University of Nevada, Reno

Starved State Dependent Modulation of Olfactory Receptor Neuron Function in *Drosophila melanogaster* Larvae

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology

by

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Abstract

The ability of olfactory neurons to locate food sources underlies survival in most species of the animal kingdom. This ability of olfactory neurons to process environmental information is often modulated by the animal's internal state such as hunger. The peripheral end of the olfactory circuit consists of first order olfactory receptor neurons (ORNs), that synapse onto the second order projection neurons (PNs), and regulatory local neurons (LNs) that innervate ORNs and PNs. While a considerable amount of information has been generated, in various animal systems, regarding sensory neuron responses to food odorants and modulation of these responses by hunger, much less is known about the extent of modulation that exists among individual sensory neurons and its impact on driving behavioral output. We hypothesized that starvation differentially alters the sensitivity of individual first-order Olfactory Receptor Neurons (ORNs). To test this hypothesis, we exposed starved or non-starved third instar Drosophila larvae to specific odorants to analyze the effect of individual ORN activity on chemotaxis. We used two different behavioral paradigms to analyze the chemotaxis response of larvae to odorants. When tested with odorants that elicit strong physiological responses from individual ORNs, starved and non-starved larvae showed different behavioral responses in these behavioral paradigms. However, the extent of behavioral differences among starved and non-starved larvae varied when different odorants were tested in the assays. Further, we provide evidence that this modulation of ORN function by starvation is mediated by GABA signaling pathway. To investigate the molecular basis for this differential modulation, we used immunohistochemistry

and gene expression analysis. We developed an antibody against the GABA (B) receptor to look at the localization of GABA receptors in the olfactory neurons. We found that GABA (B) receptors are localized at the ORN synapses. We used qRT-PCR analysis to identify other molecular players that are involved in starvation control. We conclude that an animal's internal state such as hunger differentially modulates the functions of individual ORNs to impact olfactory information processing. Our results support recent studies from our lab and other groups that suggest that ORNs are functionally diverse. Overall, this research thesis has implications for understanding peripheral odor coding.

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Introduction

Evolutionarily, olfaction - the sense of smell - is considered one of the oldest known senses, yet it is one of the least understood senses. For many members of the animal kingdom, olfaction is extremely crucial in finding food, avoiding predators, and finding mates for reproduction. Olfaction is also very useful for humans, as the sense of smell can alert us to danger by serving as a warning system. For instance, the smell of rotting food signifies that it is inedible, or the smell of additives in natural gas which can alert us of gas leaks. Humans need olfaction to maintain a good quality of life. Furthermore, olfactory dysfunction has been reported in various diseases, such as Alzheimer's, Parkinson's, and diabetes, among others (Ruan, Zheng et al. 2012). Strikingly, there is great conservation of genes among humans, various animals and insects. About 75% of genes that are known to cause disease in humans are also found in *Drosophila melanogaster*, the common fruit fly (Pandey and Nichols 2011). Beyond this, the architecture of the olfactory system among insects and mammals also appear to be similar. For instance, there is noted closeness between glomeruli in the olfactory bulbs of mammals, and antennal lobes of adult *Drosophila* (Kim, Su et al. 2017). Within each glomerulus, signals from an olfactory receptor neuron (ORN) are transmitted onto a second-order projection neuron (PN), which in turn processes and sends signals to higher olfactory regions of the brain (Masuda-Nakagawa, Tanaka et al. 2005, Masuda-Nakagawa, Gendre et al. 2009, Masuda-Nakagawa, Awasaki et al. 2010), (Ramaekers, Magnenat et al. 2005). Between

glomeruli, local neurons (LNs) innervate both ORNs and PNs. These local neurons are primarily GABAergic (Wilson and Laurent 2005).

Animals exhibit various robust behaviors, many of which are essential for their survival. These behaviors, although innate, can be modulated by the animal's internal state and the external environment (Kim, Su et al. 2017). This ability of neurons to process information is often modulated by the animal's internal state such as hunger, aggression, disease, or stress (Anderson et al., 2016). From this we make a case that the ability of neuronal circuit to sense internal and environmental stimuli and translate that information into a behavioral response underlies survival in the animal kingdom.

Various laboratories around the world have studied starvation in various animal systems such as mouse, worms, fish and fruit flies (Howard, Lord et al. 1999) (Drew, Rodnick et al. 2008) (Slocumb, Regalado et al. 2015). Knock out studies and overexpression studies have helped researchers in understanding the roles of important genes, neurotransmitters and other pathways involved. Helped by effective genetic tools, scientists have gained exceptional ground in *Drosophila* to address how input-output relationships would affect the way information is processed in the brain. Studying an animal's behavior as an output factor in response to inputs such as odorants, light, heat, or shock, help in understanding the hardwired circuits and the processing of information in these circuits.

The various mechanisms of starvation control have been extensively studied in *Drosophila* and other animals. Olfaction is tightly linked to feeding behavior – many genes in the olfactory pathway are transcriptionally regulated in response to starvation, including short neuropeptide F (sNPF) and insulin like peptides (ILPs), both of which are highly conserved in *Drosophila* (Farhadian, Suárez-Fariñas et al. 2012). There was upregulation of these pre-synaptic activity in *Drosophila* olfactory receptor neurons (ORNs) in the starved state. GABA receptors localize on ORNs and PNs, and various levels of GABA receptor (GABAR) expression has been reported among appetitive and aversive ORNs (Root, Masuyama et al. 2008). While these results explain a general mechanism of starvation's effect on olfaction, its effects on the contributions of each individual ORNs have not yet been studied - there is a gap in knowledge regarding functional diversity among olfactory receptor neuron channels and their role in causing behavioral changes (Fishilevich, Domingos et al. 2005) (Kreher, Mathew et al. 2008) (Fishilevich, Domingos et al. 2005, Kreher, Mathew et al. 2008, Louis, Huber et al. 2008, Montague, Mathew et al. 2011, Mathew, Martelli et al. 2013). Understanding the specific roles of individual ORNs would elucidate specific mechanisms in how starvation affects the olfactory system. Furthermore, these findings could be translated into research in various disease processes that have specific effects on the olfactory system in mammals.

The *Drosophila* larva is a splendid model system to use because it has a numerically simple olfactory system with only 21 ORNs and approximately 21 PNs;

projection neurons. Odorants are sensed by the larval dorsal organ, which lies in the head of the larvae, where ORN's innervate the dorsal organ and send axons to glomeruli in the larval antennal lobe (Hertweck, 1931), (Louis, Huber et al. 2008), (Oppliger, M Guerin et al. 2000), (Singh and Singh 1984), (Stocker 1994). Within each glomerulus, signals from a single ORN are relayed to a second-order projection neuron, which then sends the information to higher olfactory centers in the brain (Masuda-Nakagawa, Tanaka et al. 2005, Masuda-Nakagawa, Gendre et al. 2009, Masuda-Nakagawa, Awasaki et al. 2010) (Ramaekers, Magnenat et al. 2005). Odor molecules in environment are sensed by a class of proteins called odor receptors (Ors). Proper functioning of the Ors requires the expression of a co-receptor called as Orco (Couto, Alenius et al. 2005) (Fishilevich, Domingos et al. 2005) (Kreher, Mathew et al. 2008). Or and Orco proteins together form a ligand-gated ion channel in each ORN (Sato, Pellegrino et al. 2008) (Wicher, Schäfer et al. 2008). Additionally, *Drosophila* larvae are amenable to a wide variety of genetic, molecular, and behavioral techniques. Olfaction in the third instar *Drosophila* larvae is a well defined genetic system of 21 receptors that directly respond to sensory stimuli. Given the rich history of *Drosophila melanogaster* research and the availability of the plethora of experimental tools, it is one of the best systems to study genetics, behavior, molecular signaling, and epigenetics.

Previous results from our lab indicate that individual ORNs contribute differently to the olfactory circuit to produce discreet behavioral outputs (Mathew,

Martelli et al. 2013) (Newquist, Novenschi et al. 2016). Mathew et al., 2013, carried out a screen of all 21 functional larval odor receptors to a panel of ~500 diverse odorants. For 19 of the 21 receptors, they identified an odorant that excites each one strongly and specifically. This study also suggests that ORN's are functionally nonequivalent units. In other words, each of these ORN's are functionally diverse from each other. Our primary aim is to understand how different ORN's respond to various odorants under starved conditions. In other words, we wanted to look at the information processing in individual ORN's when excited by their cognate odorants within a starved state. Our next aim was to understand the molecular mechanisms underlying the starvation modulation among ORNs. To address this, we ask three specific questions: Is information processing during starvation modulated differently among ORNs? Does individual activity of ORNs differentially affect the composition of navigational behavior under conditions of starvation? What genes and proteins are involved in the differential contributions of ORNs?

We hypothesize that changes in an animal's internal state (starved vs. fed) alters the ability of olfactory neurons to process information and that differences in GABAR expression underlie the differences in the contributions of ORNs to larval chemotaxis in the starved state. Our working model suggests that GABA release from LNs bind to GABARs on ORNs, which increase presynaptic inhibition in the fed state. Thus, in the starved state, we suggest GABA signaling decreases to allow increased ORN to PN transmission, driving food seeking behavior. Our working model is that individual ORNs have different levels of

GABAR expression that leads them to being differentially modulated by the starved state.

To quantify larval migration towards or away from odorants we employ a classical behavioral two-choice assay (Rodrigues and Siddiqi 1978, Monte, Woodard et al. 1989). This 2-choice assay allows larvae to discriminate and migrate towards an appetitive odorant, yielding a response index value that can be compared among groups. To conduct more expansive analyses of behavior of larvae and define olfactory computations, quantitative behavioral analyses was performed using larval tracking assay (Gershow, Berck et al. 2012, Mathew, Martelli et al. 2013). In this larval tracking analysis, we will be able to measure discrete navigational parameters such as number runs per track, run speed, run length, run ratio and many more in the presence of various odors. We examine the effects of GABA on larval behavior by using RNA interference lines to disrupt GABAR expression in various loci within the olfactory circuit. Furthermore, we use qRT-PCR and immunohistochemistry to quantify and localize the expression of various starvation related genes and proteins.

With the results from our study, we can expand on our lab's previous findings of functionally nonequivalent ORNs, and build a detailed working model to explain the nuances of information processing under control of starvation. Understanding olfaction plays a large role in pest control, and these findings could be directly involved in discovering novel approaches to pest control, by the means

of improving mathematical odor coding models. As previously mentioned, various human diseases have specific effects to the olfactory system. The highly conserved nature of genes among Drosophila and other animals can allow for translation of these findings into a study of various disease processes, and how they might be affected by various internal states. Our findings touch on the fundamental question of neuroscience – how does a brain convert signals from the environment and organize it into a tangible behavioral output? By studying the mechanisms through which outside information is processed by the brain and modulated by internal states, our results may lay a framework for better understanding how simple neuronal networks can significantly alter behavioral responses to the environment.

MATERIALS AND METHODS

Drosophila Stocks

Drosophila melanogaster stocks used for the behavior assays (2-choice assay and tracking assay) and molecular analysis (qRT-PCR and immunohistochemistry), include the wild type *Canton-S* (CS) line, the UAS-GABA (B)-R_X-RNAi (Root et al., 2008) (where X represents receptor subtype 1, 2, or 3), and Or *X*-Gal4 lines (Or 47a Gal4 and Or 42b Gal4), GH-146 Gal4, Orco-Gal4, 10x; UAS-CD8; GFP were all purchased from the Bloomington stock center (http://flystocks.bio.indiana.edu). Virgin female flies from UAS-GABA (B) R1-RNAi line were crossed to males from Or 47a Gal4, also UAS-GABA (B) R1-RNAi females were crossed to males of Or 42b Gal4. Similar crosses were made using UAS-GABA (B) R2-RNAi and UAS-GABA (B) R3-RNAi lines and Or 47a and Or42b lines. We used *Orco* mutant (*Drosophila* Stock Center, Bloomington, IN), which was backcrossed to a *wCS* line for 10 generations to generate the two empty larva genotypes (Fishilevich, Domingos et al. 2005).

Odorants and reagents

We used a panel of seven different odorants that were obtained at the highest purity (≥98% purity; Sigma-Aldrich). Paraffin oil bought from Sigma-Aldrich was used as a diluent for our studies. Agarose (Apex Bioresearch) gel was used as a crawling surface for larvae during behavioral experiments. Filter paper discs / odor discs were purchased from VWR Inc.

