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The Molecular Basis of Olfactory Driven Behaviors in *Drosophila melanogaster*

A Dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

in

Genetics, Genomics and Bioinformatics

by

Sarah C Perry

June 2016

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ABSTRACT OF THE DISSERTATION

The Molecular Basis of Olfactory Driven Behaviors in *Drosophila melanogaster*

by

Sarah Perry

Doctor of Philosophy, Graduate Program in Genetics, Genomics and Bioinformatics
University of California, Riverside, June 2016
Dr. Anandasankar Ray, Chairperson

The organization and functionality of the fly olfactory system is orchestrated by complex molecular events, and the *Drosophila* model offers the tools necessary to understand the genetic basis of these events. This work aims to further characterize the molecular basis of olfactory driven behaviors in *Drosophila*. I will explore several topics to this end: The reliance of innate chemotaxis behavior on single receptor genes, the regulation of receptor gene choice, and a novel molecular mechanism required for olfactory learning and memory.

In Part I, I demonstrate that loss of single receptor genes can disrupt innate avoidance behaviors. First, I identify a novel aversive channel in the larvae governed by Or7a. Or7a mutants lose the ability to detect and respond to low concentrations of the food odorant E-(2)-hexenal. Secondly, I show that loss of Gr63a's co-receptor Gr21a completely disrupts neuronal and behavioral response to carbon dioxide in adult flies. In

both cases, I employed the emergent CRISPR/Cas9 system to create targeted knock-out mutations in *Drosophila*.

In Part II, I show that members of the chromatin modifying complex MMB/dREAM regulate expression of carbon dioxide receptors Gr63a and Gr21a. Using RNA-seq to analyze antennal transcriptomes, I observe differential expression of several other olfactory receptor genes in dREAM complex mutants, suggesting a broader role in olfactory receptor regulation for this multi-protein complex.

In Part III, I find that HDAC6, a largely cytoplasmic protein, is required for retention of olfactory memory in both larvae and adults. *HDAC6* mutants also exhibit defects in synaptic plasticity at the larval neuromuscular junction, suggesting an interesting connection between molecular events at active zones and behavioral plasticity.

Table of Contents

Introduction	1
Part I: Single olfactory receptors mediate innate chemotaxis behaviors.	
Chapter 1: Or7a mediates an aversive olfactory channel in <i>Drosophila</i> larvae.	6
Chapter 2: <i>Gr21a</i> is required for neuronal and behavioral responses to carbon dioxide.	24
Part II: Epigenetic regulation of receptor gene choice.	
Chapter 3: Epigenetic regulation of carbon dioxide receptor gene expression by members of the MMB/dREAM complex	36
Chapter 4: Antennal transcriptome analysis from dREAM complex mutants.	77
Part III: Chapter 5: The role of HDAC6 in synaptic plasticity and memory.	94
Appendices	
Appendix I: Additional data related to HDAC6.	123
Appendix II: Supplementary protocols	
A. RNA <i>in situ</i> hybridization for whole-mount antennae.	129
B. 24-hour benzaldehyde appetitive learning assay.	132
Acknowledgements	137
References	139

Figures

Chapter 1

Figure 1.1	18
Figure 1.2	20
Figure 1.3	22
Figure 1.S1	24

Chapter 2

Figure 2.1	32
Figure 2.2	34
Figure 2.3	36

Chapter 3

Figure 3.1	58
Figure 3.2	60
Figure 3.3	62
Figure 3.4	64
Figure 3.5	66
Figure 3.6	68
Figure 3.7	70
Figure 3.8	72
Figure 3.9	74
Figure 3.10	76

Chapter 4

Figure 4.1	86
Figure 4.2	88
Figure 4.3	90

Chapter 5

Figure 5.1	111
Figure 5.2	113
Figure 5.3	115
Figure 5.4	117
Figure 5.5	119
Figure 5.S1	121

Appendix I

Figure AI.1	127
Figure AI.2	129

Tables

Chapter 3

Table 3.S1	77
------------	----

Chapter 4

Table 4.1	91
Table 4.S1	92
Table 4.S2	93
Table 4.S3	94

Chapter 5

Table 5.S1	122
Table 5.S2	123

Introduction

Insects rely on chemosensation, especially olfaction, to find food, identify mates, select oviposition sites and avoid predators. For insect disease vectors such as mosquitoes, understanding how human host identification occurs is crucial in disease prevention. The fly model offers a comprehensive genetic tool set with which to approach this problem, and the *Drosophila* olfactory system serves as a powerful model for studying neurological development and function.

