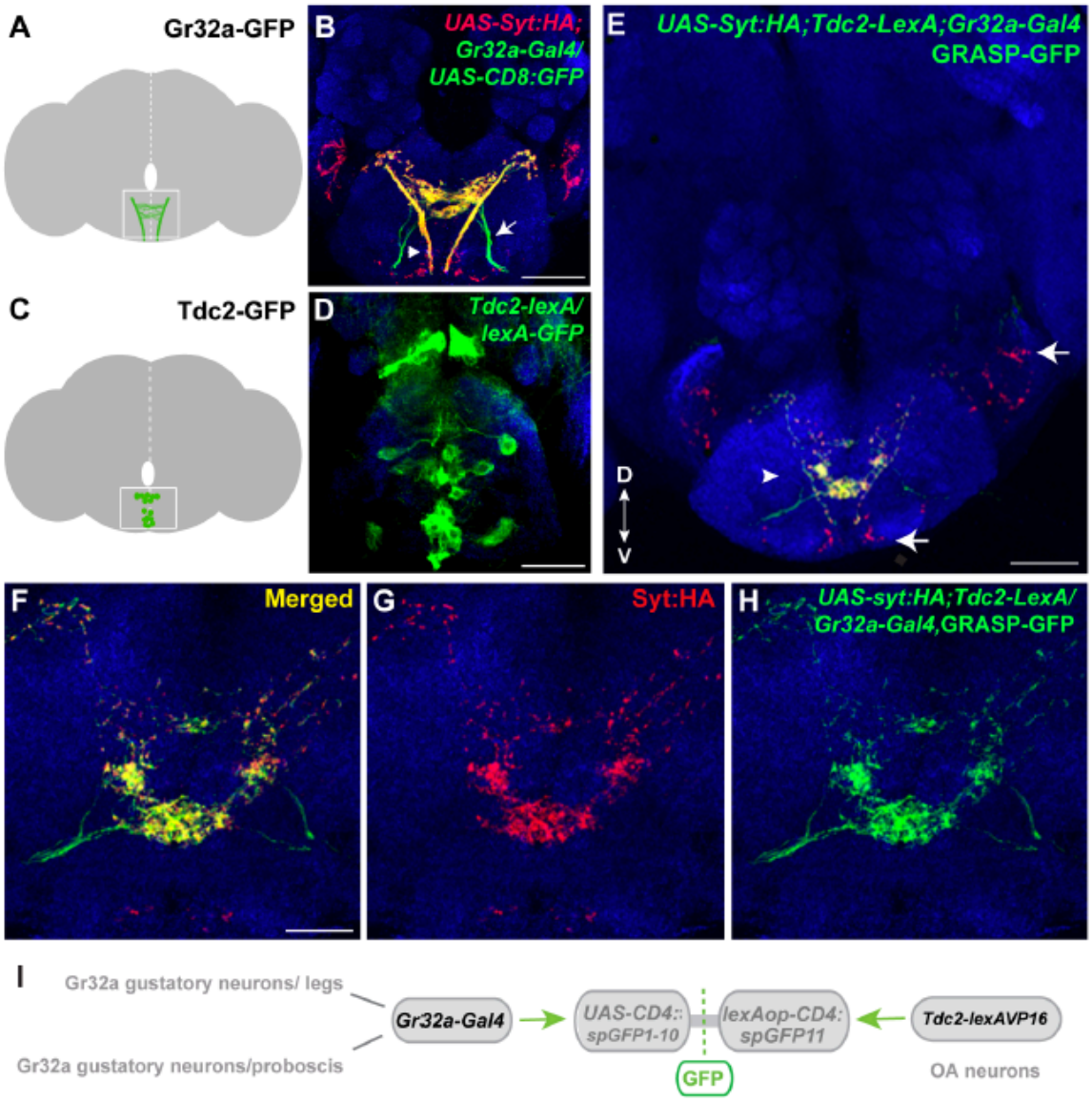

Figure 1: Gr32a neurons contact OA neurons in the suboesophageal ganglion. **(A-B)** Axons and presynaptic terminals of Gr32a-expressing neurons identified by immunofluorescence to CD8:GFP and the synaptotagmin:HA fusion protein in *UAS-sytHA;;UAS-CD8:GFP/Gr32a-Gal4* progeny (green, anti-CD8, Invitrogen; red, anti-HA, Roche). Sensory neurons from the labellum project through the labial nerve (arrow), mouthpart neurons project through the pharyngeal/accessory nerve, and neurons from thoracic ganglia project via the cervical connective (arrowhead). **(C-D)** GFP expression driven by the Tdc2-LexA line in a cluster of SOG neurons

visualized in *Tdc2-LexA;lexAop-rCD4:GFP* progeny. Extensive arborizations within the SOG are apparent in a series of optical sections ventral to the cell bodies (arrows, D). **(E)** GRASP-mediated GFP reconstitution is observed between Gr32a neurons expressing CD4::spGFP1-10 and synaptotagmin:hemagglutinin (*UAS-syt:HA*) (red, anti-HA, Roche) and OA neurons expressing CD4::spGFP11. GRASP reconstitution is detected by immunofluorescence using a rabbit monoclonal GFP antibody (Life Technologies). Regions in the SOG with only synaptotagmin:HA expression are indicated (arrows) in addition to GFP-reconstitution contacts that show co-localization with syt-HA expression (arrowhead). Scale bar is 50 μm. **(F-H)** Optical sections of the same brain at higher magnification showing GRASP-mediated GFP reconstituted expression (H), synaptotagmin:HA localization (G) and clear overlap or close association at synaptic-like puncta in the merged channel (F). Scale bar represents 20 μM. **(**See also Figure S1 and S2. **(I)** Schematic representation of the GRASP reporter lines combined with the *Gr32a-Gal4* and *Tdc2-lexA* driver lines.

demonstrating specificity in the GFP reconstitution pattern and specificity in the Gr32a to OA neuronal connections. This anatomical data is consistent with a recent study suggesting a close, possibly synaptic, apposition of Gr32a-expressing axons with male mAL neurons (Koganezawa 2010).

Gr32a expression is seen in all bitter-sensing neurons within the sensilla of the labellum, usually accompanied by many additional gustatory receptors in most of the neurons (Wang 2004, Thorne 2005, Weiss 2011). In one subgroup of chemosensory neurons, the Gr22e (9 neurons) and Gr59b (4 neurons) receptors co-localize with Gr32a as has been reported previously (Stocker 1981), while in another distinct group Gr32a and Gr47a co-localize (3 neurons) (Weiss 2011). Expressing *Gr22e-Gal4* or *Gr59b-Gal4* with *Tdc2-lexA* and the GRASP reporter transgenes resulted in split-GFP reconstitution in the SOG region as described above (**Fig. 1**) albeit with reduced GRASP expression likely due to co-expression in only a subset of the population of Gr32a neurons (**Fig. S3**). We also examined whether OA neurons might receive synaptic input from the Gr47a/Gr32a neurons, a different subgroup of bitter-responsive neurons (Miyazaki 2010, de Brito Sanchez 2011). GFP reconstitution was not observed between the *Gr47a-Gal4* labeled axons and OA neurons (**Fig. S4**). Although definitive verification of the GRASP signals will require electron microscopy, our results suggest that a number of octopaminergic SOG neurons may serve as neuromodulatory links in the information pathways between specific Gr32a-expressing neurons and taste-related behavioral outputs.

Removing OA neurons changes Gr32a SOG axonal targeting

If a subset of Gr32a gustatory neurons are in synaptic contact with octopaminergic SOG interneurons, then removing the OA neurons might cause changes in the branching patterns of incoming Gr32a axonal projections. To test this hypothesis, we eliminated OA neurons by driving expression of the programmed cell death gene, *head involution defective (hid, UAS-hid)*, coupled with the *UAS-Red Stinger* reporter transgene in OA/TA neurons. The *Tdc2-Gal4/UAS-hid UAS-Red*

Stinger combination allowed us to identify transgenic brains that retained OA neurons (DsRed expression was observed) and brains that were devoid of OA neurons (DsRed and Tβh expression was absent (**Fig. S5**)). Gr32a neuronal projections entering the SOG were visualized using the Gr32a-I-GFP reporter construct (**Fig. 2A-C**) which drives GFP expression as a direct promoter fusion (Wang 2004). The resulting GFP fluorescence is weaker than when amplified through the Gal4/UAS system, however when all OA neurons were eliminated, we observed a range of axonal projection defects including an absence of Gr32a-I-GFP immunoreactivity in the SOG (data not shown, 31%) or a severe reduction and disorganization of Gr32a leg and labellum termini in 69% of preparations (n=21, **Fig. 2D**). Since the adult brains were dissected 1-5 days after eclosion, the differing severity of the Gr32a projection phenotypes could be due to increased axonal disorganization in the absence of OA neuronal targets as flies age. No similar disorganization of Gr32a axonal projections is observed in control brains during the 1-5 day time frame.

We next asked if Gr32a axonal morphology is altered if OA neurons are present but lack OA due to a null mutation in *Tyramine ß-hydroxylase* (*tβh^{nM18})*. Using *Gr32a-Gal4* to drive reporter GFP expression, the stereotypical projections of Gr32a-expressing neurons from control and OA deficient males were examined. Gr32a axons terminated in the SOG (**Fig. S6**) in heterozygous control adult brains *(tβh^{nM18}/+;Tdc2-Gal4;20XUAS-6XGFP*). Compiling the same number of confocal sections in controls and OA deficient male brains *(tβh^{nM18};Tdc2-Gal4;20XUAS-6XGFP)* indicates the majority of Gr32a projections reach the SOG as in controls. However, we observed aberrant termination of Gr32a axons in the antennal lobe region of OA deficient brains (**Fig. S6C-E**) that is distinct from previously described projections into the ventro-lateral protocerebrum (Miyamoto 2008). The effects of eliminating production of OA on individual Gr32a-expressing neurons remains to be determined but results from these experiments suggest the correct differentiation of OA

Figure 2

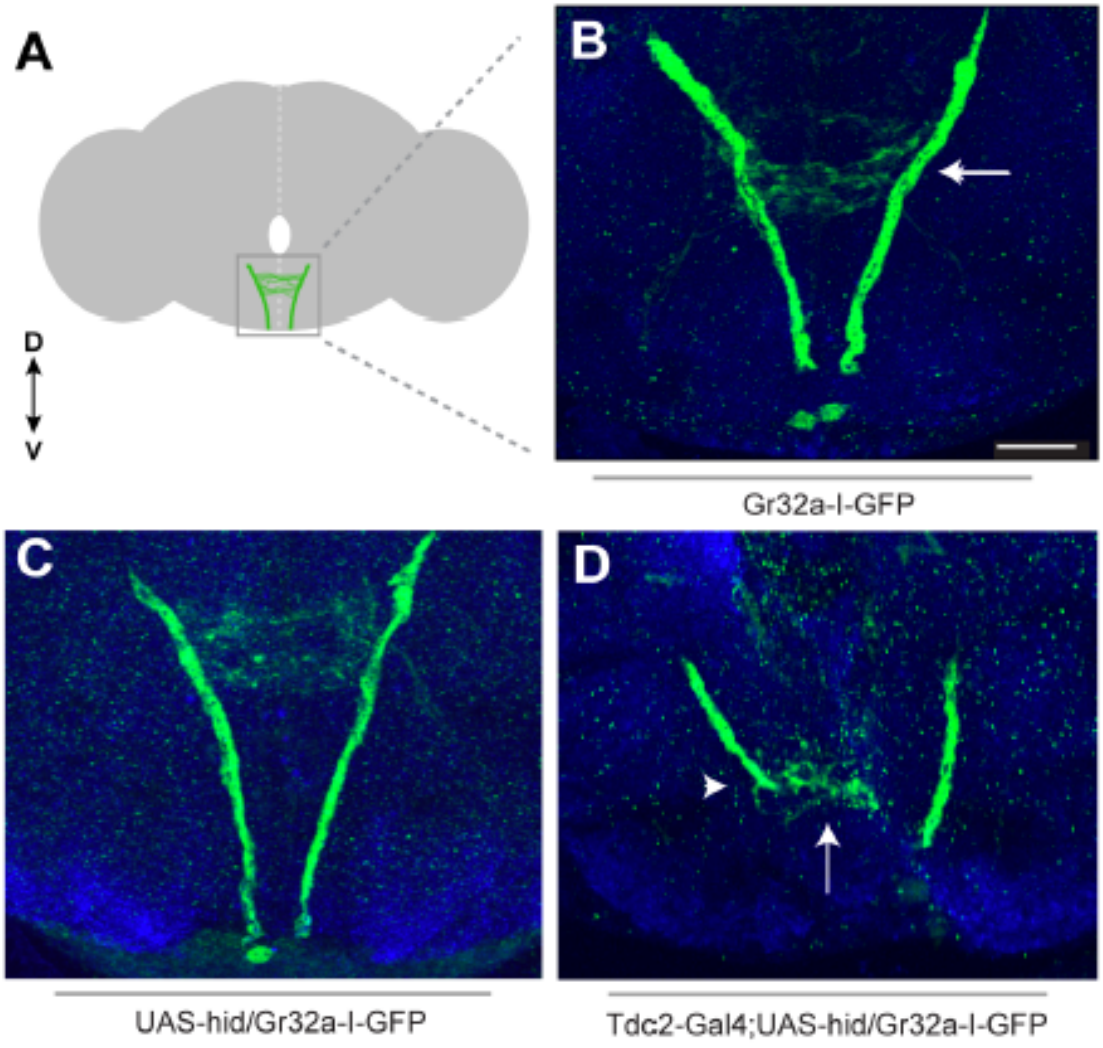

Figure 2: Removing OA neurons significantly alters Gr32a axonal projections.

(A) Schematic representation of the adult brain with Gr32a-expressing axonal arborizations in the SOG. **(B)** *Gr32a-I-GFP* expression in a typical wildtype adult brain. The Gr32a-expressing neurons located in the tarsi, labellum, and mouthparts all terminate in the SOG (arrow). **(C)**

Confocal sections of a *UAS-hid UAS-Red Stinger* control brain verifying wildtype organization of *Gr32a-I-GFP* projections (**D)** Confocal sections of transgenic *Tdc2-Gal4/UAS-hid UAS-Red Stinger;Gr32a-I-GFP* adult brains. When all OA neurons are eliminated, a range of axonal projection defects was observed including a severe

reduction and disorganization of Gr32a leg and labellum termini (arrow, arrowhead). Scale bar represents 30 μm.

neurons is required for precise axon targeting by at least a subset of Gr32a chemosensory neurons.

Gr32a expressing neurons mediate onset of aggression via OA signaling

A previous study reported that the Gr32a receptor mediates aggression-inducing and courtship suppression effects of the male-enriched cuticular hydrocarbons, (*z*)-7-tricosene (Wang 2011). Results presented here indicate that Gr32a-expressing neurons contact OA neurons and suggest that octopaminergic signaling is one of the pathways through which Gr32a-mediated pheromonal information is conveyed to other brain or possibly ventral cord regions. To test this hypothesis, we first analyzed fighting defects in males with impaired Gr32a function in our aggression chambers. This data provides a baseline for calculating how removal of OA neuromodulation in addition to eliminating Gr32a-mediated pheromonal information may or may not further alter male aggression or courtship. We ablated Gr32a-expressing gustatory neurons through expression of Diphtheria Toxin *(UAS-DTI)* via the *Gr32a-Gal4* driver line (Thorne 2004). Pairs of *UAS-DTI;Gr32a-Gal4* or transgenic control males were placed in an aggression chamber and latency to the first lunge (a key aggressive pattern essential for the establishment of hierarchical relationships) and total numbers of lunges were quantified. Consistent with a role of Gr32a-expressing neurons in perceiving pheromones utilized for sex and species recognition in males, the latency to first lunge was significantly longer in males without Gr32a neurons compared to parental controls (**Fig. 3A**). Moreover, a significant reduction in the number of lunges was also observed (**Fig. 3B**). Males without Gr32a neurons exhibited a reduction in aggressive behavior when paired with a single control male as demonstrated by few lunges per fight and a failure to initiate aggression (**Fig. S7A-C**).

Figure 3

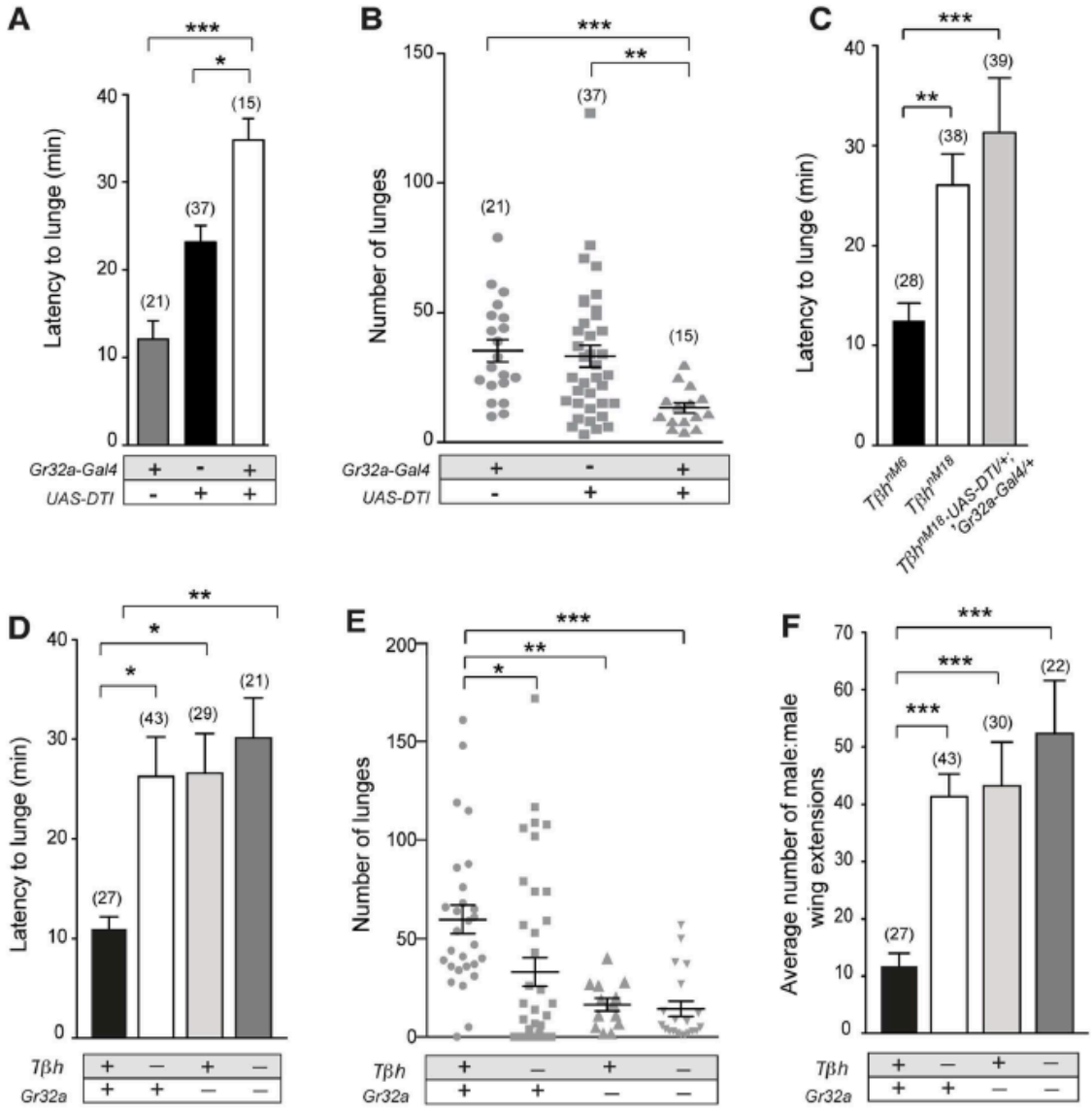

Figure 3: Gr32a-expressing neurons promote aggression via OA signaling. **(A-B)** Fights between males with Gr32a-expressing neurons removed by expressing Diptheria Toxin *(UAS-DTI;Gr32a-Gal4)* and individual transgenic controls, *UAS-DTI* or *Gr32a-Gal4.* **(A)** The latency to first lunge was significantly higher in *UAS-DTI/+; Gr32a-Gal4/+* males as compared to controls (all statistical tests are Kruskal-Wallis with Dunn's multiple comparison test except where noted, ***p<0.001, *p<0.05). **(B)** Number of lunges (represented by each dot) performed in a 30 min period after the first lunge by any control or experimental male in a