Experimental Preparations

Flies were reared on a standard medium at 25°C at 12-hour dark-light cycle. 3rd instar larvae (~96 hours AEL) were extracted 6 days after allowing the parental generation to mate for 24h. A 15% sucrose (Sigma Aldrich Inc.) solution was used to extract larvae from the food, and larvae were washed 4 times with ddH_2O. These washes are done to make sure that the larvae are free of nutrifly food particles.

Starvation Protocol

For starvation experiments, washed larvae were evenly distributed onto a 2x2cm square of a Kim wipe paper within a 60x15mm petri dish containing 350 µL of 0.2M sucrose (König, Schleyer et al. 2015) which serves as non-starved condition or ddH_2O that serves as our starved condition for exactly 2 hours. Starved and fed larvae were washed in a drop of ddH_2O in different weigh boats three times prior to behavioral analysis.

Behavioral assays

Two-choice assay

The two-choice assay was conducted as described (Kreher, Mathew et al. 2008) (Monte, Woodard et al. 1989). Two filter paper discs were placed at diametrically opposite ends of the petri dish containing 1.1% agarose. The

experimental odorant was added on one of the filter disc while the control diluent (paraffin oil) was added on the other filter disc. Odorants were serially diluted to $1:10^{-2}$ in paraffin oil for two choice assays. The response index (RI) was calculated by allowing ~50 larvae in the center of the plate and allowing them to migrate for exactly 5 mins within the odor gradient. RI is measured by counting the number of larvae after five minutes on each half of the petri plate. RI is calculated by using the formula (#larvae towards the odorant half (-) #larvae towards the control half) / (Total number of larvae). Two-choice assay was done under highly controlled conditions of humidity, temperature and light. For behavioral assays, the temperature was maintained at 22-23 degrees C and 45%-50% relative humidity.

Tracking assay

The tracking assay was conducted as previously described (Mathew, Martelli et al. 2013). Five filter paper discs holding either a test odorant or the control diluent (paraffin oil) were placed on opposite ends of a 22x22cm square tracking plate containing 1.5% agarose. Odorants were diluted to 10^{-1} for tracking assays. Approximately 20 larvae were placed along the central axis of the plate parallel to the discs for five minutes. Video microscopy of the tracking plate was performed by using dark-field illumination via 850nm red LEDs (outside the range of larval photo taxis). Larval video tracking images were measured at 2.3 frames per second with the help of a simple monochrome CCD camera, USD 3.0 camera (Basler Ace series, JH technologies). Each pixel in the captured image corresponded to a 0.119mm × 0.119mm square of the experimental arena. We

measured the larval tracking for exactly five minutes which produced overall 650 frames (130 frames per minute). Parameters of larval navigation and positions were extracted from video recordings using custom scripts written in MATLAB. The RI <Vx>/<s> was defined as the mean velocity of the larva in the x direction (<Vx>) divided by the mean crawling speed (<s>). Other tracking parameters include: run speed (measured in pixels/second), run length (pixels) and number runs per track.

Data Analysis

For analyzing larval navigation in the Tracking assay, positions of larvae for the entire duration of the assay were extracted from video recordings and larval 'trajectories' were reconstructed by using custom routines written in MATLAB (Mathworks Inc., Natick, MA, RRID: SCR_001622). Statistical analyses were performed using an advanced statistical package called Statistica (Statsoft Inc. Tulsa OK, RRID: SCR_014213).

For all behavioral parameters, a one-way ANOVA and Man-Whitney U tests were performed. Statistical significance for the ANOVA was set at $P = 0.05$. Quality figures were produced from Graph Pad Prism, Excel, Adobe Illustrator and image J software.

Immunohistochemistry analysis

Third instar larvae were dissected in ice cold PBS as described (Budnik, Gorczyca et al. 2006) and fixed with Bouin's fix for five minutes at room temperature. Primary antibodies were a chicken anti-GFP (1:150, Invitrogen) and

rabbit anti- GABA (B) R1 developed by Pocono Rabbit Farms (1:75, sequence: TVAEAAKMWNLIVLC) in 0.2%PPST+5% normal goat serum (Triton X-100). Samples were incubated overnight at 4 degrees C. secondary antibodies were a goat anti-chicken Alexa 488 (1:150, Invitrogen) and a goat anti-rabbit Alexa 647 (1:75, Invitrogen) in 0.2% PBST+5% normal goat serum.

Sequence Selection

A sequence of 15 amino acids with a terminal cysteine were selected from the sequence of GABA (B) R1 for optimal antibody binding affinity. The sequence was then BLASTed against a *Drosophila melanogaster* protein sequences with a less stringent Expect value of 100 (Altschul, Madden et al. 1997). The two hits returned two isoforms of GABA (B) R1.

qRT-PCR analysis

Third Instar larvae were collected in RNAlater Solution (Invitrogen, Thermo Scientific, and Wilmington, DE) and either stored at -20ºC or placed on ice. The heads of 15-20 larvae were dissected and pooled per sample. Tissues were homogenized with a handheld homogenizer, then RNA was extracted using the RNease plus Mini Kit with gEliminator columns (Qiagen, Hilden, Germany), followed by an additional gDNA digestion with TURBO DNA-free kit (Ambion, Thermo Scientific). RNA was quantified with Nanodrop 1000 Spectrophotometer (Thermo Scientific).

First strand cDNA synthesis was performed using SuperScript VILO MasterMix (Invitrogen, Thermo Scientific) on 1µg RNA in a 20µl reaction volume, following manufacturer's protocol. Equivelant no-RT controls were performed on each sample by replacing the volume of VILO with DEPC water. Samples were then incubated at 37ºC for 20 minutes with 0.5µl RNaseH and 2µl 10X buffer. No RT controls were analyzed using qPCR analysis with EF1 Primers.

Primer sequences were derived from literature, using FlyPrimerBank (flyrnai.org), or designed with PrimerBlast and estimated specificity was confirmed with PrimerBlast. Melt curves of 0.5ºC increments every 5 seconds from 65-95ºC were added at the end of each reaction to confirm specificity with a single peak in melt curve analysis. Standard curves were used to calculate each primer efficiency and were performed using a minimum of three dilutions of cDNA corresponding to a range of 7ng to 32pg RNA. Higher concentrations were omitted due to PCR inhibition.

Quantitative PCR (qPCR) reactions were done on ice with SsoAdvanced Universal SYBR Green Supermix (2X) (Bio Rad) in 10µl reactions, 0.4µM respective primer and cDNA corresponding to 1ng/µl RNA. Thermal cycling protocol includes a single 95ºC activation/denaturation step for 30 seconds followed by 40 cycles of 95ºC for 10 seconds and 60ºC for 30 seconds, followed by the melt curve protocol. Most primers were designed flanking introns (EF1, APPL, GABA (A) receptor, GABA (B) R1, GABA (B) R2, GABA (B) R3 and GAD

1) and therefore primer specificity and RNA contamination were controlled for with single-peak melt curve confirmation. Each technical sample was done in triplicate. Reactions were analyzed using Bio-Rad CFX96 C1000 Touch Thermal Cycler Real-time System (Bio-Rad Laboratories Inc., Hercules Ca) and data analysis done using Bio-Rad CFX Manager Software version 3.1. Reactions within the triplicates with a standard deviation >0.3 were omitted from analysis. Efficiencies were accounted for in the data analysis CFX Manager Software. Individual samples' expression data normalized to EF1, APPL, Nrv2, and Syt1 were exported and analyzed in excel. Expression differences were analyzed using two tailed T-Test comparisons.

Results

Starvation impacts larval behavior as measured in the two-choice assay

a) 3rd instar larvae:

From previous studies in our lab (Newquist, Novenschi et al. 2016) we learnt that each larval ORN is functionally diverse and each ORN differentially contributes in driving behavior in the *Drosophila melanogaster* larvae. Based on these results, we postulated that individual ORNs might be differentially modulated under starved state conditions. To begin to address this question we started with a panel of seven different odorants from the panel of 19 different odorants published in the study by (Mathew, Martelli et al. 2013). Each of the seven odorants elicits a strong and specific response from a single ORN (Or 47a :: pentyl acetate, Or 30a :: anisole, Or 42b :: acetal, Or33b/Or47a :: 2,5 dimethyl pyrazine, Or 59a :: 4,5 dimethylthiazole Or 42a :: 4-hexan-3 one). Also, all of these odorants (except acetal) have similar volatilities and thus in a test arena they form odor gradients of similar strengths (Newquist, Novenschi et al. 2016).

Initially, we tested the olfactory responses of wild-type *Canton-S* (CS) larvae to the odorant acetal after the 2-hour starvation protocol. Followed by a simple behavioral paradigm called the two-choice assay (Rodrigues and Siddiqi 1978), (Monte, Woodard et al. 1989). For starvation experiments, washed larvae were evenly distributed onto a 2x2cm square of Kim wipe paper within a 60x15mm petri dish containing 350 µL of 0.2M sucrose (König, Schleyer et al. 2015) (that serves as non-starved condition) or ddH_2O (starved condition) for exactly 2 hours.

Starved and fed larvae were washed in a drop of ddH_2O three times prior to behavioral analysis. For performing the two-choice assay, approximately 50 third instar larvae are placed in the center of a petri plate with 1.1% agarose. Two filter discs are placed at diametrically opposite ends, with one odor disc containing 25 µl of odorant and the other odor disc with same amount of control (paraffin oil). Larvae were allowed to migrate in the odor gradient for 5 minutes. Larvae on each half are counted, RI is calculated using simple equation RI =(S-C)/(S+C) [S denotes number of larvae on the odorant/test side and C denotes number of larvae towards the control side]. The data from this experiment is presented in Figure 1. When tested for odorant acetal, starved larvae showed higher RI when compared to non-starved larvae. In other words, starved larvae have a stronger attractive response towards acetal compared to the non-starved larvae.

Next, we looked at the olfactory responses of the remaining six odorants from the panel of seven that were mentioned above. As mentioned in the previous paragraph, starved and non-starved larvae were subjected to the two-choice assay after 2 hours of the starvation protocol. Data collected for all seven different odorants are shown in Figure 2. Of all the seven odorants tested, only three odorants showed significant changes in response indices upon starvation. Acetal (Or 42b), 4, 5-dimethylthiazole (Or 59a), and pentyl acetate (Or 47a) were the three odorants that showed higher RI values for starved state larvae when compared to non-starved larvae. In other words, for these three odorants starved larvae had higher attractiveness towards odorants compared to non-starved larvae. This

supported our hypothesis that individual ORNs might be differentially modulated by the animal's starved state.

b) 2nd instar larvae:

Next, we looked at the olfactory behavioral response of 2nd instar larvae under starved conditions. We chose to use 2nd instar larvae for this experiment since they are at a different developmental stage, and also their metabolic requirements differ from those of 3rd instar larvae. We wanted to ask whether the results we observed with 3rd instar larvae are also observed during an earlier developmental stage. As in the case of 3rd instar larvae, we used the same panel of seven odorants. Larvae were subjected to two-choice assay (as described previously) after a two-hour starvation protocol. In addition to the three odorants that showed differences in 3rd instar larvae, two more odorants showed significant differences when tested for second-instar larvae. Those two odorants were 4-hexan 3-one (Or 42a) and 2, 5-dimethylpyrazine (Or 33b). Data is represented in Figure 3. This experiment suggested that like what we have observed in 3rd instar larvae, the starved state of the animal also impacts olfactory behavior in the 2nd instar larvae.

