UNIVERSITY OF CALIFORNIA, SAN DIEGO

Propagation and modulation of activity in early olfactory processing and its relevance to odor-driven behavior

A dissertation submitted in partial satisfaction of the
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in

Biology

by

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The Dissertation of Cory Matthew Root is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

Chair

University of California, San Diego

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EPIGRAPH

The Astonishing Hypothesis is that "You," your joys and your sorrows, your memories and your ambitions, your sense of personal identity and free will, are in fact no more than the behavior of a vast assembly of nerve cells and their associated molecules."

Francis Crick

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Chapter 2, in part, was published in PNAS in 2007, under the title "Propagation of olfactory information in *Drosophila*". The dissertation author and Julie Semmelhack contributed equally to this paper as primary authors. Allan Wong, Jorge Flores, and Jing Wang were co-authors.

Chapter 4, in part and with modifications, was published in Neuron in 2008, under the title "Gain control by presynaptic inhibition fine tunes olfactory behavior". The dissertation author was the primary author of this paper with Kaoru Masuyama, David S. Green, Lina E. Enell, Dick R. Nässel, Chi-Hon Lee, and Jing Wang as co-authors.

Chapter 5, in part, was published in PNAS in 2009, under the title "Presynaptic peptidergic modulation of olfactory receptor neurons in *Drosophila.*" The dissertation

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Chapter 6, is in preparation for publication, under the title "Presynaptic facilitation by neuropeptide signaling mediates odor-driven food search." The dissertation author is the primary author of this paper with Kang I. Ko, Amir Jafari, and Jing Wang as co-authors.

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ABSTRACT OF THE DISSERTATION

Propagation and modulation of activity in early olfactory processing and its relevance to odor-driven behavior

by

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Doctor of Philosophy

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Professor Jing W. Wang, Chair

The olfactory system has evolved over hundreds of millions of years to perform odor recognition and discrimination across a large range of odor concentrations. One problem that is not well understood is how activity propagates and is modulated at the first synaptic transformation. The first olfactory relay of most organisms receives input from odorant receptor neurons (ORNs), whereby ORNs expressing a given odorant receptor send axons to a specific stereotyped glomerulus. ORNs synapse onto second order neurons that propagate olfactory information to higher brain areas. A hallmark of the first olfactory relay, is the presence of GABAergic local interneurons as well as a

number of neuromodulators. We have therefore investigated how different components of the early olfactory circuit contribute to the olfactory representation and odor-driven behavior in *Drosophila*.

We have asked three primary questions. 1) Is there lateral excitation between glomeruli? Using receptor gene mutations to silence ORN input to a given glomerulus, we observed that the projection neurons (PNs) of the same glomerulus have dramatically reduced odor-evoked action potentials. Thus, ORNs are the main drivers of PNs and lateral excitation is minor and potentially a modulatory mechanism. 2) How is gain control achieved in the antennal lobe? We found that the $GABA_B$ receptor is expressed in the presynaptic terminal of ORNs and mediates a feedback gain control of the early olfactory circuit. This gain control is important for pheromone-mediated mate localization. 3) Does internal state shape olfactory processing? We have found that starvation alters olfactory representation by upregulation of a neuropeptide receptor in select ORNs, which mediates starvation-dependent presynaptic facilitation. This neuropeptide signaling is important for starvation-dependent food search behavior. Together, these findings reveal how information propagates through the first olfactory relay and how it can be modulated to enhance odor-driven behavior.

Chapter 1: Introduction

1.1 Historical perspective on a journey to understand the mind

What is the nature of nature? The quest to understand the fabric of our existence has been a unifying desire from the ancient philosophers of antiquity to the pioneers of the scientific revolution to modern molecular biologists. Indeed, we've come a long way in this quest. Early philosophical descriptions of the natural world lacked experimentation, however, it is widely believed that the writings of Aristotle and Plato during the 4th century BC laid a foundation for deductive reasoning[1]. The seventeenth century is marked as the beginning of a scientific revolution with physicists and chemists such as Newton, Galileo, Boyle and Pascal who are largely credited with establishing a mathematical approach to characterizing aspects of natural world[1]. During the nineteenth and twentieth centuries an explosion of knowledge about the biological world took place with Darwin's theory of evolution, the discovery of DNA and the advent of molecular and cellular biology. One of the great remaining mysteries of the natural world is our own behavior. We are biological creatures formed from a single cell following a molecular developmental program, yet, we become self-aware and capable of emotions and thought. Great strides have been made towards understanding how neural circuits produce behavior, but we are quite far from a full understanding of cognition. Below I describe some key findings in our quest to understand the biological underpinnings of our thoughts and behavior.

The first steps toward a true understanding of our nervous system began in the seventeenth century when it became widely accepted that the brain was the substrate of emotions, perception and thought[2]. A century later, in 1791, Luigi Galvani found that electrical stimulation of a frog muscle caused it to twitch, provoking the idea that

electricity is an important component of the nervous system. Nearly sixty years later, Hermann Von Helmholtz recorded a nerve impulse traveling along the frog sciatic nerve. Subsequently, his student, Jilius Bernstein, measured the electrical potential of a cell and hypothesized that electricity flows by the opening of a gate. And in the early 1900s, Henery Dale and Otto Loewi discovered the neurotranmitter acetylcholine, revealing that a diffusible chemical can open the electrical flow (for review[3,4]).

According to Kandel and Squire, the emergence of cellular and molecular neuroscience began with two pivotal advances: the neuron doctrine of Ramon y Cajal (1906) and the ionic hypothesis from Hodgkin and Huxley (1940s)[4]. In 1839, Theodor Schwann proposed the cell theory, that the entire body is made of individual cells, which was widely accepted for all tissue and organ types except the brain[2]. This was largely due to an inability to clearly see cells in brain tissue until Cajal's use of the Golgi stain. The neuron doctrine established the idea that the brain is made up of discreet units, or neurons, rather than a continuous reticular net. Furthermore, Cajal proposed that information flow is unidirectional from one receiving pole to an output pole, and with Sherrington, he proposed that communication between neurons occurs through a discreet synapse. Studying the ionic currents in the squid giant axon, Hodgkin and Huxley, proposed the ionic hypothesis that could explain a cell's resting potential in terms of potassium ions and the action potential in terms of sodium influx followed by potassium efflux. "The ionic hypothesis unified a large body of descriptive data and offered the first realistic promise that the nervous system could be understood in terms of physiochemical principles common to all of cell biology." [4]

During the later half of the twentieth century fundamental insights occurred in our further understanding of neurophysiology (for review[3,4]). In the 1960's Hille and Armstrong demonstrated that ions flow through selective pores, or channels, in the cell membrane. In 1976, Neher and Sakmann developed the patch-clamp technique that revealed ion flow occurs when single channels transition from closed to open states. Katz and Fatt discovered that neurotransmitter receptors were ion channels, and they revealed that neurotransmitters are released in defined quantal events. Later, Llinas found that calcium controls the release of synaptic vesicles. In the 1980's voltage gated sodium, potassium, calcium, and various ligand-gated channels were cloned. The discovery of metabotropic receptors revealed that neurotransmitters can alter properties of neurons by second messenger signaling, and strikingly, these seven transmembrane domain receptors are used as the primary sensors for sight, taste and smell. By the turn of the century, nearly 100 different chemical transmitters and their receptors had been identified.

Advances in cellular and molecular physiology provided important insight into the function of neurons, however, before one can fathom understanding how the nervous system processes sensory input to produce behavioral output, one must investigate how neurons participate in neural circuits. Traditionally, approaches to understanding neural circuits fall into two general categories with different advantages[3]: Holistic, or top-down, approaches focus on activity of higher order neurons and attempt to relate their activity to a sensory stimulus and behavioral task of an animal. The advantage is that one can extract correlations between the activity of particular brain regions and particular stimuli or behavioral action; however, a disadvantage is that one learns little about the circuit driving the activity in those neurons. Reductionist, or bottom-up, approaches attempt to

analyze the nervous system in elementary components often starting from the periphery. The advantage is that one can understand how neurons participate in a circuit to shape sensory representation; however, a disadvantage is that tracking the flow of information beyond the periphery has been a daunting task.

One classic example of the top-down approach is the work of Wilder Penfield who in the 1950s established the legendary motor and sensory homunculi – stylized cartoons of the of the body surface with the relative prominence of different parts reflecting the amount of cortical area dedicated to those parts[5]. To generate these maps, Penfield stimulated the exposed cortex of epilepsy patients prepared for surgery. By stimulating and asking what the patient experienced, he was able to infer which cortical areas of S1 and M1 represent which parts of the body. Another classic example of a holistic approach is the experiments of David Hubel and Torsten Wiesel, which revealed the receptive field properties of cortical neurons in V1. By recording from random neurons in area V1 of the anesthetized cat, they observed that neurons had receptive fields corresponding to areas of the visual space, and more importantly, that they were tuned to moving bars of specific orientations. A more recent example of this approach is the work of Bill Newsome and colleagues in cortical area MT of the monkey. In elegant work, they found that the firing rate of neurons in MT correlated with the ability of the monkey to discriminate direction of motion for a visual stimulus. Furthermore, local destruction eliminated the monkey's ability to perceive motion, and local stimulation of clusters of neurons sensitive to a specific direction was able to bias the monkeys perception of motion[3]. All three examples reveal how a top-down approach can provide important insights into the feature detection and function of different brain areas.

One classic success story of the reductionism approach is that used by Eric Kandel in an attempt understand memory storage. In the beginning, Kandel thought it would be best to tackle the problem in a complex and interesting form, but soon realized that the mammalian hippocampus was too complicated to make progress in any reasonable amount of time[6]. Rather, Kandel proposed that one should study the simplest instances of memory storage and he turned to the sea slug, *Aplysia*, because of the simplicity and accessibility of its nervous system. With this new model organism, Kandel was able to demonstrate that with only a few neurons, the animal's behavior could be modified by experience. The neurons involved have cell bodies nearly 1 mm in diameter and could produce access to enough cytoplasm for molecular studies. With this approach, he was able to determine the biochemical signaling pathways involved in long-term sensitization of the gill-withdrawal reflex – most of these signaling molecules have since been found to be utilized in higher organisms. A modern example of the bottom-up approach is that of Charles Zuker and Nicholas Ryba in an effort to understand the logic of the taste system[7]. Their approach has been to ask what are the molecular sensors of taste and what cells in the tongue express them. Using this technique they have identified the receptors and cells responsible for the perception of sweet, sour, bitter, salty and umami. By genetically swapping different kinds of receptors in different taste cells, they were able to demonstrate that activation of particular taste cells generates the perception of flavor in a labeled line manner. Thus, the reductionism approach allows one to extract fundamental principles that can be applied to larger systems or set the frame work for moving deeper into the brain.

Another approach to understanding how neural circuits produce behavior is that of neurothology. A central dogma of neuroethology is that nervous systems have evolved not as general information transmitters, but as highly tuned circuits that extract specific features of environmental stimuli unique to the survival of a given organism[8]. For instance, in a crab's attempt to avoid looming predators from above, its visual system likely extracts different features of the environment than that of a cat chasing a mouse. Investigating how the visual systems of either of these animals responds to artificial stimuli, such as spots of light or moving gradients, could inform us about properties of the neural circuit, but these properties may not be relevant to the ecological function of the sensory system. Indeed studies have observed that natural stimuli trigger different patterns of activity than do artificial stimuli[9,10]. Perhaps the best example of this is from recordings in H1, the motion detecting neuron of the blow fly[11]. In these experiments a fly was mounted on a freely rotating pin such that the fly could be rotated at controlled speeds while recording from H1. Lewen and colleagues recorded from the same neuron in the forest where the fly was captured and in the laboratory with artificial stimuli mimicking that of the natural world. Strikingly, they found that in the forest the tuning curve of the neuron's response to rotational speeds was shifted by an order of magnitude, revealing that some aspect of the natural scene alters the firing of these visual neurons.

Another point made by neuroethology is that a comparative approach to understanding circuit function can inform us of nature's diverse ways to build a circuit capable of extracting particular features. Many neuroethologists prefer to study invertebrates because there is considerable diversity of uniquely evolved behaviors controlled by nervous systems that are numerically simple and accessible for

experimentation. Because the nervous systems are reduced, in many cases it is possible to determine the function of each neuron in the circuit for a specialized behavior[12]. An elegant example of the explanatory power of a simple invertebrate nervous system comes from an analysis of the leech sensory-motor transformation in the local bend response to body wall touch[13,14]. Lewis and Kristan revealed that four sensory neurons, P cells, are able to capture the Cartesian coordinates in the cross-section of the leech body wall. Briefly, two of the four P cells fire proportional to the cosine and sine of the angel between each neurons maximal receptive field. The local bend away from the touch stimulus is produced by a series of 25-30 interneurons each able to produce bends at different angles. The synaptic weights of P cells onto these interneurons are proportional to the appropriate cosines and sines of the bend angle. Thus, proportional activation of interneurons that produce bends of specified angles can generate the reflexive bend at the appropriate body wall location. Such a beautiful understanding of a sensory-motor transformation would have been nearly impossible in a more complicated organism such as a mouse.

In the spirit of reductionism and neuroethology, we reason that the study of olfaction in the fruit fly, *Drosophila*, should be a powerful model for elucidating how sensory input elicits behavior. Chemosensation is perhaps one of the most important senses to a fly in its natural environment because olfaction play an indispensible role in the search for food and mating partners[15]. In addition, *Drosophila* offers a simple neural circuit that is amenable to genetic dissection through the use of optical imaging paired with molecular manipulations and quantitative behavioral analysis. A similar strategy is being used in the mouse, however the ability to genetically define subsets of neurons is

not as exquisite and the number of neurons participating in a behavior is far greater. This emerging strategy of coupling optical imaging in defined populations of neurons with molecular dissection of circuit function and behavior, represents a powerful new approach to the study of neural activity underlying behavior[16]. Rather than simply observing correlations in ensemble activity, we now have the tools to make testable hypotheses and demonstrate causal links between neural activity and behavior.

1.2 The olfactory system

The sense of smell is one of the most ancient of the senses, and the organization of the olfactory system is highly conserved from flies to humans[17]. For most animals in their natural environment, the sense of smell is critical to their ability to find food and mating partners, as well as avoid predators. Thus, this sensory modality should elicit robust behavioral responses and provide a good model to study the neural circuit underlying behavior. Furthermore, the highly conserved anatomy should allow establishment of fundamental principles in simple organisms that are transferable to more complex nervous systems.

The basic logic of the olfactory system remained a mystery for a number of years because there did not appear to be a spatial gradient of odorant representation. For instance, in the visual and somatosensory systems there is a spatial organization reflecting the position in visual or body space. The olfactory system is not organized such that similar molecular structures activate nearby areas of the olfactory epithelium or bulb. In the olfactory bulb, the spatial organization appears to be by combinatorial code, such that different odors activate partially overlapping patterns of glomeruli. The first

examples of a glomerular map were generated by 2-deoxyglucose autoradiography of odor-evoked activity in the rat olfactory bulb[18,19]. In these studies, odors activated distinct but overlapping sets of glomeruli and increased odor concentration resulted in an increased number of activated glomeruli. Thus, it was suggested that odor identity and concentration are encoded in the spatial pattern of activity.

A paradigm shift occurred with the discovery of the multigene family of odorant receptors[20], which opened the door to a molecular genetic dissection of the olfactory system. Probing the olfactory bulb for odorant receptor mRNA revealed that individual receptor probes labeled single glomeruli[21], indicating that odorant receptor neurons expressing the same receptor gene send axons to discrete glomeruli. This was further demonstrated with transgenic mice that express a marker under the promoter of specific receptor genes[22]. Thus, it can be concluded that a pattern of activated glomeruli reflects the types of odorant receptors that are activated by a given odorant. Functional imaging experiments in mice have demonstrated that individual glomeruli respond to multiple odorants and a given odorant activates multiple glomeruli[23-25].

If odor identity is encoded by the combination of activated glomeruli, the activity of different glomeruli must be integrated somewhere in the olfactory circuit. In mouse, one possible site of integration is the periform cortex, where mitral/tufted cells, the output neurons of the olfactory bulb, synapse onto pyramidal neurons. This idea was tested using the *Arc* catFISH (compartment analysis of temporal activity by fluorescence *in situ* hybridization) technique to compare the response of cortical neurons to individual components of binary odorant mixtures[26]. Zou and Buck found that many neurons responded to the binary mixture but not the individual components indicating that these

neurons get input from multiple glomeruli and could encode the combination of glomeruli. Recently, functional imaging experiments in the periform cortex revealed a highly distributed organization in which different odorants activate unique but dispersed ensembles of cortical neurons without any apparent spatial preference[27]. Furthermore, neurons in piriform cortex, responsive to a given odorant, also exhibit discontinuous receptive fields unlike the somatosensory cortex.

Despite the progress of understanding the olfactory system in mice, establishing a causal link between elements of the olfactory circuit and behavior has been a challenge. For this reason, a number of researchers have turned to the fly to probe basic principles about circuit function and behavior. The *Drosophila* olfactory system is a powerful model because of the well-characterized stereotypic structure and plethora of genetic tools available to dissect circuit function. The antenna and maxillary palp contain primary olfactory receptor neurons (ORNs) that typically express one type of odorant receptor in addition to the non-canonical Or83b[28,29]. ORNs expressing the same odorant receptor gene project axons to one of 43 glomeruli in the antennal lobe where they synapse onto glomerulus-specific projection neurons (PNs)[30-34]. In addition to the direct ORN excitation onto PNs, there is also a dense network of multi-glomerular excitatory and inhibitory interneurons (LNs) whose role in olfactory processing remains elusive[35,36]. PNs propagate olfactory information to third order neurons in the mushroom body and lateral horn for further processing[37-40]. Individual elements of this circuit can be identified and manipulated with neuron specific *Gal4* lines to elucidate the flow of information.

Significant advances have occurred toward establishing a link between the olfactory circuit and behavior in *Drosophila*. For instance, the identification of the neural

circuit responsible for detecting the aversive odorant, CO_2. Its ecological role is not entirely clear, but CO_2 is known to be released from unripe fruit and may be important for signaling the richness of a food source[41]. Consistent with this, it was recently found that other odors of ripe fruit block detection of CO_2 to prevent aversion[42]. In addition, flies emit CO_2 in response to stress and are strongly repelled by it. Using two-photon calcium imaging in the drosophila antennal lobe, it was discovered that CO_2 activates only the V glomerulus. Furthermore, silencing the ORNs that project to the V glomerulus eliminates CO_2 avoidance[43], and synthetic activation by channelrhodopsin is sufficient to trigger avoidance behavior[44]. Thus activation of the V glomerulus mediates CO_2 avoidance. The full circuit from sensory input to behavioral output remains to be determined, but the tools are now available for such experiments.

Another key set of findings towards elucidating neural circuits underlying behavior is that of the fly pheromone system. The male cuticular hydrocarbon, cis-vacenyl acetate (cVA) inhibits male-male courtship and increases female receptivity to male courtship. This is due to the activation of the Or67d neurons that project to the DA1 glomerulus[45]. Suppression of these neurons eliminates the pheromone effects on courtship behavior[46,47]. This poses the question of how input to the same olfactory glomerulus in males and females produces sexually dimorphic behaviors (courtship inhibition vs. receptivity). Recent work from the Axel lab investigated where in the neural circuit sexual dimorphism exists[48]. They observed no difference in the cVA-evoked activity of the DA1 PNs. However, when they examined projections of these PNs to protocerebrum, they observed male specific axon branches in the lateral horn. Strikingly, they found that the male specific arborization requires the transcription factor

Fru^{M}, which is well characterized as a molecular switch between male and female courtship behavior[49,50].

It is thought that the pattern of activated glomeruli conveys information about the identity of an odorant, but directly testing this hypothesis has not been possible in other organisms. In a recent set of elegant experiments in *Drosophila*, Julie Semmelhack and Jing Wang performed a molecular deconstruction of the odor-evoked pattern of glomeruli responsible for innate attraction to an ecologically relevant food odor[51]. They found that apple cider vinegar is robustly attractive to flies and that the odor activates five glomeruli in the antennal lobe. Using molecular manipulations they were able to selectively remove individual glomeruli from the pattern to ask which glomeruli are necessary for the attraction. In addition, they removed all glomeruli and selectively restored function in single glomeruli to ask if any glomerulus is sufficient for the behavior. Indeed they found that the DM1 glomerulus is necessary and sufficient. Strikingly, they found that increased concentration of cider vinegar made the odor repulsive to flies and this was due to the recruitment of the DM5 glomerulus that is necessary and sufficient to mediate aversion. Thus, particular glomeruli that make up odor-evoked patterns appear to be hard-wired for attraction and aversion.

Another challenge in the study of neural circuits has been monitoring the propagation of activity between layers of sensory systems. For instance, what is the role of inhibition in the first olfactory relay? Studies have investigated lateral inhibition[52-54], context-dependent olfactory response[55], network oscillation[56], and synchronous firing for fine odor discrimination[57,58], but it remains a challenge in most organisms to determine a causal link between synaptic modulation in a specific population of neurons and its

function for the neural circuit and behavior. Recent work in *Drosophila* observed subthreshold oscillations in the antennal lobe that were phase locked to the local field potential. Using genetic drivers for different populations of antennal lobe LNs, Stopfer and colleagues were able to determine that one class of LNs, with distinct morphology, produced this oscillatory activity[59]. Circuit level experiments such as this, when coupled with behavioral tests, should provide a powerful framework for testing hypotheses about the function of synaptic modulation.

In the research presented in this thesis, we use two-photon imagining of genetically expressed probes and electrophysiology coupled with molecular manipulations to investigate the propagation of information in the first olfactory relay and odor-guided behavior. We investigate how gain modulation is achieved in the antennal lobe and find that presynaptic inhibition of ORN input is important for odor-guided behavior. Lastly, we investigate synaptic modulation by internal state. We reveal a neuropeptide mechanism for starvation-dependent presynaptic facilitation of ORN input that mediates a starvation-dependent food search behavior.

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Chapter 2: Propagation of Olfactory Information at the First Relay

2.1 Abstract

Investigating how information propagates between layers in the olfactory system is an important step toward understanding neural circuits. The second order projection neurons (PNs) of the antennal lobe receive two sources of input: the ORNs of the same glomerulus and interneurons that innervate many glomeruli. In order to determine how these inputs interact to produce PN output, we used receptor gene mutations to silence the ORNs innervating a specific glomerulus, and recorded PN activity with two-photon calcium imaging and electrophysiology. In the absence of direct ORN input, PNs exhibit very few odor-evoked action potentials. We next investigated whether silencing ORN input to a cognate glomerulus affects the response of other PNs. We used receptor gene mutations, to silence ORNs that innervate particular glomeruli, and monitored glomerular calcium responses with two-photon microscopy. In the absence of input to single glomeruli, odor-evoked responses in other glomeruli are largely unchanged. Thus, ORNs of the same glomerulus are the main drivers of PN firing.

2.2 Introduction

Understanding how information propagates between hierarchical layers of the nervous system is a fundamental question in systems neuroscience. The stereotypic organization of the *Drosophila* olfactory system and the identification of the odorant receptor genes make the fly an attractive model system in which to study the successive processing of sensory information. *Drosophila* olfactory receptor neurons (ORNs) in the antennae detect odors and relay neural activity to the antennal lobe in the brain. An adult fly expresses about 50 odorant receptor genes[1-6]. In the antennal lobe, axons of ORNs

expressing the same receptor gene project with precision to spatially invariant glomeruli [5-7]. In addition, there is a rich and complex network of local interneurons (LNs)[8-12]. How LNs contribute to the propagation of information has been poorly understood.

There are two general models for the function of local interneurons in olfactory coding. In one scenario, the interglomerular connections serve to modulate the PN response to odors rather than drive activity. In this glomerular propagation model, the main source of PN excitation comes from the ORNs of the same glomerulus (its cognate ORNs). In another scenario, lateral and receptor inputs are two independent sources of strong excitatory input for each PN. A powerful lateral input would give rise to a distributed representation of olfactory information in PNs. Studies investigating this transformation have found apparently conflicting results. One study found that PNs were more broadly tuned than their cognate ORNs[13], implying that interglomerular connections in the antennal lobe activate PNs without ORN input. In contrast, another study found a strong correlation between activity of ORN axon terminal and PN dendrites of the same glomerulus[14].

In order to discriminate between these two models, it is necessary to dissect the relative contributions of ORNs and interneurons to PN output. We use OR mutant flies to remove ORN input and ask whether PNs receive lateral excitation in the absence of activity in their cognate ORNs. In addition, we ask how removal of input to one glomerulus affects activity in other glomeruli. We find that PNs lacking ORN input fire few or no action potentials and removal of input to one glomerulus has little or no effect on the activity in other glomeruli. These results are consistent with the model that direct

ORN input provides the main excitatory drive to PNs, while excitatory and inhibitory interneurons modulate PN output.

2.3 Recording from PNs in the absence of ORN input

Every glomerular output projection neuron (PN) receives two sources of synaptic input–the cognate olfactory receptor neurons (ORNs) of the same glomerulus and interneurons that innervate multiple glomeruli[15]. What is the main source of neural drive for projection neurons? We reasoned that measuring action potentials of the cognate PNs in mutant flies with silenced ORNs should allow us to address this question. If PN activity comes only from the cognate ORNs, it will be eliminated when the ORNs are silenced by receptor gene mutations. Conversely, if some PN activity derives from interglomerular connections, we should still see robust odor-evoked activity in the PNs as a result of the activation of other glomeruli. Genetic tools are available to perform this experiment in the VM2 glomerulus, which is innervated by ORNs that express the *Or43b* gene[5,6]. Elmore et al. have generated a targeted mutation of the *Or43b* gene[16]. Furthermore, an enhancer trap line that labels just the two VM2 PNs (NP5103) permits us to identify them in different samples. By recording from the labeled VM2 PNs in *Or43b* mutant flies, we can investigate PN firing properties in the absence of direct ORN input.

Recordings of PN action potentials can be obtained by several techniques, including whole cell patch clamp, intracellular recording with sharp electrodes, and loose-patch extracellular recording. It is difficult to target specific PNs with sharp electrode recording, and whole cell recording may alter the excitability of PNs because their electrical properties have not been well characterized. We therefore decided to use

the less invasive method of loose-patch recording. The VM2 PNs were identified with a fluorescent microscope in flies bearing the *NP5103-Gal4* and *UAS-GFP* transgenes. In these experiments, we used two odorants, isoamyl acetate and hexanol, each of which excites different sets of glomeruli in the antennal lobe. Each odorant was administered at low, medium and high concentrations to cover the dynamic range of the VM2 PNs. At low concentrations, these odorants activate a relatively sparse pattern of glomeruli, and as the concentration is increased, a larger number of glomeruli respond[14]. At medium and low concentrations, both isoamyl acetate and hexanol elicit a robust response in VM2 PNs of wild type flies. In contrast, at these concentrations, VM2 PNs in the *Or43b* mutant flies exhibited little or no detectable response to odor stimulation (Figure 2.1A). High concentrations of both odorants did elicit a small response (Figure 2.1B), however, the wild type response was five to six times greater than that of the mutant (Figure 2.1C). The dramatic difference in PN firing between wild type and mutant flies reveals that cognate receptor input makes a greater contribution to the PN firing than any interglomerular connections. However, the residual excitatory response in the absence of direct receptor input indicates that PNs can be excited via lateral interactions.