fighting pair. Males without Gr32a neurons exhibited a significant reduction in lunges as compared to controls (***p<0.001, **p<0.01). **(C)** Fights between control male pairs (revertant $tβh^{M6}$ allele), experimental males without OA (revertant null mutation, $tβh^{nM18}$), or experimental males without OA and without Gr32a-expressing neurons ($tβh^{nM18}$;*UAS-DTI/+; Gr32a-Gal4/+).* The latency to first lunge was significantly higher in males without OA and in experimental males compared to control males (**p<0.01) and not statistically different between males without OA and experimental $tβh^{nM18}$;*UAS-DTI/+; Gr32a-Gal4/+* males. **(D-F)** Fights between control male pairs (revertant $tβh^{M6}$ allele) and three groups of experimental males; without OA= $tβh^{nM18}$, without Gr32a receptors= $tβh^{M6}$;;$Gr32a^{-/-}$, and without OA and Gr32a receptors= $tβh^{nM18}$;;$Gr32a^{-/-}$). **(D)** The latency to first lunge was significantly higher in males without OA ($tβh^{nM18}$) and in experimental males without OA and the Gr32a receptor ($tβh^{nM18}$; $Gr32a^{-/-}$) or without only the Gr32 receptor ($tβh^{M6}$; $Gr32a^{-/-}$) males as compared to control $tβh^{M6}$ males (One way ANOVA, *post hoc* Tukey's comparison, *p<0.05, **p<0.01). **(E)** The number of lunges by pairs of experimental males were significantly less than exhibited by control males but not when compared to each other (***p=0.0002, **p=0.002, *p=0.01). **(F)** The average number of wing extensions directed toward the second male in each aggression assay. The number of wing extensions exhibited by males without the Gr32a receptor and without OA, and males without Gr32a receptors were significantly greater than control $tβh^{M6}$ males (***p<0.001) but not males without OA ($tβh^{nM18}$). Error bars denote s.e.m.

To test the behavioral consequences of removing both Gr32a-expressing neurons and OA, we added the *UAS-DTI;Gr32a-Gal4* transgenes to males with either the w^+ $tßh^{nM18}$ null recombinant chromosome) or the w^+ $tßh^{M6}$ recombinant control chromosome (Certel 2007). The resulting experimental males do not produce OA yet retain OA neurons and the Gr32a-expressing neurons are ablated. Similar to what was observed for flies without Gr32a neurons, flies without OA show a 2-fold increase in latency when compared to genetic control males (**Fig. 3C**). If the function of Gr32a and OA neurons in setting the timely onset of an aggressive response were independent, the absence of both Gr32a receptors and OA function should result in an additive effect on aggression latency as compared to single mutants (flies lacking Gr32a-expressing neurons or OA only). Removing Gr32a signaling and OA via the $tßh^{nM18}$ mutation did result in a small increase in the latency to the first lunge when compared to control males (**Fig. 3C**). However, the increased latency was not significantly different from that observed in males without OA only (**Fig. 3C**), (Mann-Whitney U test, p=0.4). This equivalent aggression initiation delay exhibited by males without Gr32a neuronal function and $tßh^{nM18}$;*UAS-DTI;Gr32a-Gal4* males is the expected result if the aggression-promoting pheromonal signals transmitted by Gr32a neurons are at least partially conveyed via OA neurons. When males without OA and Gr32a neurons fight, the total lunges per fight are decreased (**Fig. 3D**), though, the reduction in lunge number is not substantially different from *UAS-DTI;Gr32a-Gal4* males (**Fig. 3B**). Removing Gr32a neurons in males without OA significantly decreased lunge number (**Fig. S7D**), however this additive value in lunge number reduction is not observed in males with only the Gr32a receptor eliminated (see below, **Fig. 3E**).

Males with lowered levels of OA have been reported to exhibit lower numbers of lunges (Hoyer 2008, Zhou 2008). Results in this study indicate that $tßh^{nM18}$ mutant males take twice as long as controls to display their first lunges in fights (**Fig. 3C, D**, **S7D**). We previously demonstrated that males without detectable OA exhibited elevated courtship behavior towards other males (Certel 2007). One possible

explanation of these results is that OA deficient males have difficulty recognizing the sex or species of a second fly. A similar delay in initiation observed in fights between males lacking Gr32a receptor neurons may be for this same reason (this study and (Wang 2011)). Given such a large delay in the onset of aggression in OA mutant flies (**Fig. 3C, D** and (Zhou 2008)), at least two factors can impact how lunge numbers are counted. First, counting lunges for a set period of time beginning when flies are first introduced to a chamber can yield very different results from counting at the start of lunging behavior (**Fig. S7D**). A second consideration is the inclusion of male pairs that did not display lunges. If fights without lunges are scored as "zeros", the numbers of lunges seen in fights between pairs of $tβh^{nM18}$ males are significantly lower than the numbers seen in the genetic controls (**Fig. S7E**), when fights that do not exhibit lunging are excluded, significant differences between *tβh* control and experimental are not found (**Fig. S7F**). $tβh^{nM18}$ males that exhibited low numbers of lunges also engaged in elevated levels of male-male courtship, which was not observed in $tβh^{M6}$ controls while OA deficient males that exhibited high numbers of lunges engaged in male-male courtship at low levels. These results are displayed as a ratio of wing extensions (singing) divided by lunges (**Fig. S7G**). Thus the affects of removing OA on the intensity of aggression also include a critical delay in the onset of aggression and an increase in male-male courtship.

To support the hypothesis that Gr32a receptor function itself is a key transducer of the aggression-enhancing stimuli regulated by OA, we tested males containing the $Gr32a^{-/-}$ mutation (Miyamoto 2008) in the $tβh^{nM18}$ (null for OA) and $tβh^{M6}$ (control) backgrounds. Males without the Gr32a receptor and males without OA and Gr32a exhibited a similar 2-fold increase in the latency to lunge (**Fig. 3D**). The number of lunges displayed by males without OA ($tβh^{nM18}$), without Gr32a ($tβh^{M6};;Gr32a^{-/-}$), or without OA and the Gr32a receptor ($tβh^{nM18};;Gr32a^{-/-}$) were each significantly reduced as compared to control males ($tβh^{M6}$) (**Fig. 3E**). Differences in lunge number between groups of experimental males were not observed (**Fig. 3G**) providing further support that OA may be downstream of Gr32a sensory signaling processes.

As separately removing OA and Gr32a receptor function has been reported to increase male-male courtship toward intact males (Certel 2007) and decapitated males (Miyamoto 2008), we quantified the occurrences of courtship to the second male within the aggression paradigm. Males without the Gr32a receptor, males without OA, and males without OA and Gr32a all displayed a significantly greater amount of male-male courtship to the second intact male compared to controls (**Fig. 3F**). As with parameters of aggression, removing OA in the context of the *Gr32a*$^{-/-}$ mutation does not increase the already elevated levels of male-male courtship suggesting that OA may modulate Gr32a sensory input related to suppressing conspecific male courtship and promoting male aggression as these two processes have been suggested to reflect independent, parallel processes (Wang 2010).

The intracellular Ca^{2+} response of OA SOG neurons to male CHCs requires Gr32a neurons

To determine if OA-expressing neurons modulate male aggression and courtship behavior by responding to sensory information concerning sexual recognition, we expressed the genetically encoded calcium indicator GCaMP6 (Chen 2013), and assayed changes in intracellular Ca^{2+} responses evoked by application of CHC extracts to the male legs. Male CHC extracts evoked significant increases in GCaMP6s fluorescence in subsets of OA SOG neurons of *Tdc2-LexA;13XLexAop2-IVS-GCaMP6s* males (**Fig. 4A-B, G**), n=23. The response to male CHCs was abolished in males with Gr32a neurons eliminated via DTI expression (*Tdc2-LexA/UAS-DTI;Gr32a-Gal4/13XLexAop2-IVS-GCaMP6s)* (**Fig. 4E-F, H**, n=12) or through *UAS-hid* expression *(Tdc2-LexA/UAS-hid UAS-RedStinger;Gr32a-Gal4/13XLexAop2-IVS-GCaMP6s,* data not shown). Male CHC extracts were also applied to the forelegs of males expressing GCaMP3.0 in Gr32a neurons *(UAS-GCaMP3.0/Gr32a-Gal4)*, however Ca^{2+} changes were not reliably detected in these foreleg neurons. As the cellular transduction mechanisms involved in Gr32a signaling are currently unknown, it is possible that Ca^{2+} changes may be near or below the detection

Figure 4

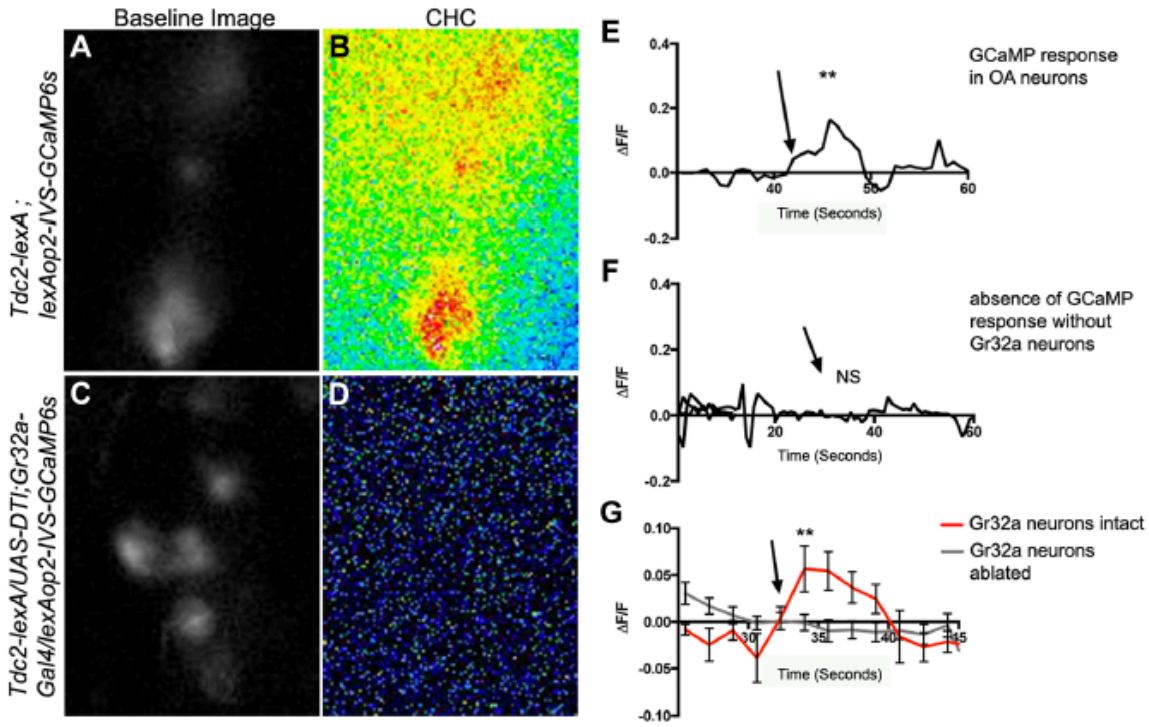

Figure 4: Male CHCs evoke intracellular Ca^{2+} responses in OA neurons that are dependent on Gr32a neurons.

(A) Greyscale image (background subtracted) of GCaMP3 fluorescence in OA neurons located within the SOG in a *Tdc2-lexA;lexAop2-IVS-GCaMP6s* male. **(B)** Pseudocolored subtraction image demonstrating an increase in fluorescence in response to male CHC application. Intensity of pseudocoloring is Red> Yellow> Green> Blue, from highest to lowest fluorescence values. **(C)** Greyscale image (background subtracted) of baseline fluorescence in the SOG of a male with Gr32a neurons eliminated (*Tdc2-lexA; UAS-DTI;Gr32a-Gal4/LexAop2-IVS-GCaMP6s).* **(D)** No changes in fluorescence are observed in the pseudocolored subtraction image of OA SOG neurons when male CHC extract is administered to the legs of males lacking Gr32a neurons. **(E)** The calcium signal trace of OA neurons expressing GCaMP6s in panels A-B in response to male CHC extract application (arrow), unpaired t-test $^{**}p<0.006$. **(F)** A representative trace demonstrating the lack of calcium response in OA neurons after male CHC extract application (arrow) to the

legs of males without Gr32a neurons (*Tdc2-LexA;UAS-DTI;Gr32a-LexA/13XLexAop2-IVS-GCaMP6s*). n=12.

threshold or that a response may not include a Ca^{2+} influx. Nevertheless, our physiological data support the hypothesis that sensory information received by Gr32a neurons is directly relayed to OA neurons in the SOG.

Subset-specific effects of Gr32a neuronal function on male aggression and courtship selection

Although a single receptor subtype, Gr32a, appears to mediate key pheromonal responses that inhibit interspecies courtship, promote male aggression, and suppress conspecific male:male courtship, different subpopulations of Gr32a-expressing neurons may be involved in each case. To test this idea, we selectively ablated Gr32a-expressing chemosensory neurons located in the mouth without removing the leg Gr32a neurons. For this purpose, we used the homeotic *teashirt* promoter driving Gal80 expression (Roder 1992) to significantly block Gal4-mediated activation in regions outside of the head. Via this route Diphtheria Toxin expression *(UAS-DTI)* was prevented resulting in males lacking Gr32a-expressing neurons only in the labellum or mouth (**Fig. S8**). As in experiments presented above, the latency to lunge was significantly longer in males without labellar Gr32a neurons (**Fig. 5A**) and a significant reduction in lunge number was also observed (**Fig. 5B**). As increased male-male courtship to a second intact male is exhibited by males without the Gr32a receptor and without OA (**Fig. 3G**), we quantified the occurrences of courtship behavior (wing extensions and abdomen bending). The male-male courtship levels of *UAS-DTI;teashirt(tsh)-Gal80/Gr32a-Gal4* male pairs are lower than control levels (**Fig. 5C**) yet experimental males court females and successfully copulate in courtship assays (92%, n=13) albeit with a longer latency to initiate courtship (**Table S1**). The ability of experimental males to successfully copulate is in agreement with a report indicating the ablation of the entire Gr32a neuron population does not alter the courtship of conspecific females (Fan 2013). Our results thereby indicate that there are functional differences on male social behavior served by the two separate populations of Gr32a-expressing

Figure 5

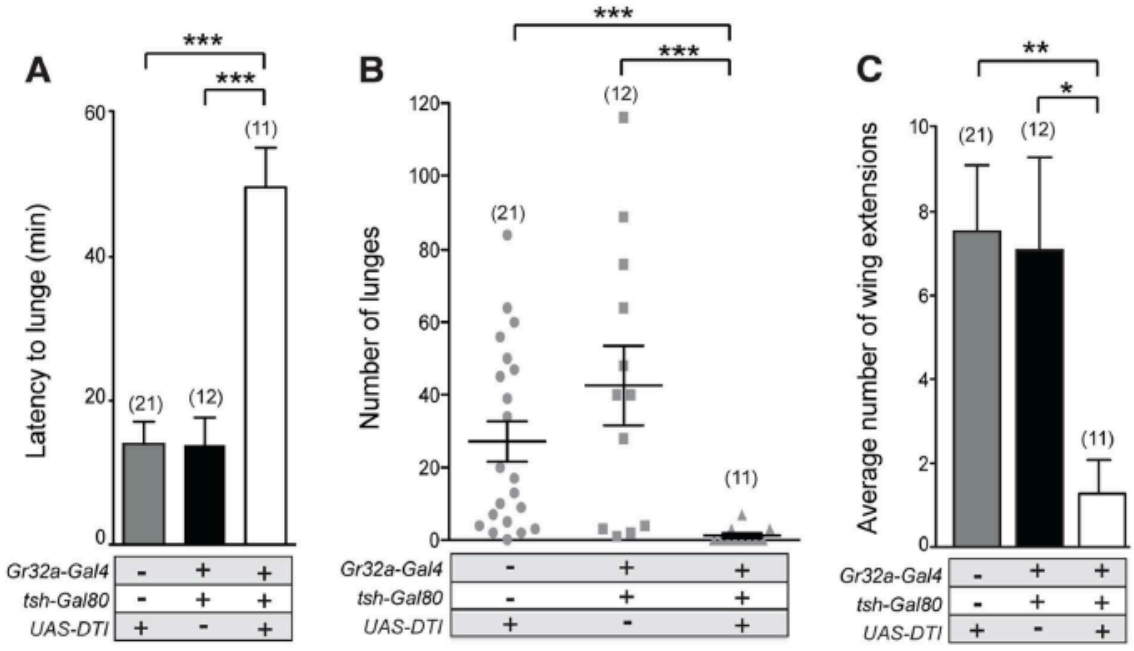

Figure 5: Gr32a chemosensory neurons located in the mouth promote aggression without an elevation in male:male courtship.

(A-B) Fights between males with the Gr32a-expressing mouth neuronal population removed by expressing Diptheria Toxin *(UAS-DTI)* through the *Gr32a-Gal4* line with Gal4 activity in the legs blocked by *tsh-Gal80*. Separate transgenic controls, *UAS-DTI/+* and *tsh-Gal80/+; Gr32a-Gal4/+* were scored. **(A)** The latency to first lunge was significantly higher in *UAS-DTI/tsh-Gal80; Gr32a-Gal4/+* males as compared to controls (Kruskal-Wallis with Dunn's multiple comparison test, ***p<0.001). **(B)** Number of lunges performed per 30 min period after the first lunge by controls or experimental *UAS-DTI/tsh-Gal80; Gr32a-Gal4/+* males. Each dot represents the numbers of lunges performed by either male in a fighting pair. Males without Gr32a-expressing mouth neurons exhibited a significant reduction in lunges as compared to controls (Kruskal-Wallis test with Dunn's multiple comparison test, ***p<0.001). **(C)** The average number of wing extensions directed toward the second male in each aggression assay. The number of wing extensions exhibited by males without mouth Gr32a neurons were less than control males (Kruskal-Wallis with Dunn's multiple comparison test, *p<0.05, **p<0.01). Error bars denote s.e.m.

chemosensory neurons and that the labellar Gr32a subpopulation is important for male aggression. Experiments in this study do not exclude a role for Gr32a leg neurons in male aggression, however the functional importance of the tarsal Gr32a subpopulation on male interspecies courtship behavior has recently been described (Fan 2013).