The *Drosophila* peripheral olfactory system is composed of the third antennal segments and the maxillary palps. Each of these organs is covered with porous, hair-like sensilla housing the dendrites of one to four olfactory receptor neurons (ORNs). Morphologically distinct classes of sensilla are arranged on the surface of the organs in a stereotypical manner, and the ORNs housed within each sensilla are also grouped stereotypically. Each ORN expresses a single receptor or receptor pair and ORNs expressing the same receptor send axonal projections to a single glomerulus in the antennal lobe in the brain (Couto et al., 2005; Fishilevich and Vosshall, 2005; Fishilevich et al., 2005). Sensory input is relayed through projection neurons that synapse with primary olfactory neurons in the antennal lobe and transmit information to higher brain centers such as the lateral horn and mushroom bodies, and behavioral output is eventually determined (Caron et al., 2013; Strutz et al., 2014).

The organization and functionality of the fly olfactory system is orchestrated by complex molecular events, and the *Drosophila* model offers the tools necessary to understand the genetic basis of these events. This work aims to further characterize the molecular basis of olfactory driven behaviors in *Drosophila*. I will explore several topics to this end:

The reliance of innate chemotaxis behavior on single receptor genes, the regulation of receptor gene choice, and a novel molecular mechanism required for olfactory learning and memory.

Part I. Single olfactory receptors mediate innate chemotaxis behaviors.

Activation of a single ORN class is sufficient to drive innate olfactory behaviors in both the larval and adult stage (Bellmann et al., 2010a; Semmelhack and Wang, 2009; Suh et al., 2004). Because each ORN generally expresses a single receptor, this makes these receptor genes critical for insect behavior. Loss of a particular receptor could result in the complete loss of an attractive or aversive channel and the animal could conceivably lose the ability to detect an odorant or respond correctly to a concentration gradient. While many concentrated odorants activate numerous receptor types (de Bruyne et al., 2001; Hallem and Carlson, 2006; Kreher et al., 2008), there are some odorants such as geosmin (Stensmyr et al., 2012), carbon dioxide (Jones et al., 2007; Kwon et al., 2007) and 9-tricosene (Lin et al., 2015) that are known to activate single receptors. In addition, low concentrations of common food odorants will often activate single receptors (Kreher et al., 2008), suggesting that loss of one these receptors may greatly impact the insect's ability to respond correctly to volatile cues.

In Part I, we demonstrate that loss of single receptor genes can disrupt innate avoidance behaviors. First, we identify a novel aversive channel in the larvae governed by *Or7a*. *Or7a* mutants lose the ability to detect and respond to low concentrations of the food odorant E-(2)-hexenal. Secondly, we show that loss of Gr63a's co-receptor Gr21a completely disrupts neuronal and behavioral response to carbon dioxide in adult flies. In

both cases, we employed the emergent CRISPR/Cas9 system to create targeted knock-out mutations in *Drosophila*.

Part II. Epigenetic regulation of receptor gene choice.

The one-receptor-to-neuron map is precise and clearly necessary for correct detection of and response to odorants. However, the mechanisms governing receptor gene choice are not completely understood. In general, the fly must select and maintain expression of a single receptor in 50 classes of ORNs arranged in a complex and stereotypic map. It has been previously shown that cis-acting motifs upstream of odorant receptor (*Or*) genes can recruit combinations of trans-acting activators and repressors (Bai et al., 2009; Jafari et al., 2012; Ray et al., 2007; Tichy et al., 2008). One theory is that the promoter sequence of *Or* genes acts as a “barcode” delineating where a receptor may and may not be expressed. However, this would require at least 50 non-redundant transcription factor codes for each ORN class. Furthermore, many of the factors known to be involved are thought to be expressed in most ORNs, making specificity difficult. It is likely that another layer of regulation is involved for precise control of the receptor to neuron map.