It is interesting to note that the PN response in wild type flies decreased at the high odor concentrations, particularly for isoamyl acetate (Figure 2.1D). This is consistent with the idea that a larger number of activated glomeruli may induce more lateral inhibition. Indeed, it has been shown that blocking GABA receptors increases PN firing in response to odors[10], demonstrating a role for lateral inhibition in the antennal lobe. The prevalence of lateral inhibition in these conditions raises the question of whether the inhibition may be masking the full extent of the lateral excitation. We thus

asked whether blocking inhibition would increase the residual excitatory response observed in the *Or43b* mutant flies. In the presence of picrotoxin and CGP54626, which block $GABA_A$ and $GABA_B$ receptors respectively, the PN response increased dramatically (Figure 2.1E). For isoamyl acetate, we observed an increase from an average of 4 spikes to 23 spikes in the first second (n=2), and from 5 ± 1 spikes to 23 ± 9 spikes (n=3) for hexanol, revealing a strong lateral excitatory connection. Thus, the small residual response observed in Figure 2.1B reflects a balance between opposing excitatory and inhibitory lateral interactions.

To rule out the possibility that the residual PN response in the mutant flies was due to ORN activation, it was important to directly record from the Or43b receptor neurons under the same conditions. By performing extracellular electrical recordings on single bristles, we verified that the Or43b ORNs of mutant flies did not respond to isoamyl acetate at the high concentration, while wild type Or43b ORNs responded robustly (Figure 2.2A-C). Thus, the residual response we observed in VM2 PNs of mutant flies does not derive from the cognate receptor neurons.

There is a possibility that synaptic efficacy in the VM2 glomerulus is enhanced in the *Or43b* mutant flies; for example, in the *Drosophila* neuromuscular junction, activity influences synapse function[17]. In order to measure synaptic function in the mutant flies, we measured calcium activity in PNs while electrically stimulating the olfactory nerve[14]. Two-photon microscopy was used to measure calcium activity in flies bearing *GH146-Gal4*[15] and *UAS-GCaMP*[14] transgenes, in which the calcium sensor G-CaMP[18] is expressed in most PNs. Calcium activity in the dendrites of the VM2 PNs of the *Or43b* mutant flies was indistinguishable from that of wild-type flies (Figure 2.2D-G),

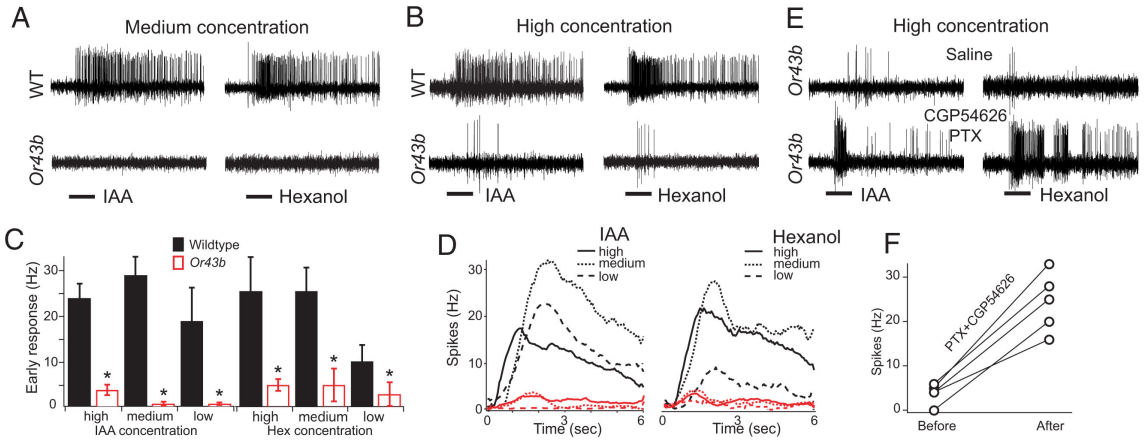

Figure 2.1. Cognate receptor neurons provide most of the input to the action potential firing of the glomerular output projection neurons.
(A-B) Representative traces of the VM2 PNs in response to isoamyl acetate and 1-hexanol, respectively, in the wild type and *Or43b* mutant flies at medium concentrations (A) and high concentrations (B). Odor application for duration of 1 second is indicated by the horizontal bars. (C) The number of action potentials in the first second of response. (D) Averaged instantaneous firing frequency of the VM2 PNs in response to isoamyl acetate and 1-hexanol in wild type (black traces) and *Or43b* mutant flies (red traces). (E) The effect of GABA receptor blockers on the odor evoked response in VM2 PNs from *Or43b* mutants. Representative traces for before (top) and after (bottom) addition of both picrotoxin (125 μM) and CGP54626 (25 μM). n=2 for isoamyl acetate, n=3 for hexanol. Isoamyl acetate, high = 1 μl/ml, medium = 50 nl/ml, low = 12 nl/ml. Hexanol, high = 5 μl/ml, medium = 1 μl/ml, low = 50 nl/ml. The medium concentration of isoamyl acetate in this experiment was ~8.8% saturated vapor concentration (SV), determined by a bioassay (see Methods). All data are presented as mean ± SD unless otherwise noted.

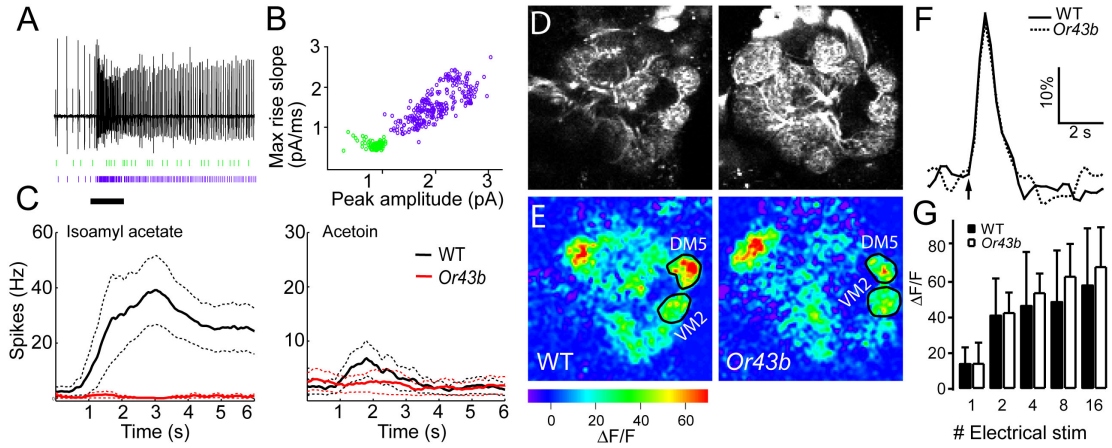

Figure 2.2. ***Or43b*** **mutation abolishes olfactory responses in the receptor neurons of the VM2 glomerulus without developmental defect in synaptic formation.**
(A-C) Extracellular recording was used to obtain action potentials from single small basiconic sensilla. Responsivity to acetoin was used to identify the ab8 bristles, each of which contains two receptor neurons. Or43b is expressed in the ab8A receptor neurons, which are located in the ab8 antennal bristle. Each ab8 bristle can be identified by morphological features and its response to acetoin[16,19,20]. (A) Representative trace from an ab8 bristle of a wild type fly in response to acetoin. Raster below shows responses of ab8A and ab8B separated by spike sorting analysis. Odor application for duration of 1 second is indicated by the horizontal bar. (B) Action potentials in the two neurons were separated based on spike amplitude and shape. Purple symbols denote ab8B and green symbols denote ab8A responses. (C) Averaged instantaneous firing frequency of the Or43b ORNs in response to isoamyl acetate (left) and acetoin (right) in wild-type (black traces) and *Or43b* mutant flies (red traces). Mean frequency was calculated in 100 ms bins and smoothed with a Gaussian filter. n=3 cells. (D-G) Electrical stimulation of the olfactory nerve. (D) Prestimulation images from wild type (left) and *Or43b* mutant (right) flies show glomerular structure. (E) Eight short electrical stimuli, 1 ms in duration in 10 V in amplitude, were delivered to the ipsilateral antennal nerve at frequency of 100 Hz. ΔF image of wild type (left) and *Or43b* mutant (right) flies at the peak response is shown in pseudocolor and reveals that the VM2 glomerulus was responsive to electrical stimulation. (F) Time course of ΔF/F in the VM2 glomerulus in response to eight electrical stimuli in wild type (solid line) and *Or43b* mutant (dashed line) flies. (G) Averaged peak amplitude of ΔF/F for the VM2 glomerulus in response to different number of electrical stimuli. n = 5. Error bars indicate standard deviation.

suggesting that a lack of odor-evoked activity does not change the efficacy of the synapse between the Or43b ORNs and the VM2 PNs.

2.4 Imaging PN activity in the absence of ORN input to one glomerulus

We next investigated whether silencing the Or43b ORNs affects the odor response of PNs that innervate other glomeruli. If the impact of lateral excitation is substantial, we should be able to detect a reduction in calcium activity in many glomeruli when input to one glomerulus is removed. We monitored PN dendritic calcium by imaging flies bearing *GH146-Gal4* and *UAS-GCaMP* with two-photon microscopy. This technique allows us to record activity in the entire antennal lobe. In wild type flies, 4-heptanol, 1-hexen-3-ol and isoamyl acetate each excite one, two and four glomeruli in this optical plane (Figure 2.3C, E and G). The VM2 glomerulus of the *Or43b* mutant flies did not show any detectable change of intracellular calcium in response to these three different odorants (Figure 2.3D, F and H). The non-cognate glomeruli (DL1, DM3, DM2, DC2) of this optical plane showed little or no reduction in odor response in the mutant flies compared to the wild type flies. The DM2 glomerulus showed a small reduction (19%, $p < 0.05$, t-test) in response to isoamyl acetate, suggesting that the Or43b ORNs may make a small contribution to the activity in non cognate PNs. Results from these imaging experiments corroborate those from electrical recordings from the VM2 PNs, suggesting that the cognate ORNs make a much greater contribution to the PN firing than any interglomerular connections.

Can these conclusions be expanded to other glomeruli? The DM1 glomerulus is innervated by ORNs that express the *Or42b* gene[5,6]. We used a fly line with a P-element insertion in the *Or42b* gene[16] to silence the ORNs that innervate the DM1 glomerulus.

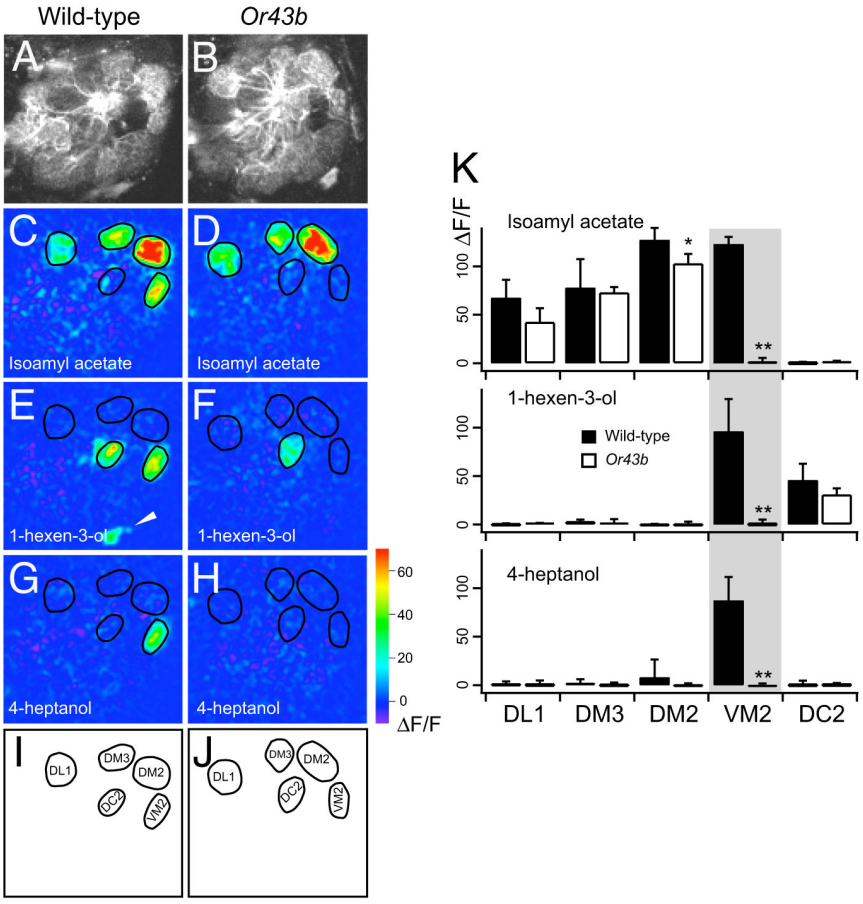

Figure 2.3. Silencing the cognate receptor neurons by the *Or43b* mutation eliminates odor-evoked calcium activity only in the VM2 PNs and has little or no effect on other glomeruli.

Brain preparations from a wild-type and an *Or43b* mutant fly were exposed to isoamyl acetate, 1-hexen-3-ol and 4-heptanol at 8% SV. (A-B) Prestimulation images show glomerular structure. The average of 10 frames before odor exposure is shown. (C-H) The glomerular responses to the three different odors are compared between the wild-type and the *Or43b* mutant (C, E, G versus D, F, and H). Arrow head points the cell body of a PN with dendrites in a different optical plane. (I-J) Specific glomeruli were identified anatomically using the established antennal lobe map (41). (K) Statistical analysis of glomerular response. The olfactory responses in five different glomeruli (DL1, DM3, DM2, VM2, DC2) were compared between the wild-type and the *Or43b* mutant. The mutant causes significant change of odor response in the VM2 and DM2 PNs (*, $p<0.05$; **, $p<0.01$). n=4.

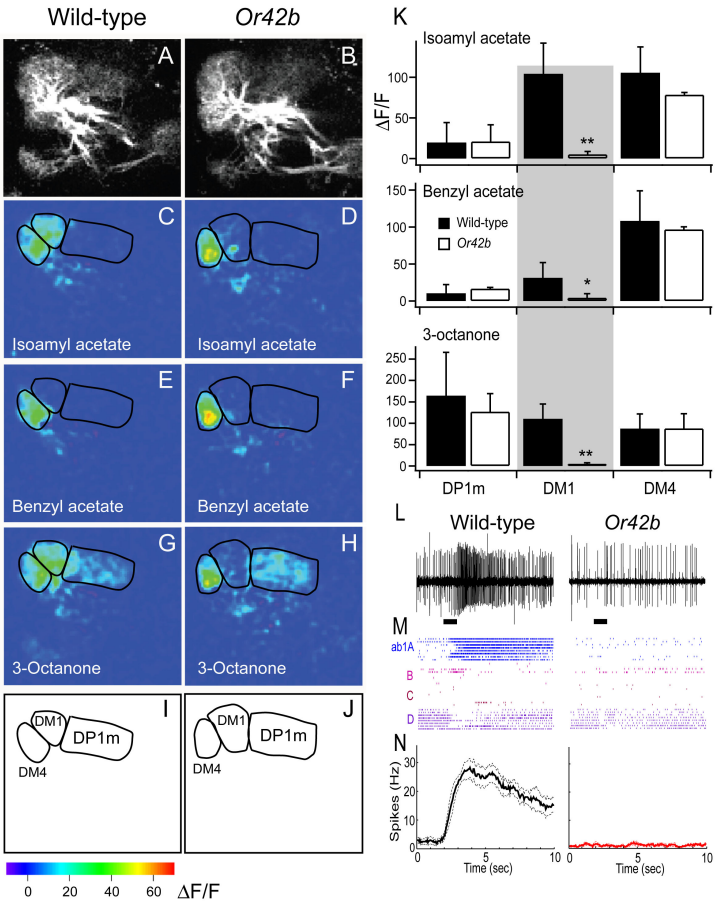

Figure 2.4. The *Or42b* mutation eliminates odor-evoked calcium activity in the cognate DM1 projection neurons.

Brain preparations from a wild-type and an *Or42b* mutant fly were exposed to isoamyl acetate, benzyl acetate and 3-octanone at 8% SV. Flies bearing the GH146-Gal4 and UAS-GCaMP transgenes express G-CaMP in many PNs, allowing us to visualize olfactory response in the glomerular output PNs. The dendritic calcium activity of the two specimens at similar optical planes is shown. (A-B) Prestimulation images show glomerular structure. The average of 10 frames before odor exposure is shown. (C-H) The glomerular responses to the three different odors are compared between the wild-type and the *Or42b* mutant (C, E, G versus D, F, H). (K) Statistical analysis of glomerular response (*, $p<0.05$; **, $p<0.01$). ΔF/F values are presented as mean ± SD. n=6. (L) Extracellular recordings of action potentials from single large basiconic sensilla. Responsivity to methyl salicilate was used to identify ab1 bristles, each of which contains four ORNs. Representative traces for ab1 bristle from wild type (left) and *Or42b* mutant (right) flies. Odor application for 1 second is indicated by the horizontal bar. (M) Raster showing the response of the four ab1 ORNs separated by spike sorting analysis using amplitude and shape. The *Or42b* receptor is expressed in ab1A ORNs, indicated in blue (top raster). Raster includes two responses for each of four bristles. (N) Averaged instantaneous firing rate of the *Or42b* ORNs in response to isoamyl acetate in wild type and *Or42b* mutant flies.

Single sensillum recordings showed that the Or42b neurons did not respond to high concentrations of isoamyl acetate (Figure 2.4L-N). With calcium imaging, we found that silencing the Or42b ORNs dramatically affected the odor response of the cognate PNs and had little or no influence on the non-cognate PNs. In wild type flies, 3-octanone, benzyl acetate and isoamyl acetate each evoked different levels of calcium activity in the DM1, DM4 and DP1m glomeruli (Figure 2.4C, E, and G). The DM1 glomerulus in the *Or42b* mutant flies did not show any detectable response to these three different odorants (Figure 2.4D, F, and H). A quantitative analysis of 6 wild type and 6 mutant flies showed that the non-cognate glomeruli DM4 and DP1m did not show any significant difference in odor response between the mutant and wild-type flies. These results suggest that the Or42b ORNs are the main drivers of activity in the DM1 PNs, but make little or no contribution to non-cognate PNs.

2.5 Population Sparseness of Projection Neurons

Our earlier data suggest that PNs are no more broadly tuned than their cognate ORNs. Thus, given the sparse representation at the ORN level[13,20], we would expect a given odor to excite relatively few PNs. To test this hypothesis, we performed loose-patch recording on a random sample of projection neurons and analyzed the population sparseness. We expressed GFP in GH146 PNs and examined the electrical response to two different odorants by loose-patch recordings of 48 randomly chosen cells (Figure 2.5A). To quantify odor response we counted the number of spikes in the first second of the response (Figure 2.5B). A large population of cells had little or no response (<5 spikes) to either odor. The average number of spikes in response to 1-octen-3-ol was 9,

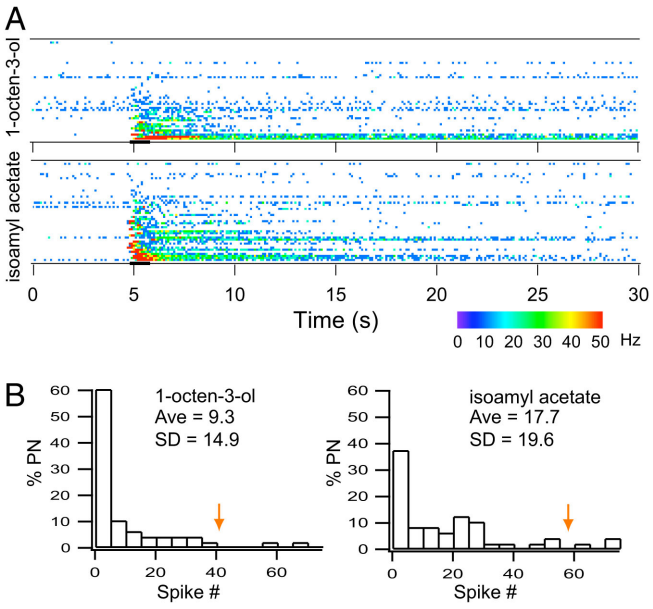

Figure 2.5. Random sampling reveals a sparse population of briskly responsive PNs. (A) Instantaneous firing rate shown in color scale for two different odorants. Bin size = 100 ms. Odorants were diluted in saline at 40 and 3.6 nl/ml for isoamyl acetate and 1-octen-3-ol, respectively, and delivered via pressure injection. Odor concentration was equivalent to 7% SV, determined by a bioassay (see Methods). (B) Histogram of spike frequency in the first second of odor response for 1-octen-3-ol (left) and isoamyl acetate (right). The arrow indicates two standard deviations above average.

while that for isoamyl acetate was 19. For both odors, only two or three cells had a firing rate more than two standard deviations above the average (>57 spikes for isoamyl acetate and >39 spikes for 1-octen-3-ol). If firing rate is important for olfactory coding, these briskly firing outliers may be the major carrier of olfactory information. We used the Treves-Rolls sparseness measure[21] to quantify the population distribution of PN responses. An index of 1 indicates that all neurons in the population respond equally, while an index near zero indicates only a single neuron responds; in other words, the response is extremely sparse. In the first second of the odorant response, the sparseness index for 1-octen-3-ol was lower than 0.19, and the sparseness index for isoamyl acetate was lower than 0.29. Isoamyl acetate excites more glomeruli than any other odorants in our collection[14], thus the sparseness index we obtained for this odorant probably represents the upper limit for the population sparseness. Given the fact that the ORN population response is sparse[20], if lateral excitation is a powerful driving force for PN activity, we should see a more distributed odor response in PNs. The sparse population response in PNs is consistent with our findings that ORN receptive fields, rather than lateral connections, are the main driving force for PN output.

2.6 Discussion

In this study, we examined propagation of olfactory information in the antennal lobe. We used two different receptor gene mutations to remove all receptor input to specific glomeruli. Silencing the ORNs of the VM2 glomerulus with the *Or43b* mutation allows us to investigate the relative contribution of the cognate receptor input to the PN output activity. Exciting many glomeruli and recording from PNs with no direct receptor

input revealed evidence of lateral excitation. However, the cognate receptor input makes a much greater contribution to the PN firing than any interglomerular connections. Furthermore, we found that removal of one glomerulus did not significantly alter the response of other glomeruli. Thus we conclude that ORNs drive PNs, consistent with the model of glomerular propagation.

Our data could seem to contradict a study suggesting that PN tuning curves are much broader than those of their cognate ORNs[13]. That conclusion was based on the finding that in some cases a large PN response can result even when the cognate ORN response is quite small. However, each glomerulus in *Drosophila* on average receives the axons of 30 ORNs and the dendrites of 3 PNs[11]. Thus, the PN output may reflect the pooling of all ORN inputs to that glomerulus, which may make each PN more sensitive to odor stimulation than any single ORN. Furthermore recent studies have found that some ORNs have an extremely high synaptic strength capable of producing much larger PN responses than ORN responses[22]. Nonetheless, in the absence of input PNs are only moderately activated.

We analyzed ensemble PN activity and found that odors are sparsely represented by the PN population. Our analysis shows that the responses of a few neurons are much greater than the average response. If these robustly responding neurons play an important role in representing olfactory information, the response is indeed very sparse. In contrast, Wilson and colleagues found a very broad PN response[13]. In their analysis, spike activity greater than two standard deviations above baseline is considered a positive response, which results in the conclusion that a given PN responds to 60% of all odors tested, and a given odor excites 69% of all PNs. Although it remains to be determined what type of

PN activity is detected by the third order neurons, the results from a recent paper[23] suggest that a high firing rate is required to mediate an olfactory behavior in *Drosophila*. Suh and colleagues found that there is a tight correlation between the level of ORN activity and the robustness of the CO_2 avoidance behavior. Further experiments addressing what type of PN activity is behaviorally relevant will be crucial to understand odor representation in the antennal lobe.

The results from a recent study have suggested that lateral excitation may play a major role in PN activity[9]. In a mutant fly with dramatically lower ORN activity, PNs showed a modest reduction in the odor response. The study by Shang and colleagues used synaptopHluorin to measure synaptic release from PN dendrites in the antennal lobe, which may not reflect PN output to higher brain centers. The relationship between PN local synaptic release in the antennal lobe and PN spike activity remains to be determined. Similarly, another paper investigating the role of lateral connections in the antennal lobe was published[24]. The findings of Olsen and colleagues are qualitatively similar to ours, in that PN output is dramatically reduced in the absence of direct ORN input. However, their study consistently showed a more robust PN response in both wild type and mutant flies. This can be attributed to several differences in recording conditions. First, we used the loose patch recording technique, while they used the whole cell patch method. Until the neuronal electrolyte composition is known, the more invasive whole cell recording method may alter the excitability of the cell. Second, differences in PN responsiveness may be due to different preparations; Olsen and colleagues used a whole fly preparation while we used an antennae-brain preparation that maintains only the olfactory sensory input. The antennae-brain preparation allows for

greater accessibility for optical and electrical recording, but the lack of non-olfactory sensory input and hormonal modulation may affect antennal lobe excitability. On the other hand, the stress associated with the dissection and immobilization required for the whole fly preparation may alter antennal lobe excitability as well. Third, for the electrical recordings we applied odorants in the liquid phase instead of gas phase, which may affect ORN responsiveness. Finally, differences in saline composition may also affect excitability; for example, Olsen and colleagues used 130 mM sodium, while we used 113 mM. Despite the fact that our experimental conditions lead to quantitative differences in numbers of spikes, these two studies both support the notion that cognate ORNs are the main source of PN activity, while lateral excitatory connections make a relatively small contribution to the response. Future experiments will be required to determine whether lateral activity is read by the third order neurons and contributes to behavioral output.

Implications for Olfactory Processing

Receptor activation by odorants results in spatial patterns of activity in the *Drosophila* antennal lobe, and constitutes the main driving force for PN excitation. These patterns are modulated by interglomerular interactions, resulting in PN action potentials that are ultimately conveyed to higher olfactory centers. This conceptual framework is reminiscent of the notion of drivers and modulators in the mammalian visual system[25,26]. Drivers are defined functionally as the transmitters of the receptive field, while modulators can alter the efficacy of the transmission, without altering its basic properties. Drivers typically project from one layer of a sensory system to another, whereas modulators are confined to a single layer. The anatomy of the antennal lobe and our

results suggest that the receptor neurons can be seen as the drivers of PNs, and the interneurons as the modulators.

The finding that PNs receive excitatory as well as inhibitory input from interneurons raises the question of what role these lateral interactions play in olfactory coding. Lateral inhibition has been proposed as a mechanism for olfactory processing, either by sharpening tuning[27,28] or generating synchrony[29,30]. One plausible function of lateral excitation – in concert with lateral inhibition – is to enhance PN synchrony. Synchronized activity could facilitate the readout of the combinatorial code by the third order neurons[31]. Another possible function could be to regulate PN sensitivity. Concurrent excitation and inhibition could be useful for fine-tuning neuronal responsiveness. Indeed, it has been demonstrated that injecting more balanced excitatory and inhibitory inputs can act to reduce the sensitivity of pyramidal neurons while a reduction in the amount of the balanced input can increase sensitivity[32]. It is possible that balanced inputs in the antennal lobe could have a similar role in modulating PN responsiveness, for example to allow these neurons to respond optimally to a wide range of odor concentrations. These balanced inputs could also mediate top-down modulation of PN sensitivity to reflect the behavioral state of the organism.