Tissue-specific refinement of Gr32a to octopamine neuron synaptic contacts

To identify subpopulation-specific synaptic contacts between Gr32a and OA neurons, we used the *teashirt-Gal80* line in combination with the GRASP system. Recent studies using the Gr32a-Gal4 driver to express GFP indicated at least 38 neurons in the mouth (19 neurons per labial palp) and 11 neurons located in the legs express the reporter (Weiss 2011, Thorne 2004). Adding the *teashirt-Gal80* transgene significantly blocked Gal4-mediated activation in the thoracic region resulting in a reduction of GFP expression in the SOG. Thoracic ganglia neuronal projections via the cervical connective are reduced or absent (arrowhead in **Fig 1A**, compare **Figure 1A** to **Figure 6A**). The reduction of GFP-expression in leg sensory neurons of *UAS-nlsGFP; tsh-Gal80/Gr32a-Gal4* progeny (0.38 neurons per front leg, n=8), versus males without Gal80 expression (5 neurons per front leg, n=8) is shown in Figure 6F, G.

With the addition of *teashirt-Gal80* to restrict split-GFP expression to mouth Gr32a neurons, GFP reconstitution is visible in a highly reproducible pattern that appears to be part of the GRASP reconstituted pattern observed when the entire Gr32a-Gal4 expressing population is labeled (compare 6D with 1E). Furthermore, GFP reconstitution co-localizes with the *UAS-syt:HA* reporter added to visualize the presynaptic terminals of Gr32a-expressing neurons. (**Fig. 6H-J**). As Gr32a and OA neuronal function strongly influence male-selective social behaviors, the GRASP patterns of male and female progeny were carefully examined. No apparent sex-specific differences were observed. Results from these experiments suggest that distinct behavioral responses to sex pheromone(s) are provided by separate

Figure 6

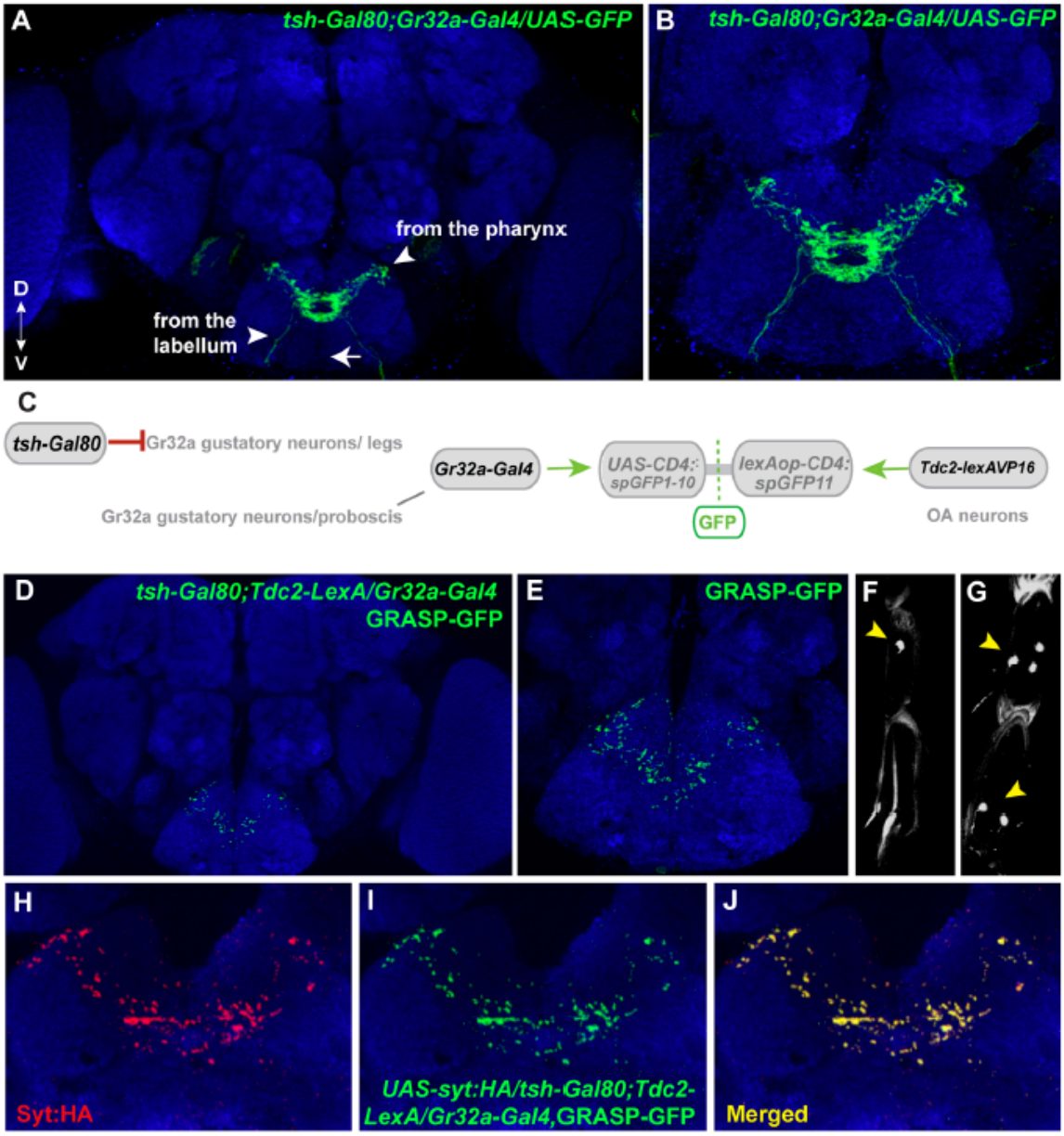

Figure 6: Mouth-specific Gr32a neurons contact OA neurons in the suboesophageal ganglion.

(A) Axons of Gr32a-expressing neurons located in the mouth identified by immunofluorescence to CD8:GFP in *tsh-Gal80;UAS-CD8:GFP/Gr32a-Gal4* progeny (green, anti-CD8, Invitrogen). Note the absence of axonal projections from the legs via the thoracic ganglion (arrow, compare to Figure 1A). **(B)** Higher magnification of Gr32a mouth neurons expressing CD8:GFP. **(C)** Schematic representation of the

GRASP reporter lines combined with the *tsh-Gal80*;*Gr32a-Gal4* and *Tdc2-lexA* driver lines. Gal80 driven by the *tsh-Gal80* line prevents Gal4 activity and subsequent expression of the *UAS-CD4::spGFP1-10* GRASP reporter. **(D-E)** Two different confocal image magnifications of a male brain with the same number of optical sections as in panel A. A reduced amount of GRASP-mediated GFP reconstitution is observed reflecting Gr32a neurons located only in the mouth expressing CD4::spGFP1-10 and OA neurons expressing CD4::spGFP11. GRASP reconstitution is detected by immunofluorescence using rabbit monoclonal GFP antibody (green; Life Technologies). **(F-G)** *Tsh-Gal80* blocks GFP expression in Gr32a-expressing leg neurons. Less than one neuron per leg of *UAS-nlsGFP; teashirt-Gal80/Gr32a-Gal4* progeny is observed (arrowhead, 0.38 neurons per front leg, n=8), versus males without Gal80 expression (arrowhead, 5 neurons per front leg, n=8). **(H-J)** Optical sections of a female brain (*UAS-syt:HA; tsh-Gal80;UAS-CD8:GFP/Gr32a-Gal4)* at higher magnification showing GRASP-mediated GFP reconstituted expression (I), synaptotagmin:HA localization (H) and clear overlap or close association at synaptic-like puncta in the merged channel (J).

subsets of Gr32a-expressing chemosensory neurons, in both cases involving potential direct reinforcement by OA.

Cell-specific refinement of octopamine neuron connections to Gr32a neurons

We previously demonstrated that three OA neurons express the male form of Fruitless (Fru^M), a neural sex determination factor that is a key determinant of male patterns of courtship and aggression (**Fig. 7A**) (Certel 2010, Manoli 2005, Stockinger 2005). The necessity of Fru^M expression in this small subset of OA neurons was evident as the absence of Fru^M resulted in an increase in male:male courtship in an aggression setting (Certel 2010). These results suggested that sexual specification of certain OA neurons may be involved in reliably establishing mate selection (or reliably suppressing conspecific male-male courtship). To determine if Gr32a-expressing neurons establish synaptic contacts with Fru^M-OA neurons, *Tdc2-LexA* was used in conjunction with the recently generated restrictable split-GFP component, *lexAop>stop>CD4::spGFP11* (María Paz Fernández, unpublished data). Selectively activating split-GFP11 expression in Fru^M neurons was achieved through the production of the FLP enzyme in Fruitless-expressing neurons via the fru^{FLP} (Yu 2010) line and putative synaptic connections were observed in male and female brains also expressing *Gr32a-Gal4* driven *UAS-CD4::spGFP1-10* (**Fig. 7B,C**). At this time, we cannot simultaneously restrict Gr32a-expressing and OA neuronal populations or as yet quantify any sex-specific connection differences that may exist. However, our experiments indicate the Fru^M-OA neurons that account for increases in male-male courtship are anatomically connected to Gr32a neurons and these may form a microcircuit that contributes to the context-specificity of male courtship behavior.

Figure 7

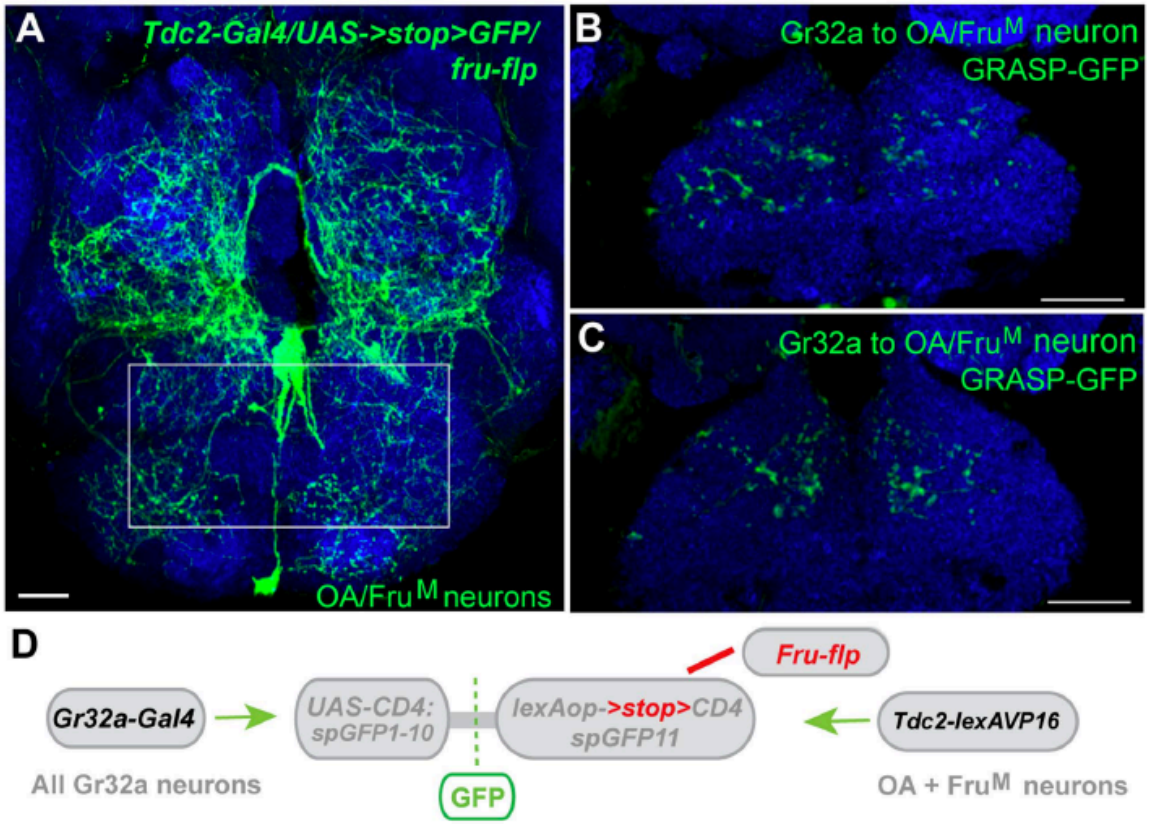

Figure 7: Gr32a neurons anatomically contact three Fru^{M}-OA neurons

(A) The morphology of three Fru^{M}-OA neurons located in the subesophageal ganglion identified by immunofluorescence to CD8:GFP in *Tdc2-Gal4/UAS->stop>CD8:GFP;fru^{FLP}* progeny (green, anti-GFP, Life Technologies). The box outlines the area of putative synaptic connections observed in B and C. Scale bar represents 20 µm. **(B, C)** Two different optical sections of a male brain exhibiting GRASP-mediated GFP reconstitution as a result of Fru^{M}-OA neurons expressing CD4::spGFP11 and the entire Gr32a neuron population expressing CD4::spGFP1-10. GRASP reconstitution is detected by immunofluorescence using rabbit monoclonal GFP antibody (green; Life Technologies). Scale bar represents 30 µm. **(D)** Schematic representation of the GRASP reporter lines combined with *fru^{FLP}*, *Gr32aVP16-Gal4,* and *Tdc2-lexA*. The FLP recombinase enzyme driven by *fru^{FLP}* excises the stop codon and permits expression of the *lexAop>stop>::spGFP11* GRASP reporter. **(E)** Expression of *fru^{FLP}* can result in a single Fru^{M}-OA neuron expressing CD8:GFP in

Tdc2-Gal4/UAS->stop>CD8:GFP;fru^{FLP} progeny. **(F)** Optical sections of a female brain (*Tdc2-LexA; lexAop>stop>CD4::spGFP11/Gr32aVP16-Gal4;UAS-CD4::spGFP1-10/ fru^{FLP})* showing GRASP-mediated GFP reconstituted expression in a restricted expression pattern potentially representing the single neuron expression shown in panel E. Scale bar represents 30 μm.

Discussion

Studies on animal behavior have been ongoing for decades and these have resulted in identifying pheromones, hormones and neurohormones, neurons, circuits and more recently, genes, that cause or contribute to the expression of social behavior. Yet a broad gap still exists between the identification of neurons and circuits suspected of involvement in specific behaviors and an understanding of how these circuits orchestrate the many context-dependent complex decisions animals routinely make in their daily lives. In this study, we demonstrate a direct early sensory link to a neuromodulatory-signaling element concerned with male aggression and courtship behavior and show that the two are interconnected in the suboesophageal ganglion. Our results show that sensory neurons expressing Gr32a, a widely distributed gustatory receptor that plays a critical role in male social behaviors (Koganezawa 2010, Miyamoto 2008, Wang 2011, Fan 2013), relays primary sensory information to the SOG where octopaminergic interneurons are contacted. The high density of putative GRASP connections we observe between receptor neurons expressing Gr32a, 22e, and 59b, and OA neurons in the SOG (these are co-expressed in a subset of the labellar sensory receptor neuron pool) (Weiss 2011)), suggests that amine-dependent modulatory steps may serve as important second order components in connecting signals from taste receptor neuron subtypes to taste-evoked behavior in flies (Miyazaki 2010, Sinakevitch 2011) (in vertebrates and other invertebrate systems see (Brezina 2010, Delaney 2007, Mellon 2000)). A separate study also identified putative synaptic connections between Gr32a axons and the total population of Fru^M-expressing neurons (Fan 2013). Whether Gr32a-expressing neurons solely contact the OA-Fru^M neurons or whether they contact additional Fru^M neurons remains to be determined. We do observe regions of Gr32a-driven syt:HA expression without GFP reconstitution to OA neurons suggesting the Gr32a-expressing neuron population likely contacts additional neuron subsets.

The Gr32a receptor is categorized as a contact-based chemoreceptor and is required for physiological responses to caffeine and other aversive, bitter-tasting compounds (Weiss 2011, Lee 2010, Lee 2009, Moon 2009). Gr32a is also reported to mediate the behavioral effects of the male pheromone *(z)*-7-tricosene and regulate interspecies courtship [Wang 2011, Fan 2013]. *(z)*-7-tricosene application to male legs evoked an increase in Ca^{2+} signaling in OA neurons (Andrews and Certel, unpublished data), although we were unable to identify a reliable response to *(z)*-7-tricosene in Gr32a foreleg neurons at this time. Reconciling behavioral and physiological roles of Gr32a-expressing leg and labellar neurons to individual CHCs will require further investigation. Nevertheless, application of male CHCs to male legs evokes significant increases in Ca^{2+} signaling in OA neurons and this response is eliminated in males with ablated Gr32a neurons (**Fig. 4**). These results support the behavioral data that indicates male aggression is promoted through the *Gr32a* receptor (this study and (Wang 2011) and suggests that at least a portion of the sensory information mediated by Gr32a receptor-bearing sensory neurons and OA modulatory interneurons operate in a single circuit.

The manipulation of neuronal populations by altering the expression of single molecular products like the Gr32a gustatory receptor or one of the monoamines, commonly yields multiple behavioral phenotypes (Koganezawa 2010, Miyamoto 2008, Wang 2011) indicating that such populations are heterogeneous in function. Separation of the grouped neurons into small subgroups can clarify the roles of these neurons in behavior and ultimately is essential in defining the circuitry involved. Recent findings indicate the tarsal Gr32a neurons are necessary to mediate species recognition (Fan 2013). Our data demonstrate that the foreleg tarsi and mouth populations of Gr32-expressing neurons may exert separable functional differences on male aggression and courtship behavior with both populations involving direct reinforcement by OA. Although Gr32a-expressing neurons do not exhibit any obvious sexual dimorphism, it has been postulated that their postsynaptic targets are sexually dimorphic (Koganezawa 2010). With the

increasing genetic capabilities of individual neuron manipulation, it will be interesting to determine if sexually dimorphic connectivity between single Gr32a and Fru^M-OA neurons regulate distinct differences in social behaviors. Results from further anatomical studies could provide insight into how potential sexual modification of OA signaling links chemosensory input to sex-specific behavioral output.

Neural networks mediating ever-changing environmental stimuli, context-specific social behavior, and internal states challenge us with the overwhelming structural and functional complexity of their interactions. To attempt to reduce network complexity, one common approach is to define network subunits and demonstrate their functional role by selective removal. It is well known that amine neurons can signal through hormonal volume transmission and act on targets at a distance [Agnati 2010, Fuxe 2012]. However, biogenic amines are also released synaptically and act on local targets (Agnati 2011, De-Miguel 2005, Huo 2009, Umbriaco 1995, Varga 2009). Whether amine neurons function in separate modulatory circuits that run parallel to and interact with hard-wired circuitries directing behavior, or whether they are an integral part of such circuitry remains to be determined. However, understanding the presynaptic sources or postsynaptic targets of OA neurons should provide useful insight into the "structural" embeddedness of single cells within a network. An anatomical analysis of individual components will be necessary as proximity-based neuron groupings break down with the addition of cell-specific markers (like Fru^M) and within amine neuron populations (Mao 2009). Network anatomical characterization that includes neuromodulatory neurons may also provide insight into the reinforcing or opposing actions of amines through second amines or peptide modulators (Burke 2012, Flavell 2013). For example, Burke et al., recently demonstrated plausible sites of synaptic contact between OA and DA neurons in the *Drosophila* mushroom body and a role for OA in providing appetitive reinforcement by OA receptor-mediated actions on DA neuron populations (Burke 2012). Our study offers a valuable framework in which to

undertake the characterization of sensory-driven neural circuits and the underlying neuromodulation of sexually dimorphic patterns of social behavior.