Starvation impacts larval behavior as measured in the tracking assay

a) 3rd instar

The two-choice assay gives information about the attractiveness/repulsiveness of an odorant. However, we wanted to test the impact

of starvation on larval behavior in more detail. To study the larval navigational metrics such as number runs per track, run speed, run length we used a second behavioral assay, a larval tracking assay. Third-instar CS larvae were subjected to larval tracking assay after the 2-hour starvation protocol as mentioned previously. Briefly, approximately ~20 larvae were placed in the central axis of a 22 x 22 cm^2 agarose plate. Five odor discs containing odorant are placed at even spacings along one side of the plate, and five odor discs containing a control odor/ diluent are placed at even intervals along the opposite side. A simple closed-circuit camera records the larval movements for 5 minutes, and their relative positions are analyzed as a function of time (Mathew, Martelli et al. 2013). Of all the seven odorants tested, three odorants elicited statistically significant differences in: number runs per track, run speed, and run length. In case of acetal, starved larvae had greater number runs per track than non-starved larvae and for pentyl acetate it was the non-starved larvae having higher number runs per track than starved larvae. While for acetal and 2, 5-dimethylpyrazine non-starved larvae had greater run speed and run length while pentyl acetate had reversed trends. This trend between acetal, 2, 5 DMP when compared to pentyl acetate was the exact reverse affect. Tracking analysis for parameters: run speed and run length are shown in Figure 4 and Figure 5 respectively. As of now, we are not sure about the reason for this reverse effect. Just like the two-choice assay results, we observed differences for only some odorants and not all.

b) Empty larvae

The panel of seven odorants tested were the ones that elicit a strong activation of single ORN's. We wanted to see if starvation effects would be observed if we silence all but a single pair of ORN's in larvae. A recent study (Fishilevich and Vosshall 2005) suggested that larvae with only a single pair of functional ORNs are able to chemotax robustly toward a subset of odorants that activates it. We postulated that a single pair of functional ORNs could still be modulated under starved conditions. In order to address this question, we genetically created larvae with only a single pair of ORN's intact. This was made possible by using the *Orco* mutation. The functional insect odorant receptor complex consists of a common co-receptor, ORCO (Pellegrino, Steinbach et al. 2011). *Orco* acts in concert with odor receptors to mediate responses to all odors (Larsson, Domingos et al. 2004). *Orco* function was retained in specific ORNs by crossing animals with specific *OrX*-Gal4 (X represents specific odor receptor) drivers to UAS-*Orco* animals (Fishilevich and Vosshall 2005).

We constructed two different *Or-empty larvae* lines. Since we have observed changes with acetal, 2, 5-dimethylpyrazine, and pentyl acetate in the tracking assay, we wanted to check the chemotaxis for these odorant specific ORN's. Also, since Or 33b and Or 47a are expressed in the same ORN (Masuda-Nakagawa, Gendre et al. 2009). We used Or 47a Gal4 to construct Or 47a empty larvae, and for acetal: we used Or 42b Gal 4 to make Or 42b empty larvae. We

tested behavioral responses using a tracking assay for the above mentioned empty larvae lines. Of all the three tested, only Or42b-Gal4 (x) UAS-Orco empty larvae showed statistically significant changes under starvation. This observed trend was similar to the results seen in larval tracking assay. However, Or47a-Gal 4 empty larvae when tested separately against odorants pentyl acetate and 2, 5-dimethylpyrazine showed no statistical significance between starved and non-starved conditions. Data is shown in Figure 6. From our results, we conclude that in the case of Or 42b Gal4 (x) UAS-Orco, the ORN expressing Or 42b is modulated under starved conditions. In other words, a single pair of ORNs are modulated under starved state conditions only in ORN::Or 42b empty larvae and not ORN::Or 33b and ORN::Or 47a.

Starvation dependent modulation of ORNs is regulated by the GABA signaling pathway

ORN's have the receptors for GABA (B) (Root, Masuyama et al. 2008). We postulated that the different levels of GABA (B) receptor on individual ORN's might be the cause for functional diversity of ORN's under starved conditions. ORNs that sense the aversive odorant Co (2) do not express GABA (B) receptors and in contrast, pheromone-sensing ORNs express high levels of GABA (B) receptors (Root et al., 2008). We used all three subtypes of GABA (B) receptors R1, R2 and R3 for our studies. We decreased the levels of GABA (B) R1 in ORN::Or 42b and also ORN::Or 47a. Similarly, we decreased the levels of receptor R2 in ORN::Or 42b and ORN::Or 47a. In order to decrease the levels of GABA (B)

receptors in individual neurons we used specific RNAi lines. When GABA (B) R1 receptor was decreased in ORN::Or 42b and ORN::Or 47a neuron separately, we did not observe any statistically significant changes. In other words, decreasing the levels of GABA (B) R1 in individual neurons didn't affect starvation dependent modulation. We used UAS-GABA (B) R1, one of our parent line as a control. However, when GABA (B) R2 was decreased in ORN::Or 42b and ORN::Or 47a neuron, the effect of starvation was abolished in both the cases. Changes were observed in larval tracking assay for parameters run speed and run length. We used one of the parent line UAS-GABA (B) R2 as the control. Then we wanted to look at the insect specific GABA (B) receptor subtype R3. We used UAS-GABA (B) R3 RNAi line and crossed with ORN::Or 47a Gal4 and ORN::Or 42b Gal4 separately. We did not observe any differences in this case. This is shown in Figure 7 and Figure 8. From these results, we conclude that decreasing GABA (B) receptor levels in individual neurons abolished the effects of starvation. Also, we can conclude by saying that starvation dependent modulation is regulated by the GABA signaling pathway.

The relative gene expression levels of GABA (A) and GABA (B) receptors in larval brain are not changed during the starved state

Since we have established the working model for starvation based modulation we wanted to confirm it using molecular techniques. Firstly, we wanted to check the levels of relative gene expression of a few important genes between starved and non-starved larvae. We performed qRT-PCR analysis with larval

whole heads. We looked at relative expression levels of these genes: Orco, GAD1 (a rate limiting protein in GABA production), GABA (A) receptor, GABA (B) receptor R1, GABA (B) receptor R2 and GABA (B) receptor R3. We haven't found any significant difference between starved and non-starved larvae for any of the genes except Orco. The relative gene expression of Orco was significantly much higher in the case of non-starved condition. Since qRT-PCR analysis was done for whole head samples, we have not observed changes in any of the GABA receptor genes. We predict this might be because GABA is localized in many other regions of the head and not just in the olfactory circuit. However, we have seen significant changes in Orco gene expression because Orco is present only in the olfactory circuit and not anywhere else. Our qRT-PCR results are represented in Figure 9. When we compared the relative expression levels of Orco gene between starved larvae and nutrifly food fed larvae, we have observed that nutrifly fed larvae had similar level of Orco expression as starved larvae.

GABA (B) receptor is localized at the ORN synapses

To look at the GABA (B) receptor localization in the olfactory circuit, we made an antibody against GABA (B) R1. A sequence of 15 amino acids with a terminal cysteine were selected from the sequence of GABA (B) R1 for optimal antibody binding affinity. The sequence was used to run P BLAST (protein BLAST) search against *Drosophila melanogaster* sequences with a less stringent Expect value of 100 (Altschul, Madden et al. 1997). The two hits returned were two isoforms of GABA (B) R1. GABA (B) R1 antibody was developed by Pocono Rabbit

Farms (1:75, Sequence: TVAEAAKMWNLIVLC). Immunohistochemistry analysis were performed on dissected larval preparations. First, we wanted to look at ORN's in general. We used Orco-Gal4 and 10x; UAS-CD8; GFP to drive GFP into the ORNs. We used primary antibody that was a chicken anti-GFP (1:150, Invitrogen) and rabbit anti GABA (B) R1 (sequence mentioned above) in 0.2%PPST+5% normal goat serum (Triton X-100). Samples were incubated overnight at 4 degrees C. Secondary antibodies were a goat anti-chicken Alexa 488 (1:150, Invitrogen) and a goat anti-rabbit Alexa 647 (1:75, Invitrogen) in 0.2% PBST+5% normal goat serum. GFP staining was clearly observed in ORN's which is represented in Figure 10. This result suggests that ORN's project much deeper into the brain than thought previously. Next, we looked for the presence of GABA (B) receptors in the larval antennal lobe. Immunohistochemistry analysis were performed in animals expressing GFP in the synaptic terminals of ORNs. We found that GABA (B) receptor R1 localized to the ORN synaptic terminals. A clear co-localization of GABA (B) R1 and ORN terminals was observed. We conclude that the GABA (B) R1 is localized at the ORN synapses. Root et al., 2008 observed GABA (B) receptors on ORNs in adult flies. Here with our results, consistent with Root et al, we are seeing GABA (B) receptors staining in larval stage too. Staining image is represented in Figure 11.

GABA (B) receptor is not found in the uniglomerular PN's

Next, we looked at the uniglomerular projection neurons for the presence of GABA (B) receptor R1. We don't know if uniglomerular PNs have receptors for

GABA (B) in the larval stage. To address this question, we used GH-146 gal4 that labels a subset of uniglomerular PN's (PN's that are synapsed by a single ORN). Projection neurons are labeled using 10x; UAS-CD8; GFP. Immunohistochemistry analysis was performed as mentioned above. We did not observe GABA (B) receptor R1 localization in uniglomerular PN's. Furthermore, we have seen that the cell bodies were not expressing GABA (B) R1. This is represented in Figure 12. With this evidence, we conclude that GABA (B) receptor is present at the ORN synapses and not at uniglomerular projection neurons.

Consumption rates of starved and non-starved larvae are similar

To see if larvae were consuming 0.2 M sucrose (fed condition) or water (starved condition) we wanted to measure the amount of food in their guts. We performed this experiment to see if larvae were consuming the sucrose in fed condition and water in the starved condition. We added 0.50ml (5%) of food dye (Kroger red food color) to 10ml of 0.2 M sucrose and 10ml of water. Two hours of starvation protocol was performed as previously mentioned. After the starvation protocol, instead of performing behavioral assays, we measured the number of larvae with red colored dye in their guts. We have observed that the feeding rates for starved and non-starved larvae are similar. We conclude by saying that larvae were consuming sucrose and water in relatively equal quantities as shown in Figure 13.

Discussion

Starved state of an animal differentially impacts individual ORNs

The major conclusion from this study is that individual *Drosophila* larval ORN's are differentially modulated by an animal's starved state. We conclude this based on the following experimental evidence: First, when we tested the olfactory responses of *Drosophila* larvae under starved conditions, using the two-choice assay (Monte, Woodard et al. 1989), (Kreher, Mathew et al. 2008) we observed changes in RI for only three out of the seven odorants tested. This experimental result supports previous work from Mathew et al., 2013, which demonstrated functional diversity among individual ORNs. Starved larvae had higher attractiveness to odorants acetal (Or 42b), pentyl acetate (Or 47a) and 4, 5 dimethylthiazole (Or 59a). Each of these odorants elicit strong and specific responses from single ORNs (Mathew, Martelli et al. 2013). With this evidence, we conclude that, each of these individual ORN's are differentially impacted under starved conditions. In other words, not all ORN's show a generalized response to starvation.

When we tested starvation driven behavior in second instar larvae using the two-choice assay, we observed that five out of the seven odorants had higher response indices. In addition to the three odorants that showed differences in third instar larvae, two more odorants showed significant differences. They are: 2, 5 dimethylpyrazine (Or 33b) and 4 hexan 3 one (Or 42a). Second instar larvae's metabolic requirements and feeding stages are different from third instar larvae.

With this experimental evidence, we conclude that like the third-instar larvae, individual ORN's in the second instar larvae are also differentially impacted by starvation. Not all ORN's show a generalized response upon starvation.

To monitor larval olfactory behavior in greater detail, we employed the tracking assay (Gershow, Berck et al. 2012, Mathew, Martelli et al. 2013). We tested the impact of starvation on navigational behavior towards all seven odorants that were tested in the two-choice assay. Consistent with results observed in the two-choice assay, we observed changes in behavioral parameters such as run speed, run length and number runs per track in only three of the seven odorants tested. We have observed that acetal (Or 42b), 2, 5 dimethylpyrazine (Or 33b) had higher run speed and run length in the case of non-starved larvae while for pentyl acetate (Or 47a), the starved larvae had higher run speed and run length. As of now we cannot explain the reason for this reversed trend in the case of pentyl acetate. However, with the trends that we have observed for acetal and 2, 5 dimethylpyrazine we conclude that not all ORN's show similar tracking effect under starvation. Each ORN is functionally diverse and act as functionally non-equivalent units. From our results, overall, we conclude that ORNs contribute differently to the olfactory circuit under changes in internal states such as starvation. These results further support previous studies regarding existence of functional individuality among neurons (Mathew, Martelli et al. 2013).