2.7 Methods

Experimental Animals and Preparations. The following transgenic lines were used: *UAS-GCaMP56*[14], *GH146-Gal4*[15], *Or42b* mutant[33] and *Or43b* mutant[16], *NP5103-Gal4*[34], *Or43a-Gal4*[4], and *UAS-GFP*. Flies were raised on standard medium at 22-25°C.

Female flies age 2-5 days after eclosion were used for experiments. Isolated brain preparations[14] were obtained by micro dissection of decapitated flies to remove head cuticle and connective tissues. Neural activity of the fly brain was reduced by dissecting in chilled calcium free AHL saline. For odor stimulation experiments, the preparation was mounted on a slide and the brain was embedded in 2% agarose in AHL saline leaving the antennae exposed to air, as originally described[14]. For nerve stimulation experiments, the antenna and brain preparation was pinned in a sylgard dish and the olfactory nerves were carefully severed near the base of the antenna with fine forceps. After dissection the preparations were rinsed and kept in AHL saline (108 mM NaCl, 5 mM KCL, 2 mM $CaCl_2$, 8.2 mM $MgCl_2$, 4 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM trehalose, 10 mM sucrose, 5 mM HEPES [pH 7.5, 265 mOsm]).

Electrophysiology. The dissecting procedure was the same as for the imaging preparation, except that the sheath covering the antennal lobes was mechanically removed using a pair of fine forceps. The electrical signal output from EPC-7 was amplified and band-filtered at 100-200 Hz by a signal conditioner, and digitized by a data acquisition system (CyberAmp 320, DigiData 1320, pCLAMP v.8, Axon Instruments). Data analysis was carried out using Igor Pro 4.0 (Wavemetrics) and the macro NeuroMatic v1.3 (by Jason Rothman) was used to generate the spike rasters. Calculation of instantaneous firing rate was done by importing spike times into Matlab and arranging them into a sparse matrix. Sliding time bins averaged the number of spikes per 50 or 100 ms for PNs and ORNs respectively. Time bins were shifted by 1 or 10 ms for PNs and ORNs respectively, such that each bin overlapped 49 ms (PN) or 90 ms (ORN) with the previous bin.

Odorants in saline were applied to the antenna submerged in saline with a dual channel pressure injection system (Pressure System IIe, Toohey Company) via a micropipette of 2 μm tip diameter at 5 psi for one second. We empirically determined that 40 μl/ml of isoamyl acetate using the pressure injection system is equivalent to 7% SV for the airborne odor stimulation. In this experiment, we first performed calcium imaging to obtain a dosage curve of glomerular response to airborne odor stimulation. We then submerged the same preparation in saline and measured glomerular response to odor stimulation using the pressure injection system.

Two-photon imaging. Calcium imaging was performed with a custom-built two-photon microscope as described[14]. Images were captured at 4 frames per second with a resolution of 128 x 128 pixels. At the end of the experiment, a high resolution Z-stack of images (512 x 512 pixels) was collected for glomerular identification. Electrical stimulation of the olfactory nerve was delivered with a glass suction electrode that was made by pulling capillary glass to a fine tip, broken with forceps and then fire polished to achieve a diameter that is about 1.5x the diameter of the nerve. The nerve was sucked as a loop into the electrode. A Grass stimulator was used to stimulate the nerve with pulses at 100 Hz, 1 ms in duration and 10 V in amplitude. Analysis of imaging data was performed using Igor Pro (Wavemetrics) and a custom macro. Statistical analysis was done using Student's T test in Excel and results were considered significant if the p value was less than 0.05.

The olfactometer used to deliver airborne odors was described previously[14]. Odor concentrations are specified for each experiment in the text. Odorants were obtained from Sigma-Aldrich. HEX, hexane; IAA, isoamyl acetate; CIN, cineole; CYN,

cyclohexanone; CYL, cyclohexanol; PYR, pyridine; CAP, caproic acid; BEN, benzaldehyde; OTO, 1-octen-3-ol; OTL, 3-octanol; OTN, 3-octanone; OTA, octanal; BNA, benzyl acetate; LIN, linalool; CAR, s-carvone; OCT, 1-octen-3-ol; CAR, S-carvone.

Calculation of Population Sparseness. The Treves-Rolls calculation sparseness is defined as: $S = [\sum r/N]^2/\sum[r^2/N]$

where r is the firing rate of a given cell and N is the number of cells.

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Chapter 3: Feedback and Feedforward Inhibition

3.1 Abstract

The antennal lobe contains many GABAergic interneurons that provide inhibition to the olfactory circuit. We have investigated feedback and feedforward inhibition in the antennal lobe. Using two-photon imaging of ORN transmission or PN dendritic calcium activity, we asked if blocking $GABA_A$Rs or $GABA_B$Rs alters activity. We find that blocking $GABA_A$Rs shifts the dynamic range of PN activity with no effect on ORN transmission. In contrast, blocking $GABA_B$Rs alters the gain of ORN transmission that is reflected in PN response. Our data suggest that feedforward inhibition by $GABA_A$R shifts the sensitivity of PN response, while feedback inhibition by $GABA_B$R alters the gain of ORN input. These two forms of inhibition may allow precise control of olfactory sensitivity over a large dynamic range.

3.2 Introduction

One hallmark of the first olfactory relay in most species is the presence of local GABAergic interneurons. Many studies have addressed the functional roles of LNs in lateral inhibition[1-3], context-dependent olfactory response[4], network oscillation[5], and synchronous firing for fine odor discrimination[6,7]. Another possible function could be to regulate sensitivity and dynamic range of olfactory response. Ideally the system must be able to adjust sensitivity and contrast between glomeruli. Since cognate ORNs are the main drivers of PN output[8,9], we reason that the function of interneurons should be to modulate olfactory sensitivity.

The *Drosophila* antennal lobe is populated with GABAergic local interneurons (LNs) that release GABA in many if not all glomeruli[10]. GABA exerts its modulatory

role via two distinct receptor systems, the fast ionotropic $GABA_A$ receptor, which is sensitive to the antagonist picrotoxin, and the slow metabotropic $GABA_B$ receptor, which is sensitive to the antagonist CGP54626. Pharmacological blockade of the GABA receptors demonstrate that GABA-mediated hyperpolarization suppresses PN response to odor stimulation in a non-uniform fashion[10-12]. Electron microscopy studies of the insect antennal lobe show that GABAergic LNs synapse with PNs[13], which support the well established olfactory mechanism of lateral inhibition[2]. GABAergic LNs also synapse onto ORNs and imaging studies in mouse suggest that activation of $GABA_B$Rs in ORN terminals suppress neurotransmitter release in ORNs[14].

3.3 Imaging ORNs and PNs

We investigated the role of GABA receptors in modulation ORN synaptic transmission by two-photon imaging of synaptopHluorin (spH), an indicator of vesicle release[15], in ORN axon terminals. Flies bearing *Or83b-Gal4* and *UAS-spH* express spH in ORNs allowing the measurement of vesicle release from ORN axon terminals. Using precise electrical stimulation of the olfactory nerve, we plotted the input-output curve of ORN transmission to a range of stimulus intensities in the presence and absence of the $GABA_B$R and $GABA_A$R antagonists, CGP54626 and PTX. We find that the spH fluorescence is significantly enhanced in the presence of CGP54626, but only at high stimulus intensities (Figure 3.1A,C). The data are well fit by a logarithmic curve and we find a significant increase in the slope of the line (gain) but not the y-intercept (sensitivity) indicating a change in the gain of ORN transmission. Interestingly, application of PTX has no effect on ORN neurotransmitter release at any stimulus

intensity, indicating that $GABA_A$ receptors are not present in ORNs (Figure 3.1A,C), consistent with findings in Chapter 4, Figure 4.3A. Thus it appears that $GABA_B$Rs provide a feedback gain control for ORN synaptic transmission that attenuates response to high intensity stimuli.

We next investigated the role of GABA receptors in modulating PN dynamic range by imaging PN dendritic responses to a range of stimulus intensities. Flies bearing *GH146-Gal4* and *UAS-GCaMP* transgenes express the calcium sensor GCaMP[16] in many PNs[17], allowing the select measurement of calcium response in PN dendrites. Insect dendritic calcium increases are mostly due to influx through nicotinic acetylcholine receptors[18,19], therefore calcium influx provides an indirect measurement of synaptic potential. We plotted the input-output function of PNs to a range of olfactory nerve stimuli in the presence or absence of the $GABA_B$R antagonist (Figure 3.1B,D). Addition of CGP54626 significantly increases PN response at high stimulus intensities but not at low intensities, and significantly increased the slope of the input-output function with no effect on the offset. Thus the gain modulation observed in ORN neurotransmitter release is preserved in PNs dendritic activity. Interestingly, blockade of $GABA_A$Rs by PTX increased PN response at all intensities and significantly shifted the PN dose-response curve to the left with no significant effect on the slope. The $GABA_A$R effect is likely a feedforward modulation since ORNs are unaffected by PTX. The $GABA_B$R effect is at least partly due to feedback modulation of ORN transmission because the ORN gain modulation is matched in PN response. These results suggest that GABA modulates PN output via feedforward $GABA_A$R inhibition to offset the sensitivity and via feedback $GABA_B$R inhibition to alter the gain of antennal lobe output.

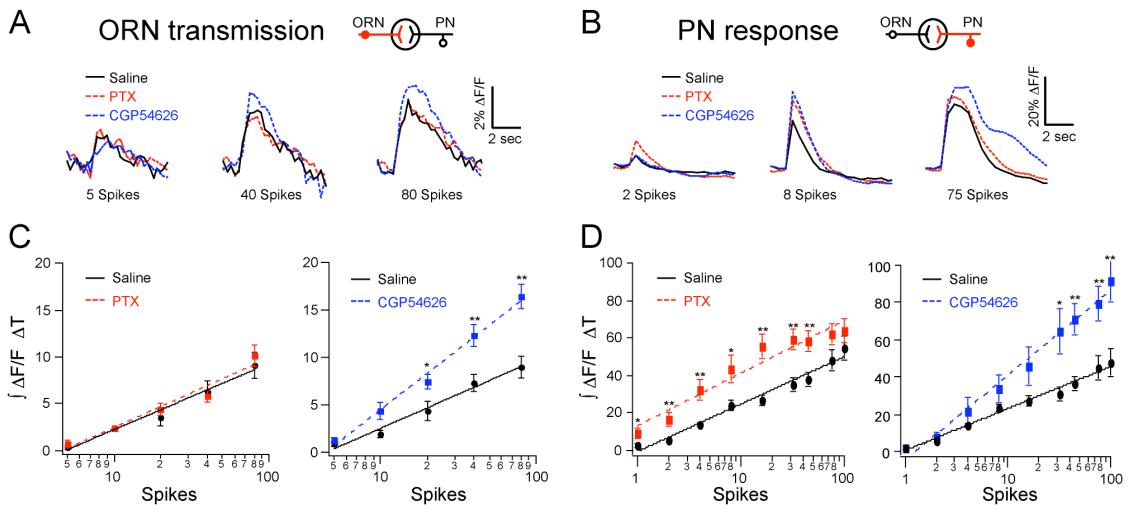

Figure 3.1. Feedback and feedforward inhibition.
Fluorescence changes over time at three stimulus intensities for two-photon imaging of ORN neurotransmitter release (**A)** and PN dendritic calcium (**B**). ORN neurotransmitter release was monitored in flies expressing synaptopHluorin in Or83b neurons. PN dendritic calcium was monitored in flies expressing GCaMP in GH146 neurons. Traces show responses in saline, and after the addition of PTX or CGP54626. SynaptopHluorin ΔF/F traces are the average of three trials, whereas GCaMP ΔF/F traces represent single trials. The dose response curve of ORNs (**C**) and PNs (**D**) to electrical stimulation of the olfactory nerve in saline and after the addition of PTX or CGP54626. Mean integrated fluorescence change over time across preparations is plotted as function of the number of stimuli. ΔF/F was measured from all mid layer glomeruli of the antennal lobe. Electrical stimulation was given at 100 Hz, 1 ms in duration and 10 V in amplitude. n, 7-8. Error bars show SEM. * $p \leq 0.05$, ** $p \leq 0.01$.

3.4 Discussion

We investigated the dose response relationship to stimulus intensity and found that $GABA_AR$ and $GABA_BR$ modulation produce different effects on PN response. $GABA_AR$ shifts the sensitivity of PN dynamic range to suppress all stimuli, while $GABA_BR$ decreases the gain of ORNs synaptic transmission and postsynaptic response to suppress only high intensity stimuli. Although we cannot rule out the possibility that PNs express some $GABA_BR$, the expanded dynamic range we observe in PN dendritic calcium activity matches the ORN modulation. The expression of $GABA_BR$ is further explored in Chapter 4. Interestingly, a paper based on computer simulation shows that presynaptic inhibition is an effective way to limit energy expenditure in the mammalian olfactory bulb[20]. Our results suggest that a feedback inhibition by $GABA_BR$ expression in ORN terminals provides a mechanism to alter the dynamic range of the system by modulating the input, while a feedfordward inhibition by $GABA_AR$ alters the sensitivity of the output.

3.5 Methods

The following transgenic lines were used: 1) *UAS-GCaMP56*[21], 2) *GH146-Gal4*[17], 3) *Or83b-LexA*[22], 4) *LexAop-GCaMP-IRES-GCaMP*[9], 5) *Or83b-Gal4*[23], 5) *UAS-synaptopHluorin*[15] with the transgene mobilized onto the third chromosome.

Picrotoxin and CGP54626 (Tocris) were dissolved as 2000x stocks in DMSO. GABA (Sigma) was prepared fresh daily and dissolved as a 1000x stock in AHL saline. SKF97541 (Tocris) was dissolved as a 500x stock in 100 mM NaCl. The appropriate

volume (1-4 mL) was first diluted with 100 mL of AHL saline and then added to the preparation to achieve the final concentration.

Two-photon imaging and nerve stimulation were performed as described chapter 2. Analysis of imaging data was performed using Igor Pro (Wavemetrics) and a custom macro. Statistical analysis was done using Student's T-Test in excel and results were considered significant if the p value was less than 0.05.

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Chapter 4: Gain Control by Presynaptic Inhibition Fine Tunes Olfactory Behavior

4.1 Abstract

Early sensory processing can play a critical role in sensing environmental cues. We have investigated the physiological and behavioral function of gain control at the first synapse of olfactory processing in *Drosophila*. We report that olfactory receptor neurons (ORNs) express the $GABA_B$ receptor ($GABA_BR$) and its expression expands the dynamic range of ORN synaptic transmission that is preserved in projection neuron responses. Strikingly, we find that different ORN channels have unique baseline levels of $GABA_BR$ expression. ORNs that sense the aversive odorant CO_2 do not express $GABA_BRs$ nor exhibit any presynaptic inhibition. In contrast, pheromone-sensing ORNs express a high level of $GABA_BRs$ and exhibit strong presynaptic inhibition. Furthermore, a behavioral significance of presynaptic inhibition was revealed by a courtship behavior in which pheromone-dependent mate localization is impaired in flies that lack $GABA_BRs$ in specific ORNs. Together, these findings indicate that different olfactory receptor channels may employ heterogeneous presynaptic gain control as a mechanism to allow an animal's innate behavioral responses to match its ecological needs.

4.2 Introduction

Presynaptic inhibition has been described in a number of systems and has been suggested to play a role in temporal processing. Mouse ORNs express the metabotropic receptor $GABA_B$[1], and it has been demonstrated that this GPCR provides a mechanism for delayed feedback inhibition of ORN output[2]. It has been hypothesized that feedback inhibition provides a mechanism for modulating input sensitivity, attenuating post-synaptic responses during repeated sniffing, and adaptive filtering of odor landscape[3-6].

The *Drosophila* antennal lobe is populated with GABAergic local interneurons (LNs) that release GABA in many if not all glomeruli[7]. GABA exerts its modulatory role via two distinct receptor systems, the fast ionotropic $GABA_A$ receptor, which is sensitive to the antagonist picrotoxin, and the slow metabotropic $GABA_B$ receptor, which is sensitive to the antagonist CGP54626. Pharmacological blockade of the GABA receptors demonstrate that GABA-mediated hyperpolarization suppresses PN response to odor stimulation in a non-uniform fashion[7-9]. Electron microscopy studies of the insect antennal lobe show that GABAergic LNs synapse with PNs[10], which support the well established olfactory mechanism of lateral inhibition[11]. GABAergic LNs also synapse onto ORNs and imaging studies in mouse suggest that activation of $GABA_B$Rs in ORN terminals suppress neurotransmitter release in ORNs[5].

We hypothesize that setting the appropriate olfactory gain for environmental cues is important for adjusting an organism's sensitivity to its environment. A recent study shows that $GABA_B$R mediated presynaptic inhibition provides a mechanism to modulate olfactory gain[12]. Electrical recordings show that interglomerular presynaptic inhibition suppresses the olfactory gain of PNs to potentially increase the dynamic range of the olfactory response. Likewise, gain modulation may not be uniform among different glomeruli, which could reflect a tradeoff between sensitivity and dynamic range in different olfactory channels. For example, high sensitivity may be crucial for some environmental cues, such as those that require an immediate behavioral response, whereas a larger dynamic range may be more advantageous for other odors where precise spatial and temporal information may be critical for optimal performance.

Here we investigated the physiological and behavioral function of gain control in early olfactory processing. We show that interneuron-derived GABA activates $GABA_BRs$ on ORN terminals, reducing the gain of ORN-to-PN synaptic transmission. Different types of ORNs exhibit different levels of presynaptic inhibition and this heterogeneity in presynaptic inhibition is preserved in antennal lobe output projection neurons. Interestingly, pheromone-sensing ORNs exhibit high levels of $GABA_BR$ expression and behavioral experiments indicate that $GABA_BR$ expression in a population of pheromone ORNs is important for mate localization, suggesting that presynaptic gain control is important for the olfactory channel-specific fine-tuning of behavior.

4.3 Odor-evoked gain control in projection neurons by $GABA_BR$ signaling

We investigated the role of $GABA_BR$ in modulating olfactory dynamic range by imaging PN dendritic responses to a range of stimulus intensities in the presence or absence of a $GABA_BR$ antagonist. Flies bearing *GH146-Gal4* and *UAS-GCaMP* transgenes express the calcium sensor GCaMP[13] in many PNs[14], allowing the select measurement of calcium response in PN dendrites. Insect dendritic calcium increases are mostly due to influx through nicotinic acetylcholine receptors[15,16], therefore calcium influx provides an indirect measurement of synaptic potential.

We investigated antennal lobe gain control with behaviorally relevant odors. The male pheromone odor *cis*-vaccenyl acetate activates the DA1 glomerulus[17]. The fruit odors ethyl hexanoate and 2-phenylethanol[18] activate the DM2 and VA3 glomeruli, respectively. Additionally, the *Drosophila* stress odorant CO_2 activates the V glomerulus to provoke avoidance behavior[19]. We monitored PN dendritic calcium in response to odor

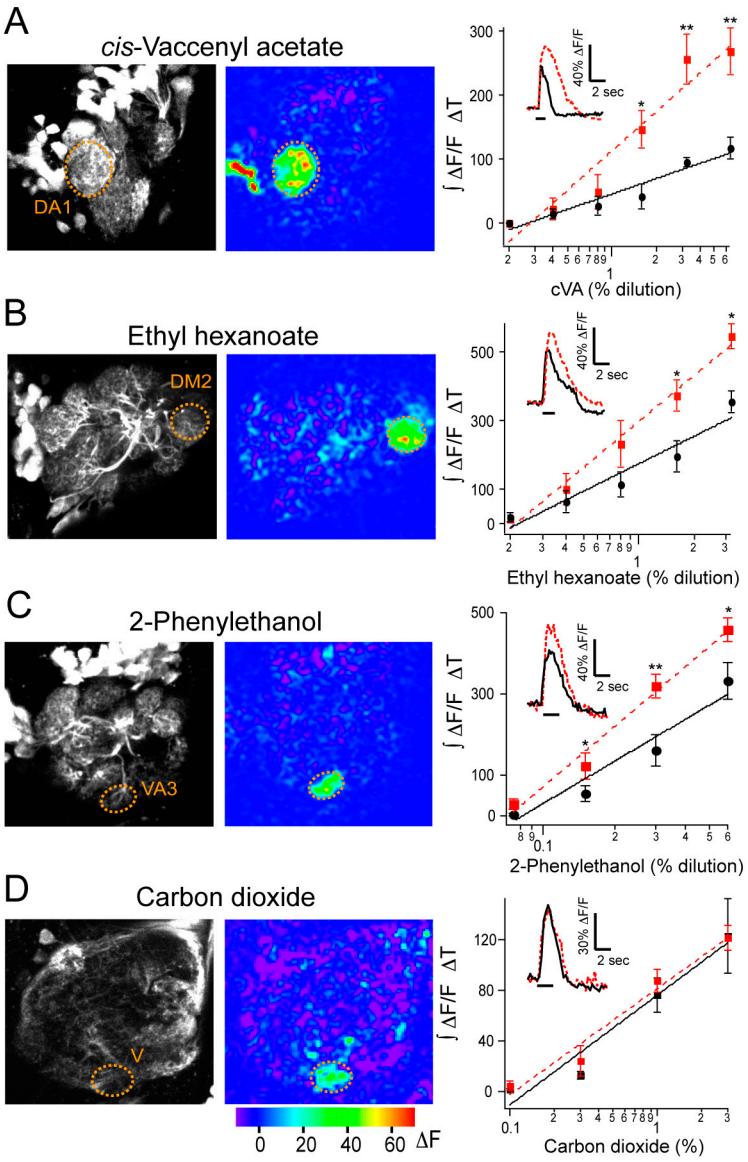

Figure 4.1. $GABA_B$ receptors alter the gain of projection neurons.
Two-photon imaging of PN activity in flies expressing GCaMP in GH146 neurons. (A) Response to electrical stimulation of the olfactory nerve. (B-E) Response to odor stimulation. Gray scale images show antennal lobe structure. Psuedocolored images reveal the response to electrical stimulation or a 2-second odor stimulation. Graphs show the input-output function of PNs in saline (black circles) and in the presence of 25 mM CGP54626 (red squares). Mean integrated fluorescence change over time across preparations is plotted as function of the number of stimuli or odor concentrations. Inset traces are representative of fluorescence change over time. ΔF/F was measured from all glomeruli in the optical section (A), or the outlined regions (B-E). Electrical stimulation was given at 100 Hz, 1 ms in duration and 10 V in amplitude. Concentrations of ethyl hexanoate and 2-phenylethanol were percent dilutions of 300 ppb and 80 ppm, respectively. n, 4-8. Error bars show SEM. * $p \leq 0.05$, ** $p \leq 0.01$.

stimulation in the presence or absence of the $GABA_BR$ antagonist. Blocking $GABA_BRs$ has different effects on PNs of different glomeruli (Figures 4.1A-D). The slope of PN response to *cis*-vaccenyl acetate, ethyl hexanoate and 2-phenylethanol is increased by 153%, 67%, and 43%, respectively, in the presence of CGP54626. In contrast, the slope of PN response to CO_2 is not altered by CGP54626. Thus, $GABA_BR$ activation has different degrees of modulation on the gain of projection neurons in different glomeruli.

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4.4 The $GABA_B$ receptor is expressed in olfactory receptor neurons and mediates presynaptic inhibition.

We next investigated the antennal lobe circuitry underlying this $GABA_BR$-mediated gain change. We first determined which populations of neurons in the antennal lobe express $GABA_BR$. Mammalian $GABA_BR$ is an obligatory heterodimeric G-protein

coupled receptor[20]. Similarly, co-expression of *Drosophila GABA$_B$-R1 and -R2* is necessary to produce a functional GABA$_B$R in heterologous systems[21]. To visualize neurons that express GABA$_B$R, we therefore generated a transgenic fly line that contains the fusion DNA of the yeast transcription factor *Gal4* with 1.5 kb of genomic DNA immediately upstream to the open reading frame of the *GABA$_B$R2* gene. The expression of GF in flies bearing *GABA$_B$R2-Gal4* and *UAS-CD8-GFP* within the antennal lobes appears to be in axon terminals of ORNs derived from the antennae and maxillary palps (Figure 4.2A). Surgical removal of the olfactory appendages causes complete degeneration of ORN axons within three days[22], and we observed a dramatic reduction of GFP detection in the antennal lobes (Figure 4.2A). Expression of *GABA$_B$R2* in the antennae was further confirmed by RT-PCR of isolated antennal tissue. Primers for *Drosophila GABA$_B$R2* as well as *RP49*, a ubiquitous ribosomal protein, detected transcripts in both the head and the antennae, while primers for *Drosophila Rhodopsin-4* (*Rh4*), which encodes one of the ultraviolet rhodopsins, do not detect transcript in the antennae (Figure 4.2E). Thus, ORNs express mRNA for *GABA$_B$R2*.

We next mapped the distribution of GABA$_B$R with antiserum specific for *Drosophila* GABA$_B$R2[23,24]. Neurons in the antennae and maxillary palps positive for GABA$_B$R2 immunoreactivity largely overlap with the Or83b ORNs (Figure 4.2C,D). We used RNA interference to further confirm the expression of GABA$_B$R2 in ORNs. Expression of the *GABA$_B$R2-RNAi* in Or83b ORNs causes a 40% reduction in GABA$_B$R2 immunoreactivity in the antennal lobe, but no change in immunoreactivity of nc82—a neuropil specific antibody (Figure 4.2B). Immunoreactivity to GABA$_B$R2 in the antennal lobe is distributed mainly on ORN axonal terminals, as surgical removal of the olfactory

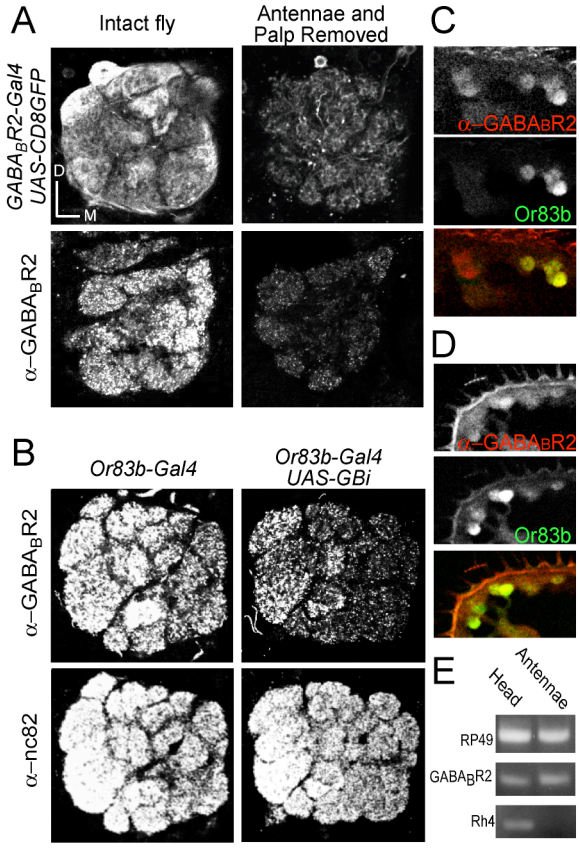

Figure 4.2. Molecular expression of GABA$_B$ receptors in olfactory receptor neurons. (A) Images of the antennal lobe in flies bearing *GABA$_B$R2-Gal4* and *UAS-CD8-GFP* (top) and cryosections stained with a GABA$_B$R2 antiserum (bottom). The Antennal lobe of normal flies is shown (left) in comparison to the antennal lobe in flies three days after the antennae and maxillary palps were surgically removed (right). (B) Whole mount immunostaining for GABA$_B$R2 and the neuropil marker nc82 in the antennal lobe of control flies expressing *Or83b-Gal4* (left) and flies expressing *Or83b-Gal4* and *UAS-GABA$_B$R2-RNAi* (*UAS-GBi*) (right). Immunostaining for GABA$_B$R2 and GFP in cryosections of the antenna (C) and the maxillary palp (D) in flies expressing *Or83b-Gal4* and *UAS-CD8-GFP*. (E) Products of RT-PCR reactions from entire fly heads or isolated antennae, with primers for GABA$_B$R2, RP49 (gene for a ubiquitous ribosomal protein), and Rh4 (a rhodopsin gene).

appendages three days prior eliminates most of the staining (Figure 4.2A). Residual $GABA_BR$ expression in the absence of ORN axons could be in LNs or PNs, as previously suggested[8]; our data are not conclusive but suggest that the residual staining is from a population of local interneurons because neurites and cell bodies are confined to the antennal lobe. Nevertheless, $GABA_BR$ within the antennal lobe is mostly distributed on ORN axonal terminals.