Materials and Methods

The following strains were used in this study: *Gr32a*$^{-/-}$ (Miyamoto 2008), *Gr32a-Gal4* (Dunipace 2001), *Gr32a-I-GFP* (Wang 2004), *UAS-DTI* (obtained from Leslie Stevens), *UAS-transformer* (BL 4590), *UAS-synaptotagmin:hemagglutinin* (*UAS-syt-HA* (Robinson 2002)), *w*$^{+}$ *Tßh*^{nM18} (Certel 2007), *w*$^{+}$ *Tßh*^{M6} (Certel 2007), *dTdc2-Gal4* (Cole 2005), *tsh-Gal80* (provided by Julie Simpson)(Clyne 2008), *UAS-mCD8:dsRed* (obtained from Liz Gavis), *lexAop-CD4::spGFP11* and *UAS-CD4::spGFP1-10* (Gordon 2009), *UAS-Red Stinger* (BL 8545), *UAS-hid UAS-Red Stinger, UAS-Denmark* (BL 33063)(Nicolai 2010), *fru*^{FLP} (Yu 2010), *20XUAS-6XGFP-Myc* (a gift from Steve Stowers, BL 52262), *UAS-GCaMP3.0* (BL-53742), *lexAop2-IVS-GCaMP6s* (BL 44274)and the Canton-S strain from the Bloomington Stock Center, Bloomington, IN.

Statistical Tools

Statistical analysis of data was performed with Prism 6.0. Details of statistical analysis can be found in the relevant figures.

Generation of transgenic lines

The *dTdc2-lexA:VP16* transgenic line was generated by cloning the same regulatory region as described previously (Cole 2005) into the pBS_LexA::VP16_SV40 vector. In the previous construct, the GAL4 was inserted immediately before the coding start, and the entire construct (genomic segments interrupted by Gal4) was inserted into the polylinker of pCaSpeR4 (Cole 2005). To generate the *dTdc2-lexA:VP16* construct, genomic DNA containing the region -3459 to +4530 was amplified with the Expand Long Template PCR system (Roche Applied Science). Fragment "A" of the *dTdc2* genomic region was amplified using the following primers, Tdc2A- Forward: GTCGCGGCCGCAAAAGTTATTGCACATTG, Tdc2A-Reverse: GGCCGGCCGTTTCGGTAGGTTTTCCAAATC, and fragment "B" with the following primers, Tdc2B Forward: GTCGGGCCCATGGACAGCACCGAATTTC, Tdc2B-Reverse: GGCCGCGGCCGCTTAGAACATATCGAGTTG. The *dTdc2* fragment A PCR product was inserted directly into the pBS-LexA::VP16_SV40 vector via the Eag1 site. Fragment B

was first inserted into the TOPO vector and digested with Apa1, followed by ligation into to pBS-dTdc2fragmentA-LexA::VP16_SV40 using the Apa1 site on the vector. The fragment containing dTdc2 fragment A+ the *LexA* coding region + *dTdc2* fragment B was subcloned into the Not1 site of pCaSpeR4.

The *lexAop-FRT-STOP-FRT-::spGFP11* line was generated by amplifying the spGF11 fragment through PCR from the previously described pLOT plasmid (Gordon 2009). The FRT-STOP-FRT cassette was amplified from the pJFRC177 plasmid (#32149, AddGene) and both the STOP cassette and the spGFP11 fragment were cloned downstream of the 13XLexAop2 sequence in pJFRC19 (#26224, AddGene). The amplified fragments were verified by sequencing. Transgenic flies were raised by standard procedures and lines screened for appropriate expression.

Immunohistochemistry

Adult male and female dissected brains were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) for 25 minutes and labeled using a modification of protocols previously described (Certel 2007). The following primary antibodies were used: rabbit anti-GFP monoclonal (1:200) (Life Technologies, G10362), mouse anti-GFP (1:200) (Invitrogen, A-11120, Lot 764809), rabbit anti-FruM (1:2000) (Stockinger 2005), rat anti-CD8 (1:100), rat anti-HA (Roche, 1:1000), mAb nc82(anti-bruchpilot) (1:30) (Hofbauer 2009), anti-Tβh (1:400) (Koon 2011). Secondary antibodies include Alexa Fluor 488-conjugated goat anti-rabbit, Alexa Fluor 488-conjugated donkey anti-mouse, Alexa Fluor 594-conjugated donkey anti-mouse, Alexa Fluor 594-conjugated goat anti-rabbit, Alexa Fluor 647-conjugated donkey anti-mouse (Invitrogen). Goat anti rabbit fluorescein-conjugated secondary antibodies a cross-adsorbed for use in multi-labeling experiments. Images were collected on an Olympus Fluoview FV1000 laser scanning confocal mounted on an inverted IX81 microscope and processed using ImageJ (NIH) and Adobe Photoshop (Adobe, CA).

Behavioral Assays

All fly strains were reared on standard fly food (medium containing agar, glucose, sucrose, yeast, cornmeal, propionic acid, and Tegosept). Flies were grown in temperature- and humidity-controlled incubators (25°C, 50% humidity) on a 12-h light/dark cycle. To collect socially naïve adults, pupae were isolated in individual 16 x 100-mm glass vials containing 1.5 ml of food medium. Upon eclosion, flies were anesthetized with CO_2, painted on the thorax with acrylic paint for identification and returned to their isolation vials to allow for recovery from anesthesia a full 24 hours before testing.

Calcium Imaging

Live brain preparations were made by anesthetizing a fly on ice followed by placement within a pipette tip with the head protruding. The pipette was then sealed with nail polish and allowed to dry. Flies thusly secured were placed in a 1 mL well for electrophysiology at an angle and the region containing the head was flooded with 400 μL of oxygentated HL3 solution. Removal of the proboscis and front of the head cuticle allowed for imaging. Each preparation was equilibrated for 5 min after proboscis and cuticle dissection. Male cuticular hydrocarbon extract (hexane extract from 150 male flies 3 days post eclosure), (*z)*-7-tricosene (Cayman Chemical #9000313 Lot# 0406404-32), or quinine (Sigma-Aldrich #6119-47-7 Lot # STBD3004V) dissolved in oxygentated HL3 solution were administered via syringe into the rear of the pipette tip. Administration of each compound occurred a minimum of 15 seconds apart. Flies received either male cuticular extract or (*z)*-7-tricosene first, followed by quinine. Analysis of F/F values in regions of interest was calculated using Fiji. Regions of interest were selected via identification of TDC2 neuronal cell bodies, and fluorescence from these regions was measured in a frame-by-frame manner. Background fluorescence was subtracted, and regions of interest were examined before and after compound administration. Adjusted fluorescence values resulting from this process were entered into Prism 6.0, and plotted for analysis.

Image Analysis

Epifluorescene images were acquired at the rate of 1 image/.750s by Hamamatsu camera (ORCA ER series, model C4742-95-12ERG). Acquired images were registered (StackReg plugin, Fiji software) and regions of interest were selected within the subesophageal ganglion. Image processing and analysis was accomplished with ImageJ version 1.44 / Fiji version 1.43. Image subtraction was performed in Fiji using the image calculator. Intensity tables were exported to excel and (F – F) / F calculated for each series of images. Traces were generated in Prism 6.0. Peak analysis was performed between regions no more than 5 seconds post compound administration (for post CHC) and no later than 4 seconds prior to compound administration (for pre-CHC).

Aggression and Courtship Paradigms

Aggression assays were performed in individual chambers of 12-well polystyrene plates containing a food cup in the center [67]. 4-5 day old males were transferred in pairs to assay chambers by aspiration. Experiments were performed at 25 °C in a humidity controlled room (50%). Fights were videotaped for 90 minutes and lunges counted for 30 minutes from the first lunge unless otherwise specified. The time between introduction into the chamber and the onset of aggression (first lunge) was defined as the fighting latency. Lunging behavior was determined as previously described [68]. Courtship assays were performed in a 12 well polystyrene plate (VWR #82050-930) with one Canton S virgin female (aged 7-10 days) and one 4-5 day old male. The period between introduction into the courtship chamber and the first male wing extension (singing) was defined as courtship latency.

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Lou Herrit for technical expertise. The nc82 antibody was developed by Eric Buchner obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biology, University of Iowa (Iowa City, IA).

Figure S1

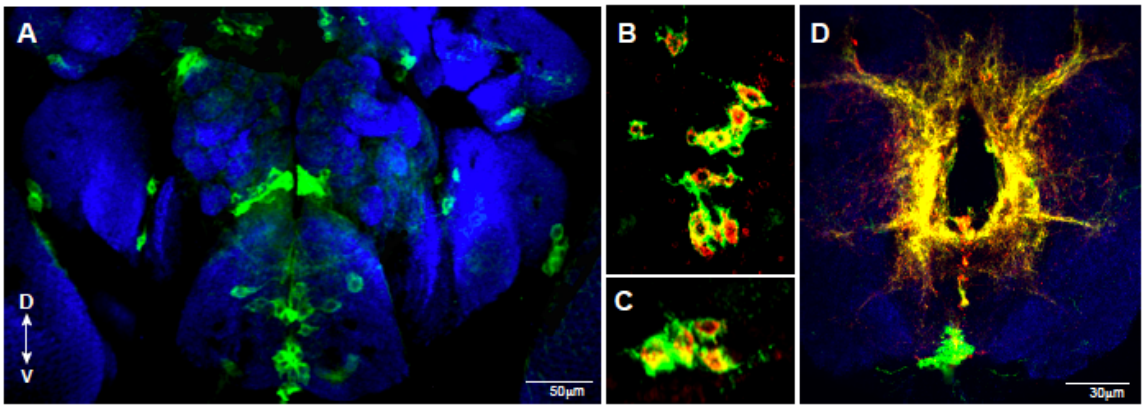

Figure S1: Characterization of the *Tdc2-LexA* line.

(A) GFP expression drive by *Tdc2-LexA* in the adult brain maintains the same pattern as the *Tdc2-Gal4* driver. The SOG region shown in panels B and C from a separate brain is outlined with the white box. **(B-C**) Complete overlap is observed between Tβh immunoreactivity and GFP in Tdc2-lexA SOG neurons (*Tdc2-lexA;lexA-rCD2:GFP* progeny). **(D)** Selected optical sections identifying a subset of putative input regions of Tdc2-Gal4 SOG neurons visualized by the postsynaptic reporter *UAS-DenMark* with the membrane marker UAS-mCD8::GFP *(UAS-DenMark;Tdc2-Gal4;UAS-CD8:GFP* progeny, green, anti-CD8, Invitrogen).

Figure S2

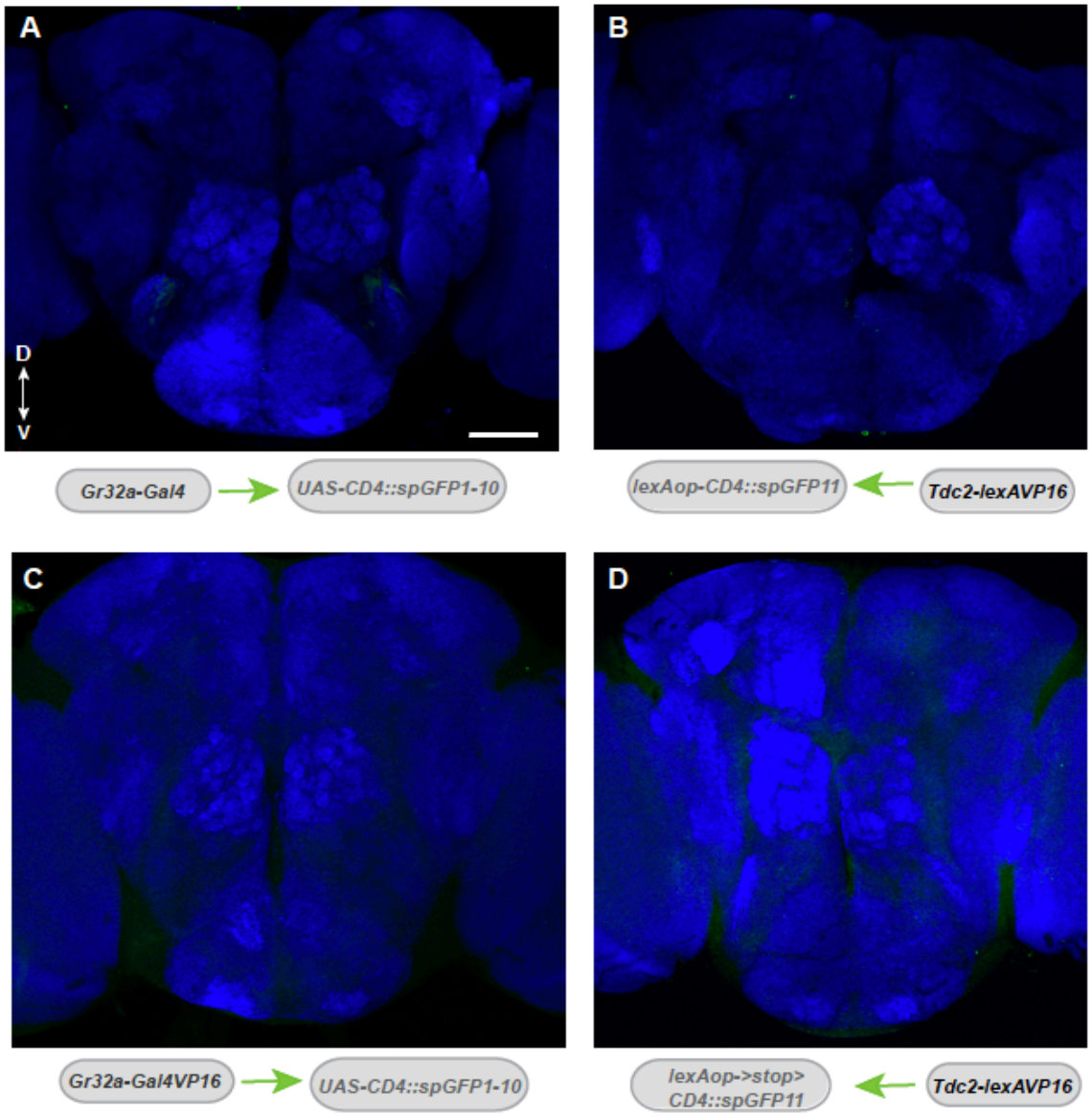

Figure S2. Single GRASP component control brains demonstrate an absence of GFP expression.

(A-D) Control brains were imaged for immunofluorescence against GFP in brains containing one component of the GRASP system. **(A)** No signal was observed in *Gr32a-Gal4/ UAS- CD4::spGFP1-10* controls. **(B)** Fluorescence was not detected in *Tdc2-lexA:VP16/lexAop-CD4::*spGFP11 control brains. **(C)** The *UAS-CD4::spGFP1-10* GRASP component driven by *Gr32a-Gal4VP16* did not generate a signal. **(D)** The addition of a flp-out stop codon in progeny containing *Tdc2-lexA:VP16/lexAop-*

*>stop>CD4::*spGFP11 did not result in detectable fluorescence. All brains were labeled with rabbit monoclonal GFP, Life Technologies. Scale bar represents 20 μm.

Figure S3

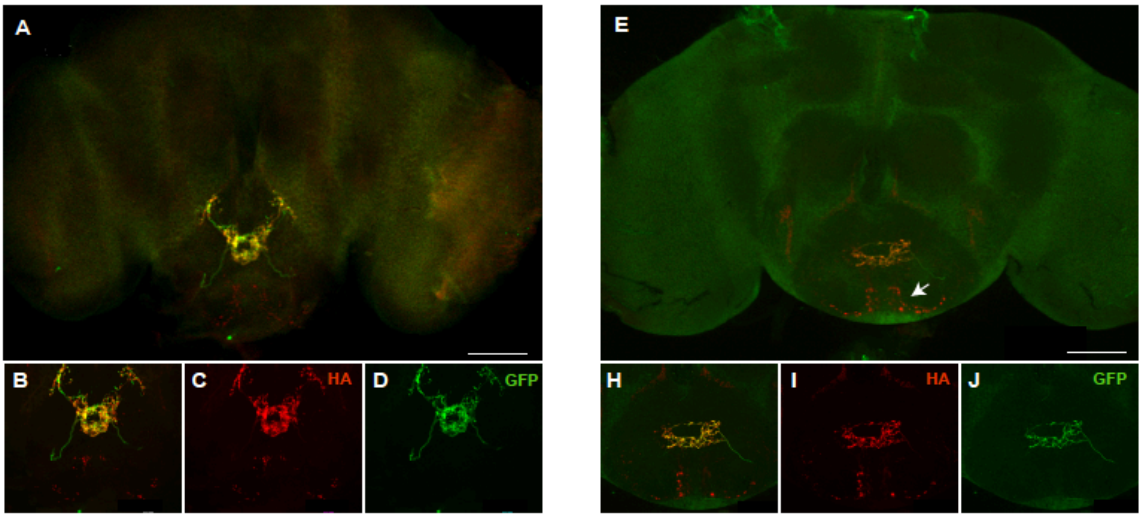

Figure S3. Gr22e and Gr59b neurons contact OA neurons in the suboesophageal ganglion.

(A) GRASP-mediated GFP reconstitution specifically in the SOG is observed between Gr22e neurons expressing CD4::spGFP1-10 and synaptotagmin:hemagglutinin (*UAS-syt:HA*) (red, anti-HA) and OA neurons expressing CD4::spGFP11. GRASP reconstitution is detected by immunofluorescence using a monoclonal GFP antibody (green, Invitrogen, A-11120, Lot 764809). **(B-D)** Optical sections at higher magnification showing GRASP-mediated GFP reconstituted expression (D), synaptotagmin:HA localization (C) and clear overlap or close association at synaptic-like puncta in the merged channel (B). **(E)** GRASP-mediated GFP reconstitution between Gr59b neurons expressing CD4::spGFP1-10 and synaptotagmin:hemagglutinin (*UAS-syt:HA*) (red, anti-HA) and OA neurons expressing CD4::spGFP11. Regions in the SOG with only synaptotagmin:HA expression are indicated (arrow) in addition to GFP-reconstitution contacts that show co-localization with syt-HA expression. **(H-J)** Higher magnification view of optical sections with GRASP-mediated GFP reconstitution (J), synaptotagmin:HA localization (I), and the observed overlap in punctate patterns (H). Scale bars represent 20 μm.

Figure S4

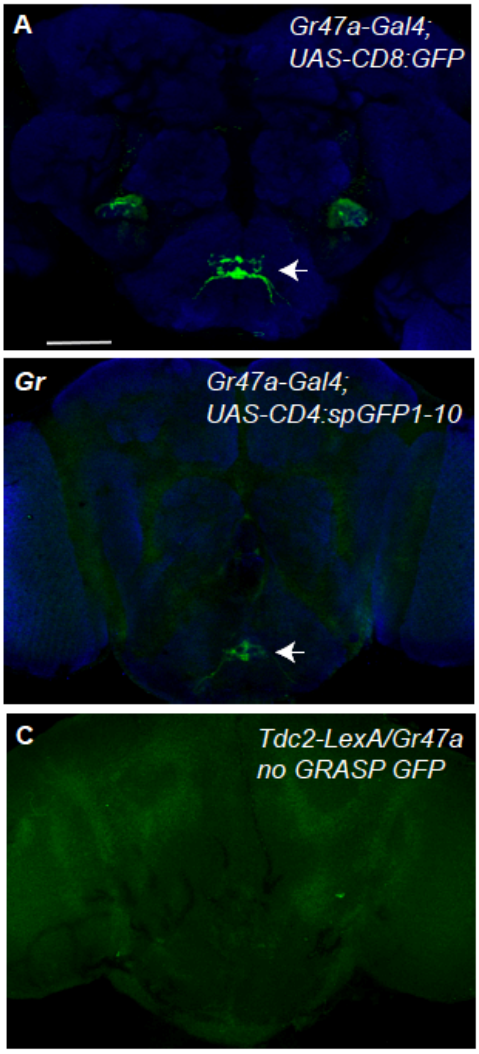

Figure S4. GRASP-reconstitution between Gr47a neurons and OA-expressing neurons is not observed.
(A) The *Gr47a-Gal4* line drives GFP expression via the *UAS-CD8:GFP* reporter in the SOG (arrow). **(B)** The single GRASP line *UAS-CD4::spGFP1-10* is expressed by *Gr47a-Gal4* and detected by a polyclonal rabbit anti-GFP that recognizes this split-GFP fragment (Invitrogen, A6455). **(C)** GRASP-mediated GFP reconstitution was not observed between Gr47a neurons expressing CD4::spGFP1-10 and OA neurons expressing CD4::spGFP11 (monoclonal GFP, Invitrogen, A11120, Lot 764809).