Starved state of the animal impacts olfactory behavior via GABA signaling

As mentioned previously, local neurons secrete GABA and are primarily GABAergic interneurons (Ng, Roorda et al. 2002). Olfactory receptor neurons express GABA (B) receptors (Root, Masuyama et al. 2008) and strikingly each ORN channel has different levels of GABA (B) receptor expression in adult flies (Root et al., 2008). When the levels of GABA (B) R1 in ORN::Or 42b and in ORN::Or 47a were decreased we observed no effect on starvation dependent behavior changes. However, decreasing the levels of UAS GABA (B) R2 in ORN::Or 42 b and ORN::Or 47a abolished the impact of starvation on corresponding larval behavior. Due to the weakness of the UAS GABA (B) R3 RNAi line, we couldn't perform any control experiments with receptor R3. *In-situ* hybridization shows that two of the GABA (B) receptors GABA (B) R1 and R2 are expressed in regions that are similar, indicating the likeliness of expression among the two receptors which indicates that these GABA (B) receptors function as heterodimers (Fritschy, Sidler et al. 2004). From our findings, we see that reducing the levels of GABA (B) R1 has not abolished the starvation regulation. However, decreasing GABA (B) R2 in specific ORN's abolished the effect of starvation. We conclude that GABA (B) R1's absence might be masked by GABA (B) R2, while GABA (B) R2's decrease is not masked by GABA (B) R1. We conclude that GABA (B) receptor inhibition is involved in starvation control of olfactory behavior.

GABA (B) receptors are localized in the terminals of ORNs

As mentioned earlier, one of the limitation with qRT-PCR analysis with larval heads is that we cannot concentrate exactly on the olfactory circuit. To

understand more specifically about the olfactory circuit, we concentrated on immunohistochemistry analysis. From the confocal image shown in Fig 10 we conclude that ORN's project all the way into the brain. Our counterstain image between GABA (B) receptor and larvae expressing GFP in the ORN's show that GABA (B) receptors are expressed in the ORN synapses. We conclude that in the *Drosophila* larval stage GABA (B) receptors are expressed in the ORN synapses as in adults. This study supports evidence from Root et al., 2008 findings where GABA (B) receptors were shown in the ORNs

In adult flies' GABA (B) receptors are present on the olfactory receptor neurons and its expression expands the vital range of ORN synaptic communication that is stored in projection neuron responses (Root, Masuyama et al. 2008). We looked for the presence of GABA (B) receptors in the uniglomerular projection neurons. Uniglomerular PN's are PN's that are synapsed by single ORN. We have not seen any GABA (B) receptor staining in the uniglomerular PN's. From this evidence, we conclude that GABA (B) is not found in the uniglomerular projection neurons.

Starved state of an animal leads to changes in Orco gene expression

We looked at the relative expression of Orco, GAD1, GABA (A) receptor, GABA (B) receptor subtypes R1, R2 and R3 genes using qRT-PCR analysis. We haven't seen any significant changes in any of the above-mentioned genes between starved and non-starved conditions except for the Orco gene. This is

interesting and can be tied directly to the olfactory system. Orco gene is greatly enriched in non-starved state compared to the starved state. Feeding high sugar diet is involved in the increase of Orco gene expression. This leads to an interesting aspect in understanding whether different feeding protocols (feeding sugars and other complex foods) affect Orco gene expression. We haven't seen any significant changes in the GABA (A) or GABA (B) gene expression. Since the qRT-PCR analysis was performed using whole larval head samples, we expect any small changes in gene expression in the olfactory neurons to be diluted by the high expression of these genes in the larval brain. Orco on the other hand is only specific to the olfactory circuit, which allows us to observe changes in Orco gene expression in starved and non-starved larvae. With our qRT-PCR study we conclude that: starvation doesn't lead to significant changes in GABA (A) or GABA (B) gene expression in the whole brain. Orco gene expression is enriched in non-starved condition.

Limitations of this study

We accept the potential limitations with this study. Most of our tracking assays were performed at concentration of 10^{-1}. It might be possible that at higher concentrations, odorants elicit responses from more than one ORN (Hallem and Carlson 2006). However, we observe significant changes at concentrations of 10^{-2} in our behavioral two-choice assays. Our data from the 2nd instar two-choice assay under starved conditions is to see how larvae respond to starvation. However, we are not sure about the number of ORNs and its processing in 2nd

instar larvae. We have observed reverse trends in run speed and run length for pentyl acetate when compared to 2, 5 dimethylpyrazine and acetal, we acknowledge the fact that there are some discrepancies over this data. As of now we are not able to answer that aspect but future experiments that use a metabolizable sugar such as glucose, fructose etc. and a non-metabolizable sugar such as L glucose and comparing it to starved conditions might answer this question. Only tracking analysis was performed for all the experiments involving GABA receptors. We haven't performed any two-choice assay experiments with GABA receptor RNAi lines. However, it should be kept in mind that each two-choice assay plate require about 50 larvae and for a normal two-choice assay we use 10 plates for each condition (n=10) which would be around 1000 larvae per experiment. It is relatively not easy to get such high number of larvae for genetic crosses.

Final conclusions

- Starvation affects larval behavior
- Starvation affects individual ORNs differently
- GABA (B) R inhibition is involved in the starvation control of olfactory behavior
- GABA (B) R1 is found at the ORN synapses

  Individual ORNs are differentially modulated by the animal's starved state!

Future directions

- Check for GABA (B) R staining in ORN::Or47a and ORN::Or42a (Or 47a showed changes in starvation dependent modulation and Or 42a never showed any differences under starvation) under starved and non-starved conditions.
- Drive GABA (A) R in the PNs (Since PNs have receptors for GABA (A)) and see if it affects starvation.
- Since Orco expression was enriched in non-starved state, determine if different feeding protocols affect gene expression.
- Build a model that determines the specific neurons in the olfactory circuit that are controlled by GABA (B) receptors.

Figures

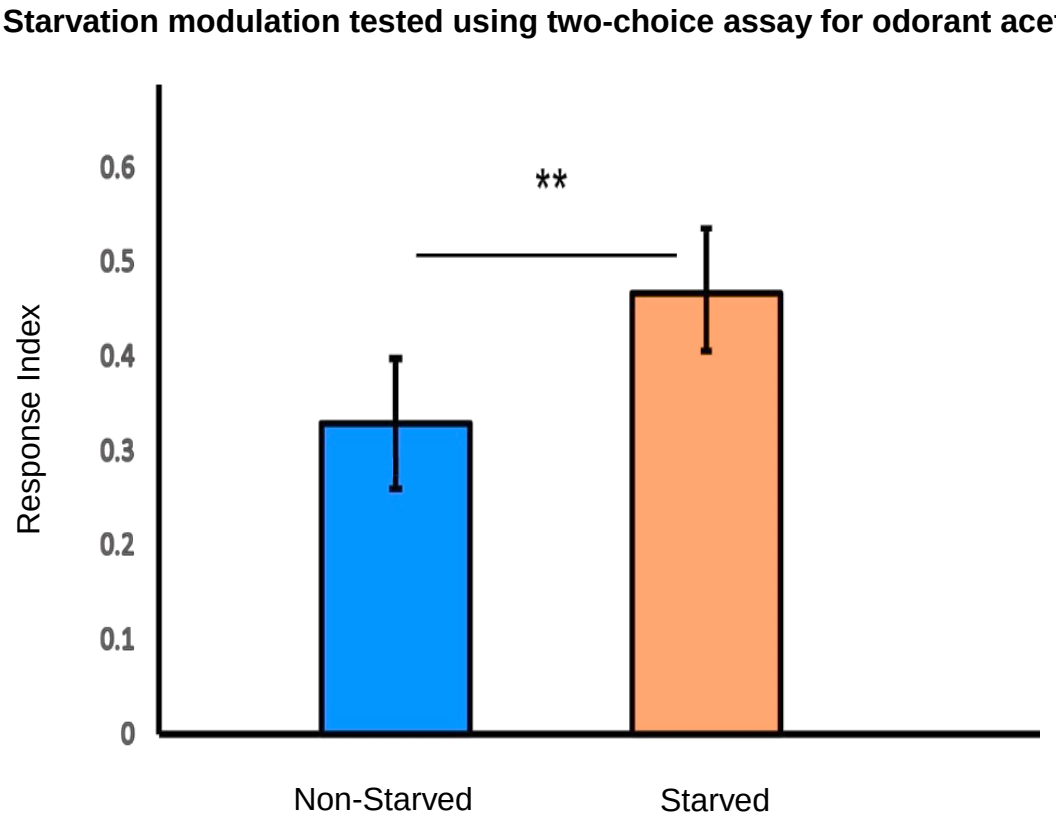

Figure 1. Behavioral changes in non-starved and starved larvae when tested with odor acetal. Third instar larvae were fed and starved as described and exposed to odorant acetal and subjected to simple two-choice assay. Starved larvae showed increased RI. Blue bar represents non-starved larvae while orange represent starved larvae. (ANOVA P, **P<0.01)

Individual ORNs are modulated differently under starvation in the 3rd instar larvae

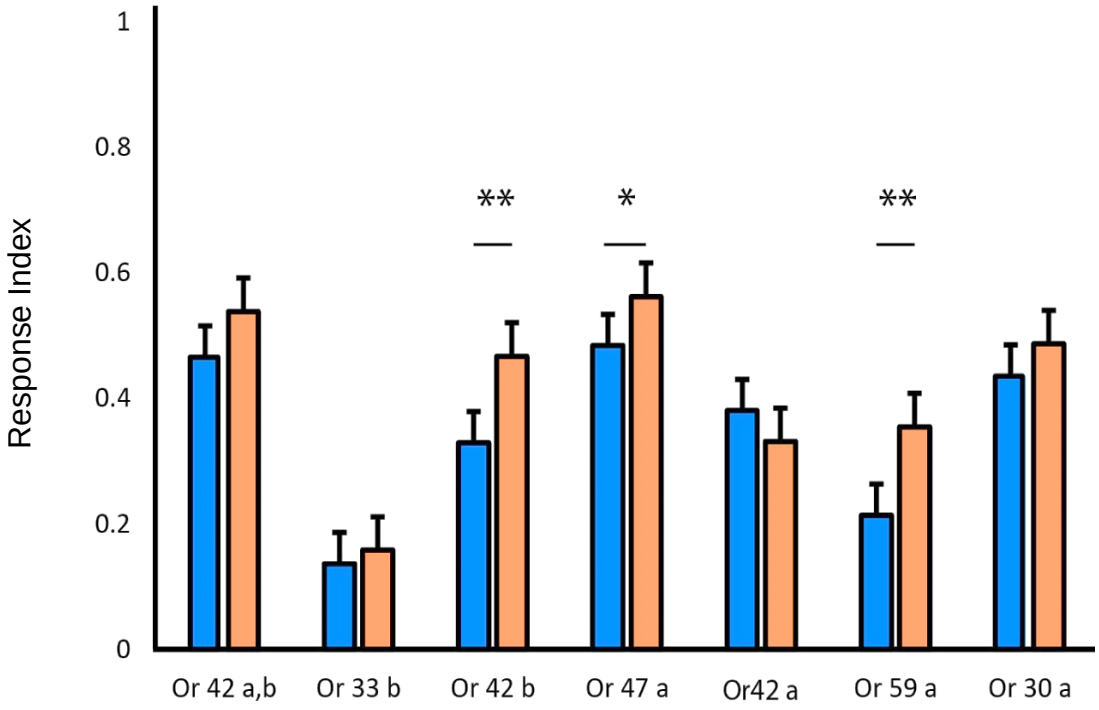

Figure 2. Behavioral changes in non-starved and starved larvae when tested for seven different odors. Third instar larvae were fed and starved as described and exposed to panel to seven different odorants and subjected to simple two-choice assay. Only 3 out of 7 odorants showed significant differences upon starvation. Blue bars represent non-starved larvae while orange represent starved larvae. (ANOVA P, **P<0.01)

Individual ORNs are modulated differently under starvation in the 2nd instar larvae