To investigate the function of $GABA_BR$ in ORN terminals, we next tested whether $GABA_BR$ agonists applied exogenously can induce presynaptic inhibition in ORNs. Flies bearing the *Or83b-LexA*[25] and *LexAop-GCaMP* transgenes express the calcium sensor GCaMP in most ORNs, allowing the select measurement of calcium activity in ORN axonal terminals. Electrical stimulation of the olfactory nerve elicits a calcium transient in ORN terminals that is significantly reduced in the presence of 20 μM GABA or 20 μM SKF97541, a selective $GABA_BR$ agonist[23] (Figure 4.3A). If the GABA effect on presynaptic calcium is caused purely by activation of $GABA_BR$, it should be blocked by CGP54626 but not picrotoxin, the selective *Drosophila* $GABA_BR$ and $GABA_AR$ antagonists respectively[8,23]. Indeed, the reduction caused by GABA is prevented by 25 μM CGP54626, but not by 125 μM picrotoxin (Figure 4.3A-B). Given that presynaptic calcium triggers neurotransmitter release, activation of $GABA_BRs$ should serve to attenuate synaptic transmission. To test this possibility, we performed two-photon imaging of synaptopHluorin (spH), a fluorescent indicator of vesicle exocytosis[26], in flies bearing *Or83b-Gal4* and *UAS-spH* transgenes. Electrical stimulation of the olfactory nerve elicits an increase in spH fluorescence that is significantly reduced by the agonist SKF97541 and enhanced by the antagonist CGP54626 (Figure 4.3C-D). Thus,

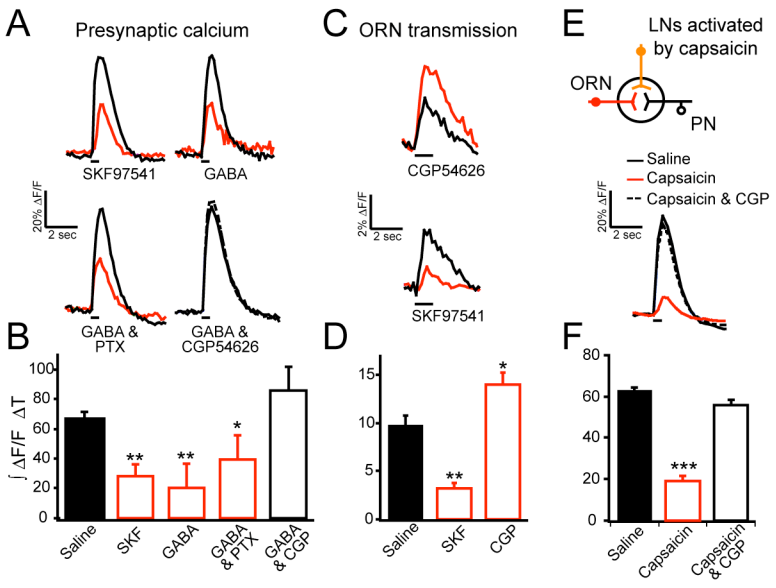

Figure 4.3. $GABA_B$ receptors mediate presynaptic inhibition in olfactory receptor neurons.

Two-photon imaging of ORNs expressing GCaMP or spH in Or83b neurons was used to monitor response to electrical stimulation of the olfactory nerve. Pharmacological effects on fluorescence change over time: black and red traces show representative responses before and after drug application respectively. (**A**) Representative traces of GCaMP fluorescence change over time. (**B**) Bar graph summary of pharmacological effect shows the integrated fluorescence change over time. (**C**) Representative traces of spH fluorescence change over time; traces are the average of three trials. (**D**) Bar graph summary of (**C**). (**E**) Two-photon calcium imaging of ORN terminals in flies expressing VR1 in LNs. Electrical stimulation of the olfactory nerve elicits a response that is suppressed with 100 μM capsaicin, but is rescued with the addition of 25 μM CGP54626. (**F**) Bar graph summary of pharmacological effect shows the integrated fluorescence change over time. For all, electrical stimulation was 1 ms in duration and 10 V in amplitude, and 45 pulses (A-B, E-F) or 80 pulses (C-D) at 100 Hz. n, 3-18. Error bars show SEM. * $p \leq 0.05$, ** $p \leq 0.01$. *** $p \leq 0.001$.

$GABA_BR$ activation suppresses ORN presynaptic calcium and neurotransmitter release in *Drosophila*.

We next asked whether a physiologic concentration of GABA released by interneurons causes presynaptic inhibition in ORNs. The *GH298-Gal4* line has been shown to label many GABAergic LNs in the antennal lobe[8,14]. We therefore examined whether activating these LNs induces presynaptic suppression in ORNs. In these experiments, we used a dual genetic expression system[25] to express GCaMP in ORNs for calcium imaging and to express the capsaicin sensitive VR1 channel in LNs for exogenous activation of these LNs. Expression of the VR1 channel endows *Drosophila* neurons with responsivity to capsaicin[27]. Flies bearing *Or83b-LexA*, *LexAop-GCaMP*, *GH298-Gal4*, *UAS-VR1* allow calcium imaging in ORN terminals and activation of many GABAergic LNs by capsaicin. In these flies, ORNs exhibit a significantly reduced calcium response in the presence of 100 μM capsaicin; this reduction is blocked by CGP54626 (Figure 4.3E-F). Control flies that do not express the VR1 channel (bearing *Or83b-LexA*; *LexAop-GCaMP*; *UAS-VR1* transgenes) do not exhibit any presynaptic suppression by 100 μM capsaicin (Supplementary Figure 2). Thus activation of the GABAergic LNs is capable of inducing $GABA_BR$ mediated presynaptic inhibition.

4.5 Presynaptic expression of $GABA_BR$ alters the Gain of projection neurons.

In a simple model, $GABA_BR$-mediated gain control in the antennal lobe revealed by pharmacological experiments is entirely attributed to the activation of $GABA_BRs$ in ORNs. We addressed this issue first by measuring vesicle release in ORNs that lack $GABA_BR2$ expression. We performed two-photon imaging of spH in flies bearing *Or83b-Gal4, UAS-spH* and *UAS-$GABA_BR2$-RNAi* transgenes. Electrical stimulation of

the olfactory nerve elicits an increase in spH fluorescence that is not affected by the addition of the agonist SKF97541 or the antagonist CGP54626 (Figure 4B). We plotted the input-output function in control flies and those expressing the RNAi and found that the responses to high intensity stimuli are significantly greater in RNAi expressing flies than that of control flies (Figure 4C). The data are fit with a logarithmic curve and exhibit a 110% increase in the slope, thus indicating an increase in the gain of ORN transmission in flies expressing *$GABA_BR2$-RNAi*, suggesting that $GABA_BR$ provides a scalable inhibition to ORN presynaptic terminals.

To ask whether knock-down of $GABA_BR$ in ORNs has a concomitant impact on PN output, we next performed loose patch electrical recordings to monitor PN action potential firing in response to ORN stimulation in the presence or absence of *$GABA_BR2$-RNAi*. To do this, we investigated the ORN to PN transformation in VA1lm glomerulus which detects female odors[28]. Since the ligand for these ORNs has not been identified, we expressed the light sensitive cation channel, channelrhodopsin 2 (ChR2)[29] in Or47b neurons and activated them with light illumination. We plotted the input-output function of VA1lm PNs in control flies bearing the *Or47b-Gal4* and *UAS-ChR2* transgenes and in flies also expressing *$GABA_BR2$-RNAi*. Indeed expression of RNAi in Or47b neurons increases the gain of the post-synaptic PN firing frequency (Figure 4E). Addition of the $GABA_BR$ antagonist did not further increase PN firing in RNAi expressing flies, suggesting that $GABA_BR$ expression in other cell types (see Figure 2A) does not contribute to this gain change (n, 2; data not shown). Thus, $GABA_BR$ expression in ORNs decreases the gain of PN output.

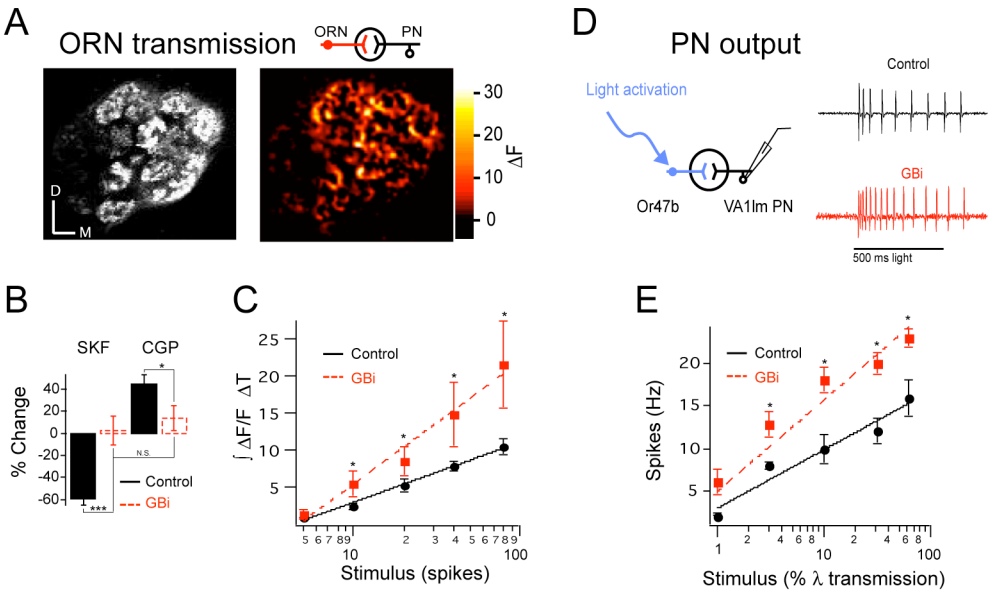

Figure 4.4. Knockdown of $GABA_B$ receptors increases the gain of receptor neuron transmission and projection neuron firing.
(**A**) Two-photon imaging of ORNs expressing spH in Or83b neurons was used to monitor synaptic transmission in response to electrical stimulation of the olfactory nerve. Gray scale image show antennal lobe structure at one optical plane. Psuedocolored images reveal the response to electrical stimulation. (**B**) The percent decrease or increase in integrated ΔF/F over time upon addition of SKF97541 or CGP54626, in control flies and those expressing two copies *$GABA_BR2$-RNAi* (*GBi*) in Or83b neurons. (**C**) The input-output function of ORN transmission in control and flies expressing *GBi* in Or83b neurons. Mean integrated fluorescence change over time across preparations is plotted as a function of the number of stimuli. ΔF/F was measured from all glomeruli in this optical section. (**D**) Loose patch recordings from PNs with dendrites in VA1lm in response to light activation of the Or47b neurons that express two copies of channelrhodopsin-2 in control flies and flies that also express *GBi* in Or47b neurons. (**E**) Input-output function of PNs plotted as a function of light intensity. n, 4-10 (**B**-**C**) and n, 8 (**E**). Error bars show SEM. * $p \leq 0.05$, *** $p \leq 0.001$.

4.6 Heterogeneity in $GABA_BR$ expression

The above experiments indicate that $GABA_BR$ expression in ORNs is necessary for presynaptic inhibition and for suppression of PN output gain. We reasoned that heterogeneity in the PN olfactory gain (See Figure 4.1) reflects heterogeneity in the level of presynaptic inhibition. To investigate this, we first asked whether specific ORNs exhibit different amounts of presynaptic inhibition. Presynaptic inhibition was quantified by the percent reduction in calcium activity in the presence of the $GABA_BR$ agonist. Stimulation of the labial nerve in flies bearing the *Or83b-LexA* and *LexAop-GCaMP* transgenes exhibit calcium transients in the palpal axonal terminals that is dramatically reduced by 20 μM SKF97541 (Figure 4.5A-D, column 3). Similarly, stimulation of the antennal nerve causes a calcium transient that is dramatically reduced in glomeruli receiving axons from pheromone detecting ORNs, while other glomeruli exhibit significantly less reduction in presynaptic calcium (Figure 4.5A-D, column 1). At the middle layer of the antennal lobe, presynaptic calcium suppression in these ORNs is less than that of the palpal ORNs on the same level (Figure 4.5A-D, columns 2&3). In the extreme case, the Gr21a CO_2 sensing ORNs exhibit no significant suppression of presynaptic calcium by the agonist (Figure 4.5A-D, column 4). Thus, there is heterogeneity between glomeruli in the magnitude of $GABA_BR$-mediated presynaptic suppression; the Gr21a CO_2 sensing neurons do not exhibit any significant presynaptic inhibition, while the palpal and pheromone ORNs exhibit significantly stronger levels of inhibition.

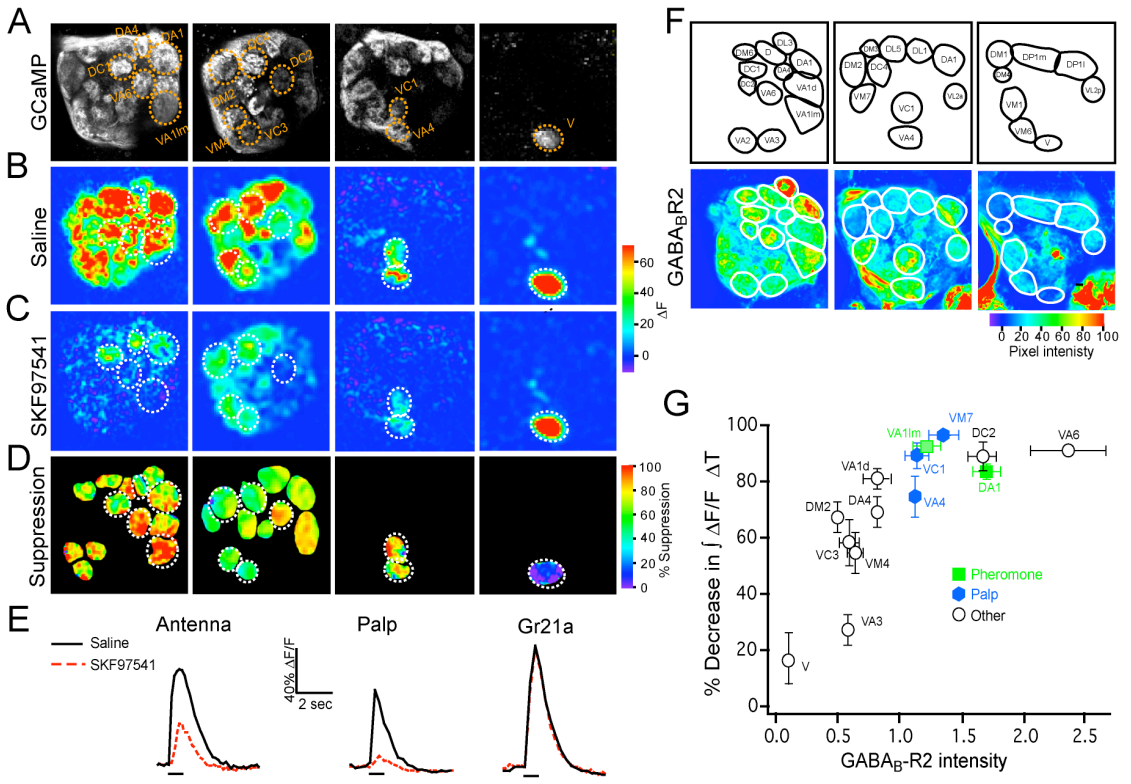

Figure 4.5. Heterogeneity of presynaptic inhibition in olfactory receptor neurons. (**A**) Two- photon images of GCaMP expression in Or83b neurons (columns 1-3) and Gr21a neurons (column 4). (**B**) Electrical stimulation of the olfactory nerve generates calcium influx in axon terminals of antennal ORNs (columns 1,2,4). Stimulating the labial nerve causes calcium influx into axon terminals of the palpal ORNs (column 3). (**C**) Addition of SKF97541 decreases the calcium response of Or83b neurons to different degrees (columns 1-3), but does not affect Gr21a calcium influx (column 4). (**D**) The percent suppression is represented by color intensity for particular glomeruli. Images were generated by subtracting images in **C** from those in **B** and then dividing the resulting images by those in **B**. Images were smoothed with a Gaussian filter and a black mask was overlaid on the non-glomerular background. (**E**) GCaMP fluorescence change is plotted over time for Gr21a and Or83b antennal and palpal neurons; traces for Or83b neurons represent responses from multiple glomeruli in one optical plane. For response to antennal nerve stimulation, ΔF/F was measured from all mid layer glomeruli. (**F**) GFP intensity from *GABA$_B$R2-Gal4* reporter line at three different optical planes through the antennal lobe. Images are psuedocolored to emphasize the differences between glomeruli. (**G**) The suppression of calcium influx by the GABA$_B$R agonist is plotted against the reporter intensity for individual glomeruli. n, 3-5. Error bars show SEM, but are not plotted when the SEM is smaller than the symbol. * $p \leq 0.05$, ** $p \leq 0.01$.

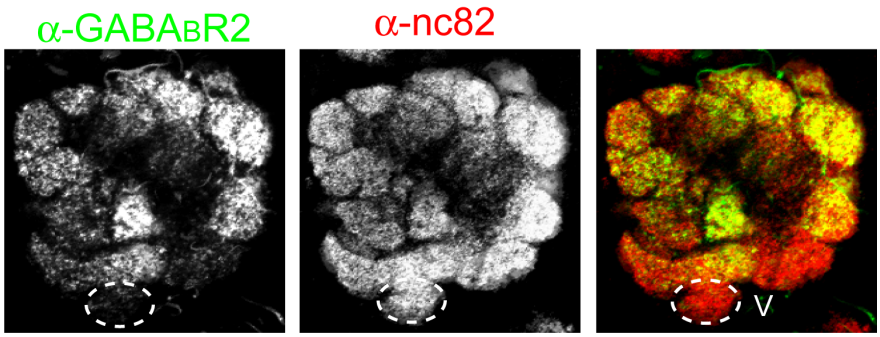

Figure 4.6. The V glomerulus is devoid of $GABA_BR2$ immunoreactivity.
Images show whole mount staining with anti-serum for $GABA_BR2$ (left), the neuropil marker nc82 (middle), and merge (right). The V glomerulus is circled.

We next asked whether the heterogeneity in presynaptic inhibition matches the heterogeneity observed in the putative $GABA_BR2$ expression level. We first addressed this question by correlating the level of presynaptic inhibition with the reporter expression level in flies bearing the *$GABA_BR2$-Gal4* and *UAS-CD8GFP* transgenes. GFP fluorescence intensity shows that different glomeruli exhibit different levels of expression (Figure 4.5F). In the extreme case, the Gr21a ORNs projecting to the V glomerulus have little or no fluorescence intensity (Figure 4.5F, 4.6). ORNs innervating the VC3 and DM2 glomeruli have relatively weak intensity. In contrast, the VC1, VA4 and VM7 glomeruli that have ORN axons from the maxillary palp via the labial nerve[30,31], exhibit very high intensity. Additionally, the pheromone sensing glomeruli VA1lm and DA1[17,28,32] also exhibit high intensity. Thus, there is a tight correlation between the $GABA_BR2$ promoter driven reporter level and the extent of presynaptic inhibition (Figure 4.5G).

The above experiments suggest that the $GABA_BR$ level dictates the magnitude of presynaptic inhibition, if so we expect that varying reduction in receptor expression should lead to matching changes in presynaptic inhibition. Using a promoter such as Or83b to express varying levels of *$GABA_BR2$-RNAi* in different ORN types should create a condition to assess the link between receptor and presynaptic inhibition. When two copies of the RNAi transgenes are expressed in ORNs using the *Or83b-Gal4* driver, presynaptic inhibition is completely abolished (Figure 4.4B). However, the expression of one copy of the RNAi transgene in the same condition only partially reduces presynaptic inhibition (Figure 4.7A). The level of presynaptic inhibition was measured by calcium imaging as percent suppression by SKF97541, in which electrical stimulation of the olfactory nerve was used to elicit calcium transients in ORN terminals in flies bearing

Or83b-Gal4 and *UAS-GCaMP*. The promoter strength of *Or83b* was measured by the baseline fluorescence. We plotted the percent suppression for individual glomeruli against the *Or83b-Gal4* expression level. In control flies without expression of the RNAi, we find that there is no correlation between Gal4 level and presynaptic inhibition; however in flies bearing one copy of the *UAS-GABA$_B$R2-RNAi* transgene, we find an inverse correlation between the Gal4 level and presynaptic inhibition (Figure 4.7A). Thus differential reduction in GABA$_B$R2 expression level with RNAi suppresses presynaptic inhibition accordingly, suggesting that the level of GABA$_B$R expression determines ORN sensitivity to presynaptic inhibition.

We further examined whether the heterogeneity in presynaptic inhibition is preserved in the antennal lobe output PNs. We compared the percent change in slope, or gain modulation, in PNs (see Figure 4.1) with the degree of presynaptic inhibition (Figure 4.7B) and also with the *GABA$_B$R2-Gal4* intensity (Figure 4.7C) for four different glomeruli. In the extreme, the V glomerulus ORNs have little or no presynaptic inhibition or *GABA$_B$R-Gal4* labeling, and blocking GABA$_B$R does not alter the PN gain. Conversely, the ORNs projecting to DA1 have a high level of presynaptic inhibition and *GABA$_B$R-Gal4* intensity, and blocking GABA$_B$R causes an increase of 153% in the gain of PN response. Thus, there is a tight correlation between the level of presynaptic inhibition in ORNs and the degree of gain modulation in PNs, suggesting that glomerulus-specific gain modulation may convey important information for olfactory behavior.

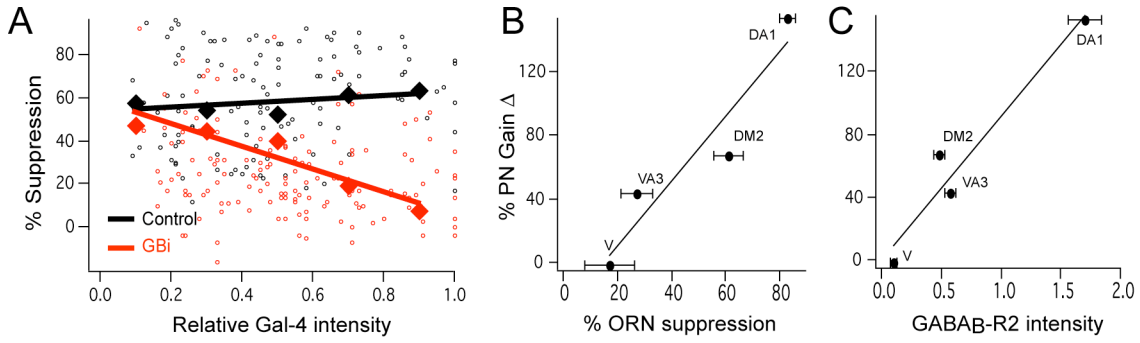

Figure 4.7. The level of $GABA_B$ receptors in ORNs sets the level of gain modulation for PN response of select glomeruli.
(**A**) *$GABA_BR2$-RNAi* (*GBi*) was differentially expressed in different ORN types by the *Or83b-Gal4* promoter. Flies expressed GCaMP and one copy of *GBi* in Or83b neurons. Two-photon imaging of ORN presynaptic calcium was used to measure the calcium response to olfactory nerve stimulation in the presence and absence of SKF97541. The percent suppression in integrated ΔF/F over time is plotted as a function of the Gal4-intensity measured by the baseline GCaMP fluorescence intensity. The Gal4-intensity for each glomerulus is normalized to the brightest glomerulus within each preparation. Each glomerulus is plotted as a small circle and the average within bins (0.2 intensity units in size) are plotted as large diamonds. n, 118 from 5 preparations (control) and 145 glomeruli from 7 preparations (*GBi*). (**B**) The percent change in PN gain (from Figure 1) is plotted as a function of the level ORN presynaptic inhibition of the same glomeruli. (**C**) The percent PN gain change as a function of the *$GABA_BR2$-Gal4* reporter intensity. n, 4-8 (**B-C**).

4.7 $GABA_BR$-mediated gain control improves odor guided-behavior

Pheromone sensing ORNs in the DA1 and VA1lm glomeruli exhibit high levels of presynaptic inhibition. We thus hypothesized that gain control in pheromone detection is important for mating behavior. We have adopted an established mating assay in which we measure the latency of courtship exhibited by a single male fly toward a virgin female in an environment with no light. The virgin female was decapitated to prevent movement and any communication from female to male. We used a chamber (40 mm in diameter, 5 mm in height) with mesh screen bottom to create a pheromone gradient for this assay (Figure 4.8A). We monitored the male with time lapse imaging at a rate of 0.2 Hz for thirty minutes. Upon finding the female, the male exhibits robust courting behavior hallmarked by wing vibration and attempted copulations. In computer analysis, male flies are also found to spend long durations of time in close proximity to the female fly (Figure 4.8A).