Figure S5

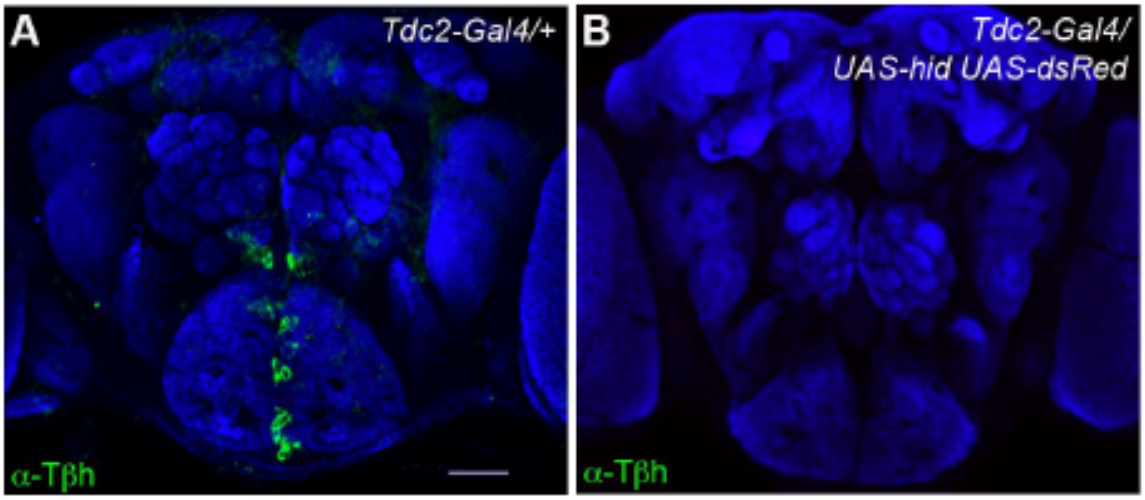

Figure S5. Tdc2-expressing neurons are ablated by *UAS-hid UAS-DsRed* expression.

(**A**) Expression of the rate-limiting enzyme, Tyrosine β−hydroxylase, is detected in OA-expressing SOG neurons in *Tdc2-Gal4/+* control brains (anti-Tβh, [66]). **(B)** Octopamine neurons are eliminated in *Tdc2-Gal4/UAS-hid UAS-DsRed* progeny as assayed by the absence of DsRed and Tyrosine β−hydroxylase production. Scale bar represents 20 μm.

Figure S6

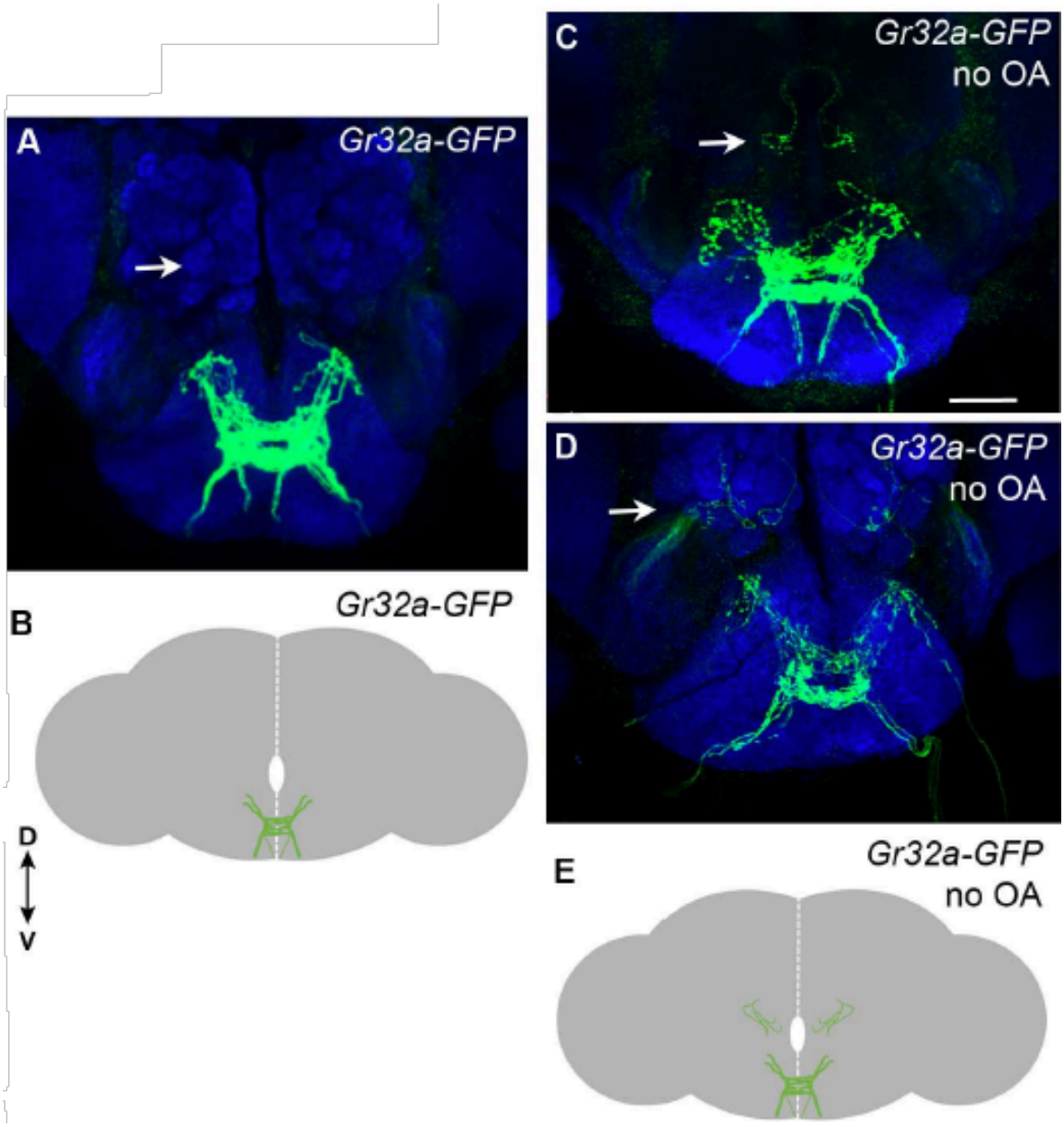

Figure S6. Eliminating OA production alters a subset of Gr32a axonal projections.

(A) GFP expression in a heterozygous control adult brain *(tβh^{nM18}/+;Tdc2-Gal4;20XUAS-6XGFP-Myc)*. The Gr32a-expressing neurons located in the tarsi, labellum, and mouthparts terminate in the SOG. **(B)** Schematic representation of the adult brain with Gr32a-expressing axonal arborizations. **(C-D)** Confocal sections of OA deficient male brains (*(tβh^{nM18};Tdc2-Gal4;20XUAS-6XGFP-Myc)*. When OA production is eliminated throughout development, a subset of Gr32a

axon projections terminate in the antennal lobe region (arrow). **(E)** Schematic representation of the adult OA deficient brain with a subset of Gr32a-expressing axons terminating in the antennal lobe region. Scale bar represents 30 μm.

Figure S7

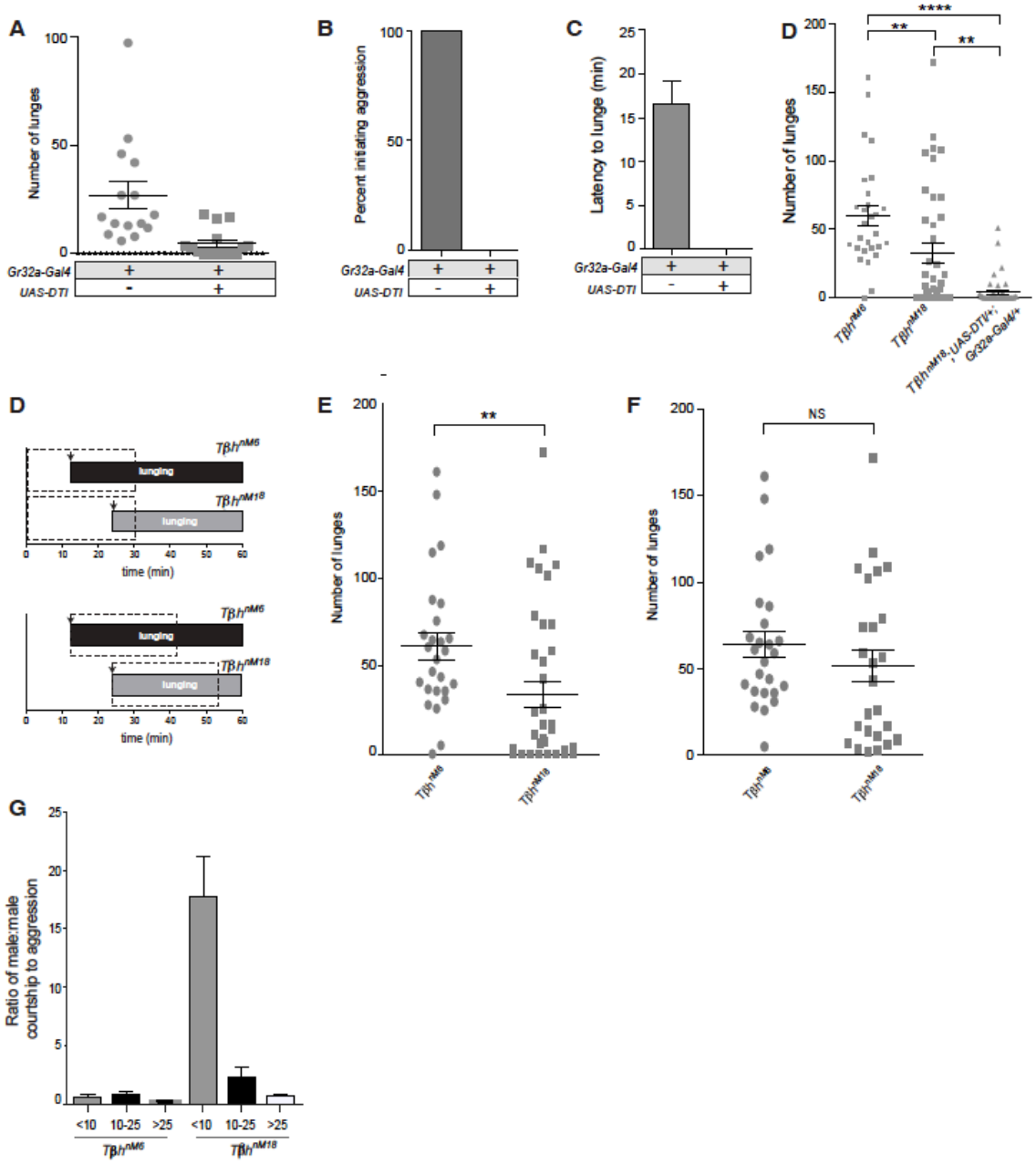

Figure S7. Defects in aggressive behavior parameters in Gr32a-expressing and OA deficient males.

(A-C) Experimental males without Gr32a-expressing neurons *(UAS-DTI;Gr32a-Gal4)* do not exhibit aggressive behavior when paired with control males. **(A)** Males without Gr32a-expressing neurons display significantly fewer lunges than control males *(+/Gr32a-Gal4)*. **(B)** Control males initiated aggression as measured by the first lunge in all assays, n=15. **(C)** The latency to first lunge by control males

is similar in pairings with experimental and control males (Figure 3). **(D)** The number of lunges by experimental *tβh^{nM18};UAS-DTI/+; Gr32a-Gal4/+* males was significantly less than exhibited by control males (*tβh^{M6})* or males without OA (*tβh^{nM18})*(****p<0.0001, **p=0.003). **(D-F)** Aggressive behavior or the component patterns that make up aggressive behavior are commonly quantified for a given period of time from the moment that pairs of flies are placed into a fight chamber (**D**, upper panel). This method of scoring does not take into account any substantial differences in the latency to begin fighting. Given the observed latency to initiate the fights, we quantified the number of lunges performed by each pair of males during a 30-minute period starting from the onset of aggression (lower panel). **(E)** If fights without lunges are scored as "zeros", the numbers of lunges seen in fights between pairs of *tβh^{nM18}* males are significantly lower than the numbers seen in the genetic controls. One outlier value of 416 is observed in a *tβh^{nM18}* pairing. In this comparison with fights that do not exhibit fighting, *tβh^{M6}* and *tβh^{nM18}* are statistically different with the inclusion or absence of the outlying value (Mann-Whitney test, p value with outlier= 0.0049, p value without outlier= 0.0023) **(F)** When pairs that did not display lunges are excluded in the quantification, significant differences are not found in the lunge frequency between *tβh^{nM18}* and *tβh^{M6}* male pairs. One outlier value of 416 is observed in a *tβh^{nM18}* pairing. In this panel *tβh^{M6}* and *tβh^{nM18}*are not statistically different with the inclusion or absence of the outlying value (Mann-Whitney test, p value with outlier= 0.2193. p value without outlier= 0.1327. Both >0.5). **(G)** Elevated male:male courtship occurs when lunge number is low in male pairs without OA (*tβh^{nM18}*). The three columns, <10, 10-25, and >25 represent the observed number of lunges per fight. For each assay, the number of wing extensions/singing was divided by the number of lunges resulting in the average ratios per column.

Figure S8

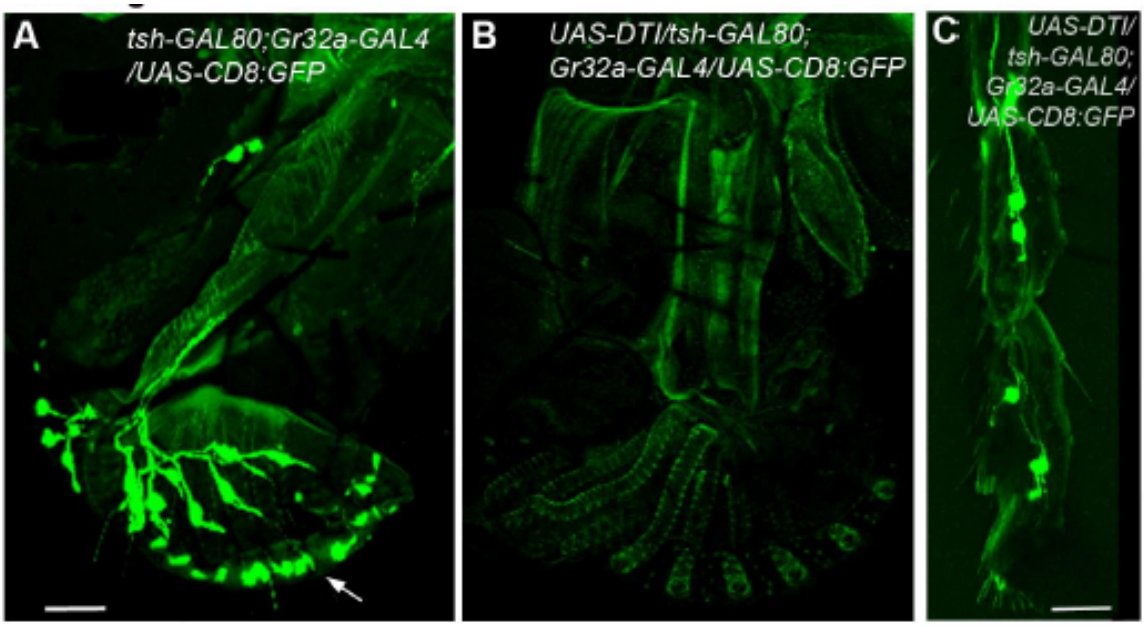

Figure S8. *tsh-Gal80* blocks Gal4-mediated expression in the leg

(A) Gr32a neurons expressing GFP in the labellum of *tsh-Gal80;Gr32a-Gal4/UAS-CD8:GFP* progeny (arrow). **(B)** The addition of *UAS-DTI* ablates the Gr32a-expressing labellar neurons. **(C)** Gr32a leg neurons still maintain GFP expression in *UAS-DTI/tsh-Gal80;Gr32a-Gal4/UAS-CD8:GFP* progeny. Scale bar represents 20 μm.

Supplemental Table 1

Supplemental Table 1: Single Male to Female Courtship

Genotype	Latency (sec) (SEM)	Copulation Rate
UAS-DTI/+	115 ± 41	96% (24/25)
tsh-Gal80/+;Gr32a-Gal4/+	43 ± 20	100% (12/12)
UAS-DTI/tsh-Gal80; Gr32a-Gal4	621 ± 158	92% (12/13)

Table S1. Analysis of male-female courtship in males with ablated mouth Gr32a-expressing neurons.

Single male to virgin female courtship parameters measured in control males and males with mouth Gr32a-expressing neurons ablated. Latency to courtship initiation is the time when a singing/wing extension event to the female is first observed after introduction into the courtship chamber. Courtship initiation differences between *UAS-DTI/+* controls, *tsh-Gal80;Gr32a-Gal4* controls, and *UAS-DTI/tsh-Gal80;Gr32a-Gal4* males were significant (Kruskal-Wallis with Dunn's multiple comparison test, $^{**}p<0.01$, $^{***}p<0.001$). However, the delay did not significantly change copulations rates. Due to the extended latency period exhibited by *UAS-DTI/tsh-Gal80;Gr32a-Gal4* males, the copulation rate equals the percentage of males mating in 60 minutes.

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Chapter III
Introduction

Survival and reproduction in a complex environment requires that an organism be able to identify signals that correspond with the presence of mates and food availability. These cues are gathered by specialized sensory organs that are rich in stimulus-sensitive neurons and encoded into patterns of neural activity, which in turn promote many animal behaviors, including aggression (Zwarts 2012, Kravitz 2015). As aggressive behavior performed while securing new resources or mates can be energetically costly or physically dangerous, animals must assess if the potential risks justify the reward (Zwarts 2012, Kravitz 2015, Schwartz 2012). This makes aggression an ideal means by which to study how organisms attenuate sensory inputs in order to respond appropriately to environmental cues.