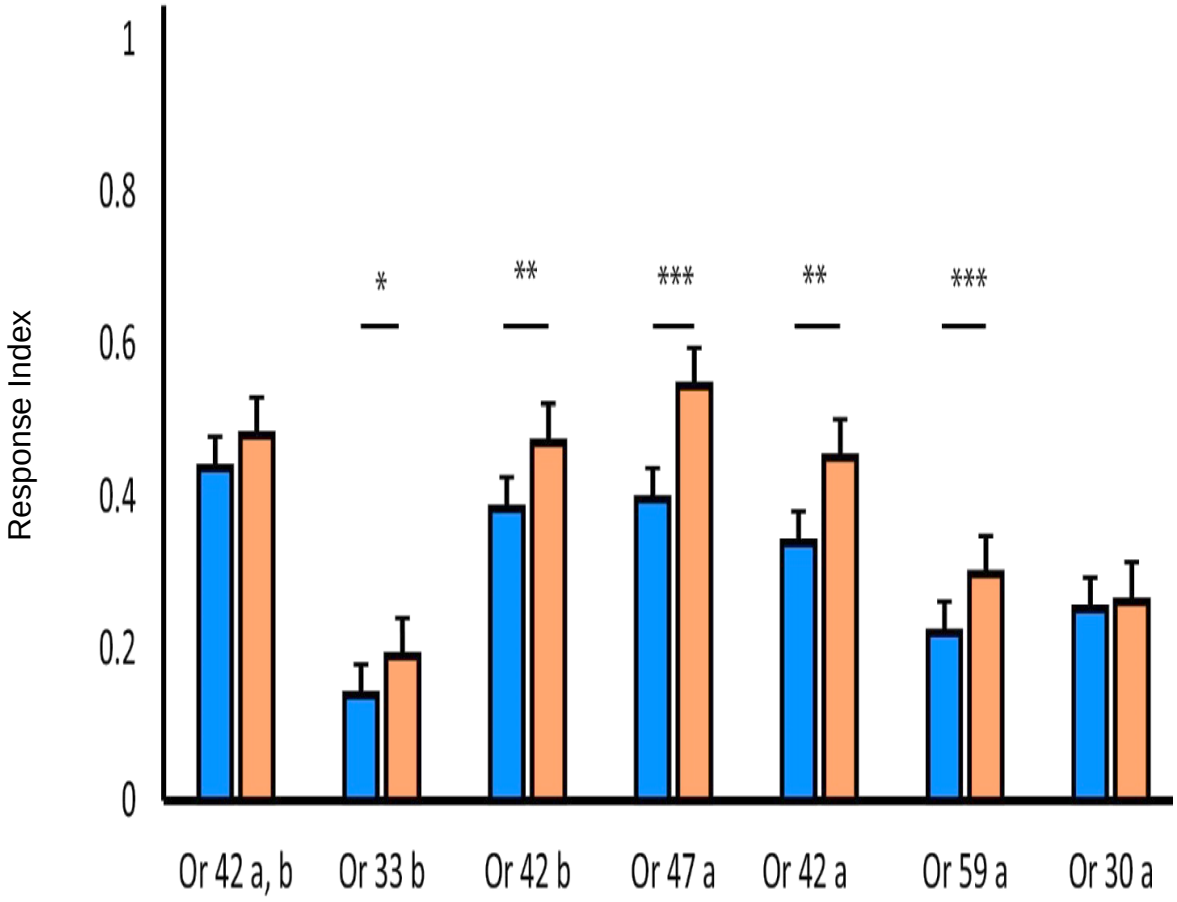

Figure 3. Behavioral changes in 2nd instar non-starved and starved larvae when tested for seven different odors. Second instar larvae were starved as described and exposed to panel to seven different odorants and subjected to simple two-choice assay. 5 out of 7 odorants showed significant differences upon starvation Blue bar represent non-starved larvae while orange represent starved larvae. (ANOVA P, **P<0.01)

Starvation impacts larval behavior as measured in the tracking assay

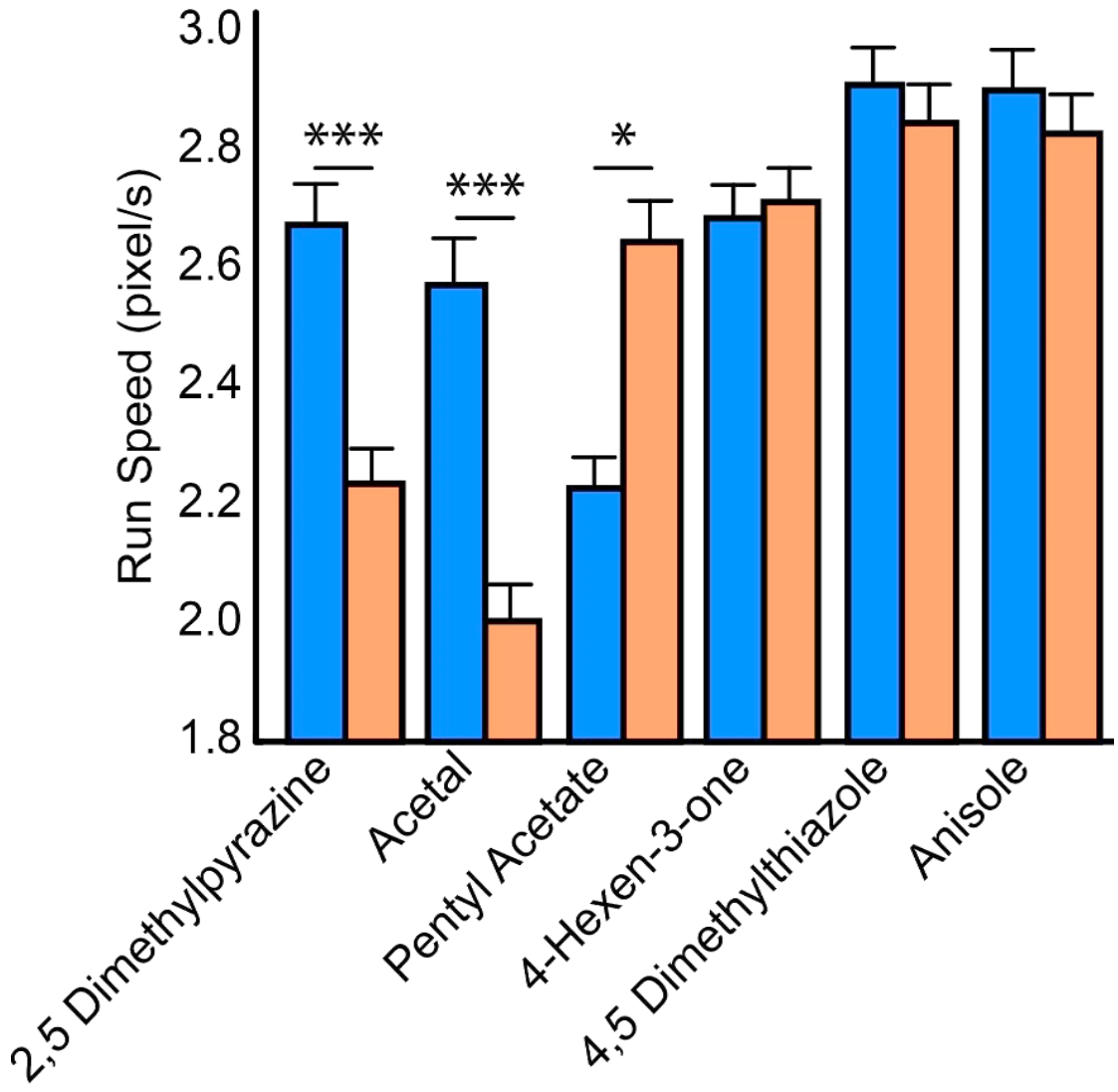

Figure 4. Individual ORNs are differentially modulated by starvation. Using 6 of the odorants from the previous behavioral two-choice assay run speed was determined using tracking assay for non-starved and starved larvae. Only 3 odorants showed statistically significant changes. (Mann-Whitney U: *, P<.05, ***, P<.005)

Starvation impacts larval behavior as measured in the tracking assay

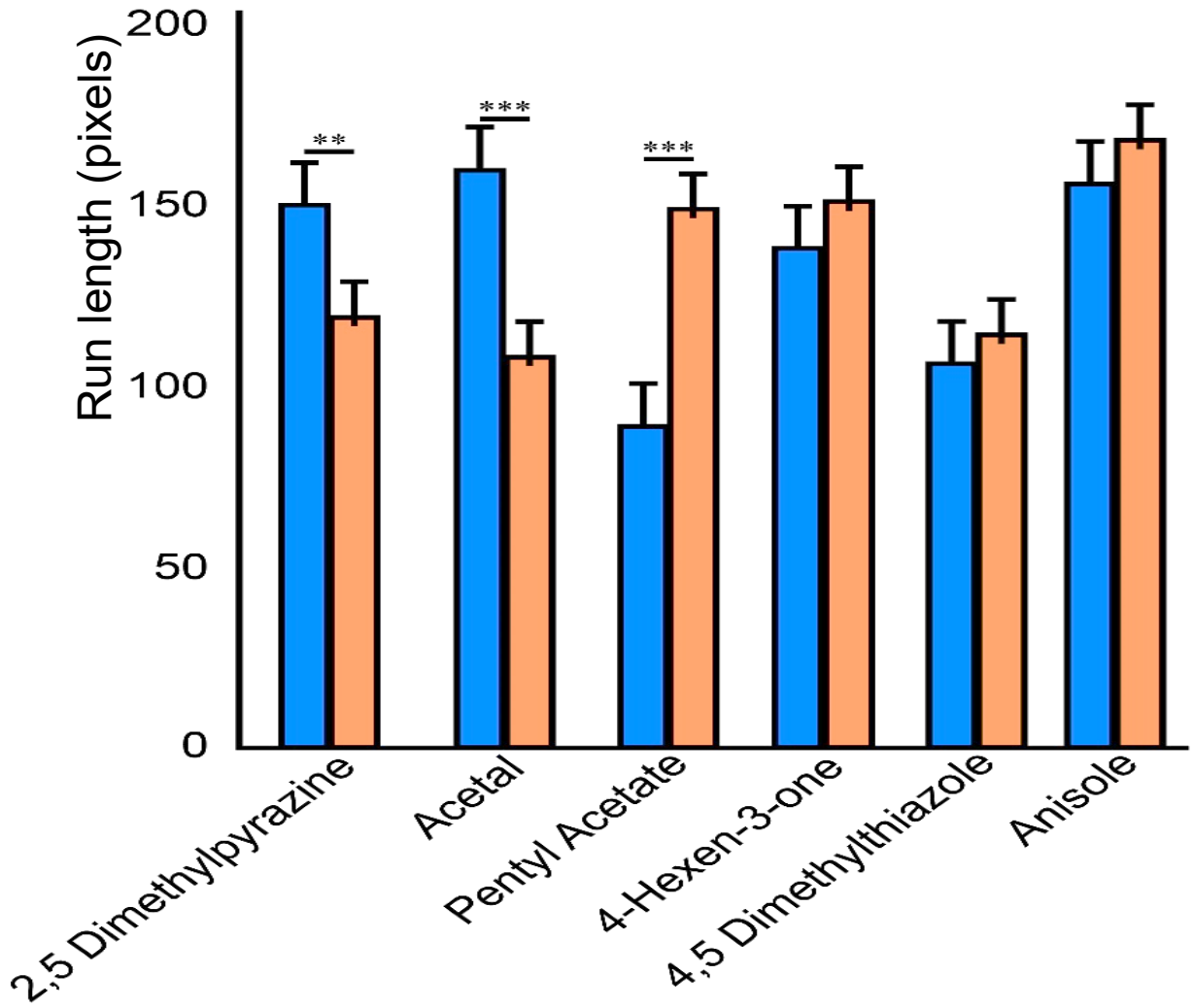

Figure 5. Individual ORNs are differentially modulated by starvation. Using 6 of the odorants from the previous behavioral two-choice assay run length was determined using tracking assay for non-starved and starved larvae. Only 3 odorants showed statistically significant changes. (Mann-Whitney U: *, P<.05, ***, P<.005)