Within 30 minutes, approximately 60% of control flies have located the female fly (Figure 4.8B). In contrast, only about 30% of the male flies expressing *$GABA_BR2$-RNAi* in Or83b ORNs. We next asked whether gain modulation in VA1lm, a glomerulus that responds specifically to female pheromones[28], affects male localization of the female fly. We measured courtship latency in male flies expressing *$GABA_BR2$-RNAi* in only Or47b ORNs. Remarkably, these flies exhibit the same courtship latency as those flies expressing *$GABA_BR2$-RNAi* in many ORNs (Figure 4.8B). We performed further analysis to ask whether impaired localization is due to a reduction in locomotor activity. During the first five minutes in this assay when most of the male flies have not found the female, there is no significant difference in travel distance among experimental groups. These

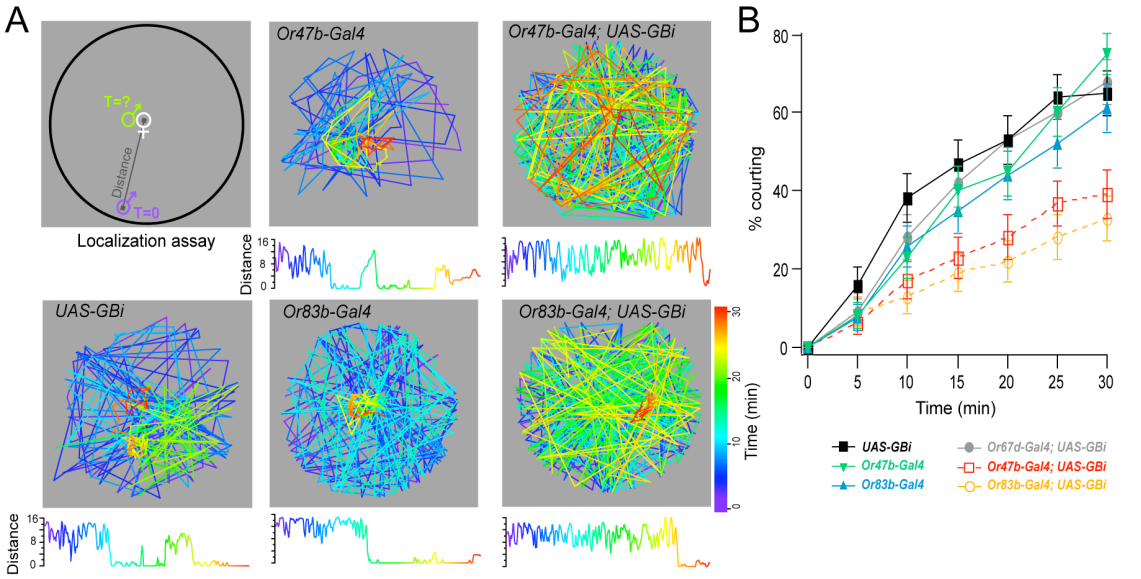

Figure 4.8. Knockdown of $GABA_B$ receptors impairs odor object localization. (**A**) A modified courtship assay was used to measure latency in odor object localization. Measurements record the location of the male fly as well as the distance between the male and immobilized female at each time point. The coordinates and distances (mm) as a function of time (color scale) are depicted for representative control flies (columns 1,2) and those expressing *$GABA_BRNAi$* (*GBi*) in ORNs (column 3). Distance trances were smoothed with a Gaussian filter. (**B**) Percent of males that initiate courtship behavior as a function of time. n, 53-71. Error bars show SEM. At the 30-minute time point all controls are significantly different for those flies expressing *GBi* in Or47b and Or83b ORNs. $p \leq 0.01$, Z-test.

results suggest that reduction of olfactory gain in pheromone sensing ORNs facilitates the process of mate localization.

4.8 Discussion

Capturing and amplifying behaviorally relevant stimuli in early sensory processing are critical steps for signal recognition and discrimination. Appropriate implementation of gain control in the first synapse plays an important role in sensory processing[33]. In this study, we investigated gain control in the first synapse of the *Drosophila* olfactory system. We used two-photon imaging to monitor activity in selective neural populations in the antennal lobe. Specific blockade of $GABA_BRs$ reveals a scalable presynaptic inhibition to suppress olfactory response at high odor concentrations. Pharmacological and molecular experiments suggest that $GABA_BRs$ are expressed in primary olfactory receptor neurons. Furthermore, the level of presynaptic inhibition is different in individual glomerular modules, which is tightly linked to the level of $GABA_BR$ expression. We have begun to investigate the importance of presynaptic $GABA_BRs$ in olfactory localization, and found that reduction of $GABA_BR$ expression in the presynaptic terminal of ORNs impairs the ability of male flies in locating potential mates.

Our study revealed that expression of $GABA_BR2$ in ORNs is necessary for presynaptic inhibition. Unexpectedly, we discovered heterogeneity in the levels of presynaptic inhibition among different glomeruli. Varying $GABA_BR2$ expression level in ORNs with molecular manipulations is sufficient to produce predictable alterations in presynaptic inhibition in specific glomeruli. Together these experiments argue that

presynaptic $GABA_BR$ expression level is a determinant of glomerulus-specific olfactory gains in the antennal lobe. A recent report revealed that there is a non-linear transformation between ORNs and PNs that is heterogeneous between glomeruli[34]. In other words, PNs innervating a given glomerulus have a unique response range for its ORN input. Given that ORNs are the main drivers of PN response[35,36], it is plausible that the heterogeneity in presynaptic inhibition contributes to the heterogeneity in ORN to PN transformations observed by Bhandawat and colleagues. Additionally, heterogeneity in GABA release by LNs[7] could also contribute to heterogeneity in presynaptic inhibition. It is interesting to note that when presynaptic inhibition is abolished, heterogeneity remains in the input-output curves of PN response to the four different odors in our experiments (Figure 4.1), suggesting that other mechanisms such as probability of vesicle release contribute to the heterogeneity as well.

Theoretical analysis of antennal lobe coding has recently suggested that the non-linear synaptic amplification in PNs observed by Bhandawat and colleagues provides an efficient coding mechanism for the olfactory system[37]. According to this model, the optimal distribution of firing rates across a range of odorants should be flat without clusters. Firing rates of a given ORN responses cluster in an uneven distribution. Conversely, PNs exhibit a more equalized firing rate distribution than ORNs[34]. According to the optimum coding theory, the high amplification of ORN to PN transformation generates a more even distribution of PN firing rates that should facilitate odor discrimination. However, this model of olfactory coding poses a potential problem. The high gain in this synaptic amplification reduces the dynamic range of PNs, causing a loss of information about concentration variation that could be important for an animal to

localize odor objects. Presynaptic inhibition may provide a mechanism to expand the dynamic range of the olfactory system. For some glomerular modules that mediate innate behaviors such as avoidance of the stress odorant CO_2[19], there is a potential trade off for odor sensitivity and dynamic range. The lack of $GABA_BR$ in the CO_2 sensing ORNs could be important to maintain high sensitivity.

Pheromones play an important role in *Drosophila* mating behaviors[38,39] and our results indicate that pheromone sensing ORNs have high levels of $GABA_BR$, which is correlated with a high level of presynaptic inhibition in these ORNs. We have found that mate localization is impaired in the absence of presynaptic inhibition in one pheromone sensing glomerulus. It is interesting to note that in addition to the pheromone sensing ORNs, the palpal ORNs also exhibit high $GABA_BR$ expression level. Although the behavioral role of the palpal ORNs has not been determined, it is possible that they are also important for odor object localization. There are two potential mechanisms for the role of $GABA_BR$ in olfactory localization. $GABA_BR$-mediated activity-dependent suppression of presynaptic transmission on a short time scale provides a mechanism for dynamic range expansion. On a longer time scale, activity-dependent suppression provides a mechanism for adaptation, hence a high pass filter to allow the detection of phasic information[5]. Further experiments will be necessary to determine which property is important for olfactory localization.

Intraglomerular and interglomerular presynaptic inhibition mediated by $GABA_BRs$ have been described in the mammalian olfactory system. Intraglomerular presynaptic inhibition was suggested as a mechanism to control input sensitivity while maintaining the spatial maps of glomerular activity[5]. Interglomerular presynaptic

inhibition was proposed as a mechanism to increase the contrast of sensory input[40]. A recent report revealed a similar gain control mechanism by interglomerular presynaptic inhibition in the *Drosophila* olfactory system[12] where $GABA_BR$ expression in ORNs was shown to scale the gain of PN responses. Interestingly, most if not all of the presynaptic inhibition was suggested to be lateral. In contrast, our study does not seek to distinguish between intra- and interglomerular presynaptic inhibition, however, we do find evidence that the VA1lm glomerulus receives significant intraglomerular presynaptic inhibition (Figure 4.4D-E). Thus, despite significant differences between the insect and mammalian olfactory systems[41], the inhibitory circuit in the first olfactory processing center appears remarkably similar.

Based on whole cell recordings of PNs in response to ORN stimulation, Olsen and Wilson[12] suggest that both $GABA_AR$ and $GABA_BR$ are expressed in ORNs to mediate presynaptic inhibition and that $GABA_AR$ signaling is a large component of lateral presynaptic inhibition. In contrast, our study, which employed direct optical measurements of presynaptic calcium and synaptic vesicle release, suggests that $GABA_BRs$ but not $GABA_ARs$ are involved in presynaptic inhibition. To resolve these discrepancies further molecular experiments will be important to determine conclusively whether ORNs express $GABA_AR$ and whether the receptor contributes to gain control. Furthermore, the antennal lobe is a heterosynaptic system comprised of at least three populations of neurons that include ORNs, LNs and PNs. Therefore, how these different populations of neurons respond to GABA signaling and what contribution they make to olfactory processing in the antennal lobe is a critical question for future investigation.

We have demonstrated differential presynaptic gain control in individual olfactory input channels and its contribution to the fine–tuning of physiological and behavioral responses. Synaptic modulation by the intensity of receptor signaling is reminiscent of the mammalian nervous system where expression levels of AMPA glutamate receptors play an important role in regulating synaptic efficacy[42]. Furthermore, presynaptic regulation of $GABA_BR$ signaling provides a mechanism to modulate the neural activity of individual input channels without much interference with overall detection sensitivity because this mechanism of presynaptic inhibition would only alter responses to high intensity stimuli. In parallel, it is tempting to speculate that global modulation of interneuron excitability should alter the amount of GABA release across channels, thus providing a multi-channel dial of olfactory gain control that may reflect the internal state of the animal.

4.9 Methods

Experimental Preparations. The following transgenic lines were used: 1) *UAS-GCaMP56*[43], 2) *GH146-Gal4*[14], 3) *Or83b-LexAVP16*[25], 4) *LexAop-GCaMP-IRES-GCaMP,* 5) *Or83b-Gal4*[30], 5) *UAS-spH*[7] with the transgene mobilized onto the third chromosome, 6) *Gr21a-Gal4*[44], 7) *UAS-VR1E600K*[27], 8) *GH298-Gal4*[14].

Preparations were made and two-photon imaging was performed as described in chapter 2. In preparations for labial nerve stimulations, the nerves were similarly preserved and carefully severed far from the brain. In experiments using ChR2 stimulation, the saline also contained 50 μM retinal. Loose patch recordings were performed as described in chapter 2.

Picrotoxin and CGP54626 (Tocris) were dissolved as 2000x stocks in DMSO. GABA (Sigma) was prepared fresh daily and dissolved as a 1000x stock in AHL saline. SKF97541 (Tocris) was dissolved as a 500x stock in 100 mM NaCl. Capsaicin (Sigma) was dissolved as a 100x stock in ethanol. The appropriate volume (1-4 μL) was first diluted with 100 μL of AHL saline and then added to the preparation to achieve the final concentration.

Odor delivery. A constant carrier airflow of 1 L/min was applied to the antennae in a pipe of 12 mm in diameter. Odor onset was controlled by solenoid valves that mixed a defined percentage of the carrier air with air re-directed through 100 mL bottles containing 20 μL of odorant on a piece of filter paper. Ethyl hexanoate was dissolved 1:10,000 (V/V) in mineral oil. The concentration of odorant inside the bottle was measured to be approximately 80 ppm for 2-phenylethanol and 300 ppb for ethyl hexanoate, using a PID calibrated to isobutylene standard reference. *cis*-Vaccenyl acetate was applied by redirecting a percentage of the airflow over a piece of filter paper with 1 μL of *cis*-vaccenyl acetate inside a glass pipette located about 5-10 mm from the antennae. CO_2 was applied by addition of a small amount of gas to the carrier airflow to achieve the final percentage concentration.

Analysis of imaging data was performed using Igor Pro (Wavemetrics) and a custom macro. Statistical analysis was done using Student's T test in Excel and results were considered significant if the p value was less than 0.05. Input-output functions were fit with y=m*log(x) + b.

$GABA_BR2$ Expression. For $GABA_BR2$ immunohistochemistry on brain cryosections and whole mounts was performed as previously described[24]. Antennal

sections were obtained by mounting live fly heads in OCT, Freezing at -20°C on the stage of a cryostat and 12 μm thick section were cut. Slides were immediately fixed with ice-cold 4% paraformaldehyde in 0.1 M $NaHPO_4$ for 10 min. Staining was performed using standard techniques with chick-α-GFP (Abcam), rabbit-α-$GABA_BR2$, and nc82 (DSHB) primary antibodies at 1:1000, 1:10,000 and 1:100 respectively. In quantification of differences between control and $GABA_BR2$-RNAi flies (Figure 2B), staining intensity was normalized to nc82 staining. The background intensity was first subtracted from Z-projections of the antennal lobe. Then the $GABA_BR2$ intensity was normalized to the nc82 to generate a ratio of $GABA_BR2$ to nc82 intensity.

RNA was prepared with the RNeasy kit (Qiagen) and RT-PCR was performed using SuperScript One-Step RT-PCR with Platinum Taq (invitrogen) according to manufacture's instructions. Sequence of Primers for RT-PCR:

GABABR2-F GCCTGGGAAACTCGACATGTTTCTA

GABABR2-R TTGCTCCAGTTCGCACACCGAGGA

RP49-F ATGACCATCCGCCCAGCATACA

RP49-R TGTGTATTCCGACCAGGTTAC

Rh4F TGTACTGCACACCGTGGGTTGTCCTG

Rh4R AGCTGAAAAAGAAGATGGTGCCCACAAAC

1.5 kb of genomic DNA upstream of the *$GABA_BR2$* gene was obtained using the following primers:

R2upXbaI AAATCTAGAATAATGTCAGCCATAAGGAT

R2downBamHI AAAGGATCCGTTGACCGCGTGGGCTGTAAA

The genomic DNA was inserted into *pTGal4* vector via the xbaI and BamHI restriction sites. The *GABABR2-RNAi* fly was made by cloning a150 bp inverted repeat of the 3' end of *$GABA_BR2$* coding region with a 50 bp linker into the pUAST vector between the EcoRI and Xba1 restriction sites. PCR using the following primers was used to obtain the PCR fragments:

GBR2downXbaI AAATCTAGAGGGACTCTTCTCGGTGAGGA

GBR2upEcoRI AAAGAATTCGTAAGGTCAGCCGGAGCTCT

GBR2upXbaI AAATCTAGAGTAAGGTCAGCCGGAGCTCT

GBR2insideDownXbaI AAATCTAGACGCCCTCGAGCAGTTCCGTC

Transgenic flies were generated by standard method.

Behavioral assay

Flies were collected within a few hours of eclosion. Males and females were housed separately in groups of 10-30 flies per vial and aged 3-5 days before experiments. The mating chamber was a small plastic dish (40 mm diameter, 5 mm height) placed upside down on a stainless steel mesh screen (0.178mm openings, Small Parts, Inc.) The mesh screen was suspended approximately two centimeter above water in a clear container. Flies were gently aspirated into the chamber through a small hole that was covered with clear plastic. Experiments were done in a light proof box with illumination from a 660 nm LED panel. Images were captured using a Logitech Quickcam and an acquisition script written in Labview (National Instruments). Significance was tested using a hypothesis test for proportions, the z-test. Movies were analyzed by eye in ImageJ and by use of the object tracker pluggin to track the position of the male over time.

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Chapter 5: Presynaptic Inhibition Induced by Neuromodulators

5.1 Abstract

Sensory systems must be able to extract features of environmental cues within the context of different physiological states of the organism. We examined the effect of a neuropeptide, *Drosophila* tachykinin (DTK), and the biogenic amine, serotonin, on antennal lobe activity. We found that a subpopulation of *Drosophila* LNs express DTK, and its receptor (DTKR) is expressed in ORNs. Using two-photon microscopy, we found that DTK suppresses presynaptic calcium and synaptic transmission in ORNs. Furthermore, serotonin enhances the responses of GABAergic local interneurons resulting in a reduction of neurotransmitter release from ORNs via $GABA_BR$-dependant presynaptic inhibition. In addition, serotonin enhances sensitivity of PNs in an odor specific manner, consistent with increased lateral interactions by LNs. Together our data indicate two neuromodulatorory mechanisms that increase presynaptic inhibition of sensory input. Thus inhibition at the first synapse is likely a powerful mechanism to alter sensory representation to meet the physiological needs of the organism.

5.2 Introduction

The response to a sensory stimulus changes with the physiological state of an organism. The nervous system must alter the acuity and resolution of different sensory systems so that processing of certain stimuli is enhanced, while less relevant stimuli can be neglected. This is accomplished, in part, by the local release of neuromodulators, substances often released within a specific context (e.g. fear, hunger and reproduction) causing alteration of the response properties and synaptic efficiency of individual neurons without directly causing excitation or inhibition[1,2]. Neuromodulation is usually mediated

by G protein-couple receptors (GPCRs). The *Drosophila* genome contains at least 44 genes coding for neuropeptide GPCRs, 3 genes for protein hormone GPCRs, and 21 genes for biogenic amine GPCRs[3].

It is likely that certain neuropeptides are employed as neuromodulators in the antennal lobe circuitry of insects[4,5], as also suggested in the olfactory bulb in mammals[6,7]. One neuropeptide gene that has been implicated in olfactory processing is *dtk* [8], a gene encoding five tachykinin-related peptides, DTKs[9]. The DTKs are expressed in about 150 neurons in the *Drosophila* brain, and in the antennal lobe glomeruli there are extensive DTK-immunoreactive arborizations derived from a subset of antennal lobe LNs[10]. Two DTK receptors, DTKR and NKD, have been identified in *Drosophila*[11,12] and one of these, DTKR, is strongly expressed in antennal lobe glomeruli[13]. Behavioral evidence for a role of DTKs in olfaction was obtained from analysis of flies where *dtk* expression was knocked down globally using RNA interference; these flies displayed diminished odor sensitivity[8].

In addition to neuropeptide modulation, there is evidence in insects to suggest that serotonin plays a role in olfactory processing. In *Drosophila*, glomeruli receive innervation from two 5HT-immunoreactive neurons[14,15]. Moths possess morphologically similar 5HT-ir neurons[15,16] and 5HT enhances the excitability and responses of AL neurons[17-20]. The increased excitability is caused by a reduction in two K^+ conductances[21]. The levels of 5HT in the ALs vary throughout the day, peaking when moths are most active[18] and 5HT increases the behavioral sensitivity of males to sex pheromone[22].

In this study, we found that the neuropeptide DTK is expressed in a subset of GABAergic LNs. Furthermore, the receptor for DTK, DTKR, is expressed in ORNs and mediates presynaptic inhibition that appears to be independent of $GABA_BR$ presynaptic inhibition. In addition, we examined the effects of 5HT on specific populations of neurons and the interactions between glomeruli in *Drosophila*. We found that 5HT enhances the sensitivity of PNs in an odor-dependant manner (indicating a modulation of the intrinsic local circuitry), and 5HT increases the responsiveness of inhibitory local interneurons. Modulation of multiple neuron types in the AL by multiple neuromodulators is a potential mechanism to alter olfactory representation in a state-dependent manner. This study lays out the foundation for future genetic dissection of neuromodulation and its contribution to olfactory information processing.

5.3 The tachykinin neuropeptide is expressed in the antennal lobe

Local interneurons (LNs) in the antennal lobe have been suggested to play a role in the transformation of olfactory information, and thus the shaping of elaborate behavioral responses to odor cues, through synaptic interactions with the ORNs and PNs in the antennal lobe circuitry[23-29]. Immunocytochemistry with a well-characterized antiserum has previously revealed that DTKs are expressed in certain LNs that form a dense supply of neuronal processes to the antennal lobe glomeruli[10]. To further analyze DTK expression in antennal lobe LNs we applied the same tachykinin antiserum to flies with GFP expression in a large population of LNs due to the transgenes *UAS-CD8-GFP* and *GH298-Gal4*. We found that about 21 (21 ± 0.9; n=5) LNs were tachykinin-immunoreactive. Of these DTK immunoreactive neurons, approximately 70% are also

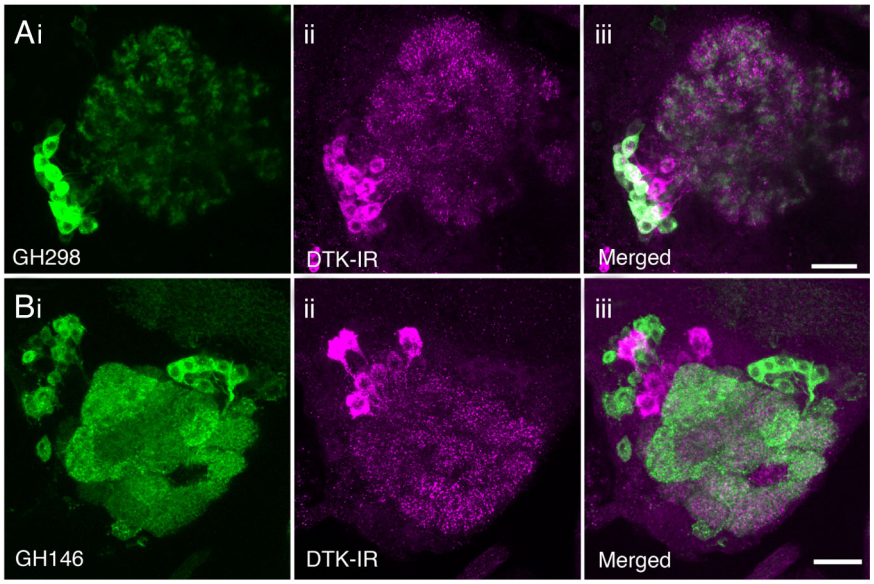

Figure. 5.1. **Distribution of DTK peptide immunoreactivity in local antennal lobe interneurons.** Using antiserum to LemTRP-1 (magenta) we localized DTK peptide distribution in relation to Gal4-driven GFP (image stacks from wholemounted specimens). **A1-3.** Many LNs of the GH298-Gal4 line express DTK-IR. **B1-3.** We did not detect DTK-IR in any of the projection neurons displayed in the GH146-Gal4 line. Scale bars 20 μm.

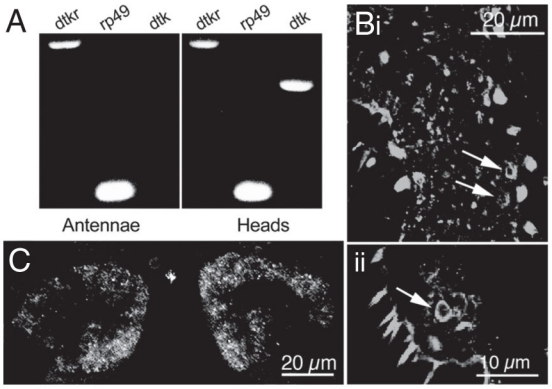

Figure 5.2. Tachykinin receptor (DTKR) expression in the olfactory receptor neurons (ORNs). A. Molecular analysis of the *dtkr* expression in ORNs. Reverse transcriptase PCR products from isolated antennae and whole heads of w^{1118} flies. Expression of *dtkr* in antennae and in whole heads is observed. However, *dtk* expression (peptide precursor) is only detected in heads. Parallel reactions with *rp49* as a template control were performed. **B** and **C**. DTKR immunoreactivity was observed in the ORNs (arrows) of the antenna (B, and details in B') and in most of the glomeruli of the antennal lobe neuropils (C) of w^{1118} flies. The two antennal lobes are shown in a frontal 9 μm thick section.

GH298-positive (15 ± 0.5; n=5) (Figure 5.1A), In contrast we found that PNs identified by the *GH146-Gal4* do not express DTKs (Figure 5.1B), confirming earlier work that suggested lack of DTK in all PNs[10].

We next investigated the synaptic target of DTK neurons. Immunocytochemistry using an antiserum to the tachykinin receptor DTKR[13] showed that expression in the antennae is localized to cell bodies of ORNs (Figure 5.2B). DTKR immunoreactivity was also detected in the glomeruli of the antennal lobes (Figure 5.2C). To verify that *dtkr* is expressed in ORNs we performed reverse transcriptase PCR analysis of isolated antennae. We found that *dtkr* transcripts are in RNA extracts from antennae as well as those from whole heads (Figure 5.2A). On the other hand, transcript of the peptide gene *dtk* is only detected in RNA from heads (Figure 5.2A). This result suggests that cells in the antenna express DTK receptors. We next expressed *dtkr-RNAi* in Or83b neurons to test whether ORNs are the antennal cells that express DTKR. The efficacy of the RNAi was tested by quantitative PCR in flies bearing the *elav-Gal4* or the *Or83b-Gal4* and *UAS-dtkr-RNAi* transgenes. We found that expression of the RNAi in Or83b neurons dramatically reduced the immunoreactivity from the DTKR antiserum. Thus, ORNs appear to be the main population of cells expressing DTKR in the antenna.

5.4 The tachykinin receptor mediates presynaptic inhibition in ORNs

We next asked whether neurotransmission of ORNs is modulated by the DTK signaling system. First we measured calcium in ORN axon terminals using two-photon imaging in flies expressing the calcium sensor GCaMP in ORNs[30,31]. We drove the expression of *UAS-GCaMP* with the ORN-specific line *Or83b-Gal4*. Electrical

stimulation of the olfactory nerve elicits a calcium influx in ORN terminals, and this calcium response was reduced by the application of DTK (Figure 5.3A,B). When quantifying the effect of DTK across preparations we found an average 38% reduction in presynaptic calcium responses (Figure 5.3C). Presynaptic calcium entry triggers the release of neurotransmitters from synaptic vesicles, hence DTK mediated reduction in presynaptic calcium should be accompanied by a decrease of synaptic vesicle release. To investigate this we used two-photon imaging of the antennal lobe of flies expressing synaptopHluorin (spH), an indicator of synaptic vesicle release[32], in Or83b neurons. Electrical stimulation of the olfactory nerve elicited an increase of spH fluorescence and applying DTK resulted in a reduction of this fluorescence signal by approximately 50% (Figure 5.3D-F). We next examined whether the DTK mediated presynaptic inhibition requires DTKR expression in ORNs. When *dtkr-RNAi* was expressed in Or83b neurons the same stimulation of the olfactory nerve produced a larger increase in spH fluorescence intensity. Furthermore *dtkr-RNAi* abolished sensitivity to DTK application (Figure 5.3F). These experiments indicate that DTKR in ORNs mediates presynaptic inhibition by reducing calcium influx into axon terminals and reducing neurotransmission.