In *Drosophila,* males exhibit stereotyped patterns of aggressive behavior towards other males (Sturtevant 1915, Zwarts 2012). These behaviors are provoked by an ensemble of environmental factors, including pheromones, the presence of food resources, body size, and the presence of female conspecifics (Chen 2002, Skrzipek 1979, Lee 2000, Jacobs 1979, Schilcher 1975, Ueda 2002). Previous studies have indicated that several biogenic amines play a significant role in the regulation of aggressive behavior (Dierick 2007, Johnson 2009, Alekseyenko 2010, 2013, 2014, Riemensperger et al. 2011, Van Swinderen 2011, Waddell 2013, Asahina 2014, Winther 2006). Of particular note is the amine octopamine (OA), the invertebrate analog to norepinephrine. Previous work in our lab has demonstrated that males with reduced levels of OA exhibit decreased levels of aggression and increased rates of male-male courtship, and is necessary for males to make the correct choice between courtship and aggression (Baier 2002, Hoyer 2008, Zhou 2008, Certel 2007, 2010, Andrews 2014). However, the full pathway by which OA regulates aggressive behavior has not been identified.

OA has also been shown to be an important player in the regulation of metabolic processes in several insect species. Its role in the regulation of muscle tension and

relaxation has been demonstrated in both the locust and cockroach, where OA is known to play a large role in switching between short-term energy reserves (typically carbohydates) and long-term stores of energy (typically lipids) during energy intensive behavior such as flight. This shift toward lipid metabolism is reliant on two events: the release of adipokinetic hormone from the *corpora cardiaca*, and OA mediated release of lipids from the insect fat body. In flies that lack OA, body fat levels are known to increase significantly (Li 2016). While these findings demonstrate that OA plays a significant role in the liberation of energy sources during fight-or-flight situations, a direct link between OA's role in aggression and its metabolic effects as yet to be established (Adamo 1995).

In addition to internal aminergic signals, external signals such as the sensation of sugar have been shown to be important players in the generation of multiple patterns of behavior (Lim 2014, Grosjean 2011, Schwartz 2012). Previous studies of fly aggression have implicated the activity of sugar sensing gustatory receptor neurons (GRNs) in the initiation of aggressive behavior. Specifically, the activity of Gr5a-expressing GRN's is necessary for normal levels of food-induced aggression, but activation of these neurons is insufficient to increase aggression in the absence of a food resource (Lim 2014, Wang 2011, Chen 2002, Yuan 2013). This effect is dependent on the amount of food present in the environment, and can be mimicked by the administration of sucrose (Lim 2014). The presence of a food substrate containing yeast or sugars has been shown to promote sexual activity (Gorter 2016). Female flies become more receptive to copulation in the presence of a nutritious food source, and subsequently increase their production of offspring (Gorter 2016, Schwarts 2012). This effect has been extensively studied and is known to be the product of both olfactory and gustatory neuron activation and an internal response to the nutritional value of the food consumed (Miyamoto 2012, Billeter 2012, Wigby 2011, Wade 1996).

In *Drosophila*, gustation and octopaminergic signaling are tightly linked. Like many other animals, fruit flies exhibit elevated levels of locomotion in response to

starvation (Yang 2015, Lee 2004, Meunier 2007). This response has been shown to be dependent on both OA signaling and peripheral Gr5a-expressing GRN's (Erion 2012, Yang 2015, Yu 2016, Inagaki 2014), but the mechanism by which these two systems interact has yet to be elucidated. However, OA signaling has been shown to modulate sensory input and the outcome of sensory pathways in several different invertebrates. In the silkworm (*Bombyx mori*) and honeybee (*Apis mellifera*) OA signaling is used to fine tune responses to olfactory stimuli (Farooqui 2003, Pophof 2002). Likewise, a similar effect has been observed in *Drosophila* vision, where OA acts to modulate visual processing during flight (Suver 2012). Given that flies must detect a territory containing food in order to escalate their aggressive behavior or reduce locomotion during periods of starvation, it is possible that OA neuromodulation could act to sensitize food-detecting neurons, either as a means of encouraging defense of a food territory or priming the fly for food consumption post aggressive bout.

In this study, we provide evidence that octopaminergic modulation of GRN signaling may play an important role in the generation of stereotyped social behavior by demonstrating that a subset of previously undocumented neurons expressing the OA receptor Oaβ1R in the forelegs and proboscis also express known sugar receptors, including Gr5a and Gr64f. We also demonstrate that administration of OA is sufficient to increase the firing frequency of these neurons.

We used behavioral assays, EPAC1-CAMPs imaging and a series of co-localization trials to demonstrate the presence of peripheral Oaβ1R expression, and its presence within known GRN's. Ablating Oaβ1R-expressing neurons resulted in noteworthy deficits in male aggressive and courtship behavior. A role for labellar Oaβ1R neurons was also established. Flies lacking Oaβ1R in their proboscis displayed a greater latency to initiate aggression and fewer wing-based aggressive and courtship related behaviors than control flies. A series of EPAC1-CAMPs experiments were also used to demonstrate that the neurons are OA sensitive, and that exposure to OA resulted in an increase in intracellular CAMP levels. Finally, our

work shows that Oaβ1R receptor-expressing neurons also express sugar-sensitive receptors, and that OA acts to sensitize these neurons via a cAMP and PKA dependent process. The results presented here demonstrate that OA signaling plays an important role in tuning how external stimuli are detected, based on the internal state of the fly.

Results

Oaβ1R-expressing neurons are present in the legs and proboscis

In order to explore how OA signaling translates into aggressive behavior, we first needed to identify which neurons expressed *Oaβ1R.* To this end, our lab has generated an *Oaβ1R-Gal4* line. Previous studies of the *Oaβ1R* receptor have identified a pattern of expression within the adult brain, ventral nerve cord, crop, malpighian tubules, and larval neuromuscular junction (El-Kholy 2015, Koon 2012, Li 2016). Here we report an additional pattern of expression within the neurons of the forelegs and labellum (a gustatory organ of the proboscis and pharynx) (**Fig. 1**). Male *Drosophila* expressing *Oaβ1R-Gal4 ; 20x-UAS-6xGFP* display clusters of neurons within the fifth, fourth and third segments of all six legs (**Fig. 1**), and expression in the mouthparts was widespread across both palps (**Fig. 1**). These neurons send projections into taste bristles on the surface of the proboscis. Further quantification of the neurons within the proboscis found that males had an average of 18 (± 2.8) *Oaβ1R* neurons per palp, and displayed an average of 13.9 (± 1.1) neurons within the foreleg. Mid and hindlegs also contained *Oaβ1R* neurons, with an average of 9.4 (± 0.8) and 14.2 (± 1.8) neurons per leg, respectively.

As this pattern of expression had yet to be identified, we wished to confirm the validity of our *Oaβ1R-Gal4* line. To do this, we ablated Oaβ1R-expressing neurons by expressing diphtheria toxin (*UAS-DTI*). Proboscis and legs were removed from both experimental (*Oaβ1R-Gal4* ; *UAS-DTI*) and control (*UAS-DTI/+*) adult males between three and five days post eclosure, and *Oaβ1R* transcript levels were then quantified via QPCR. These experiments demonstrated that the ablation of *Oaβ1R*-expressing neurons resulted in a ninety-six percent reduction in *Oaβ1R* transcript levels (**Fig.**

Figure 1

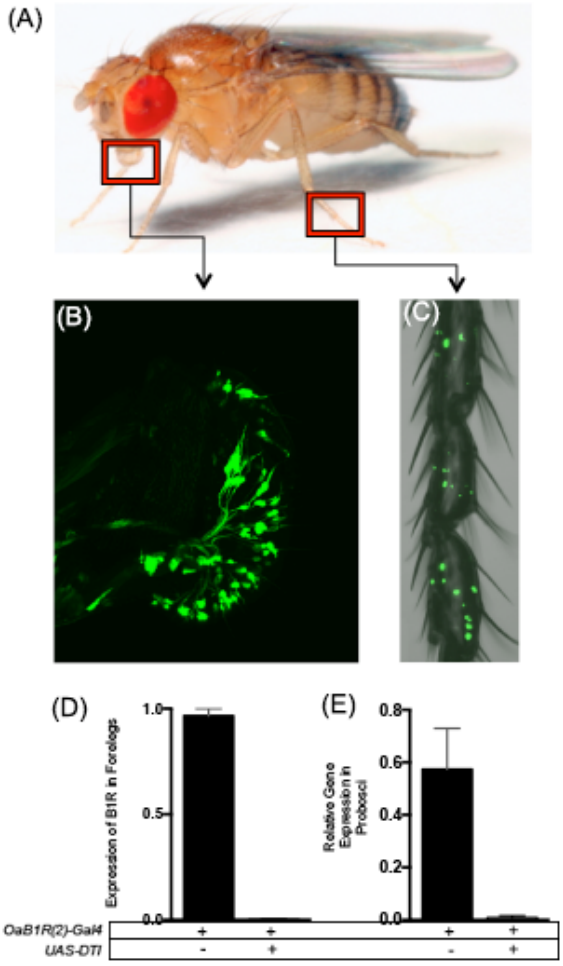

Figure 1: *Oaβ1R* neurons within the tarsi and labellum

(**A**) *Drosophila melanogaster*. Red squares indicate the proboscis/labellum and tarsi. (**B**) Labellar Oaβ1R-expressing neurons identified by GFP in an *Oaβ1R-Gal4 ; 20x-UAS-6xGFP* adult male. (**C**) *Oaβ1R* neurons identified within the fifth, fourth, and third tarsal segments of a *Oaβ1R-Gal4 ; 20x-UAS-6xGFP* adult male. (**D**) QPCR analysis of *Oaβ1R* expression within the forelegs. *Oaβ1R-Gal4 ; UAS-DTI* flies demonstrate a 98% reduction in Oaβ1R transcript level. (**E**) QPCR analysis of Oaβ1R expression within the proboscis. *Oaβ1R-Gal4 ; UAS-DTI* flies demonstrate a 96% reduction in Oaβ1R transcript level.

1). These results suggest that our *Oaβ1R-Gal4* line is an accurate representation of *Oaβ1R* expression in the periphery.

Octopamine administration causes an increase in cAMP levels in Oaβ1R-expressing neurons

If the neurons identified by our *Oaβ1R(2)-Gal4* line express functional octopamine receptors, then exposure to octopamine should generate an observable intracellular change. To determine if *Oaβ1R* neurons increase intracellular Ca^{2+} levels, we expressed *UAS-GcAMP6* and assayed changes in intracellular Ca^{2+} in response to the administration of octopamine (Chen 2013, Svoboda 2009, Akerboom 2012, Nakai 2001, Jahagirdar 1987). Neurons in both the forelegs and the proboscis failed to reliably demonstrate an increase in intracellular Ca^{2+} levels (data not shown).

As previous studies have identified an increase in intracellular cAMP levels in response to activation of *Oaβ1R* receptors, we next assayed for a change in intracellular cAMP in response to OA administration (Farooqui 2007, Evans 1981, Evans 1993, 1993). To confirm if neurons highlighted by our *Oaβ1R(2)-Gal4* reporter are sensitive to OA, we used *UAS-EPAC1-cAMPS*, a genetically encoded cAMP FRET sensor which detects changes in intracellular cAMP levels. This sensor uses a pair of fluorescent proteins- a yellow fluorescent protein (YFP) and a cyan fluorescent protein (CFP), which flank the cAMP-binding protein Epac. When cAMP is not bound to Epac, FRET occurs, resulting in an energy transfer from CFP to YFP. In the presence of cAMP, the CFP and YFP domains are separated, reducing FRET levels (Lohse 2011, Nikolaev 2006, Berrera 2008, Willoughby 2008, DiPilato 2004, van der Krogt 2004). Upon exposure to OA, probosci (**Fig. 2**) taken from *Oaβ1R(2)-Gal4 ; UAS-EPAC1-CaMPS* flies demonstrated a loss of FRET, indicating a rise in cAMP levels within the Oaβ1R-expressing neuron, consistent with previously reported data (Farooqui 2007, Evans 1981, Evans 1993, 1993). This change in FRET was not observed in response to vehicle administration alone, confirming that at least a portion of the neurons identified by *Oaβ1R(2)-Gal4* are OA sensitive. While it

Figure 2

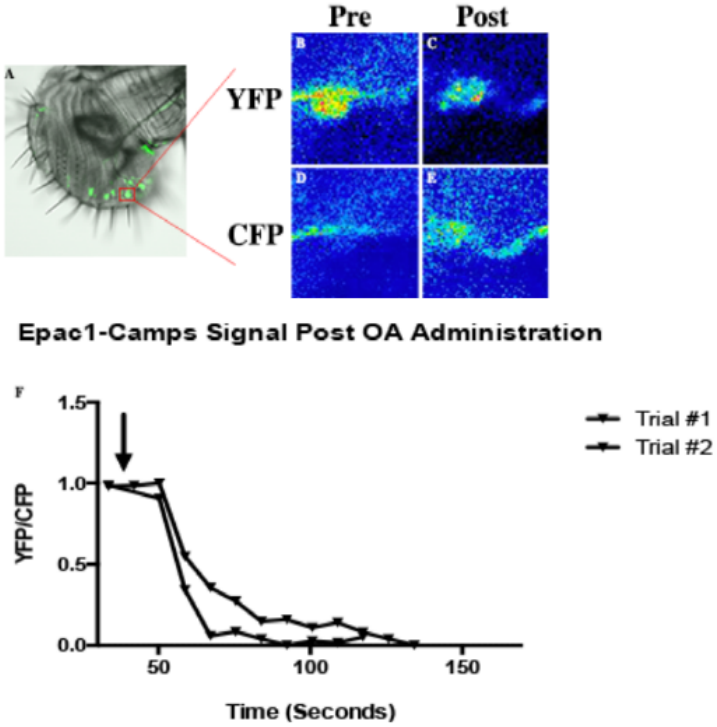

Figure 2: cAMP levels change in response to OA administration.

(**A**) Male proboscis with several neurons marked by *Oaβ1R-Gal4 ; UAS-EPAC1-cAMPS.* (**B-C**) Pseudocolored images demonstrating YFP fluorescence pre- and post-OA administration. (**D-E**) Pseudocolored images demonstrating CFP fluorescence pre- and post-OA administration. (**F**) Representative YFP/CFP signal traces of neurons expressing EPAC1-cAMPS in response to OA administration (arrow).

is possible that the lack of observed Ca^{2+} changes may result from changes in Ca^{2+} levels below a detectable threshold, the observed changes in cAMP levels indicate that OA signaling may otherwise perform a modulatory role in *Oaβ1R*-expressing neurons, similar to what has been shown in molluscan feeding (Vehovszky, 2005)

Oaβ1R-expressing neurons also express sugar sensors

As the presence of the Oaβ1R receptor does not indicate the function of the neuron, we asked if the neurons identified by our Oaβ1R-Gal4 line also expressed known sugar, water or pheromone receptors. To answer this question, we used a *lexAop-nucRFP; 20xUAS-6xGFP* line to screen ten lexA driver lines for co-localization with our newly identified Oaβ1R-expressing neurons. Of the lines we tested, six were found to be present in Oaβ1R-expressing neurons (**Fig 3, 4**). Pickpocket 28, a known water sensor, was observed to co-localize with 8 Oaβ1R neurons in the forelegs (Cameron 2010, Meunier 2009) (**Fig. 3**). This suggests that *Oaβ1R*-bearing neurons may also have some effect on *Drosophila* metabolism, as evidenced by *Ppk28*'s effect on adipokinetic hormone (AKH) signaling. (Waterson, 2014).

Furthermore, Gr64f, a coreceptor responsible for the detection of sucrose, maltose, trehalose and other sugars, was found to co-localize with 2 leg neurons and ~15 neurons in the proboscis (**Fig. 4**) (Jiao 2008, Carlson 2001, Thorne 2004). The presence of Gr64f and *Oaβ1R* within neurons in the proboscis and legs represent a point where octopameniergic modulation could alter the ability to recognize food resources. Further exploration of other sugar sensors revealed that *Oaβ1R*-expressing neurons also co-localized with Gr64B and Gr64C in both the legs and proboscis (**Fig. 4**). Gr64B was found to co-localize with two neurons in the proboscis and one in the foreleg, while Gr64C co-localized with only a single neuron in both locations. *Oaβ1R* was also found to co-localize with two Gr5a-expressing neurons in the foreleg, and 3 in the proboscis (**Fig. 4**) (Fujii 2015, Dahanukar 2007, Jiao 2007, Slone 2007). Given these findings, the increased level of cAMP caused by octopaminergic signaling may influence how flies detect food and water in their

Figure 3

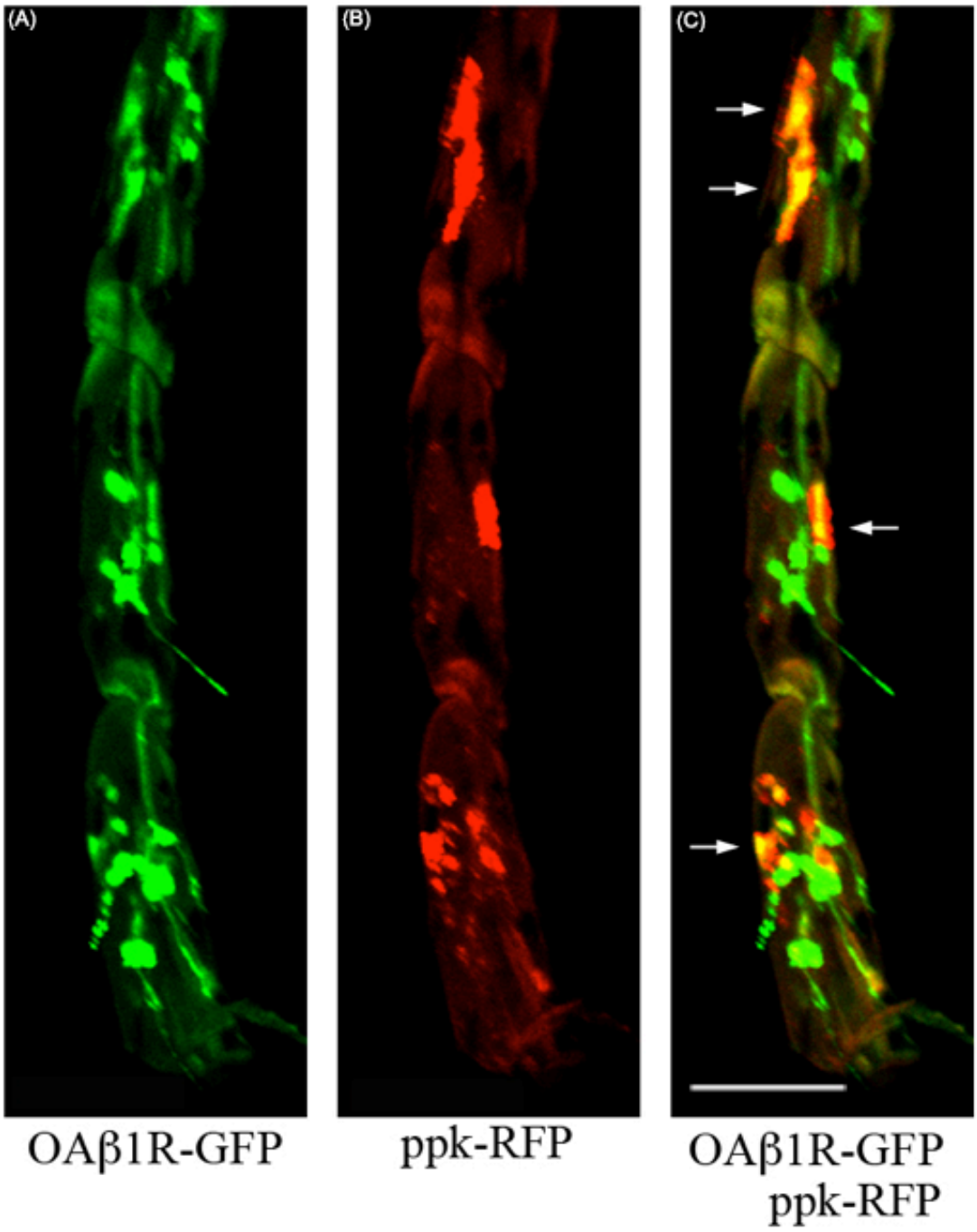

Figure 3: *Oaβ1R* neurons express water sensors.
Images of *Oaβ1R-Gal4/LexAop-nucRFP ; Ppk28-lexA/20x-UAS-6xGFP* tarsi. (**A**) *Oaβ1R*-expressing neurons alone. (**B**) Ppk28 expressing neurons alone. (**C**) Co-localization between Oaβ1R and Ppk28.