Starvation modulation in empty larvae system measured in tracking assay

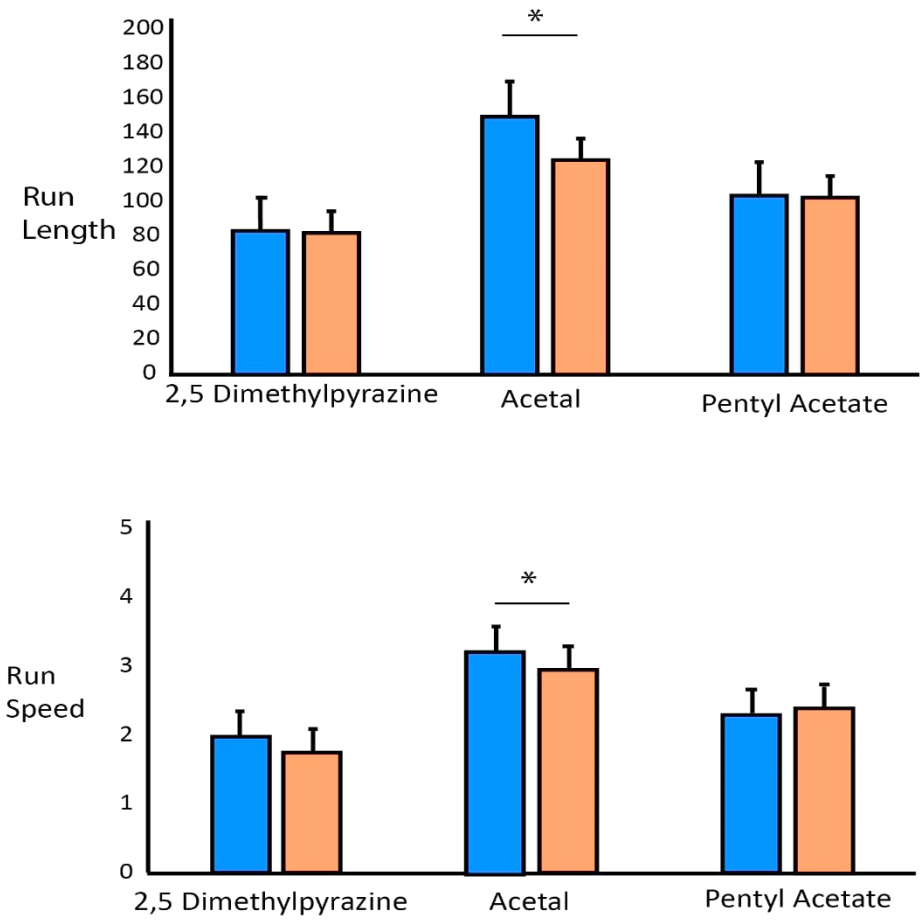

Figure 6. Single pair of ORNs are being modulated under starved state conditions only in ORN::Or42b empty larvae. Using 3 of the odorants that showed changes from previous tracking assay we measured run speed and run length in empty larvae system. (ANOVA P, *P<0.05)

Starvation dependent modulation of ORN::Or 42b is regulated by the GABA signaling pathway

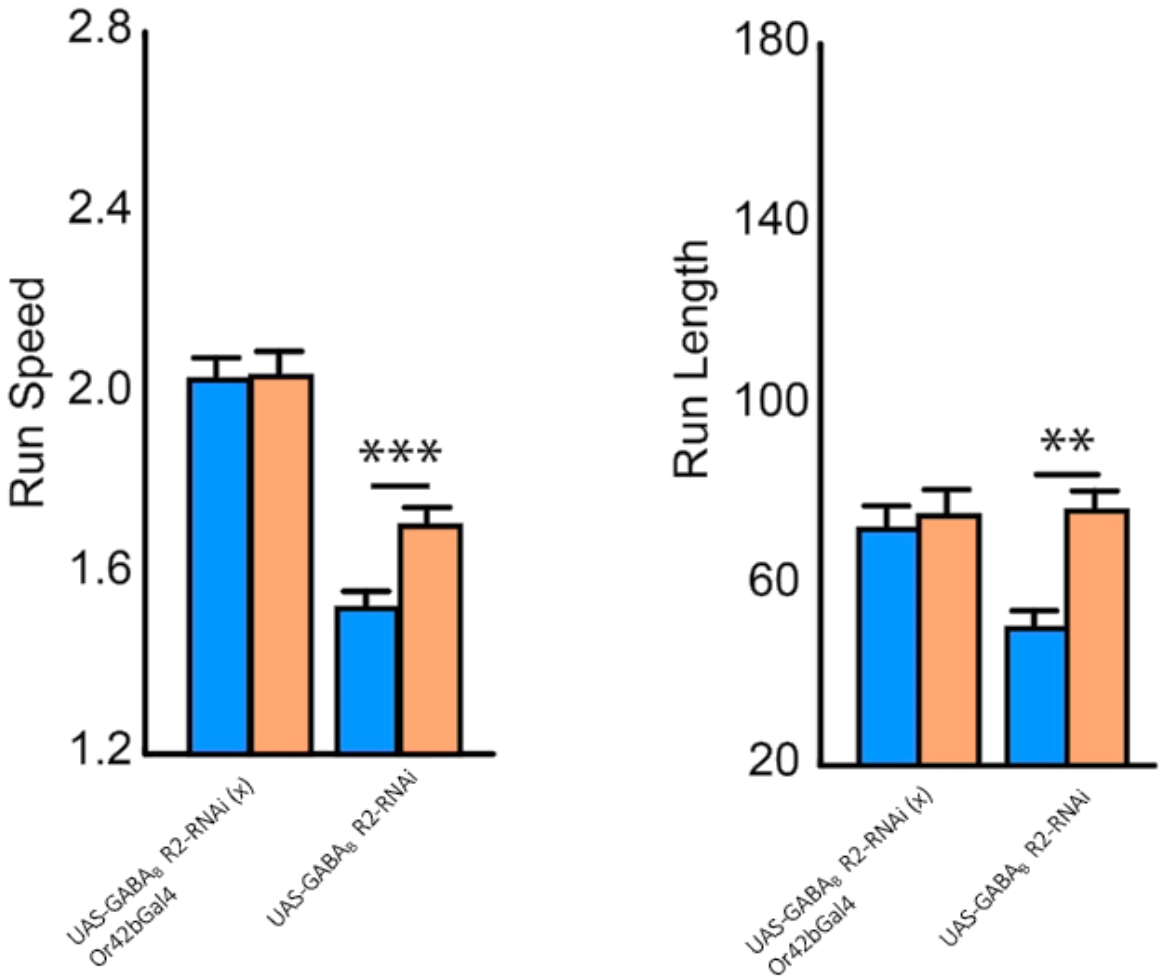

Figure 7. Reduction of GABA (B) R2 in ORN::Or 42b affects starvation dependent modulation. RNAi knockdown of GABA (B) R subunits was performed in ORN::Or 42b. Control was homozygous for the RNAi driver. Non-starved and starved larvae of both the genotypes were exposed to acetal in tracking assay. (Mann-Whitney U: **, P<.01, ***, P<.005)

Starvation dependent modulation of ORN::Or47 a is regulated by the GABA signaling pathway

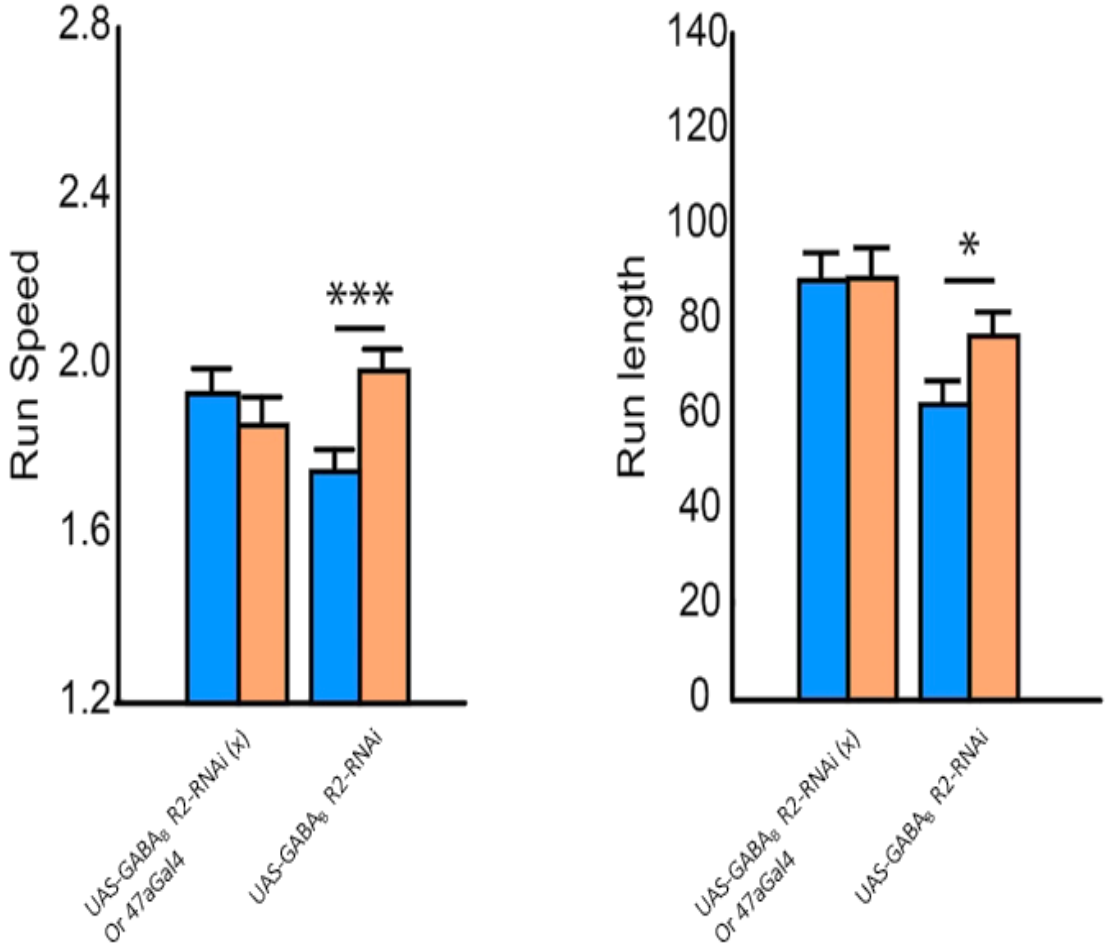

Figure 8. Reduction of GABA (B) R2 in ORN::Or 47a affects starvation dependent modulation RNAi knockdown of GABA (B) R subunits was performed in ORN::Or 47a. Control was homozygous for the RNAi driver. Non-starved and starved larvae of both the genotypes were exposed to pentyl acetate in tracking assay. (Mann-Whitney U: *, P<.05, ***, P<.005)

Gene expression levels of GABA (A) and GABA (B) receptors in larval brain were not changed during starvation

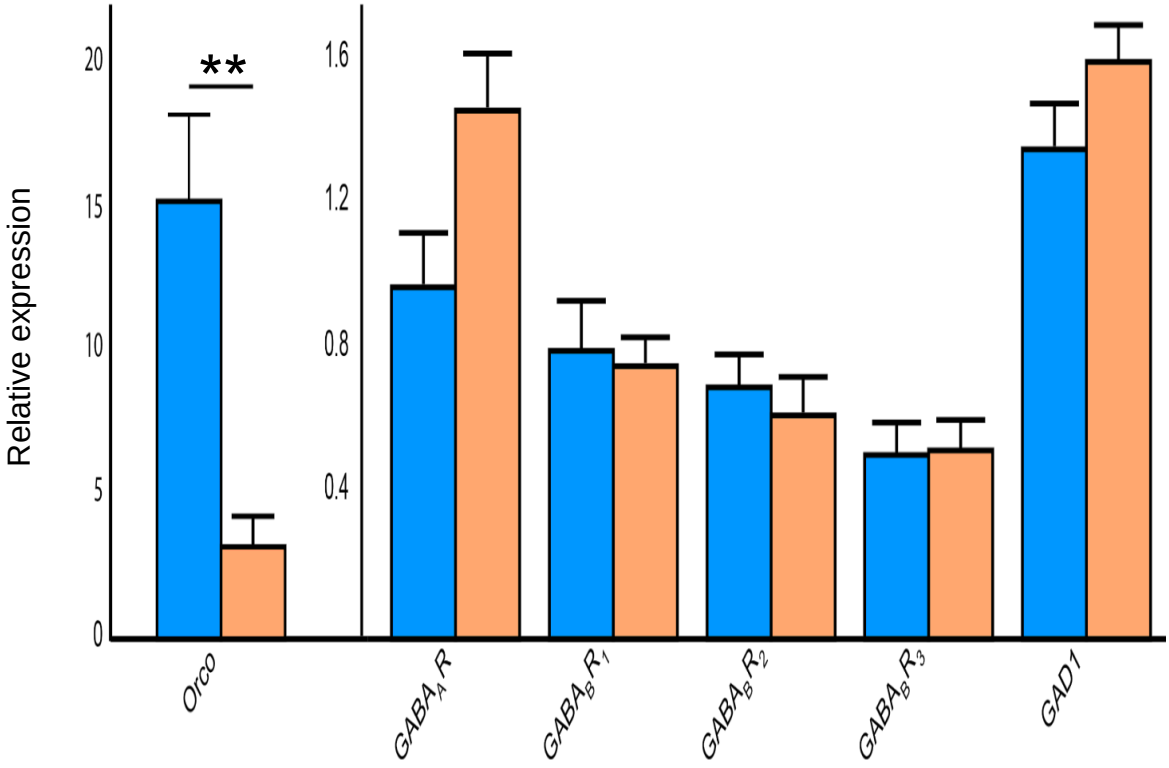

Figure 9. Orco has higher expression in the non-starved state. Non-Starved and starved third instar larvae heads were used for qRT-PCR analysis. Orco, shows a higher expression in the non-starved state compared to starved state. There was no global change in the expression of either GABA receptor subunit or GAD 1. (T-Test: **, P<.01)