Next we investigated the role of DTKR in the modulation of neurotransmission from the ORNs in responses to high (10^{-1}) and low (10^{-4}) concentrations of the food related odors: ethyl-3-hydroxybutyrate and methyl hexanoate. To monitor the activity in the ORN axon terminals we imaged flies expressing synaptophluorin in Or83b neurons in control flies and flies that also express *dtkr-RNAi*. High concentration of ethyl-3-hydroxybutyrate mainly activated five glomeruli (DM1, DM2, DM5, VM2 and VM3)

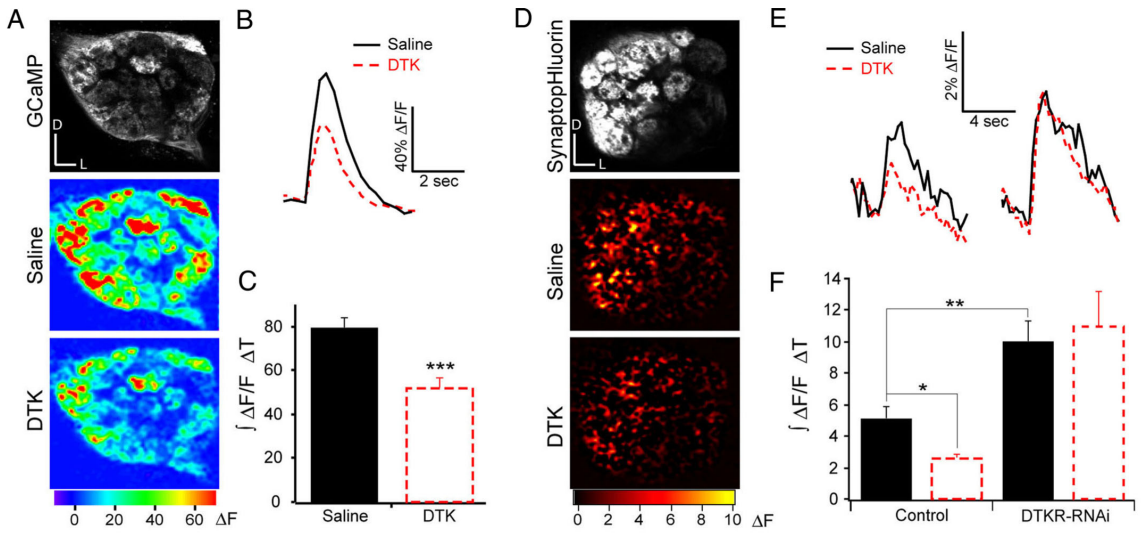

Figure 5.3. Tachykinin receptors mediate presynaptic inhibition in *Drosophila* olfactory receptor neurons. (**A**) Two-photon images of the antennal lobe of a fly expressing GCaMP in Or83b neurons (top). Pseudocolored images reveal the response to electrical stimulation of the olfactory nerve in saline (middle) and after addition of 10 mM DTK (bottom). (**B**) Fluorescence change over time. Black and red traces show representative responses before and after drug application, respectively. (**C**) Effect of DTK on presynaptic calcium response quantified as the integrated fluorescence change over time (area under the curve in [B]) across preparations. (**D**) Two-photon images of the antennal lobe of a fly expressing synaptopHluorin in Or83b neurons (top). Pseudocolored images reveal the response to electrical stimulation of the olfactory nerve in saline (middle) and after addition of 10 mM DTK (bottom). (**E**) Fluorescence change over time; traces are the average of three trials. (**F**) Effect of DTK on presynaptic calcium response quantified as the integrated fluorescence change over time across preparations. Electrical stimulations were 1 ms in duration and 10 V in amplitude, 45 pulses A-C or 80 pulses (D-F) at 100 Hz. n, 16 (C) and 5 (F). TTest * $p \leq 0.05$, ** $p \leq 0.01$*** $p \leq 0.001$.

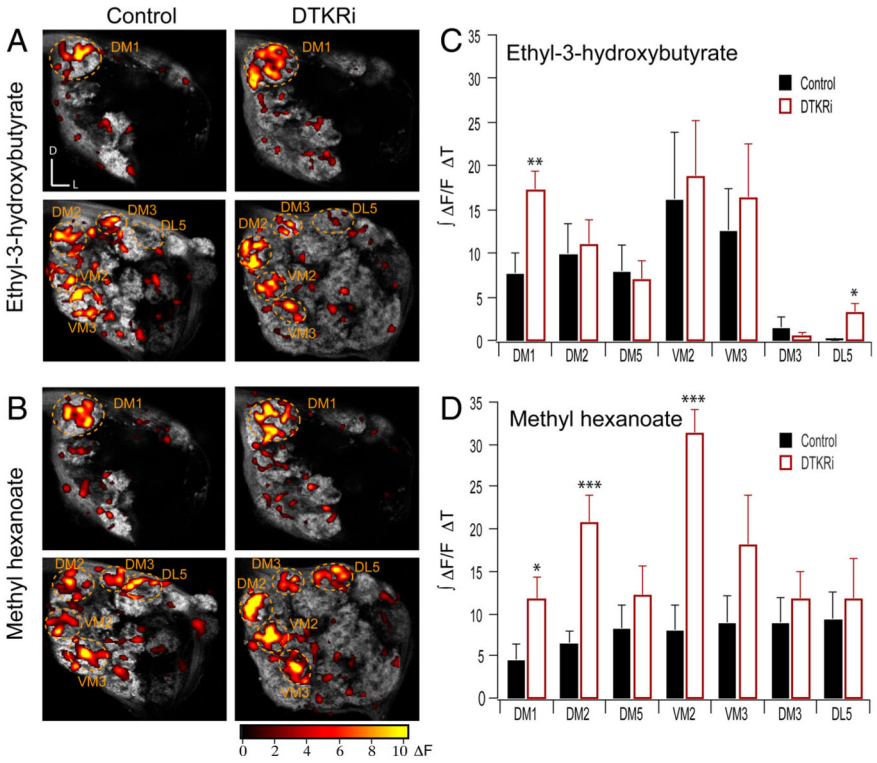

Figure 5.4 Presynaptic tachykinin receptors modulate odor-evoked olfactory receptor neuron transmission. Two-photon imaging of ORN synaptic transmission elicited by odor stimulation at high odor concentration (10^{-1}) in control flies and flies expressing DTKRi in Or83b neurons. (**A, B**) Two-photon images of the antennal lobe from flies expressing spH in Or83b neurons in control flies (left column) and flies that also express DTKR-RNAi in ORNs (right column). Pseudocolor overlays reveal the change in fluorescence in response in response to (**A**) ethyl-3-hydroxybutyrate and (**B**) methyl hexanoate at two different optical planes. (**C**) Ethyl-3-hydroxybutyrate and (**D**) methyl hexanoate evoked responses quantified as the integrated fluorescence change over time for each glomerulus. Odors were delivered for 2 seconds. n, 7-8 preparations. TTest * $p \leq 0.05$, ** $p \leq 0.01$*** $p \leq 0.001$.

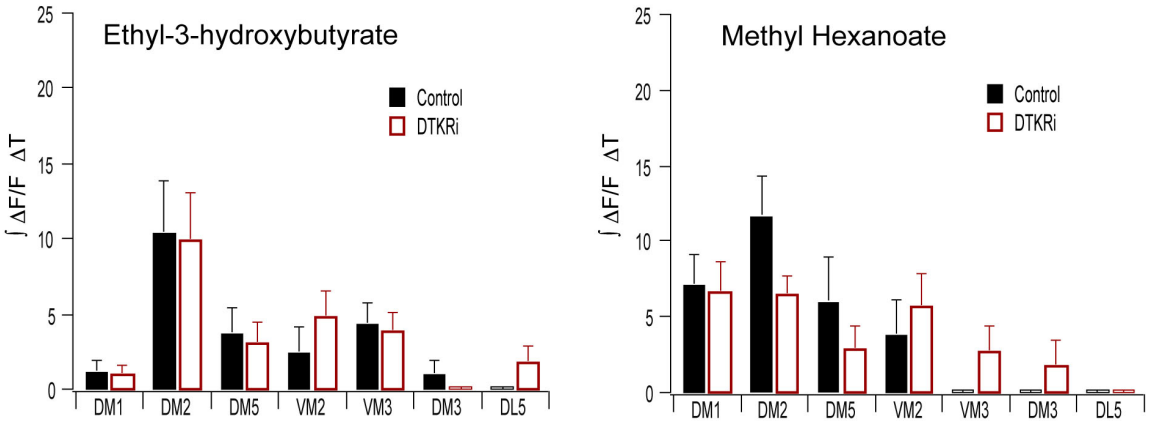

Figure 5.5. Presynaptic tachykinin receptors do not affect odor-evoked response to low odor concentration.

Two-photon imaging of ORN synaptic transmission elicited by odor stimulation at low odor concentration (10^{-4}) in control flies and flies expressing DTKRi in Or83b neurons. Odor-evoked responses for ethyl-3hydroxybutyrate and methyl hexanoate are quantified as the integrated fluorescence change over time for each glomerulus. No significant differences (TTest) were detected between control and DTKRi flies. Flies express spH in Or83b neurons. Odors were delivered for 2 seconds. N = 7-8 preparations.

(Figure 5.4A,C). Reduction of DTKR levels in Or83b neurons, significantly increased olfactory responses in the DM1 and DL5 glomeruli (Figure 5.4A,C). High concentration of methyl hexanoate activated seven glomeruli (DM1, DM2, DM5, VM2, VM3, DM3 and DL5) in control flies and knocking down DTKR expression in Or83b neurons significantly increased the response in the DM1, DM2 and VM2 glomeruli (Figure 5.4B,D). Stimulating the flies with the lower odor concentration (10^{-4}) did not result in any significant change in response between control and DTKR-RNAi expressing flies (Figure 5.5). Thus, olfactory response in antennal lobe glomeruli is modulated by the DTK signaling pathway in some but not all glomeruli.

5.5 Tachykinin receptor signaling is independent of $GABA_B$ receptors

Previous work has found that expression of the metabotropic $GABA_BR$ in ORNs of *Drosophila* provides a similar presynaptic inhibition[33]. Since many of the DTK expressing LNs also appear to produce GABA we investigated whether the peptide and amino acid transmitters interact presynaptically on the ORNs. We used two-photon imaging of calcium or synaptic transmission in ORNs to monitor DTKR and $GABA_BR$ modulation of the presynaptic response to electrical stimulation of the olfactory nerve.

We first asked if the two signaling pathways interact to produce a synergistic suppression of presynaptic calcium. We quantified the suppression induced by the $GABA_BR$ agonist SKF97541 (SKF), DTK or both together (Figure 5.6A). Addition of the two agonists together produces an additive effect that was not different from the sum of the independent effects. Thus, the $GABA_BR$ and DTK pathways do not produce

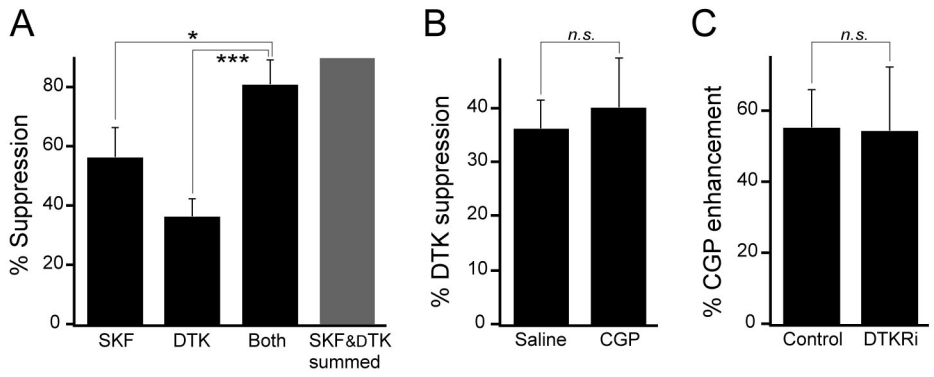

Figure 5.6. Presynaptic inhibition by tachykinin receptors is independent of $GABA_B$ receptor signaling.

A - C Two-photon imaging of ORN calcium or synaptic transmission was used to monitor DTKR and $GABA_BR$ modulation of the presynaptic response to electrical stimulation of the olfactory nerve. (**A**) Suppression of presynaptic calcium activity by application of the $GABA_B$ agonist SKF97541 (SKF) [33,34], DTK or both. The grey bar (right) shows the quantitative summation of the SKF and the TK effect. The effect does not appear to be synergistic. (**B**) Blocking $GABA_BRs$ does not alter the DTK-mediated suppression of presynaptic calcium. DTK was added in saline or in the presence of the $GABA_BR$ antagonist CGP54626 (CGP) [28,33,34]. (**C**) Blocking $GABA_BR$ with CGP enhances synaptic transmission from ORNs in control flies and flies that also express *dtkr-RNAi* in ORNs. Flies express GCaMP (A, B) or spH (C) in Or83b neurons. Suppression and enhancement are calculated by comparing the response before and after drug addition, and the values are given as the percent decrease or increase in integrated $\Delta F/F$. Electrical stimulations were 1 ms in duration and 10 V in amplitude, 45 pulses (B, C) or 80 pulses (D) at 100 Hz. n, 8 (B), 5-8 (C) and 5-13 (D). TTest * $p \leq 0.05$, *** $p \leq 0.001$, *n.s.* $p \geq 0.75$. **E** Olfactory choice behavior of flies with $GABA_B$ receptor expression reduced by $GABA_BR2$-RNAi in ORNs (Or83b). The response to ethyl-3-hydroxy butyrate is affected at 10^{-5} displaying an increased response index. ANOVA, Tukey's post-test, ** $p < 0.01$.

synergistic presynaptic inhibition. Next, we asked whether blocking either the endogenous $GABA_BR$ or the DTKR signaling alters the sensitivity to the other agonist. Blockade of $GABA_BRs$ with the antagonist CGP54626 (CGP) does not alter the DTK-mediated suppression of presynaptic calcium (Figure 5.6B). Furthermore, blocking $GABA_BRs$ with CGP enhances the synaptic transmission from ORNs to the same extent in control flies and flies that also express DTKR-RNAi in ORNs (Figure 5.6C). Taken together our data indicate independent action of GABA and DTK on calcium and neurotransmission in ORNs

5.6 Serotonin enhances the responses of projection neurons

To investigate how serotonin affects the antennal lobe activity, we first looked at its effects on PN activity. In the moth *Manduca sexta*, 5HT causes an increase in membrane resistance via a reduction in two K^+ channel conductances[21] leading to the prediction that 5HT should enhance PN sensitivity[17,18,20]. Using two-photon imaging we measured the effect of 5HT in PNs to odors that activate sparse sets of glomeruli. Flies bearing the *GH146-Gal4* and *UAS-GCaMP* transgenes express G-CaMP in many PNs of the antennal lobe. Ethyl hexanoate (Figures 5.7A, B) and 3-heptanol (Figures 5.7D, E) at low concentrations excited only the DM2 and VM2 glomeruli, respectively. The responses of DM2 PNs to ethyl hexanoate were significantly enhanced by 5HT (Figure 5.7C) as were the responses of VM2 PNs to 3-heptanol (Figure 5.7F). Thus 5HT appears to enhance PN responses.

In the above experiments, the antennal lobe was sparsely activated by odors at low concentration, and lateral interactions were likely minimally evoked. To further

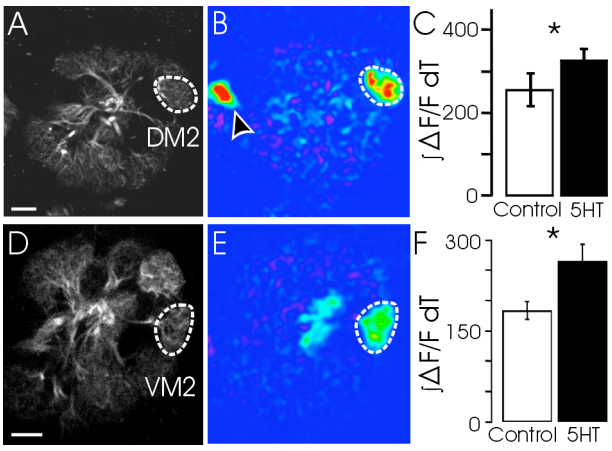

Figure 5.7. Serotonin enhances the responses of PNs to odorants activating sparse glomeruli. ***A***, Single optical plane through the AL reveals the DM2 glomerulus (hatched white outline). ***B***, Response of DM2 PNs to ethyl hexanoate (EH). Arrowhead indicates a PN cell body. ***C***, 5HT significantly enhanced the DM2 responses to EH (n=5 flies. *; $p<0.05$). ***D***, Single optical plane through the AL reveals the VM2 glomerulus (hatched white outline). ***E***, Response of VM2 PNs to 3-heptanol. ***F***, 5HT significantly enhanced the VM2 responses to 3-heptanol (n=5 flies. *; $p<0.05$). Data in ***C*** and ***F*** are represented as mean +/- SEM. Scale bar = 10µm.

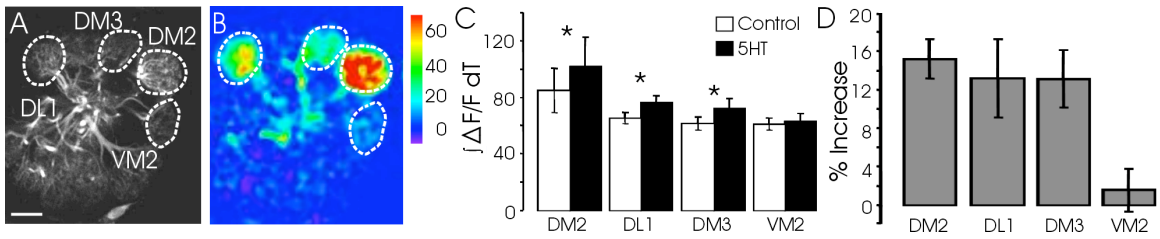

Figure 5.8. **Differential enhancement of PN responses by 5HT is odor dependant.** ***A***, Single optical plane through the AL reveals the DL1, DM3, DM2 and VM2 glomeruli (hatched white outlines). ***B***, Responses of PNs in the 4 glomeruli depicted in ***A*** to iso-amyl acetate (IAA). ***C***, 5HT significantly enhanced the responses in the DM2, DL1 and DM3 PNs to IAA (n=8 flies. *, $p<0.05$), but did not enhance the response of VM2 PNs to IAA (n=8 flies. $p=0.326$). ***D***, Percent increase in responses of DM2, DM3, DL1 and VM2 PNs after 5HT application. Data in ***C*** and ***D*** are represented as mean +/- SEM. Scale bar = 10µm.

investigate how 5HT affects interglomerular interactions, the effects of 5-HT on PN responses to isoamyl acetate (IAA), an odorant that excites multiple glomeruli, were tested. At a moderate concentration, IAA excites four different glomeruli in one optical plane (Figures 5.8A, B). The responses of PNs in three of the glomeruli (the DM2, DL1 and DM3) were significantly enhanced by 5-HT (Figures 5.8C, D) whereas the response of PNs in the VM2 glomerulus to IAA was not enhanced by 5HT (Figure 5.8C, D). This effect could not have been due to differential expression levels of 5HT receptors on VM2 PNs or the ORNs that project to the VM2 glomerulus, as the responses of VM2 PNs to 3-heptanol were enhanced by 5HT application (Figure 5.7D-F).

5.7 Serotonin induces $GABA_BR$-dependent presynaptic inhibition of ORNs

To address the possibility that the differential enhancement of PN responses by 5HT is due to a differential increase in the responses of ORNs (thus increasing the amount of input to the PNs), we examined the effects of serotonin on ORN neurotransmitter release. Flies bearing the *Or83b-Gal4* and *UAS-spH* transgenes express the synaptopHluorin, an indicator of vesicle release[35], in most ORNs. Surprisingly, 5HT significantly decreased the responses ORNs to electrical stimulation of the olfactory nerve (Figure 5.9A). This could either have been due to a direct decrease in the excitability of ORNs or an enhancement of the presynaptic inhibition impinging upon ORNs mediated by $GABA_B$ receptor expression in ORNs[36,37]. To test the possibility that 5HT enhances presynaptic inhibition, we applied the $GABA_B$ receptor antagonist CGP54626 to block any presynaptic inhibition and then applied 5HT. When 5HT was

applied to preparations that had been pre-treated with CGP54626, there was no longer any effect of 5HT on ORN response (Figure 5.9B) suggesting that the attenuation of ORN responses by 5HT was due to an enhancement of the influence of GABAergic LNs.

To test the hypothesis that 5HT modulates the activity of GABAergic LNs, we examined the effects of 5HT on the responses of flies bearing the *UAS-GCaMP* and *GAD1-Gal4* transgenes, which label most of the GABAergic LNs within the AL[35]. Serotonin application enhanced the responses of these LNs (Figure 5.10B, C) to antennal nerve stimulation (Figure 5.10D, E) while saline application did not (Figure 5.10F). Furthermore, there was no statistically significant difference in the magnitude of response enhancement for LNs in the VM2 glomerulus compared to the DM2 glomerulus (Figure 5.10G). This indicated that the differential enhancement of PN responses to IAA was not likely due to a greater enhancement of LNs innervating the VM2 glomerulus. To determine if there was a difference in the level of expression of $GABA_B$ receptors expressed by ORNs innervating the VM2 and DM2 glomeruli we measured the reporter expression levels in flies bearing the *$GABA_BR2$-Gal4* and *UAS-CD8GFP* transgenes, which had been analyzed in Root et al. (2008)[33]. There was a significantly more $GABA_BR$ reporter expression in the ORNs innervating the VM2 glomerulus than those innervating the DM2 glomerulus (Figure 5.10H). These results suggest that while 5HT enhances the responses of PNs and GABAergic LNs, the resultant increase in presynaptic inhibition impinging upon the ORNs may balanced the enhancement of PN responses in those glomeruli in which the ORNs express high levels of the $GABA_B$ receptor.

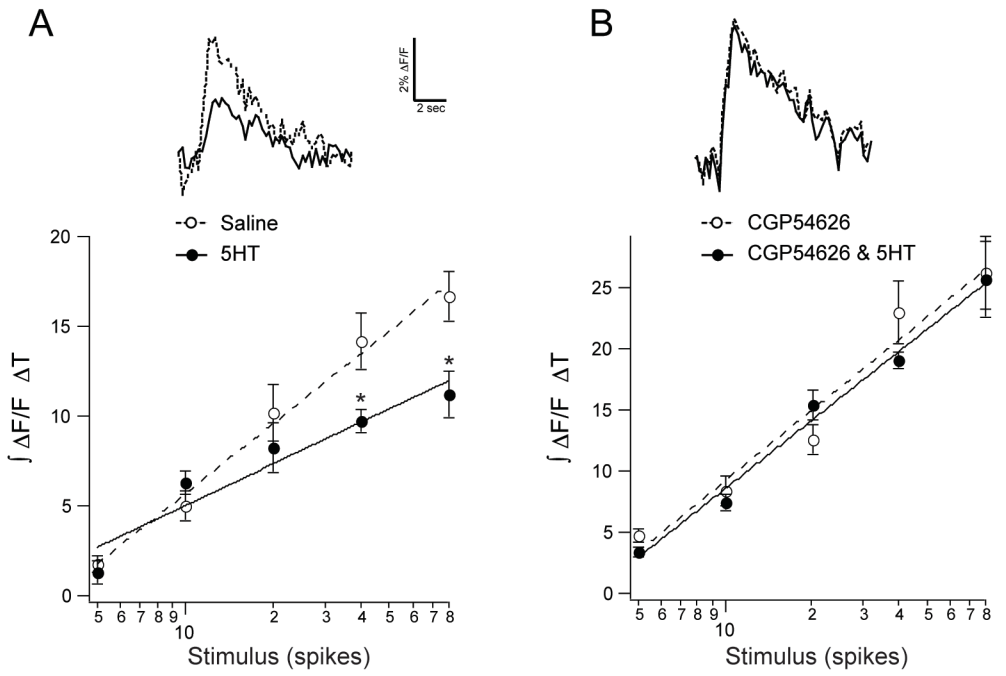

Figure 5.9. **Serotonin suppresses ORN transmission via GABAB receptor signaling.** ***A***, Serotonin attenuates ORN transmission elicited by antennal nerve stimulation (t-test, * $p< 0.05$, n=6). Upper panel; Representative traces of synaptopHluorin fluorescence change over time before (hatched line) and after (solid line) application of 5HT at 10^{-4}M; traces are the average of three trials in response to 80 spikes. Lower panel; Responses to increasing intensity of antennal nerve stimulation before (hatched line) and after (solid line) 5HT application. ***B***, The 5HT dependant attenuation of ORN responses is eliminated by the application of the GABAB receptor antagonist CGP 54626 (t-test, p=0.15-0.8, n=5). Upper panel; Representative traces of synaptopHluorin fluorescence change over time for CGP54626 alone (hatched line) and after (solid line) CGP54626 and 5HT at 10^{-4}M; traces are the average of three trials in response to 80 spikes. Lower panel; Responses to increasing intensity of antennal nerve stimulation for CGP54626 alone (hatched line) and after (solid line) CGP54626 and 5HT at 10^{-4}M. Data in lower panels are represented as mean +/- SEM.

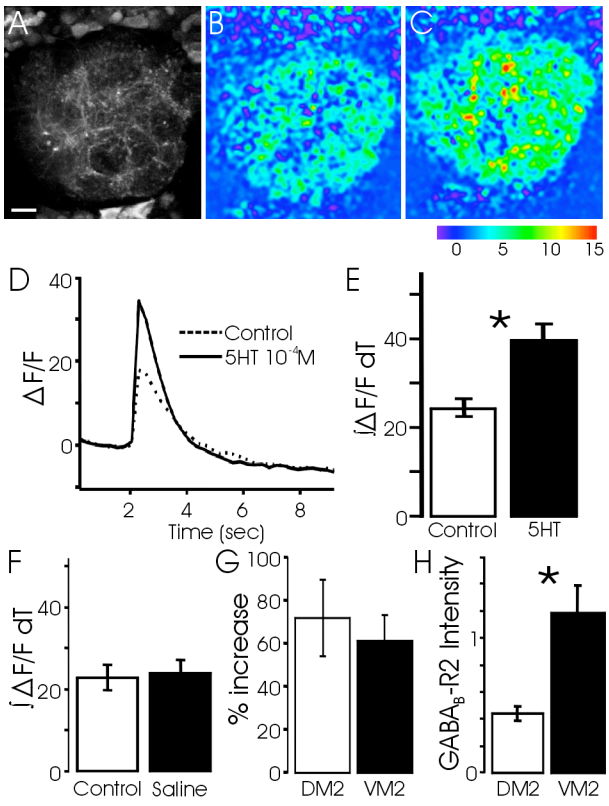

Figure 5.10. **Serotonin enhances the responses of GABAergic LNs.** ***A***, Single optical plane through the AL reveals the arborization patterns of GAD1-Gal4 LNs. LN responses to antennal nerve stimulation before ***B***, and after ***C***, 5HT application. ***D***, Average time course of LN responses across all preparations to middle intensity stimulation (8 spikes) as measured across the entire AL before (hatched line) and after (solid line) 5HT application. ***E***, Responses of GABAergic LNs to antennal nerve stimulation of 8 spikes stimulus duration were enhanced by 5HT (n=9, *; $p<0.005$). ***F***. Responses of *GAD1 Gal-4* neurons to antennal nerve stimulation of 8 spikes stimulus duration were unaffected by saline (n=7, p=0.60). ***G***. Responses of GAD1 Gal-4 neurons in the DM2 and VM2 glomeruli to electrical stimulation of the antennal nerve were equally enhanced by 5HT application (n=7, p=0.29, t-test). ***H***. Reporter intensity for ORNs innervating the VM2 glomerulus are significantly higher compared to the DM2 glomerulus (n=4, *; $p<0.05$, t-test). Data in ***B-H*** are represented as mean +/- SEM. Scale bar = 10µm.