Figure 4

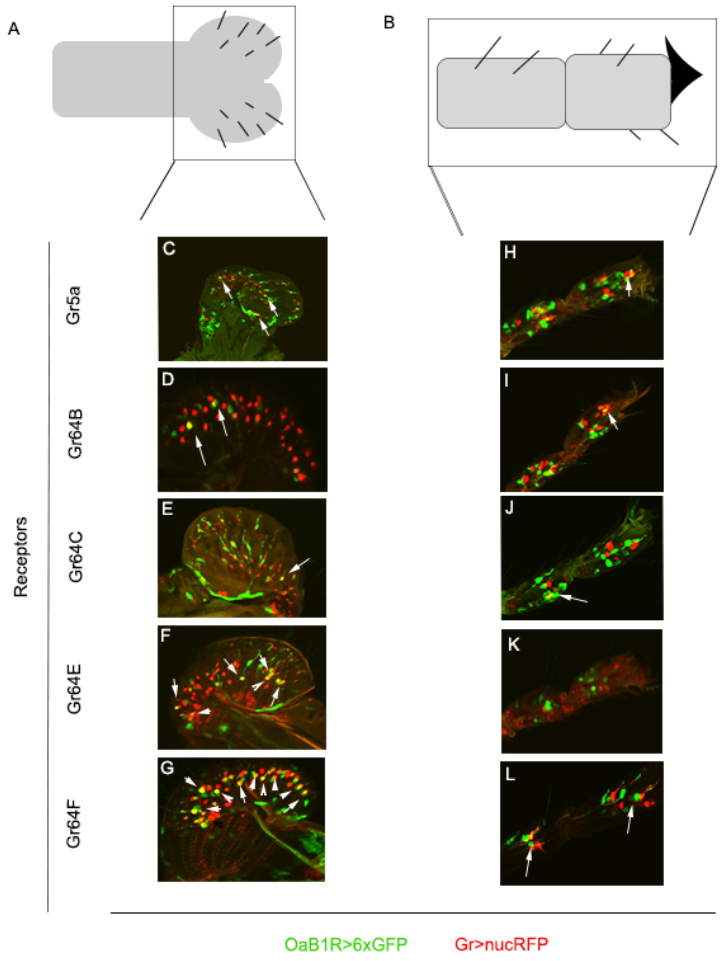

Figure 4: *Oaβ1R* neurons express sugar receptors

Schematic representation of the *Drosophila* proboscis (**A**) and *Drosophila* tarsi (**B**) displaying gustatory hair fibres. Confocal sections of proboscis (**C, D, E, F, G**) and tarsi (**H, I, J, K, L**) expressing the *LexAop-nucRFP ; 20x-UAS-6xGFP* reporters under the control of *Oaβ1R-Gal4* and *Gr5a-lexA (**C, H**), Gr64B-lexA* (**D, I**), *Gr64C-lexA (**E, J**), Gr64E-lexA* (**F, K**), and *Gr64F-lexA* (**G, L**). Co-localization is indicated with arrows.

environment, and subsequently influence the initiation of food-dependent patterns of behavior.

Octopamine administration alters neuronal excitability in the periphery

Increased levels of cAMP have been associated with an increase in neuronal excitability in both vertebrate and invertebrate models. In *Aplysia* sensory neurons, cAMP is responsible for both increased spike duration and neuron excitability, while hippocampal slices taken from rodents have demonstrated that cAMP induced excitability enhances long-term potentiation (Vehovszky 2005, Budnik 2012, Brunelli 1976, Kang 1993, Nicoll 1995, Silva 1998). In the *Drosophila* central nervous system, cAMP signaling has been directly linked to the excitability of cholinergic and GABAergic neurons (Lelito 2012, Lee 2015). This cAMP mediated increase in excitability is thought to occur via PKA dependent phosphorylation of potassium channels, which subsequently reduces their conductance (Wright 1995, Delgado 1998, Lee 2015). As we have identified *Oaβ1R* expressing neurons which also express choline acetyltransferase (**Fig. 5**) and demonstrate an increase in cAMP levels when exposed to OA, we hypothesized that this population of neurons would demonstrate a potassium mediated change in excitability in the presence of OA. To test this, we performed field recordings on the probosci of *Oaβ1R(2)-Gal4 / 20x-UAS-6xGFP* flies, using the GFP marker to identify the senscilla of *Oaβ1R*-expressing neurons (Delventhal 2014) (**Fig. 6**). Spike frequency was analyzed in both the presence and absence of fructose/sucrose and OA. In the absence of both sugar and OA, tonic spike firing was observed at a frequency of 1.94 action potentials per second. This rate of firing changed when sugars were administered, increasing to an average of 4.16 spikes per second. When OA was administered to the proboscis via the hemolymph-like solution in the recording electrode during sugar exposure, the frequency of spikes increased significantly, to an average rate of 12.27, 3 times that of sugar alone (**Fig. 6**).

As OA is hypothesized to act via cAMP dependent phosphorylation of a K^+ channel, we wanted to assess if blocking the activity of the channel with

Figure 5

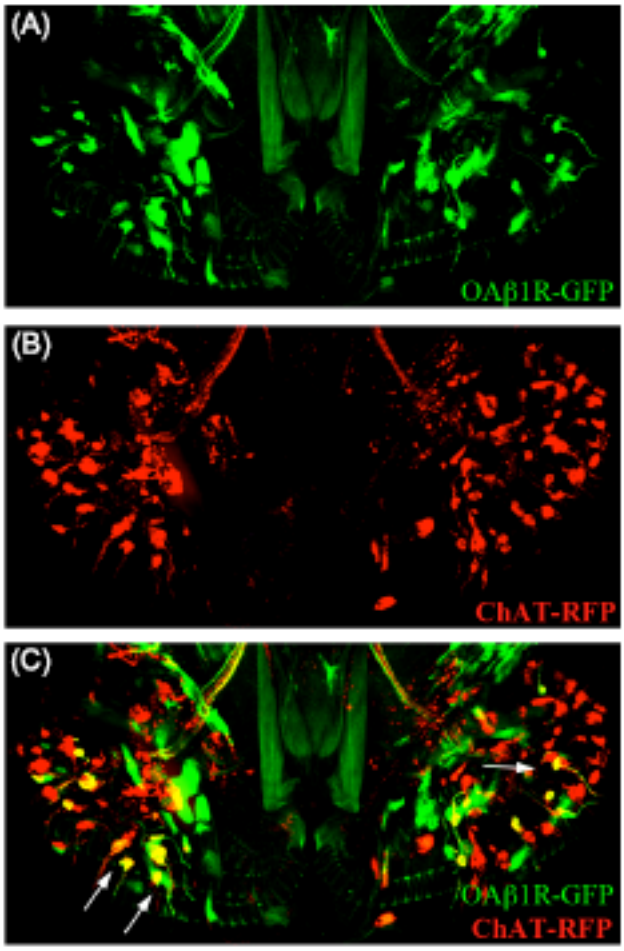

Figure 5: *Oaβ1R* neurons express choline acetyltransferase

Images of a *Oaβ1R-Gal4/LexAop-nucRFP ; Chat-lexA/20x-UAS-6xGFP* male proboscis. (**A**) *Oaβ1R*-expressing neurons alone. (**B**) Neurons expressing choline acetyletransferase alone. (**C**) Co-localization between Oaβ1R and choline acetyltransferase.

Figure 6

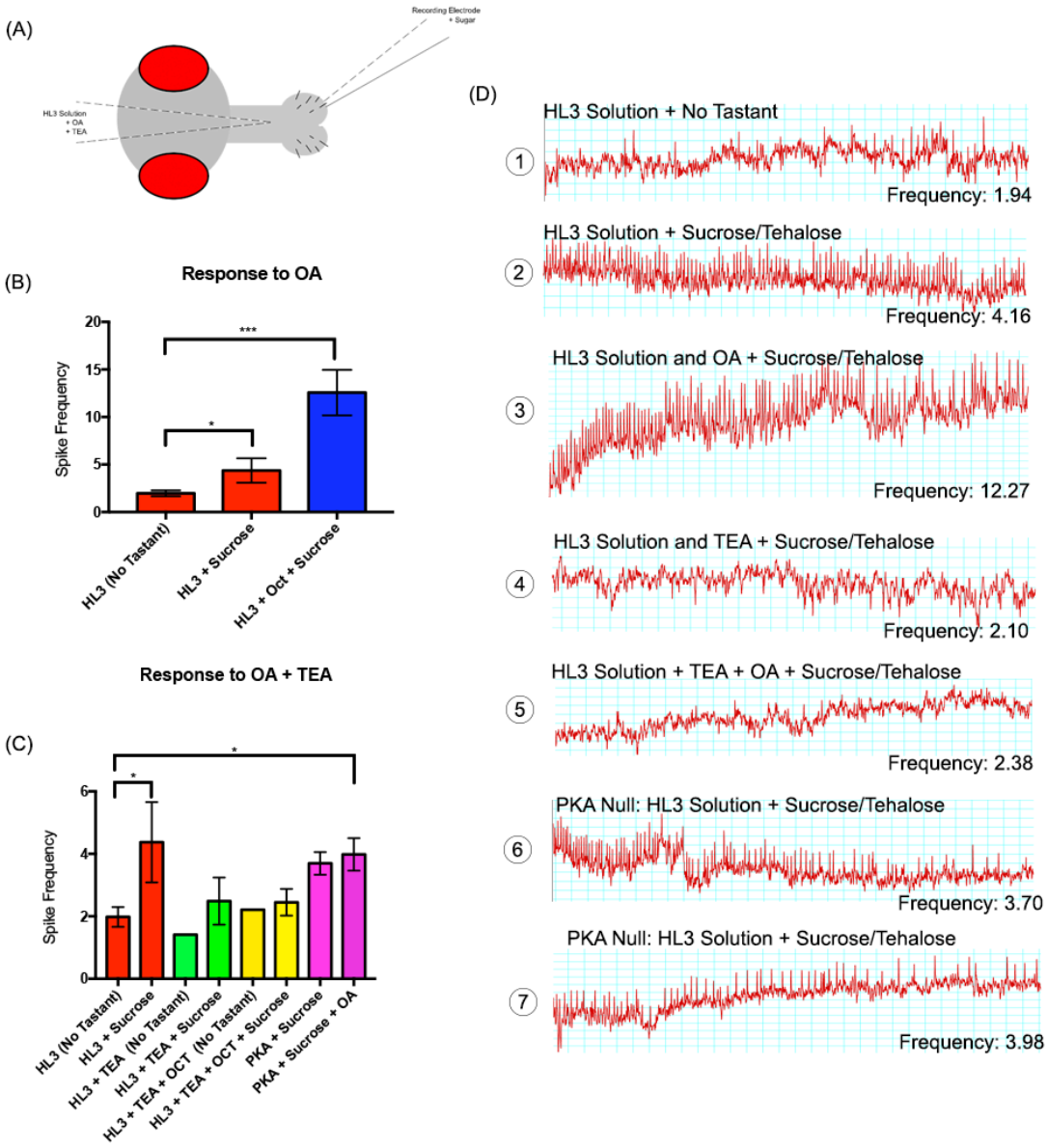

Figure 6: OA administration increases firing frequency in labellar *Oaβ1R* neurons.

(**A**) Schematic depicting recording probe placement and sites of OA/TEA administration. (**B**) Frequency of action potentials increased in response to the administration sucrose or sucrose and OA. (*p<0.0343, ***p<0.0003) (**C**) Administration of TEA or the presence of a dominant negative null PkA was sufficient to suppress OA sensitization. (*p<0.0343, *p<0.0499). (**D**) Representative samples of action potentials taken from electrophysiological recordings.

tetraethylammonium (TEA), would result in the OA signal having no effect. When we applied TEA concurrently with OA in the recording electrode in the presence of sugar, we observed an average firing frequency of 2.30 spikes per second. This was not significantly different than the administration of TEA and sucrose, without the administration of OA (**Fig. 6**). As it is possible that this effect may be due to the actions of TEA alone, we then examined *Oaβ1R(2)-Gal4 / UAS-PkA-R1-BDK* flies, which possess a dominant negative version of PkA (Kiger 1999). As this cross will lack a functional PKA, we would expect to see the same results as our TEA trials if OA signaling results in a change in neuronal excitability. In fact, this is what we observed. Trials with *Oaβ1R(2)-Gal4 / UAS-PkA-R1-BDK* showed no difference in the frequency of action potentials in the presence of sugar, or sugar and OA (**Fig. 6**). These experiments indicate that OA acts to sensitize Oaβ1R neurons within the proboscis, and that these changes in sensitivity are dependent on changes PkA levels.

Oaβ1R-expressing neurons mediate onset and frequency of aggressive behavior

As little is currently known about what role Oaβ1R may play in initiating or regulating behavior, examining how Oaβ1R-expressing neurons contribute to aggressive behavior is a logical step in understanding how OA modulates aggression (Koon 2011, 2012). To ask whether the activity of Oaβ1R-expressing neurons contributes to male aggressive behavior, we first analyzed the stereotyped patterns of aggression produced by flies lacking Oaβ1R neurons. Using the Gal4-UAS gene expression system and the *Oaβ1R(2)-Gal4* driver generated by our lab, we ablated Oaβ1R-expressing neurons by expressing diphtheria toxin (*UAS-DTI*). Flies lacking Oaβ1R-expressing neurons were then screened and several important behavioral parameters were quantified, including time required to initiate aggression, number of lunges (a form of high intensity aggressive behavior), and number of wing threats (a threat display) performed towards a single rival male. All assays were performed in a temperature and humidity controlled environment with a chamber containing a

food-based territory for the flies to fight over (Chen 2002, Lee 2000, Jacobs 1979, Schilcher 1975).

Consistent with the known roles of OA and food availability in male aggression, adult males lacking Oaβ1R-expressing neurons demonstrated a significant increase in time before committing to their first lunge when compared to their control counterparts (*Oaβ1R(2)-Gal4/+* and *UAS-DTI/+*) (**Fig. 7**). Additionally, a significant reduction in the number of lunges and wing threats was also observed (**Fig. 7**). As the patterns of aggression observed here are similar to those observed in flies that cannot detect food resources, Oaβ1R-expressing neurons may play an important role in the OA-mediated regulation of food-dependent male aggressive behavior.

Ablation of Oaβ1R-expressing neurons delays courtship and copulation

Previous results from our lab (Certel 2007, 2010, Andrews 2014) have demonstrated that males lacking detectable levels of OA display an increase in the frequency of male-male courtship behaviors. To determine if ablating Oaβ1R-expressing neurons would also significantly alter male courtship ability, we placed two *Oaβ1R(2)-Gal4; UAS-DTI* or control (*Oaβ1R(2)-Gal4/+* or *UAS-DTI/+*) males with a single virgin female and quantified the frequency of male-male courtship behaviors, and the duration of time spent in the chamber before wing extension/singing behavior and copulation occurred.

In contrast to OA-deficient flies, *Drosophila* lacking Oaβ1R neurons did not display a significant increase in male-male courtship events, but low levels of courtship were observed, consistent with previous reports of normal male-male courtship on sucrose media (Lim 2014). Males lacking Oaβ1R-expressing neurons did demonstrate a significant delay before performing wing extensions towards other flies in the arena or copulating (**Fig. 7**), however rates of successful copulation were not significantly different in experimental or control populations. These results imply that Oaβ1R-expressing neurons do not necessarily play a role in differentiating between the genders of other conspecifics, but instead reinforces the

Figure 7

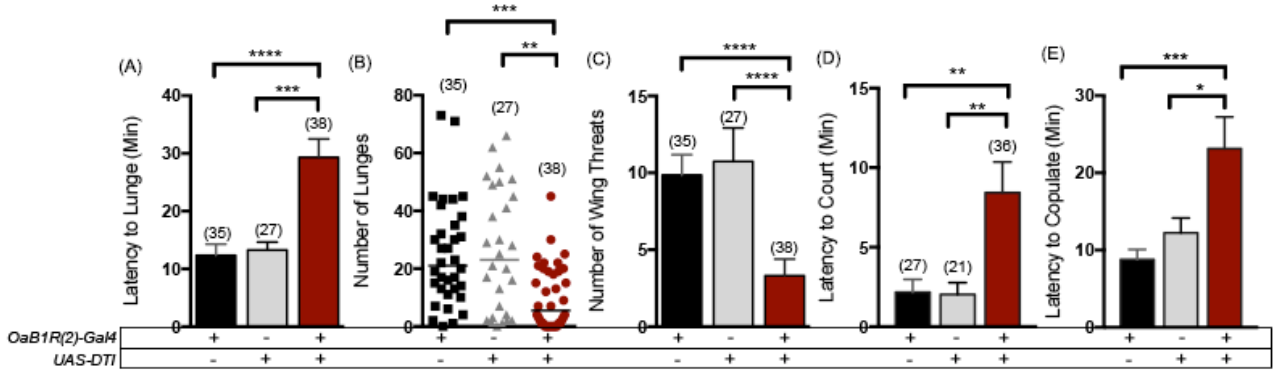

Figure 7: Oaβ1R neurons promote aggression and courtship behavior.
(**A-C**) Fights between males lacking *Oaβ1R*-expressing neurons removed by expressing Diptheria Toxin *(Oaβ1R-Gal4 ; UAS-DTI)* and individual transgenic controls, *Oaβ1R-Gal4 or UAS-DTI*. (**D-E**) Courtship assays between males lacking *Oaβ1R*-expressing neurons removed by expressing Diptheria Toxin *(Oaβ1R-Gal4 ; UAS-DTI)* and individual transgenic controls, *Oaβ1R-Gal4 or UAS-DTI* and canton-s females. All statistical tests are Kruskal-Wallis with Dunn's multiple comparison test. (**A**) The latency to first lunge was significantly higher in *Oaβ1R-Gal4 /+; UAS-DTI/+* males as compared to controls (****p<0.0001, *p<0.0041). (**B**) Number of lunges (represented by each dot) performed in a 30 min period after the first lunge by any control or experimental male in a fighting pair. Males without Oaβ1R neurons exhibited a significant reduction in lunges as compared to controls (***p<0.0007, **p<0.0019). (**C**) Number of wing threats performed in a 30 min period after the first lunge by any control or experimental male in a fighting pair. Males without Oaβ1R neurons exhibited a significant reduction in wing threats as compared to controls (***p<0.0001, **p<0.0001). (**D**) The latency to initiate courtship was significantly higher in *Oaβ1R-Gal4 /+; UAS-DTI/+* males as compared to controls (**p<0.0046, **p<0.0012). (**A**) Time required to successfully copulate was significantly higher in *Oaβ1R-Gal4 /+; UAS-DTI/+* males as compared to controls (***p<0.0009, *p<0.0315).

idea that they detect the nutritional value of a substrate. These findings imply that male flies may adjust their willingness to court females depending on the perceived value of their current environment, and may indicate that male mate choice is dependent not only on the presence of sex pheromones, but also the location upon which the female has chosen to land.