Confocal image showing ORNs labelled with GFP projecting all the way into the brain

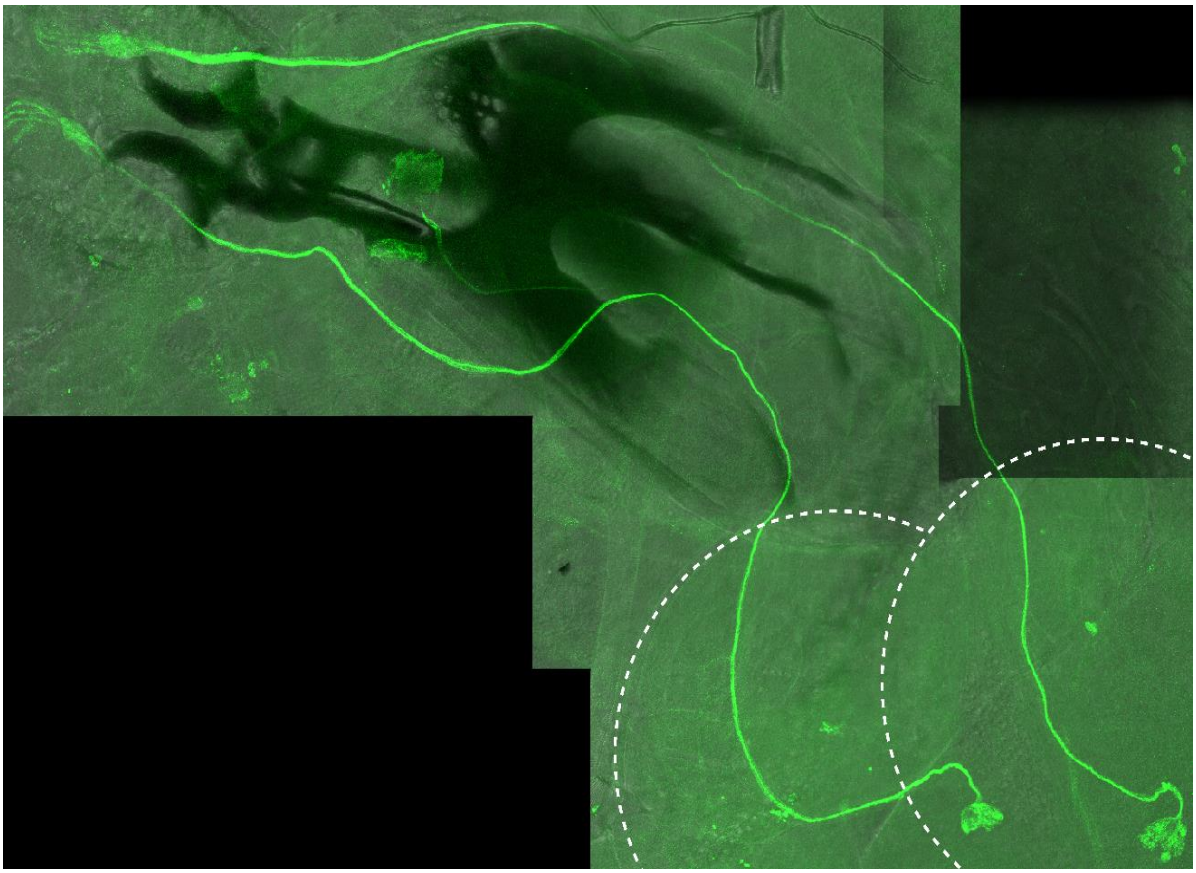

Figure 10. Olfactory Receptor Neurons labelled with GFP Confocal image showing all the ORNs labelled by 10X; CD8; GFP. Dashed circles in the figure represents the larval brain. ORNs project all the way into the brain.

Confocal image showing GABA (B) R1's localization at the ORN synapses

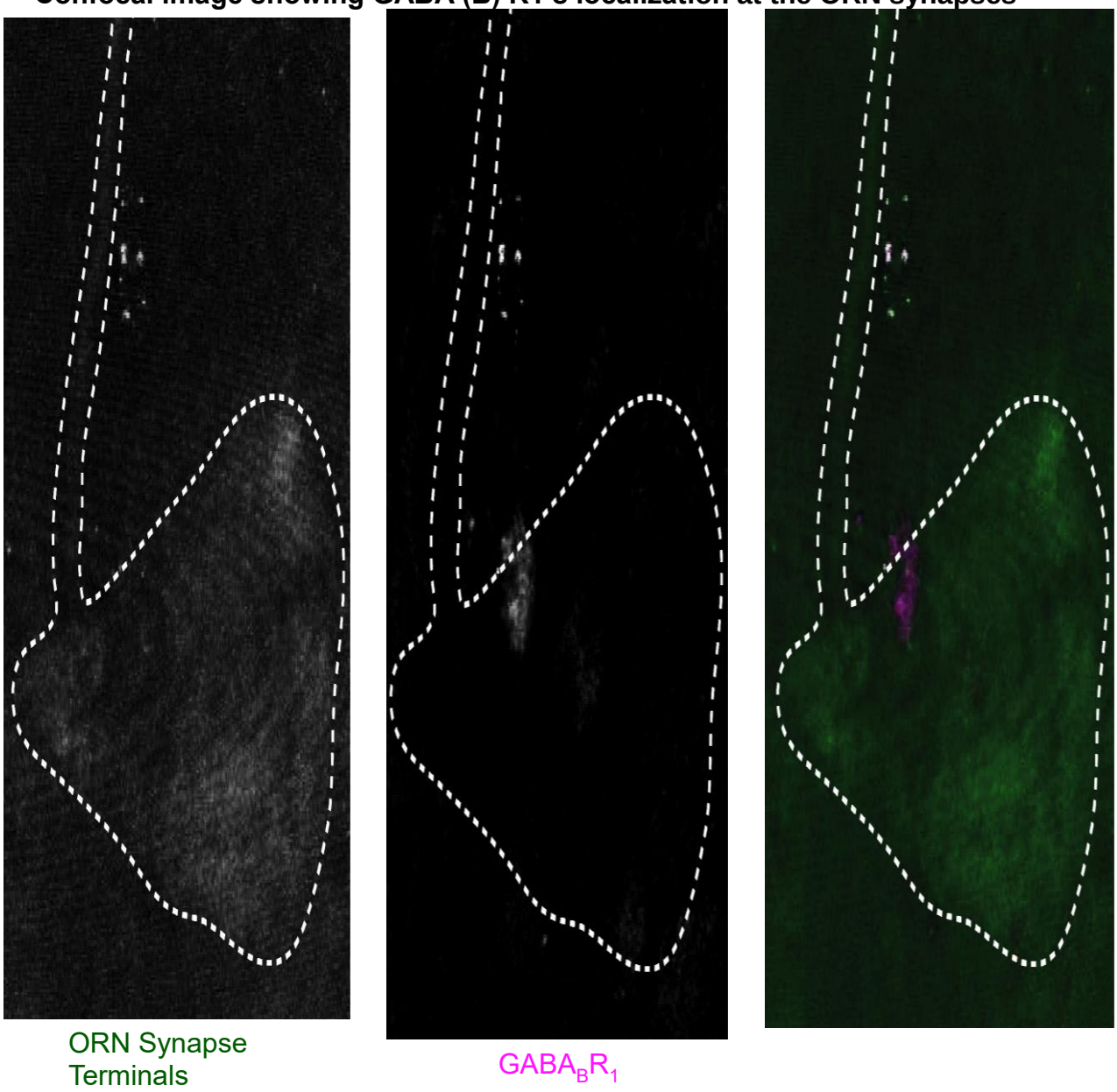

Figure 11. GABA (B) receptor is localized at the ORN synapses. Using genetic tools GFP was driven in ORNs (green) and larvae were dissected and stained with GABA (B) R antibody (Magenta). The images are a z-projection of a stack of ~ 4.5 microns in depth.

Confocal image showing GABA (B) R1 is not found at the uniglomerular PNs

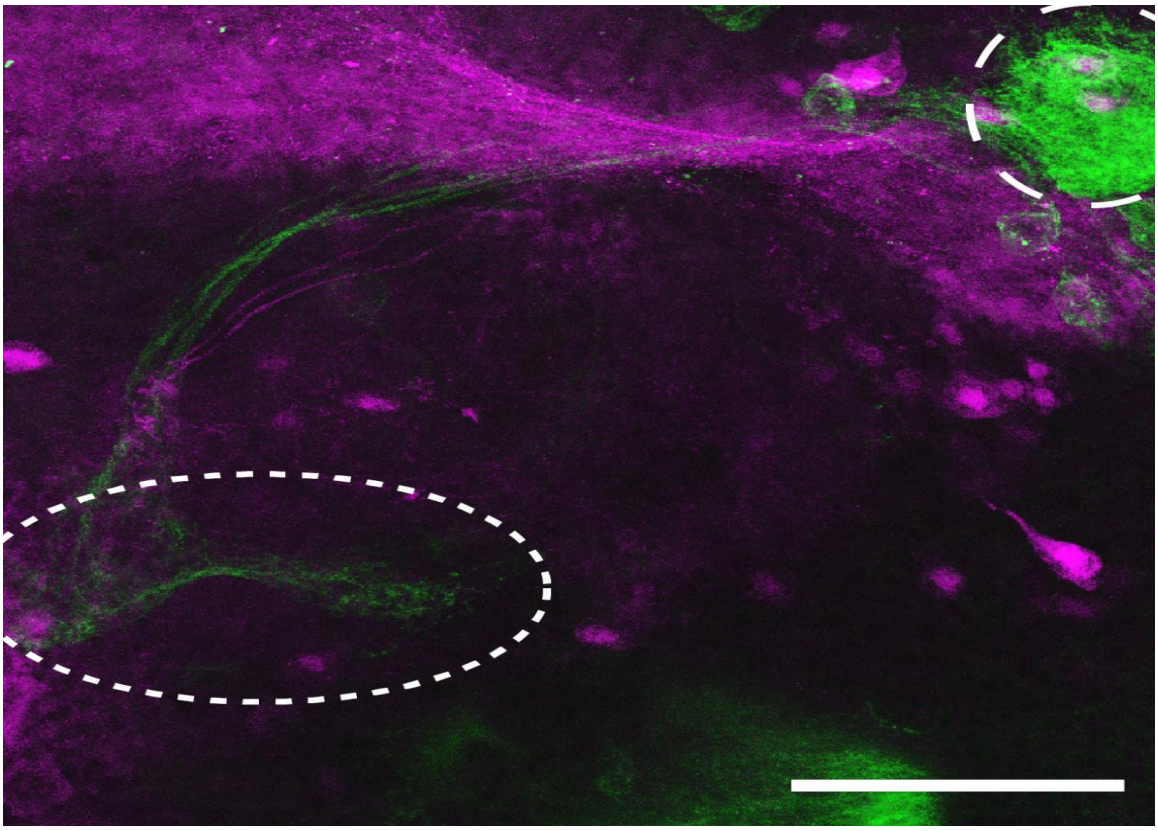

Figure 12. GABA (B) receptor is not found in the uniglomerular PNs. Using genetic tools GFP was driven in a subset of uniglomerular PNs (green) and larvae were dissected and stained with GABA (B) R antibody (magenta). Smaller circle represents the larval antennal lobe and larger circle depicts the mushroom bodies. The images are a z-projection of a stack of ~ 4.5 microns in depth.