5.8 Discussion

A novel finding in the present study is that *Drosophila* ORNs express a presynaptic neuropeptide receptor, DTKR, which appears to serve in a feedback circuit from local peptidergic interneurons, LNs, of the antennal lobe. These LNs express the peptide products, DTK1-5, of the *dtk* gene[10] and are a subpopulation of the GAD1-expressing, GABAergic LNs in the antennal lobe. Neuropeptides often colocalize with classical neurotransmitters in neurons and may act as cotransmitters at synapses[4,38,39]. We found no evidence for synergism between GABA and DTK suggesting that the two compounds act independently. This is in contrast to findings in the crayfish visual system where GABA hyperpolarizes photoreceptors and a tachykinin-like peptide potentiates this response [40].

There is ample physiological evidence to suggest that vertebrate as well as invertebrate ORN axon terminals can be presynaptically modulated by GABA and inhibitory LNs[33,41-44], but to our knowledge this study is the first to demonstrate peptidergic presynaptic inhibition of ORNs by local interneurons in any organism. In animals other than *Drosophila* there is only immunocytochemical data to suggest such circuitry. For example, in the rat, periglomerular cells, interneurons that modulate the first synaptic relay in olfactory processing, have been shown to express two neuropeptides: somatostatin and cholecystokinin[7]. Moreover, somatostatin receptor immunoreactivity in axons of the rat olfactory nerve has been demonstrated[45]. These morphological studies may suggest that presynaptic peptidergic modulation of ORNs is not exclusive to *Drosophila*. In addition, there are examples of peptidergic modulation in the olfactory system by efferent neurons. Centrifugal peptidergic modulation has been

demonstrated in the olfactory epithelia of a salamander, where neuropeptide Y was shown to enhance responses evoked by a food-related odor in hungry animals[46]. The peptide FMRFamide has been shown to modulate the activity of ORNs in the olfactory epithelium of the mouse and the salamander, but the circuitry is not clear[6,47].

In addition to peptide neuromodulation, we found that the biogenic amine, serotonin, alters antennal lobe activity. Serotonin appears to have two primary effects: an enhancement of PNs and a concomitant increase in lateral inhibition by GABAergic LNs. Studies of the AL in moths have established that the excitability of antennal lobe neurons is enhanced by 5HT[17-20], which causes a reduction of two K^+ conductances[18,21]. The resultant increase in membrane resistance should therefore decrease the amount of input current required to elicit a response, thus increasing the sensitivity of AL neurons. Our results are consistent with these effects of 5HT observed in moths. Serotonin causes *Drosophila* PNs to respond to intensities of antennal nerve stimulation that were previously sub-threshold. It is possible that 5HT causes a similar reduction in K^+ conductances in *Drosophila* AL neurons, however further biophysical studies are necessary to determine if this is the case.

When we examined the effects of 5HT on the responses of PNs to odors that activated large portions of the AL, we found evidence that 5HT modulated lateral interactions within the antennal lobe. Consistent with this we found that serotonin enhanced responses of GABAergic LNs, which in turn provide $GABA_BR$-dependent presynaptic inhibition of ORNs. In *Drosophila* different glomeruli receive variable innervation from LNs[28,48] and the lateral interactions between glomeruli are non-uniform[25,27,35,49]. Heterogeneous inhibitory interactions within the AL have been

demonstrated in other insect species[50,51] and so modulation of LN activity by 5HT likely has non-uniform effects across glomeruli.

Sensory systems must temper their function based on the physiological needs of an organism. Neuromodulation accommodates state-dependant adjustment of neural function whereby synaptic strength is altered by affecting pre-synaptic release of primary neurotransmitters or the effective synaptic current in post-synaptic cells[1,2]. By adjusting the excitability of the cellular elements within a network, neuromodulators can alter network properties[52]. In moths, the levels of 5HT in the AL cycle throughout the day, peaking when moths are most active[18] suggesting that 5HT may be a mechanism by which arousal modifies olfactory processing to suit the physiological state.

5.9 Methods

Experimental Animals and Preparations

The following transgenic flies were used: *UAS-GCaMP56*[31], *GH146-Gal4*[53], *GAD1-Gal4*[35], *UAS-spH*[35], *Or83b-Gal4*[31], *GABA$_B$R2-Gal4*[37] and *UAS-CD8GFP*. Two-photon imaging and preparations were as in described in chapters 2 and 4.

Pharmacology. Because 5HT is light sensitive, we took measures (dissecting under dim light conditions, covering preparations, etc. to decrease the light exposure to all preparations (whether exposed to 5HT or saline). In addition, 5HT was never used if it had been prepared more than 3 hours previously. Serotonin (Sigma) was applied to the bath or agarose at a final concentration of 10 μM to remain comparable with previous studies on the effects of 5HT on the AL[17,18,20,21]. Methysergide (Tocris Bioscience) was similarly applied at a final dilution of 50μM in 0.1% DMSO. CGP54626 (Tocris) was

dissolved as 2000X stocks in DMSO and applied at a final dilution of 25μM. SKF97541 was dissolved as a 1000X stock in AHL saline and used at a final concentration of 20 μM. Synthetic DTK-1 (produced by Vulpes LTD, Tallin, Estonia), was dissolved as 1000x stock in AHL and used at 10 μM. In the Results section "control" describes pretreatment responses. All pharmacological agents were applied for 10 minutes before taking measurements.

Statistical analysis. The imaging data were analyzed using Igor Pro 6.0 (Wavemetrics) and a custom macro. Tests for normality (Kolmogorov-Smirnov) were performed in SigmaStat (Systat Software Inc., Ashburn, VA). If data were fit with a normal distribution, two-tailed Student's t-tests for dependent (submerged preparations) and independent samples (embedded preparations) were performed in Statistica (Statsoft, Tulsa, OK). If data were not normally distributed, data was analyzed with a Wilcoxon matched-pairs test (Statistica). For quantification of $GABA_B$ receptors (Fig. 8*H*), data obtained by Root etl. (2008) was reanalyzed to compare the levels of $GABA_B$ receptors expressed by ORNs innervating the VM2 and DM2 glomeruli. This analysis was performed as described in Root et al. (2008).

Part of this chapter, was published in PNAS in 2009, under the title "Presynaptic peptidergic modulation of olfactory receptor neurons in *Drosophila.*" The dissertation author and Rickard Ignell contributed equally to this paper as primary authors. Ryan T. Birse, Jing W. Wang, Dick R. Nässel, and Åsa M.E. Winther were co-authors.

Part of this chapter was also published in the Journal of Neurogenetics in 2009, under the title, "Serotonin modulates olfactory processing in the antennal lobe of

Drosophila." Andrew M. Dacks was the primary author with David S. Green, Alan J. Nighorn, and Jing W. Wang as co-authors.

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Chapter 6: Presynaptic Facilitation by Neuropeptide Signaling Mediates Odor-Driven Food Search

6.1 Abstract

It has long been speculated that olfaction plays an important role in flavor perception and appetitive behavior. Here we investigate the neural mechanism for hunger modulation of olfactory representation and food search behavior in *Drosophila*. We found that sensory neurons express short neuropeptide F (sNPF) and its receptor (sNPFR1). We established an assay to measure appetitive behavior and found that expression of the neuropeptide and its receptor in odorant receptor neurons (ORNs) is necessary for starvation-induced food search behavior. Using two-photon calcium imaging, we found that starvation increases presynaptic activity in select ORNs via sNPF signaling. Furthermore, presynaptic facilitation specifically in Or42b neurons is necessary for food finding. Quantitative RT-PCR experiments demonstrated that starvation increases the transcription level of the receptor but not that of the neuropeptide. Thus, starvation increases expression of sNPFR1 to change the odor map in the antennal lobe, resulting in more robust food search behavior.

6.2. Introduction

The modulation of behavior by basic physiological need is essential for animal survival. Relevant sensory stimuli are transformed by peripheral receptors into electrical signals to form an internal representation of the external world. Internal physiological state of the organism should also play an important role in shaping sensory representation. Physiological modulation is often accomplished by release of neuromodulators that alter neuronal excitability or network properties[1-3]. In particular, hunger and energy homeostasis modulate feeding behavior in most animals. Much is

known about hormonal regulation and central control of feeding behavior[4], but much less is known about hunger modulation of sensory representation.

Appetite for food in mammals is controlled by multiple brain regions[5]. One of the critically important regions, the hypothalamus, integrates hormonal signals such as ghrelin, insulin and leptin from the gut, pancreas and adipose tissues, respectively. Activation of neurons containing neuropeptide Y (NPY) and AgRP in the arcuate nucleus of the hypothalamus augment food intake (for review[4,6]). In insects, a number of neuropeptides have been implicated in modulation of feeding behavior[7]. In particular, two independent homologs of NPY, neuropeptide F (NPF) and short neuropeptide F (sNPF)[8-10], promote feeding behavior[11,12]. Studies of hunger modulation in the *Drosophila* nervous system affords an opportunity to investigate an evolutionarily conserved mechanism for energy homoeostasis and establish a causal link between neuropeptide modulation and feeding behavior.

For most animals in their natural environment, feeding begins with a search for the appropriate food source in which the sense of smell plays an indispensible role[13]. We hypothesized that hunger modulates olfactory processing that mediates food-search behavior. We report that starvation alters olfactory representation of food odor at the first olfactory synapse. The neuropeptide, sNPF, which is implicated in feeding behavior[12] and expressed in *Drosophila* olfactory receptor neurons (ORNs)[14], mediates this change by facilitating synaptic transmission from select ORNs. Signaling by sNPF is necessary for starvation-dependent enhancement of odor-driven food search behavior. Furthermore, starvation increases the expression level of the sNPF receptor. Thus, neuropeptide

signaling causes starvation-dependent presynaptic facilitation of sensory transmission, which optimizes olfactory representation for food finding.

6.3 Starvation alters early olfactory representation

The antennal lobe is the center for early olfactory processing and is a target for many neuromodulators. Within the antennal lobe, ORNs expressing the same odorant receptor genes[15,16] converge onto a single glomerulus[17]. ORNs make synapses with local interneurons and projection neurons (PNs)[18]. The antennal lobe output PNs propagate activity from glomeruli to higher brain centers such as the lateral horn and mushroom body[19,20]. Although ORNs are the main drivers of PN output[21,22], lateral activity has been shown to control olfactory sensitivity by presynaptic inhibition[23-25]. Two neuromodulators, serotonin[26] and tachykinin[24], have been shown to alter antennal lobe activity. If hunger modulates antennal lobe neurons, we should observe a change in odor-evoked activity in PNs.

We performed two-photon calcium imaging to measure PN dendritic responses to odor stimulation in flies that were starved and fed. Flies bearing *GH146-Gal4* and *UAS-GCaMP* transgenes express the calcium sensor GCaMP in many PNs allowing the select measurement of calcium response in PN dendrites. We investigated calcium response of PNs to apple cider vinegar, which is highly attractive for *Drosophila* and is a complex odor that resembles a natural food source[27]. We imaged PN dendritic activity in flies that were fed and flies that were starved overnight (Figure 6.1A). Cider vinegar activates five glomeruli at the tested concentrations. Starvation significantly enhances odor response in three glomeruli (DM1, DM4 and DM2) but decreases odor response in two glomeruli

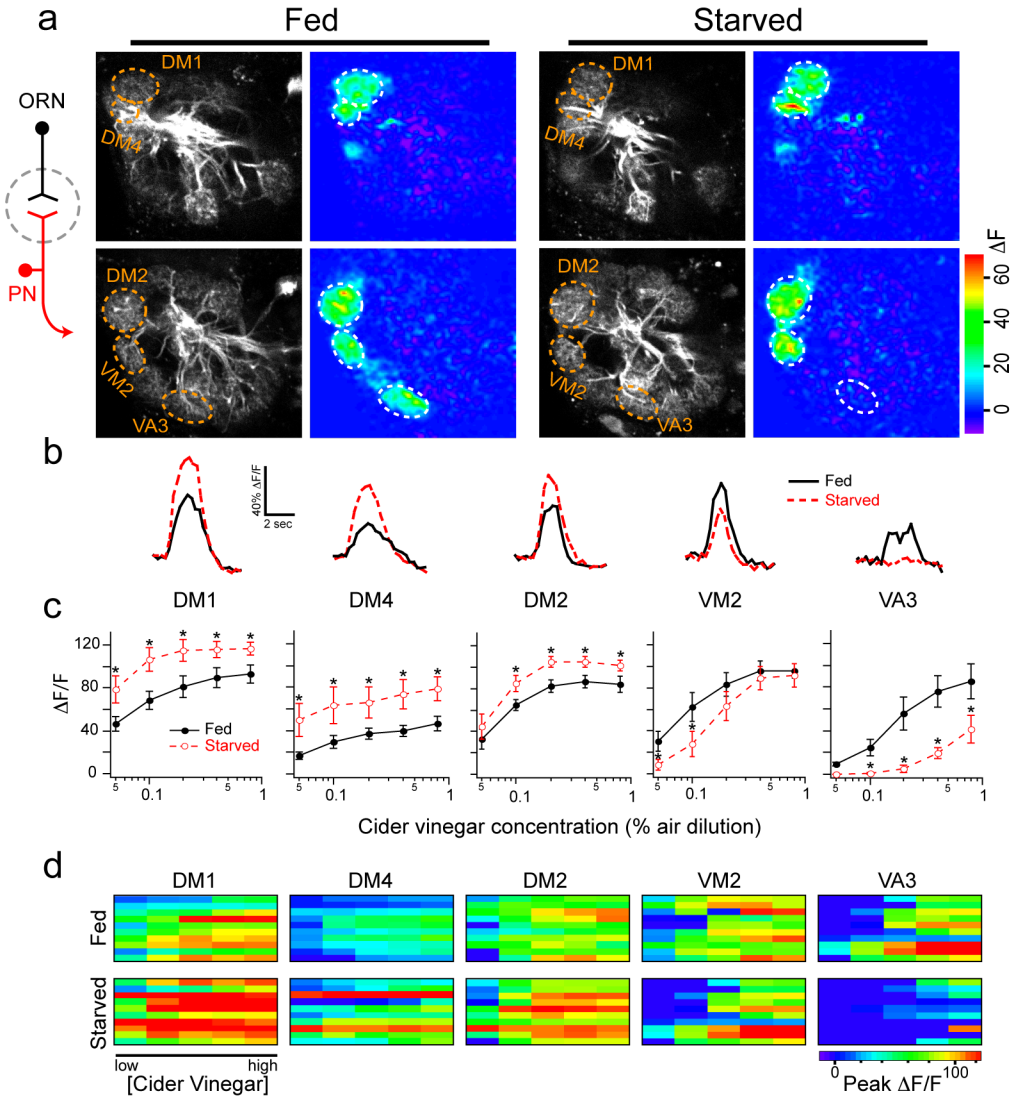

Figure 6.1. Olfactory representation in projection neurons is altered by starvation. **a,** Two-photon imaging of PN activity in response to cider vinegar stimulation on two optical planes of the antennal lobe in fed and starved flies. Gray-scale images show antennal lobe structure while pseudocolored images reveal odor-evoked activity. **b**, Representative traces of fluorescence change over time for the five glomeruli excited by cider vinegar. **c**, Peak DF/F across a range of cider vinegar concentrations for each glomerulus. **d**, Raster plots of the peak response for each glomerulus in each preparation. The raster plots for each glomerulus show the response at the five odor concentrations. n=10 for each condition; error bars show SEM. *P≤0.05, t-test.

(VM2 and VA3; Figure 6.1B-D). It is interesting to note that starvation alters the amplitude of calcium activity without changing the temporal kinetics (Figure 6.1B). In sharp contrast, our previous study shows that activation of $GABA_B$ receptors causes presynaptic inhibition and alters the temporal kinetics of PN calcium activity[25]. Therefore, a change in $GABA_B$ receptor signaling is unlikely to account for the starvation dependent change in olfactory response. Rather, our results are more consistent with an excitability change in antennal lobe neurons. We conclude that some antennal lobe neurons are subject to hunger modulation resulting in an alteration of the odor map.

6.4 Food search behavior is modulated by starvation and requires the antennae

We developed a single fly assay that allows the assessment of hunger modulation of odor driven food search behavior. We reasoned that latency to find food is a metric of food search. We developed an automatic computer system to monitor the position of individual flies from which we measured the latency required for individuals to reach an odor target. Latency is defined as the elapsed time before an individual fly spends more than 5 seconds within a 2.5 mm distance from the odor source, which minimizes false positives due to random entry into the odor zone. Individual flies were introduced into small arenas that contained a food odor, apple cider vinegar, at the center. The arenas were illuminated by LEDs at a wavelength not visible to *Drosophila*, thus forcing flies to rely on their sense of smell to navigate towards the odor source.

Does hunger as an internal state influence food search behavior? Within the 10 minute observation period, we observed that starved flies spend most of the time walking near the food source, whereas fed flies wander in the entire arena with a preference for

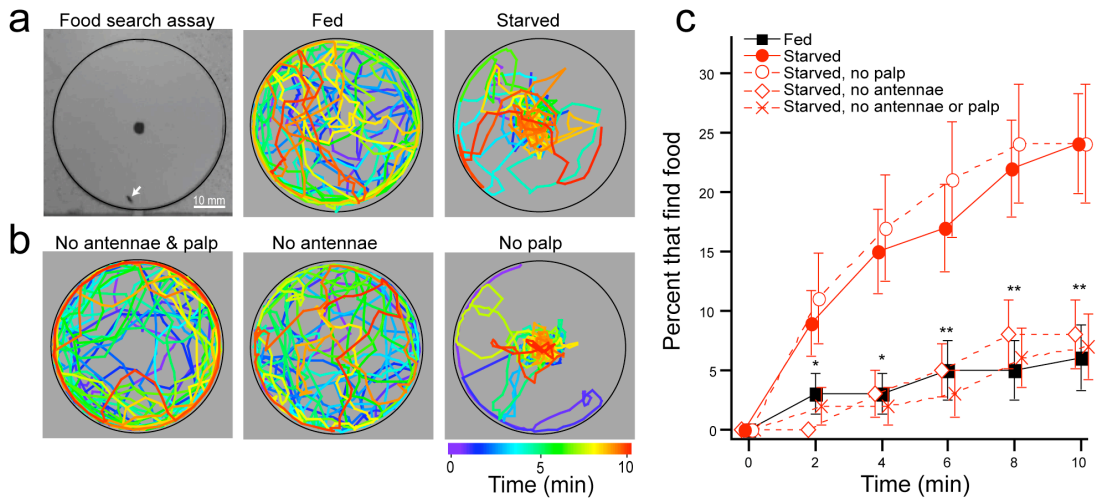

Figure 6.2. Food search behavior is modulated by starvation and requires olfaction. **a**, A food search assay was used to measure the latency of odor-guided food finding. Grayscale image (left) shows an arena with a food odor, cider vinegar, in the center and a single fly (white arrow). The coordinates of single flies are plotted as a function of time in pseudocolor for a representative fed and starved fly. **b**, Starved flies with amputations of olfactory appendages; the antennae, maxillary palp or both were removed. **c**, The latency of food search is quantified as the cumulative percentage of flies that find the odor source as a function of time. n=71-102 flies for each condition. Error bars show SEM. *P≤0.05, **P≤0.01, z-test for proportions comparing starved and starved no palp with fed, starved no antennae, and starved no antennae or palp.

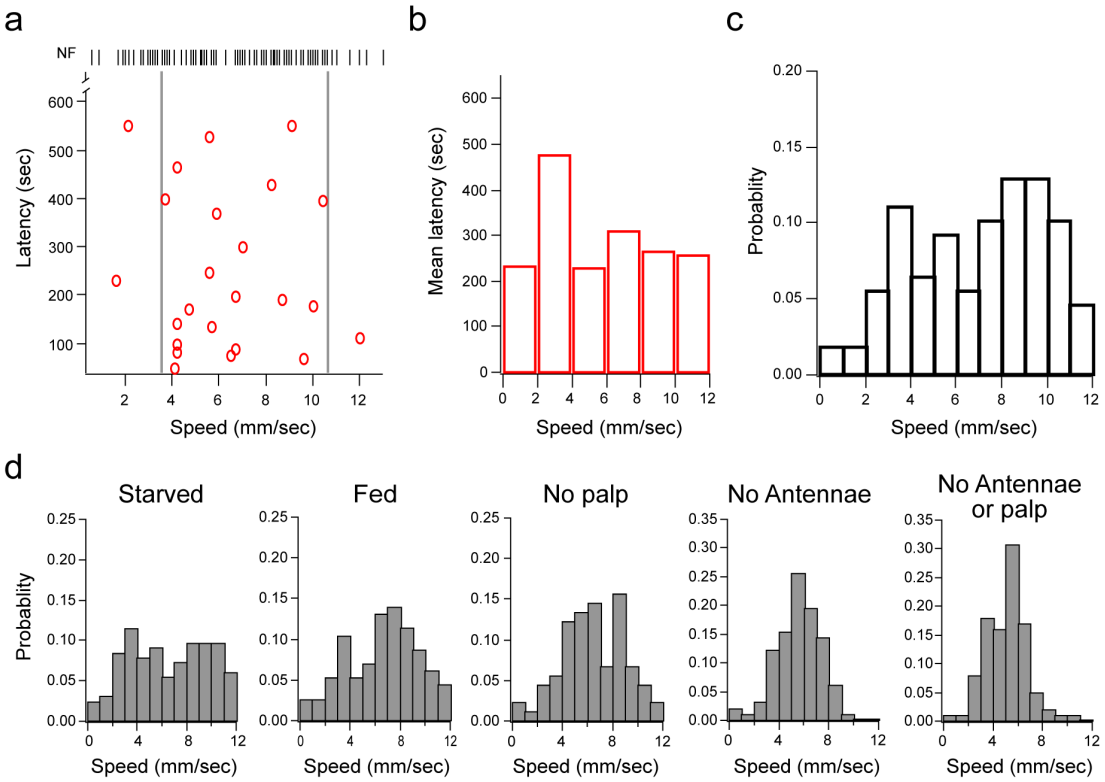

Figure 6.3. Locomotor activity during food search behavior.
To investigate the relationship between locomotion and food finding, we measured the speed of flies during the first 50 seconds after entering the arena, a time when most flies have not located the odor source. **a**, The latency of food finding plotted as a function of the speed for starved control flies (from Figure 1c). The red circles show flies that found the food at the given latencies and speeds, while the black lines above the graph show flies that did not find food at the given speeds. We find that very few flies with speeds above 10.5 mm/sec and below 3.5 mm/sec (gray vertical lines) find food, and for all of the quantification of food finding we restricted our analysis to flies with speed between 3.5-10.5 mm/sec. **b**, The mean latency of food finding for flies (red circles in **a**) in 2 mm/sec bins of speed. Within the flies that find food there is no apparent optimum speed. **c**, The speed distribution of flies that don't find the odor source (black lines in **a**). Flies that don't find food are not confined to any particular speed bin. **d**, Speed distributions for all of the flies in Figure 1. Flies lacking antennae, show a higher density of mid-range speeds than control flies, but given that there is no optimum speed for food finding, they are not disadvantaged by this narrowing of speed distribution.

the perimeter (Figure 6.2A,B). We found that starvation shortens the latency of food finding. Cider vinegar was diluted 100 times to minimize food search in fed flies. For flies that have been starved 17-24 hrs, roughly 25% of them reach the odor target within 10 minutes, whereas only about 5% of fed flies do so (Figure 6.2C). We next asked whether the olfactory system is necessary for this food search behavior. *Drosophila* has two olfactory appendages, the antennae and the maxillary palp. We surgically removed the antennae, maxillary palp or both appendages and found that the antennae are required for starvation-dependent food finding while the maxillary palp are dispensable (Figure 6.2B,C). Furthermore, we investigated walking speed of individual flies in relation to food finding latency but found no clear relationship (Figure 6.3A-C). In addition, we examined the walking speed of flies lacking olfactory appendages. Although there is a slight narrowing of speed distributions for amputation flies, the subtle change cannot account for the difference in latency (Figure 6.3D). Therefore, the sense of smell, mediated by the antennae, is required for food search behavior, and hunger enhances food finding in *Drosophila*.

6.5 sNPF signaling in ORNs mediates hunger modulation of food search

What is the mechanism by which starvation affects odor-guided behavior? The neuropeptide, sNPF, is highly implicated in hunger signaling and has recently been found to be expressed in *Drosophila* ORNs[14]. We therefore hypothesized that sNPF signaling in ORNs is responsible for the starvation-dependent enhancement of food search behavior. We expressed RNAi to knockdown ORN sNPF expression in flies bearing the *Or83b-Gal4* and *UAS-sNPF-RNAi* transgenes. If sNPF signaling is important for food search

behavior, we expect that knockdown of sNPF would eliminate the effect of starvation. We measured the latency of food finding in our behavioral assay and found that indeed starved flies lacking sNPF in ORNs exhibit a significantly longer latency in food finding (Figure 6.4A,B). Within 10 minutes, about 22% of control flies reach the odor source while only 9% of sNPF knockdown flies do so. Interestingly, sNPF knockdown flies behave similarly to fed flies, suggesting that low sNPF signaling mimics the fed state in the antennal lobe. The difference in latency between sNPF knockdown flies and control flies cannot be attributed to a change in locomotor activity (Figure 6.8A). Thus, we conclude that sNPF expression in olfactory receptor neurons mediates the starvation-dependent enhancement of food search behavior.