Males lacking the Oaβ1R receptor take longer to court and perform fewer wing threats

In addition to ablating the Oaβ1R neurons, we further verified that the defects observed in *Oaβ1R(2)-Gal4; UAS-DTI* males were due to Oaβ1R receptor activity by using RNAi interference to reduce Oaβ1R transcript levels. We first determined the efficiency of our *UAS-Dicer2* ; *UAS-OaB1R^{IR}* (RNAi- inverted repeat) line when paired with a deficiency line (*Df(3R)BSC685*) that reduced the level of Oaβ1R transcript. This cross *Oaβ1R(2)-Gal4/ UAS-Dicer2* ; *UAS-OaB1R^{IR}/ Df(3R)BSC685* successfully reduced the expression of Oaβ1R transcript by >90%, which was comparable to the level of Oaβ1R transcript found in *Oaβ1R(2)-Gal4; UAS-DTI* males (**Fig. 8**). Our aggression assays demonstrated that *Oaβ1R(2)-Gal4/ UAS-Dicer2* ; *UAS-OaB1R^{IR}/Df(3R)BSC685* males exhibited a statistically significant decrease in the number of wing threats performed towards other males, and an increase in the latency to court female conspecifics (**Fig. 8**). The lack of change in the amount of time required to initiate aggression and the number of lunges performed was noted, but is not unexpected. As OA signaling increases the excitability of Oaβ1R neurons, then the loss of the Oaβ1R receptor alone should not completely abolish signaling from the neuron in the presence of environmental tastants, such as sugar. This leaves open the possibility that the loss of OA signaling may not fully recapitulate the behavioral changes seen when Oaβ1R neurons were ablated, as the neurons remain intact and capable of reacting to the presence of environmental stimuli.

Figure 8

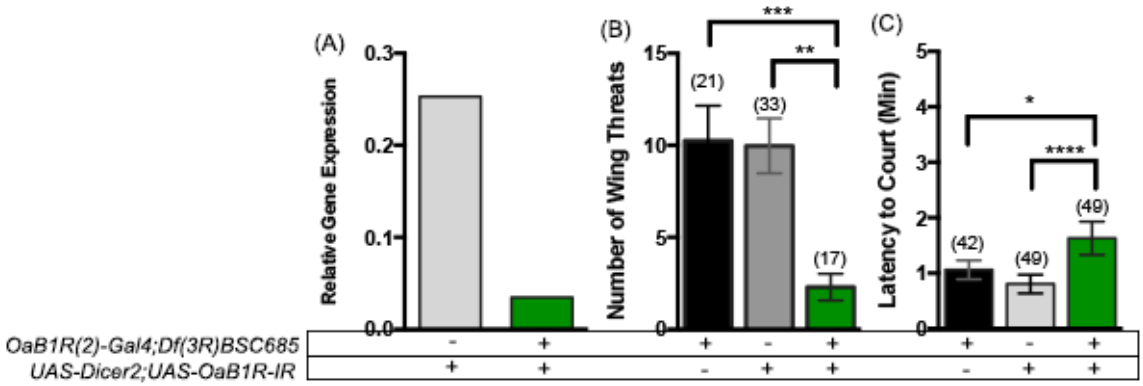

Figure 8: Loss of the Oaβ1R receptor alters wing specific behavior

(**A-C**) From Males lacking the Oaβ1R receptor, or associated controls (*Oaβ1R-Gal4;Df(3R)BSC685 or UAS-Dicer2;UAS-Oaβ1R^{IR})*. Receptor was removed by a combination of RNAi and deficiency line (*Df(3R)BSC685)*. (**A**) QPCR analysis of Oaβ1R expression within the tarsi. *Oaβ1R-Gal4/UAS-Dicer2; Df(3R)BSC685/UAS-Oaβ1R^{IR}* flies demonstrate a >90% reduction in Oaβ1R transcript level when compared to *UAS-Dicer2;UAS-Oaβ1R^{IR}* controls. (**B**) Aggression: number of wing threats performed in a 30 min period after the first lunge by any control or experimental male in a fighting pair. Males without the Oaβ1R receptor exhibited a significant reduction in wing threats as compared to controls (***p<0.0004, **p<0.0010). (**C**) Courtship: the latency to initiate courtship was significantly higher in *Oaβ1R-Gal4/UAS-Dicer2; Df(3R)BSC685/UAS-Oaβ1R^{IR}* males as compared to controls (****p<0.0001, *p<0.0361).

A subset of Oaβ1R-expressing neurons is required for male aggression

As neurons bearing the Oaβ1R receptor appear to mediate relevant nutritional information that promotes male-male aggression and male-female courtship, it is possible that anatomically disparate subpopulations may influence each behavior through different circuits. To test this hypothesis, we selectively prevented *Oaβ1R*-expressing neurons within the labellum from signaling through the use of tetanus toxin, while leaving the neurons within the legs intact using the homeotic *teashirt* promoter to drive Gal80 expression, which prevented Gal4-mediated silencing of neurons outside the head. Using the *UAS-TnT/FM7 ; tsh-Gal80/Cyo* line under the control of the *Oaβ1R(2)-Gal4* driver, we examined both aggression and courtship, using the same paradigm as presented above. In these experiments, we found that males lacking *Oaβ1R*-expressing neurons in the labellum demonstrated a significant increase in the amount of time spent in the fight chamber before committing to aggressive activity (**Fig. 9**). As an increased latency to court and copulate was observed in flies lacking *Oaβ1R*, we also quantified the time until courtship and copulation in the presence of both a female and food. We noted that the time required to initiate courtship in *UAS-TnT/+ ; tsh-Gal80/Oaβ1R(2)-Gal4* males was significantly increased when compared to controls (**Fig. 9**). Our results thereby indicate that functional differences in male social behavior are determined by different subpopulations of *Oaβ1R*-expressing neurons, and that the labellar subpopulation is important in the initiation of aggressive and courtship. This data also implies that the tarsal subpopulation of *Oaβ1R* neurons may play a role in determining the intensity of aggressive behavior separate from the labellum.

Discussion

Investigation into the means by which neurological activity translates into animal behavior has been a topic of great interest that has resulted in the identification of a plethora of pheromones, hormones, circuits, and genes that directly impact social behavior. However, little is known about how the circuits

Figure 9

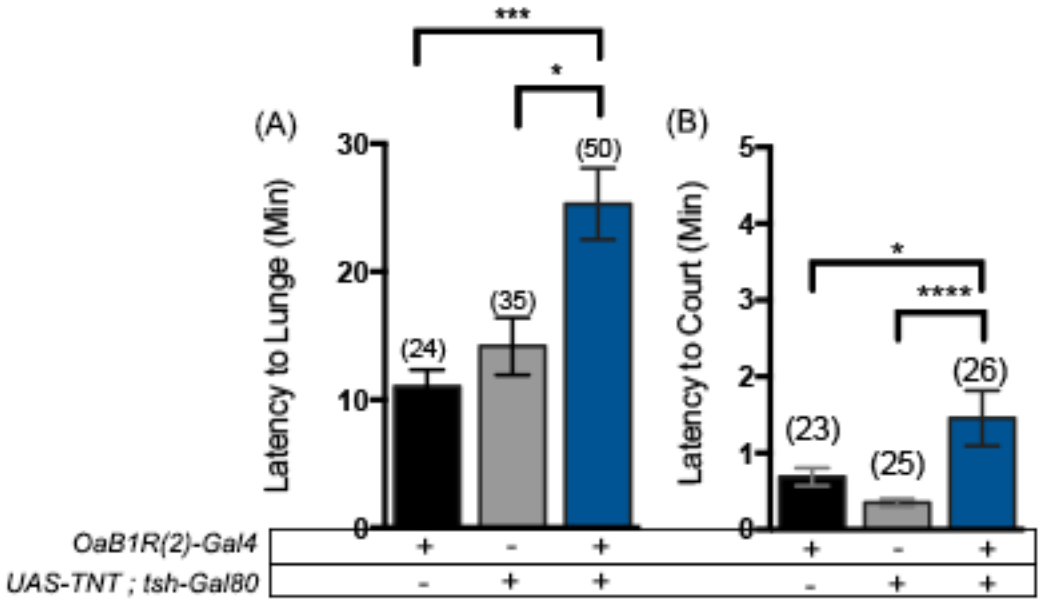

Figure 9: Oaβ1R-expressing neurons in the mouth promote initiation of courtship and aggression

(**A-B**) *UAS-TNT ; Oaβ1R-Gal4/tsh-Gal80* flies lack Oaβ1R signaling in labellar neurons. (**A**) The latency to first lunge was significantly higher in *Oaβ1R-Gal4 /+; UAS-DTI/+* males as compared to controls (***p<0.0009, *p<0.0476). (**B**) The latency to initiate courtship was significantly higher in *UAS-TNT ; Oaβ1R-Gal4/tsh-Gal80* males as compared to controls (****p<0.0001, **p<0.0391).

affecting aggression interplay with systems affecting metabolism and energy homeostasis.

In this study, we provide evidence that neurons expressing *Oaβ1R*, an octopamine receptor present in the legs and proboscis, also express a number of different sugar receptors, including Gr64B, Gr64C, Gr64E and Gr64F. The Gr64-series of receptors are categorized as contact based chemoreceptors that mediate the response to sweet taste. As octopamine signaling has been demonstrated to increase the excitability of neurons in invertebrates (Lelito 2012, Lee 2015, Prier 1994, Pophof 2002), the presence of *Oaβ1R* receptors in neurons associated with identifying food and water sources suggests that octopamine signaling may play a modulatory role in determining the approximate value of a food substrate, and therefore may act to enhance territorial behavior (Lim 2014, Hoffmann 1990). Our data supports this claim, both in demonstrating that *Oaβ1R* acts to increase neuronal sensitivity via cAMP-mediated modulation of K^+ channels, and by establishing that flies lacking *Oaβ1R*-expressing neurons exhibit behavioral deficits similar to that of flies who cannot sense sucrose (Lim 2014, Matsuda 2015). Furthermore, flies lacking only the *Oaβ1R* receptor partially recapitulate the phenotype, displaying a significant decrease in wing threats and courtship song, behaviors that are thought to be more energetically taxing (Malamud 1988, Mental 2003). As starvation and intense physical activity are both known to increase the level of octopamine present in the hemolymph in several invertebrate species (Goosey 1982, Bellah 1984, Sombati 1984), our findings suggest a means by which the internal state of an organism may influence the perception of the external environment, and how such a shift in perception may influence behavior.

As the manipulation or ablation of large populations of neurons frequently yields changes in multiple different behavioral phenotypes, the segregation of *Oaβ1R*-expressing neurons into tarsal and labellar subpopulations is necessary in order to accurately define the role of the circuitry involved. Our data demonstrates that the labellar subpopulation exerts a significant effect on the period of time required

before the initiation of aggressive or courtship behavior. As postsynaptic targets have not yet been identified for peripheral *Oaβ1R*-expressing neurons, it remains to be seen whether the circuits involved in octopamine mediated sugar sensation interact with other known olfactory or gustatory networks to influence behavioral outcomes, act independently to modify the flies internal state and subsequently behavior, or operate by a yet unknown mechanism. Taken together, our experiments suggest that octopamine neuromodulation of sugar sensing neurons in the periphery is important for the generation of energetically expensive social behavior and offers insights into how octopaminergic signaling serves to regulate social behavior in the context of environmental sources of food.

As of this time, many of the internal and external signals that contribute to the generation of social behavior have been identified and we are beginning to understand how pheromones, hormones, neurons, circuits and genes all contribute to animal behavior. Yet, the means by which each signal contributes to the final behavior produced or how a signal may become more salient than others within a complex environment is just beginning to be understood. The data presented here suggests a new role for OA as a mediator of food resource saliency, and provides an important clue as to how different neural circuits and neuromodulators may work together to determine which behaviors are appropriate in different environmental contexts. While it has been known for some time that octopaminergic neurons are capable of affecting distant targets via hormonal volume transmission, the neurons we have identified with our *Oaβ1R-Gal4* line demonstrate that long-range neurotransmission is an important player in the regulation of stereotyped social behavior. These observations hint at a more complicated regulatory role for octopaminergic signaling, both in the regulation and direct transmission of external signals indicative of environmental resources.

As previous exploration of OA signaling has identified several key behavioral changes associated with the loss of OA production, our current findings imply that the behavioral deficits observed in these flies may be due to a perturbation in the

equilibrium of a complex system of signaling that encompasses both metabolic regulation and sensory input. In order to fully map OA's influence on *Drosophila* behavior, it will be necessary to identify the postsynaptic targets of peripheral *Oaβ1R*-expressing neurons and determine if these circuits act directly, or in parallel, with the known networks directing courtship and aggression. The identification of these neurons also provides for an opportunity to explore how internal signals attenuate the perception of the environmental stimuli, and how these changes in signaling influence the production of behavior.

Future directions

As of the time of this writing, my work poses two major questions. First, what is the direct link between aggressive behavior and metabolic signaling? Performing further electrophysiological experiments on fed and starving flies or flies post aggressive bout, will allow us to address this question by evaluating if the shifts in levels of OA due to lack of food or the presence of conspecifics is sufficient to cause increased neuron sensitization (Admo 1995). Likewise, further evaluation of feeding behavior will help us to determine if the increased sensitivity from OA exposure translates into additional food intake, or if the activation of these neurons is uncoupled from satiety.

Second, what synaptic connections are made by Oaβ1R neurons? Currently, we do not know what neurons are downstream from Oaβ1R-expressing neurons, and discovering what signaling partners interact with the populations identified in this dissertation would allow for a more complete understanding of OA acts as a mediator between external information and internal state. Three possible targets within the brain are neurons expressing *Drosophila* insulin like peptide, neurons expressing tachykinin, and neurons ion transport peptide, as each of these neurons have been implicated in *Drosophila* metabolism, aggression or both (Kahsai 2010). Using the GRASP technique discussed in chapter two, it should be possible to screen for connections between Oaβ1R neurons and these potential targets.

Material and methods

The following strains were used as part of this study: *Oaβ1R-Gal4, 20x-UAS-6xGFP, lexAop-20xRFP* (gifts from Steve Stowers)*, UAS-DTI* (obtained from Leslie Stevens), *UAS-GcAMP6f (BL64204), UAS-EPAC1-cAMPS* (BL25407)*, Gr64B-LexA, Gr64C-LexA, Gr64E-LexA, Gr64F-LexA, Gr5a-LexA (*all *Gr64* and *Gr5a* lines were provided by Hubert Amrein), *Ppk28-lexA* (Provided by Robert Thistle), *UAS-PkA-R1-BDK* (BL 35550), *UAS-Dicer2* (BL 24648), *UAS-OaB1R^{IR}, Df(3R)BSC685* (BL 26537), *UAS-TnT (*BL28996)*, tsh-Gal80* (provided by Julie Simpson) and the Canton-S strain from the Bloomington Stock Center, Bloomington, IN.

Generation of transgenic lines

Our *Oaβ1R-Gal4* line was created via P-element insertion as detailed elsewhere (Dahmann 2008) using the following primers: oa2(B1R)-5': GGGCGCAAGAACATAAGAGC and oa2(B1R)-3': CGTTGACAAGCTGATGGCTA.

Imaging of legs and probosci

Whole adult male and female flies were washed in 70% ethyl alcohol for 1 minute. Following the wash, flies were rinsed three times in phosphate buffered saline for 15 minutes. Flies were then placed in 4% paraformaldehyde, and the animals legs and head were removed. The legs and heads were allowed to fix overnight in paraformaldehyde, and the proboscis was removed from each head the following morning. The fixed appendages were then washed 5 times for 10 minutes per wash in phosphate buffered saline, and subsequently mounted for imaging. Images were collected on an Olympus Fluoview FV1000 laser scanning confocal mounted on an inverted IX81 microscope and processed using ImageJ (NIH) and Adobe Photoshop (Adobe, CA).

Rearing Conditions

All fly strains were reared on standard fly food (medium containing agar, glucose, sucrose, yeast, cornmeal, propionic acid, and Tegosept). Flies were grown in temperature- and humidity-controlled incubators (25°C, 50% humidity) on a 12-h

light/dark cycle. To collect socially naïve adults, pupae were isolated in individual 16 x 100-mm glass vials containing 1.5 ml of food medium. Upon eclosion, flies were anesthetized with CO_2, painted on the thorax with acrylic paint for identification and returned to their isolation vials to allow for recovery from anesthesia a full 24 hours before testing.

Aggression and Courtship Paradigms

Aggression assays were performed in individual chambers of 12-well polystyrene plates containing a food cup in the center (Fernandez 2010). 4-5 day old males were transferred in pairs to assay chambers by aspiration. Experiments were performed at 25 °C in a humidity controlled room (50%). Fights were videotaped for 90 minutes and lunges counted for 30 minutes from the first lunge unless otherwise specified. The time between introduction into the chamber and the onset of aggression (first lunge) was defined as the fighting latency. Lunging behavior was determined as previously described (Chen 2002). Courtship assays were performed in a 12 well polystyrene plate (VWR #82050-930) with one Canton S virgin female (aged 7-10 days) and one 3-5 day old male. The period between introduction into the courtship chamber and the first male wing extension (singing) was defined as courtship latency.

Epac1-CaMPS Imaging

Live proboscis preparations were made by anesthetizing a fly on ice followed by separation of the head within a 1 mL well for electrophysiology filled with 400 µL of oxygentated HL3 solution. The head was then mounted onto a glass pipette, so that the tip of the pipette extended into the proboscis. Each preparation was then secured to the electrophysiology well, and allowed to equilibrate for 5 minutes. Octopamine (Sigma-Aldrich #770-05-8 Lot #BCBK4366V) dissolved in oxygentated HL3 solution was administered via syringe into the rear of the glass pipette. Administration of octopamine occurred after a minimum of 1 minute of imaging. Analysis of F/F values in regions of interest was calculated using Fiji and Prism 6.0.

Image analysis

Epifluorescence images were acquired at a rate of 1 image / 1.7 seconds by an Olympus Fluoview FV1000 laser scanning confocal mounted on an inverted IX81 microscope. Acquired images were registered (StackReg plugin, Fiji software) and regions of interest were selected within the proboscis. Image processing and analysis was accomplished with ImageJ version 1.44 / Fiji version 1.43. Image subtraction was performed in Fiji using the image calculator for both YFP and CFP channgels. Intensity tables were exported to excel and (Δ F – F) / F calculated for each series of images, using corrections based upon the fluorescence of a *Oaβ1R-Gal4 ; UAS-ECFP* and *Oaβ1R-Gal4 ; UAS-EYFP* cross. Traces were generated in Prism 6.0.

Electrophysiology

Live preparations were made according to slight modifications of a previously described protocol (Delventhal 2014). HL3.0 solutions were prepared both with and without the addition of sugars, and used to fill stimulating and recording pipettes as required by the experiment. The following substances were used: Octopamine (Sigma-Aldrich #770-05-8, Lot #BCBK4366V) in 5mM and 10mM concentrations, and tetraethylammonium chloride (Sigma-Aldrich #55-34-8, Lot 127H0925) in 5mM and 10mM concentrations and were added to the recording electrode as required by the experiment. Recordings were made at 1000 data points per second and a total of 3 recordings were performed per experiment. For analysis, 30 seconds of recorded data was analyzed from the time of sugar exposure, and frequency averages made from each 30 second period. Recordings were performed via labchart 8.0, and analysis was performed in prisim 6.0

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