Non-starved and starved larvae's consumption rates had no significant changes

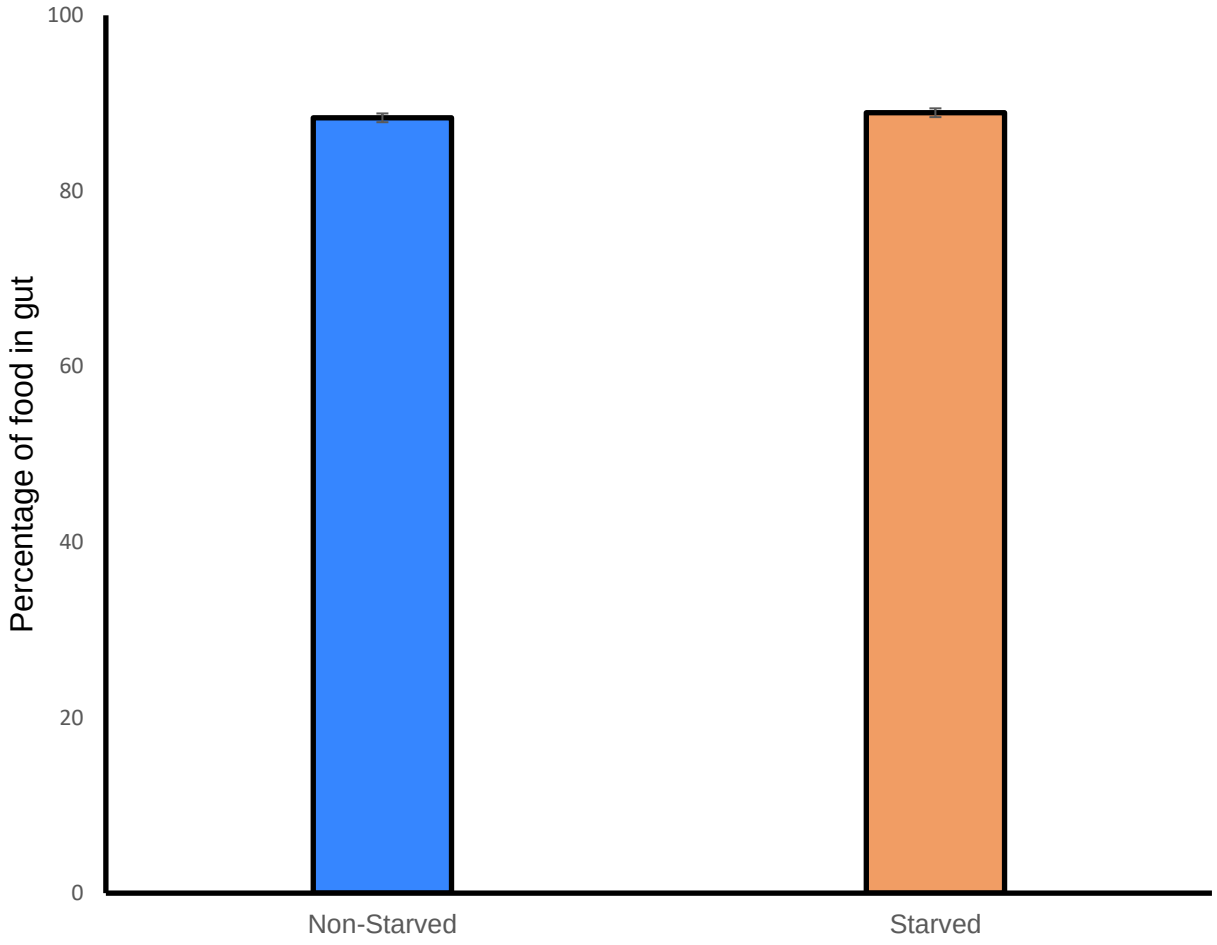

Figure 13. Consumption rates of starved and non-starved larvae are similar. Number of larvae that had food dye (red color) in their guts was measured. There was no significant difference between both the conditions.

Appendix

List of forward and reverse primers, their sequence and efficiency are shown in the following table:

Abbreviation	Primer Squence		PCR Product	Primer Pair Source	Primer Efficiency (%)
	Forward	Reverse			
Syt1	TCCCTATGTCAAGGTGTACTTGC	GTTGAAGACCGGACTCAGTGT	88	PP5891*	104.8
Nrv2	TCGAATGACTTGCCCGCGAA	GCCCTCGCACGATACCCAAA	108	Ling, 2011~	99.4
EF1-F	GCGTGGGTTTGTGATCAGTT	GATCTTCTCCTTGCCCATCC	125	Ponton, 2010~	92.1
APPL	AGTGGAGTTCGTCTGCTGTC	TGGCGCTATTGATCTGAGCTG	101	PP32134*	98.3
GABA-A	CACAGGCAACTATTCGCGTTT	GCGATTGAGCCAAAATGATACC	130	PP25107*	97.3
GABA-BR1	GATGTCAACAAGCAGCCAAATC	CGGGCTCACACTCACTGTC	76	PP15543*	104.2
GABA-BR2	CGCCTTGGGTCACGTTAATGA	GCATTGCACTGAGTGTCGTTC	84	PP22487*	103.6
Gaba-BR3	TGCTGCTCGGACTCTTTGAG	AGCTCCCAATTCGCTCAGAC	71	Primer Blast~	95.0
GAD1	TGAATCCCAACGGGTATAAACTG	TCACTGTTGTGGGCATGAGAT	75	PP383*	109.9
Orco	GATGAGGAAGCTGTTCTTTCTGG	ACCACCATTTTTACGCTGTCG	99	PP37330*	96.4

Table. Primer Information used in qPCR analysis. (*) = Fly Bank Primer Pair Number. (~) = other primer source

References

1. Altschul, S. F., et al. (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res **25**(17): 3389-3402.

2. Budnik, V., et al. (2006). "Selected methods for the anatomical study of Drosophila embryonic and larval neuromuscular junctions." Int Rev Neurobiol **75**: 323-365.

3. Couto, A., et al. (2005). "Molecular, anatomical, and functional organization of the Drosophila olfactory system." Curr Biol **15**(17): 1535-1547.

4. Drew, R. E., et al. (2008). "Effect of starvation on transcriptomes of brain and liver in adult female zebrafish (Danio rerio)." Physiol Genomics **35**(3): 283-295.

5. Farhadian, S. F., et al. (2012). "Post-fasting olfactory, transcriptional, and feeding responses in Drosophila." Physiol Behav **105**(2): 544-553.

6. Fishilevich, E., et al. (2005). "Chemotaxis behavior mediated by single larval olfactory neurons in Drosophila." Curr Biol **15**(23): 2086-2096.

7. Fishilevich, E. and L. B. Vosshall (2005). "Genetic and functional subdivision of the Drosophila antennal lobe." Curr Biol **15**(17): 1548-1553.

8. Fritschy, J. M., et al. (2004). "Independent maturation of the GABA(B) receptor subunits GABA(B1) and GABA(B2) during postnatal development in rodent brain." J Comp Neurol **477**(3): 235-252.

9. Gershow, M., et al. (2012). "Controlling airborne cues to study small animal navigation." Nat Methods **9**(3): 290-296.

10. Gershow, M., et al. (2012). "Controlling airborne cues to study small animal navigation." Nat Methods **9**(3): 290-296.

11. Hallem, E. A. and J. R. Carlson (2006). "Coding of odors by a receptor repertoire." Cell **125**(1): 143-160.

12. Howard, J. K., et al. (1999). "Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice." J Clin Invest **104**(8): 1051-1059.

13. Kim, S. M., et al. (2017). "Neuromodulation of Innate Behaviors in Drosophila." Annu Rev Neurosci **40**: 327-348.

14. Kreher, S. A., et al. (2008). "Translation of sensory input into behavioral output via an olfactory system." Neuron **59**(1): 110-124.

15. König, C., et al. (2015). "Bitter-Sweet Processing in Larval Drosophila." Chem Senses **40**(6): 445.

16. Larsson, M. C., et al. (2004). "Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction." Neuron **43**(5): 703-714.

17. Louis, M., et al. (2008). "Bilateral olfactory sensory input enhances chemotaxis behavior." Nat Neurosci **11**(2): 187-199.

18. Masuda-Nakagawa, L. M., et al. (2010). "Targeting expression to projection neurons that innervate specific mushroom body calyx and antennal lobe glomeruli in larval Drosophila." Gene Expr Patterns **10**(7-8): 328-337.

19. Masuda-Nakagawa, L. M., et al. (2009). "Localized olfactory representation in mushroom bodies of Drosophila larvae." Proc Natl Acad Sci U S A **106**(25): 10314-10319.

20. Masuda-Nakagawa, L. M., et al. (2005). "Stereotypic and random patterns of connectivity in the larval mushroom body calyx of Drosophila." Proc Natl Acad Sci U S A **102**(52): 19027-19032.

21. Mathew, D., et al. (2013). "Functional diversity among sensory receptors in a Drosophila olfactory circuit." Proc Natl Acad Sci U S A **110**(23): E2134-2143.

22. Montague, S. A., et al. (2011). "Similar odorants elicit different behavioral and physiological responses, some supersustained." J Neurosci **31**(21): 7891-7899.

23. Monte, P., et al. (1989). "Characterization of the larval olfactory response in Drosophila and its genetic basis." Behav Genet **19**(2): 267-283.

24. Newquist, G., et al. (2016). "Differential Contributions of Olfactory Receptor Neurons in a Drosophila Olfactory Circuit." eNeuro **3**(4).

25. Ng, M., et al. (2002). "Transmission of olfactory information between three populations of neurons in the antennal lobe of the fly." Neuron **36**(3): 463-474.

26. Oppliger, F. Y., et al. (2000). "Neurophysiological and behavioural evidence for an olfactory function for the dorsal organ and a gustatory one for the terminal organ in Drosophila melanogaster larvae." J Insect Physiol **46**(2): 135-144.

27. Pandey, U. B. and C. D. Nichols (2011). "Human disease models in Drosophila melanogaster and the role of the fly in therapeutic drug discovery." Pharmacol Rev **63**(2): 411-436.

28. Pellegrino, M., et al. (2011). "A natural polymorphism alters odour and DEET sensitivity in an insect odorant receptor." Nature **478**(7370): 511-514.

29. Ramaekers, A., et al. (2005). "Glomerular maps without cellular redundancy at successive levels of the Drosophila larval olfactory circuit." Curr Biol **15**(11): 982-992.

30. Rodrigues, V. and O. Siddiqi (1978). "Genetic-Analysis of Chemosensory Pathway." Proc Indian Acad Sci [B] **87**(7): 147-160.

31. Root, C. M., et al. (2008). "A presynaptic gain control mechanism fine-tunes olfactory behavior." Neuron **59**(2): 311-321.

32. Ruan, Y., et al. (2012). "Olfactory dysfunctions in neurodegenerative disorders." J Neurosci Res **90**(9): 1693-1700.

33. Sato, K., et al. (2008). "Insect olfactory receptors are heteromeric ligand-gated ion channels." Nature **452**(7190): 1002-1006.

34. Slocumb, M. E., et al. (2015). "Enhanced Sleep Is an Evolutionarily Adaptive Response to Starvation Stress in Drosophila." PLoS One **10**(7): e0131275.

35. Stocker, R. F. (1994). "The organization of the chemosensory system in Drosophila melanogaster: a review." Cell Tissue Res **275**(1): 3-26.

36. Wicher, D., et al. (2008). "Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels." Nature **452**(7190): 1007-1011.

37. Wilson, R. I. and G. Laurent (2005). "Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the Drosophila antennal lobe." J Neurosci **25**(40): 9069-9079.