Does the starvation-dependent behavioral change require feedback or feedforward synaptic modulation? Our findings, in addition to previous work[14] indicate that ORNs express the sNPF peptide, however, the population of neurons that express sNPFR1, the receptor for sNPF, is not known. Two potential mechanisms may account for the observed modulatory effects of the neuropeptide: expression of the receptor in ORNs can mediate ORN-ORN feedback modulation, whereas expression of the receptor in PNs would constitute as ORN-PN feedforward modulation. For example, in salamander, the NPY receptor appears to be expressed in sensory neurons of the olfactory epithelium[28], consistent with a feedback modulation. In the mammalian hypothalamus, NPY neurons project from the arcuate nucleus to the lateral hypothalamus[4] in a feedforward manner. Therefore, the most parsimonious hypotheses would be that the sNPF receptor is expressed either presynaptically in the ORNs or postsynaptically in PNs. To investigate these two possibilities we expressed RNAi to knockdown sNFPR1 in either the ORNs or

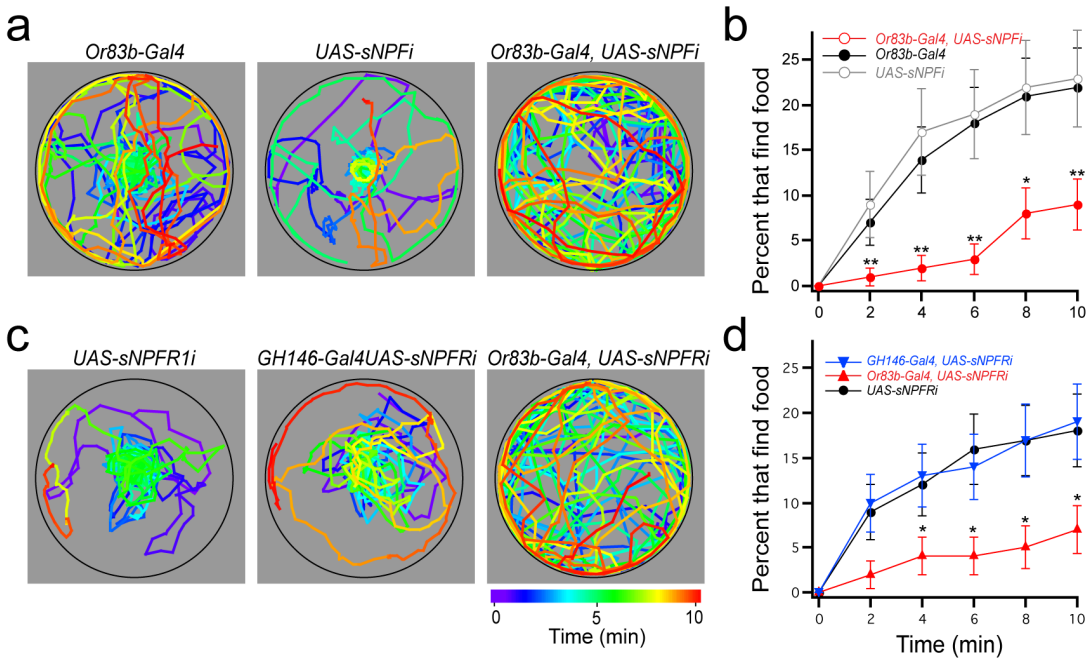

Figure 6.4. Starvation-dependent food search requires sNPF signaling in ORNs.
a, The coordinates of single flies for a representative control fly (left two plots) and those expressing *sNPF-RNAi* (sNPFi) in ORNs (right). **b**, The latency of food search is quantified as the cumulative percentage of flies that find the odor source as a function of time. **c,** The coordinates of a representative control fly (left) and those expressing *sNPFR1-RNAi* (sNPFRi) in PNs (middle) or ORNs (right). **d**, The latency of food finding. n=64-103 flies for each condition. Error bars show SEM. *P≤0.05, **P≤0.01, z-test for proportions comparing the top two curves to the bottom curve in **b,d**.

PNs in flies bearing either *Or83b-Gal4* or *GH146-Gal4,* respectively, and *UAS-sNPFR1-RNAi*. Strikingly, we found that expression of *sNPFR1-RNAi* in ORNs mimics the effect of the neuropeptide knockdown (Figure 6.4C,D). In contrast, expression of *sNPFR1-RNAi* in the PNs has no effect on food search behavior. The difference in latency between sNPFR1 knockdown flies and control flies cannot be attributed to a change in locomotor activity (Figure 6.8B). Thus, feedback modulation by sNPFR1 expressed in ORNs is necessary for starvation-dependent food search.

6.6 Presynaptic activity in ORNs is modulated by sNPF signaling

Given that knockdown of sNPF and its receptor in ORNs has a profound effect on starvation-dependent food search behavior, we reasoned that hunger should alter activity in ORN axon terminals. To investigate this, we imaged odor-evoked activity in ORNs in flies that were fed and flies that were starved overnight. Flies bearing the *Or83b-Gal4* and *UAS-GCaMP* transgenes allow the select measurement of calcium activity in ORN axon terminals. We observed that cider vinegar activates the same five glomeruli when comparing ORNs (Figure 6.5A) to PNs (Figure 6.1A). Three glomeruli (DM1, DM4 and DM2) exhibit significant increases in calcium activity, while the VM2 glomerulus exhibits significant suppression of response to low odor concentration, and the VA3 glomerulus is not affected (Figure 6.5B,C). Thus, starvation alters olfactory representation in sensory neurons, which is largely consistent with the changes observed in the antennal lobe output PNs.

We next asked if sNPF signaling in ORNs causes the hunger-induced changes in olfactory representation. To investigate this, we imaged ORN response to cider vinegar in

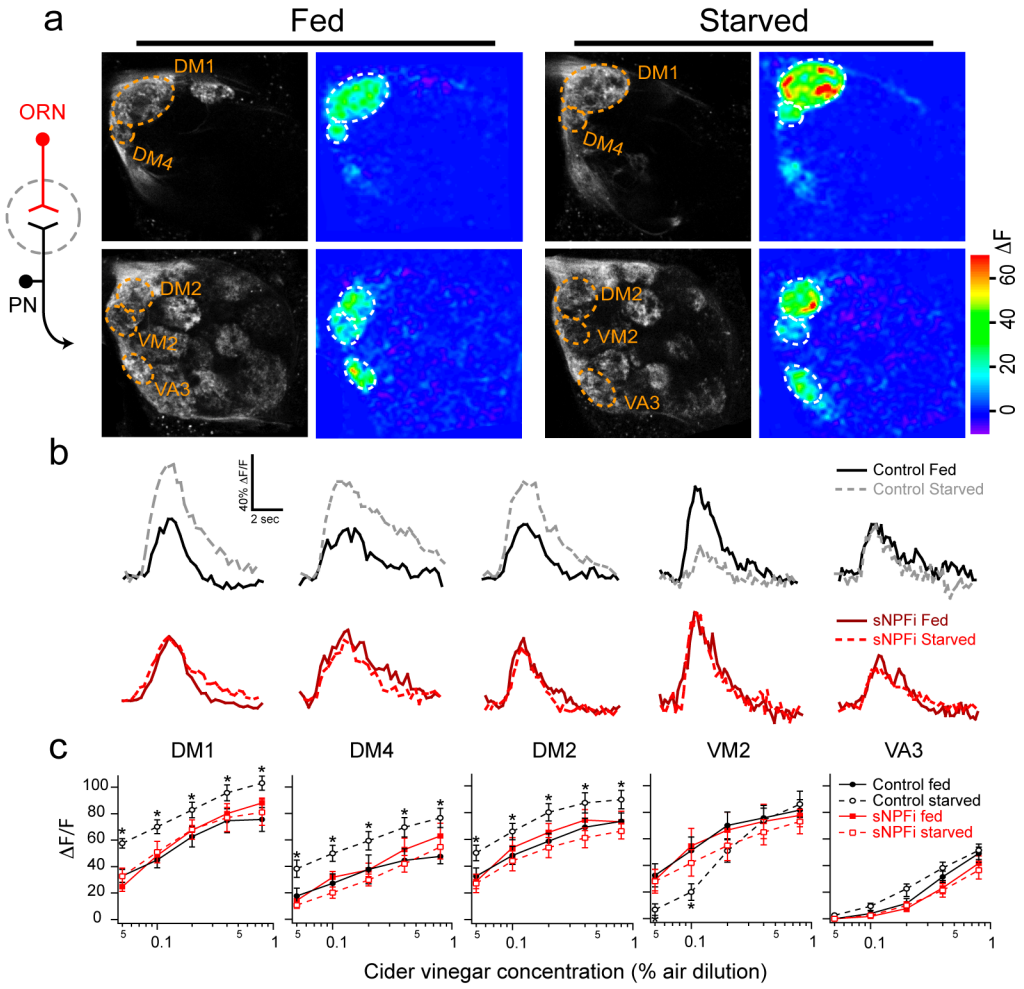

Figure 6.5. sNPF signaling alters presynaptic calcium activity in sensory neurons. **a**, Two-photon imaging of ORN axon terminal activity in response to cider vinegar stimulation. **b**, Representative traces of fluorescence change over time for the five glomeruli excited by cider vinegar in control flies (top) and those expressing *sNPF-RNAi* in ORNs (bottom), in fed (solid line) and starved (dashed line) flies. **c**, Peak DF/F across a range of cider vinegar concentrations for each glomerulus in control (black) and those expressing *sNPF-RNAi* (sNPFi) in ORNs (red). n=10-12 each condition; error bars show SEM. *P<0.05, t-test comparing starved control to fed control.

starved and fed sNPF knockdown flies. Strikingly, we found that expression of *sNPF-RNAi* in the ORNs eliminates the effect of starvation such that the olfactory representation in starved flies lacking sNPF mirrors that of fed control flies (Figure 6.5C). The overlapping curves between control fed flies and starved RNAi flies suggests that the effect of RNAi is specific to sNPF rather than a non-specific effect on neuronal properties. Furthermore, there is no difference between starved and fed sNPF knockdown flies, indicating that sNPF mediates the hunger modulation of ORN activity. Thus, we conclude that sNPF signaling causes the change in olfactory representation upon starvation.

6.7 sNPF signaling mediates presynaptic facilitation

The above results indicate that hunger enhances activity in ORNs by sNPF signaling, suggesting that the neuropeptide could act to facilitate presynaptic activity. To directly test this hypothesis we asked if exogenous application of sNPF affects presynaptic calcium activity in ORN terminals. In order to eliminate any potential modulation at ORN cell bodies, we cut the olfactory nerves and delivered precise electrical stimulation to the nerve while imaging ORN axon terminal calcium. In addition, we expressed *sNPF-RNAi* in ORNs to eliminate endogenous sNPF, which could occlude the effect of exogenously applied sNPF. Flies bearing the *Or83b-Gal4*, *UAS-GCaMP* and *UAS-sNPF-RNAi* transgenes lack sNPF expression and express GCaMP in ORNs. Electrical stimulation of the olfactory nerve elicits a calcium transient that is increased upon sNPF application (Figure 6.6A-C). Interestingly, this increase occurs only in starved flies but not in fed flies, suggesting that sNPFR1 signaling is upregulated upon

starvation. We compared the sensitivity to sNPF between the five glomeruli that respond to cider vinegar and found that the DM1, DM2 and DM4 glomeruli exhibit enhanced activity by the neuropeptide, whereas the VM2 and VA3 glomeruli do not (Figure 6.6D). This result reveals that ORNs terminating in VM2 and VA3 are not modulated by sNPF. Therefore, the suppression of calcium activity in VM2 ORNs (Figure 6.4B) could be a result of lateral presynaptic inhibition[23,25]. Furthermore, the suppression of VA3 PN calcium activity (Figure 6.1B) could be due to lateral feedforward inhibition[29,30]. Thus, the sNPF peptide and its receptor mediate presynaptic facilitation in starved flies at select glomeruli.

Is starvation-dependent ORN facilitation caused by upregulation of sNPF receptor expression? We performed quantitative RT-PCR to measure changes in transcription of the gene for sNPFR1. We measured the level of *sNPF* and *sNPFR1* transcripts in isolated antennae of fed and starved flies relative to a control gene, *rp49* (a ribosomal protein). Interestingly, we found that the level of *sNPFR1* mRNA is increased by starvation to approximately four-fold, while the level of *sNPF* mRNA does not change (Figure 6.6E). Future experiments are necessary to determine the hunger signal that increases *sNPFR1* expression. Although we do not detect a difference in *sNPF* mRNA we cannot rule out starvation-dependent changes in neuropeptide translation or release. Nevertheless, we conclude that starvation leads to increased expression of the sNPF receptor, which in turn leads to presynaptic facilitation.

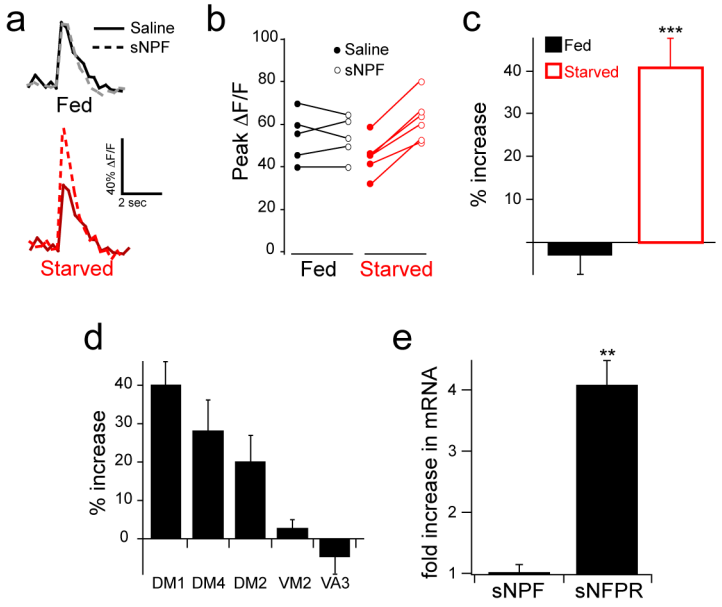

Figure 6.6. The sNPF receptor is upregulated upon starvation and mediates presynaptic facilitation.

a-d, Two-photon imaging of ORNs axon terminal activity in response to electrical stimulation of the olfactory nerve. Stimulation was 1 ms in duration, 10 V in amplitude and 16 pulses at 100 Hz. a, Representative traces of fluorescence change over time from the DM1 glomerulus in fed (top, black) and starved flies (bottom, red), in saline (solid line) and after addition of 10mM sNPF. b, Peak DF/F before and after sNPF in fed (left) and starved (right) flies. c, Percent increase in peak DF/F after exogenous sNPF addition. d, Percent increase in peak DF/F after sNPF addition for the five glomeruli that respond to cider vinegar. a-d, n=5-6; ***P<0.001, t-test. e, Quantitative RT-PCR analysis of starvation-induced changes in mRNA expression. Results are the average of four biological replicates, each replicate measured in triplicate and normalized to a control gene (*rp49*). Error bars show SEM. **P<0.01, t-test between sNPF and sNPFR1 fold change.

6.8 sNPF signaling in DM1 is necessary for food search behavior

We next investigated the contribution of sNPF signaling in individual glomeruli to the starvation-dependent food search behavior. The above results indicate that sNPF signaling is necessary for food search behavior, and sNPF signaling selectively increases activity in only three of the five glomeruli activated by cider vinegar. To determine if sNPF signaling in individual glomeruli is necessary for food search behavior, we expressed RNAi to knockdown the peptide or the receptor in each of the five ORN channels. We found that knockdown of the neuropeptide or its receptor in DM1 ORNs results in significantly decreased food finding in starved flies (Figure 6.7A). Within 10 minutes, only about 10% of the RNAi expressing flies reach the odor target, whereas about 24% of the control flies do so. This difference cannot be attributed to a difference in locomotor activity (Figure 6.8C). Strikingly, knockdown of the neuropeptide or its receptor in the other four ORN types has no effect on the starvation-dependent food search behavior (Figure 6.7B-E). These results indicate that sNPF signaling in a single ORN channel is necessary for the starvation-dependent food search behavior.

6.9 Discussion

We report here that a state of hunger modulates specific sensory activity at the first synapse in the olfactory system by sNPF-mediated presynaptic facilitation. This modulation is necessary for starvation-dependent food search behavior. Interestingly, a subset of glomeruli exhibit increased activity as a result of starvation-dependent sNPF signaling, while selective knockdown of sNPF or sNPFR1 in only the DM1 glomerulus affects food search behavior. This finding corroborates our previous work revealing that

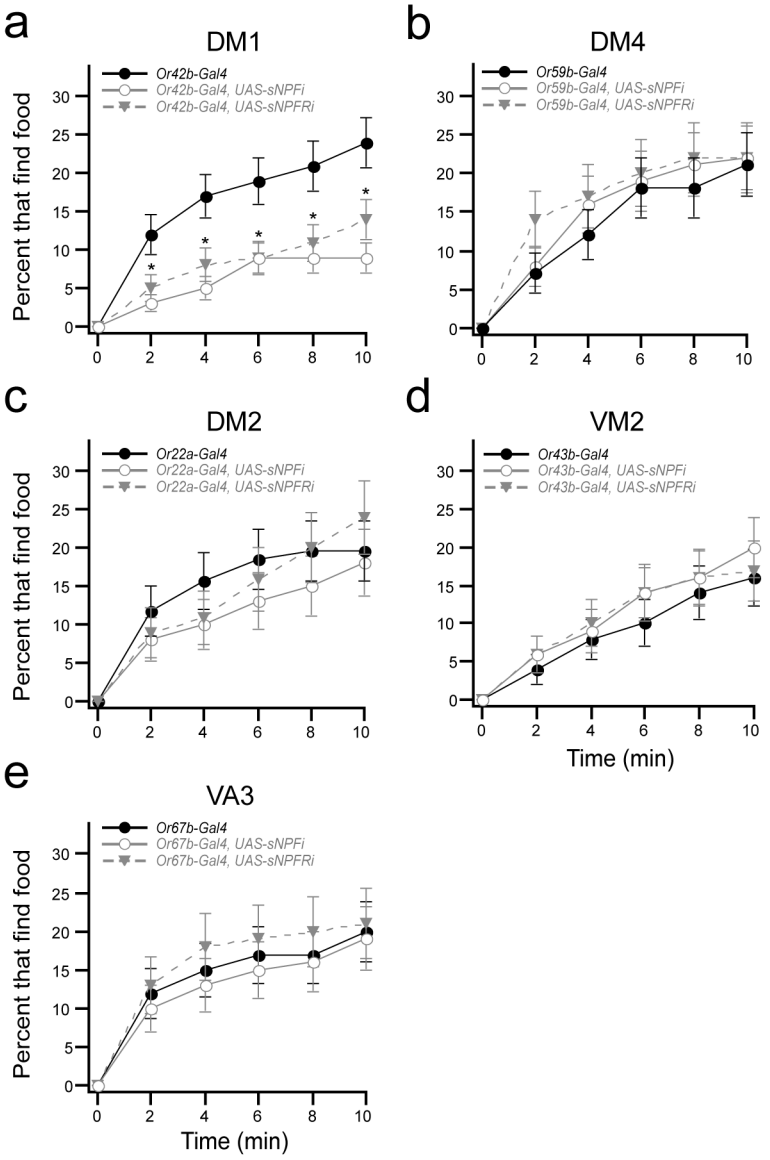

Figure 6.7. sNPF signaling in a single glomerulus is necessary for starvation-dependent food search.

a-e, The latency of food search for starved flies expressing RNAi to knockdown sNPF or sNPFR1 in individual glomeruli. **a,** RNAi expression in only the DM1 glomerulus significantly decreases food finding. **b-e**, expression in other glomeruli does not significantly affect food finding. n=80-195 flies for each condition. Error bars show SEM. *P≤0.05, z-test for proportions comparing *Or42b-Gal4* to *Or42b-Gal4*, *UAS-sNPFi* and to *Or42b-Gal4*, *UAS-sNPFRi*.

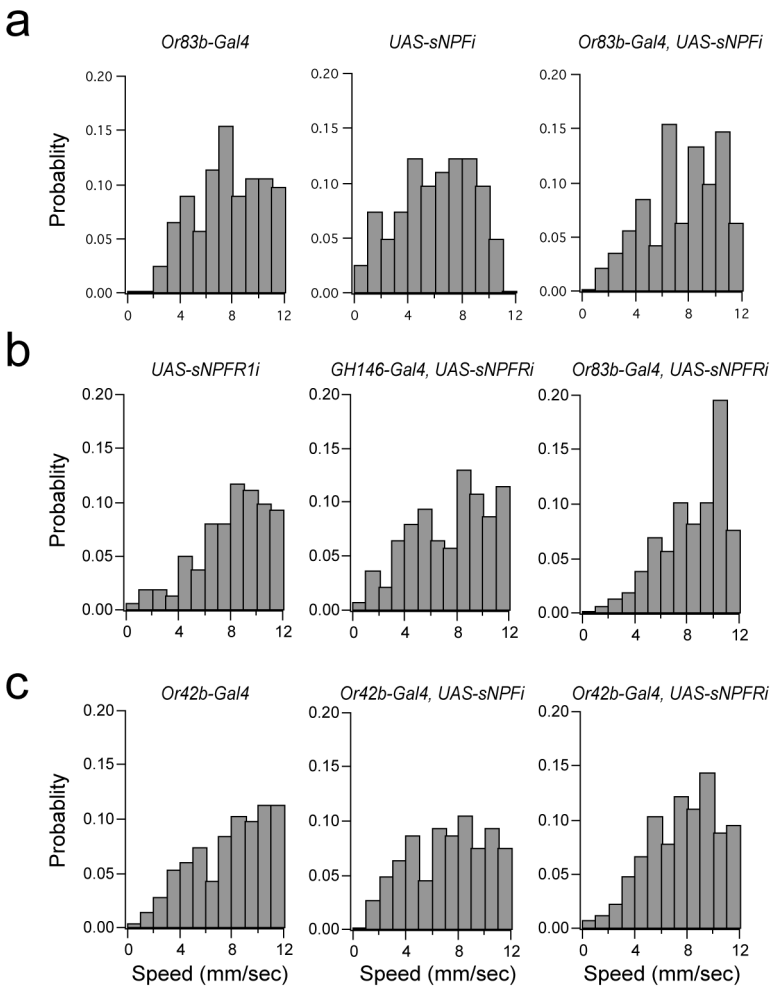

Figure 6.8. Speed distributions for flies.
Histograms of fly speeds for **a**, flies in Figure 3a-b, **b**, those in Figure 3c-d, and **c**, those in Figure 6a. There are no major differences in the distributions of fly speeds between genotypes.

the DM1 glomerulus is hardwired for innate odor attraction[27]. Furthermore, the odor map is not static, rather, it is subject to modulation by internal physiological state. Thus, an internal state of hunger, via sNPF signaling, shifts the odor map to increase the saliency of glomerular activity to match the physiological needs of an organism.

Information processing in the nervous system is a high energy expenditure process[31]. Physiological modulation of sensory information could, in principle, happen at different layers in the hierarchy of sensory processing, however, modulation at the first synapse could be more energy efficient and favored by natural selection. Consistent with this, our results and a number of other reports reveal that modulation of early sensory processing can have profound effects on stimulus detection. For instance, serotonin mediates presynaptic facilitation of mechanosensory neurons in *Aplysia* to sensitize the siphon and gill withdrawal reflex[32]. Serotonin mediates presynaptic inhibition of mechanosensory transmission in the leech to establish a behavioral hierarchy in which feeding suppresses tactile sensation[33]. In the olfactory system, serotonin modulates activity at the first olfactory synapse in mammals[34,35] and insects[26,36,37], and GABA-mediated synaptic inhibition serves as a mechanism to modulate sensitivity[23,25,38-41] and olfactory behavior [25,41].

The present results indicate that a highly conserved neuropeptide[10] plays an important role in the early olfactory system to mediate starvation-dependent neuromodulation. A similar presynaptic facilitation mechanism may exist in vertebrates as well. In an aquatic salamander, NPY has been shown to enhance electrical responses of cells in the olfactory epithelium to a food related odorant in hungry animals[28]. In addition, NPY immunoreactivity has been observed in the olfactory epithelium of

mouse[42] and zebrafish[43]. In the nematode *C. elegansf*, increased activity levels of an NPY-like receptor causes the switch from aggregation to solitary feeding[44]. Thus, modulation by NPY/sNPF in the early olfactory system could be a conserved mechanism between invertebrates and vertebrates.

Central mechanisms to control appetitive behavior, similar to the well-documented modulation of the hypothalamus by NPY, also appear to be important in *Drosophila*. A recent study demonstrates that appetitive memory requires the NPF receptor in the dopaminergic neurons that innervate specific mushroom body lobes[45]. This poses the question: what functions are subserved by hunger modulation of multiple neural substrates? Given that each mushroom body lobe receives input from many different glomeruli[46], and therefore many different odorants, the central modulation by hunger has the potential to alter responses to many different odorants. Modulation in the periphery may serve to gate an animals' sensitivity to specific food odorants, while central modulation may serve to enhance an animal's ability to remember the relevant cues in finding a particular food source. As olfaction plays an important role in our flavor perception[47], peripheral modulation of the olfactory system by hunger may thus be a potential therapeutic target to control appetite.

6.10 Methods

Experimental preparations. The following fly stocks were used: *Or83b-Gal4*[48], *Or42b-Gal4*[49], *Or43b-Gal4*[49], *Or22a-Gal4*[49], *Or59b-Gal4*[50], *Or67b-Gal4*[49], *GH146-Gal4*[51], *UAS-GCaMP*[52], *UAS-sNPF-RNAi*[12] and *UAS-sNPFR1-RNAi*[53]. Two-photon calcium imaging was performed as described in chapter 2 and 4. Starved flies in imaging

experiments were starved overnight for 17-24 hr. The sNPF peptide was generated by Celtek Peptides with a purity of 98%. The peptide sequence was: AQRSPSLRLRF-NH_2. The peptide was dissolved in AHL to produce a stock solution of 10 mM, which was diluted 1000 fold to get the final bath concentration of 10 μM. In physiology experiments, two nerve stimulations were given before the peptide was added, and then two more stimulations were given 10 minutes after addition of the peptide.

Behavior assay. Female flies aged 2-5 days old were used for all behavior. The flies were collected 1-2 days after ecolosion and were presumed to be non-virgin. The behavior apparatus contained 6 individual chambers that were 60 mm in diameter by 6 mm in height. Flies were loaded into a small holding chamber that was about 5 mm in diameter prior to starting the experiment. The holding chambers allowed us to introduce six flies into individual arenas, all at the same time by ungating the six hold chambers simultaneously. The behavior chambers were placed on a stainless steel mesh screen (0.178mm openings, Small Parts, Inc.) so that the bottom of the arena was open with a mesh bottom. The mesh screen was suspended approximately two centimeter above the surface to prevent saturation of an odor gradient. Experiments were done in a light proof box with illumination from a 660 nm LED panel. Images were captured using a digital Basler scA1390 camera (National Instruments) and an object tracking script written in Labview (National Instruments). Analysis was performed in Igor Pro (Wavemetrics) with a custom macro. We used criteria about the locomotor activity of flies to reject poorly performing flies. To be included in the data set, flies must have had an initial speed of 3.5-10.5 mm/sec during the first 50 seconds, and flies must not have been completely inactive for 5 minutes of the 10-minute experiment. The latency of food

search was defined as the point at which the flies spent at least 5 sec within 2.5 mm of the center of the chamber. Significance was tested using a hypothesis test for proportions, the z-test.

Quantitative RT-PCR. Antennal tissue was first collected by careful removal with fine forceps. Each biological sample was from the antennae of 50 female flies. The antennae were frozen at -80˚C and thawed to lyse cells. Qiagen lysis buffer was as added and then the antennae were ground up with a pestle before passing the lysate through a QiaShredder column. Total RNA was isolated using the RNeasy kit (Qiagen) and the reverse transcription was performed using the Retroscript kit (Ambion) with random decamers. This cDNA was subjected to quantitative PCR analysis using SYBR green detection on a Biorad iCycler machine. Each biological sample was run in triplicate. Primers were tested for efficiency using serial dilutions and fold changes were calculated as described[54]. Primers used are as follows:

sNPF (97 bp) F: CAAAAAGCGTGGCATACATT, R: AATGTCCGGATTTCAAGGAG

sNPFR1 (77 bp) F: CTGGCCATATCGGACCTACT, R: GGCCAGTACTTGGACAGGAT

RP49 (64 bp) F: CCAGTCGGATCGATATGCTA, R: TCTGTTGTCGATACCCTTGG

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