In mammals, it has been shown that repressive chromatin structure plays a role in *OR* gene choice. As in *Drosophila*, each olfactory neuron selects a single receptor gene to express while the rest of the family (~1500 genes) remains silenced. Recent work in mice has revealed that unexpressed ORs are maintained in a stable “OFF” state through the use of heterochromatin (Magklara et al., 2011). This raises the question of whether chromatin structure could influence receptor gene choice in flies as well.

In Part II, we show that members of the chromatin modifying complex MMB/dREAM regulate expression of carbon dioxide receptors Gr63a and Gr21a. Using RNA-seq to analyze antennal transcriptomes, we observe differential expression of several other olfactory receptor genes in dREAM complex mutants, suggesting a broader role in olfactory receptor regulation for this multi-protein complex.

Part III. The role of HDAC6 in synaptic plasticity and memory.

In addition to innate olfactory-driven behaviors, flies are also able to exercise behavioral plasticity in response to new information from the environment. Because of this, the *Drosophila* model has become an effective approach to examining cognitive behaviors such as learning and memory. In olfactory conditioning paradigms, an odor stimulus (the conditioned stimulus, CS) is paired with an unconditioned stimulus (US) such as electroshock, heat or sugar in an effort to alter the fly's innate preference for that odor. This type of associative learning has been well studied in flies for over 30 years and we now understand many of the important biochemical components and brain structures involved in this mechanism. In the case of appetitive olfactory conditioning, olfactory projection neurons send information about an odor stimulus from the antennal lobe to the mushroom bodies. The mushroom bodies are innervated by the dendrites of Kenyon cells, which also receive input from other sensory systems, such as taste. They can then send information to various down-stream behavior circuits. Kenyon cells are highly plastic "coincidence" detectors and thought to serve as substrate for learned olfactory-driven behaviors. Similar brain structures and mechanisms are involved in both adult and larval learning (Krashes and Waddell, 2008; Margulies et al., 2005; McGuire et al., 2005; Scherer et al., 2003).

We were initially interested in examining the role of histone modifications and chromatin structure in higher neuronal functions, so we employed a tractable larval learning model to explore the role of HDACs, an important class of chromatin modifiers in learning and memory. Interestingly, we find that HDAC6, a largely cytoplasmic protein, is required for retention of olfactory memory in both larvae and adults. *HDAC6* mutants also exhibit defects in synaptic plasticity at the larval neuromuscular junction, suggesting an interesting connection between molecular events at active zones and behavioral plasticity.

Part I. Single olfactory receptors mediate innate chemotaxis behaviors.

Chapter 1. Or7a mediates an aversive olfactory channel in *Drosophila* larvae.

Summary

The olfactory systems of most animals share general principles of organization. Odorants interact with receptors expressed in specialized olfactory neurons and neurons of the same class send their axons to distinct glomeruli in the brain. Many complex environmental odorants and even single odors activate multiple glomeruli and generate a spatial activity map in the brain. This stereotypic spatial activity map generates recognition and behavioral valence for the odorant. It is known in *Drosophila* that activation of a single olfactory channel is sufficient to convey valence and drive behavior. However, much work still needs to be done to uncover how individual glomeruli interact to dictate innate behaviors when more than one is activated. We use the simple *Drosophila* larval olfactory system to address how a repellant channel and an attractive channel interact with odorants and each other to drive chemotaxis behaviors. Using this approach, we identify a novel repellant channel driven by the olfactory receptor Or7a. Activation of Or7a alone using low concentrations of (E)-2-hexenal elicits an avoidance response. Interestingly, higher concentration of the same odorant activates several additional receptors and is highly attractive. We demonstrate that Or7a-driven avoidance can be overcome by activation of additional attractive channels, most notably Or42a and Or42b. These findings offer new mechanisms by which odorants from the environment are detected by a sensory system and converted into behavioral responses.

Introduction

In most animals, the olfactory system is responsible for detecting chemical cues in the environment and conveying that information to the brain so that valence – attractiveness or repellency - can be determined. In both vertebrates and flies, primary olfactory neurons (ORNs) are highly specialized cells that typically express single or few receptor proteins (Couto et al., 2005; Fishilevich and Vosshall, 2005). ORNs expressing the same receptor project their axons to the same glomeruli in the brain . In *Drosophila*, the receptor-to-glomerus map is highly stereotypical and has been extensively studied. A given odorant may activate several receptor types and therefore several ORN classes to produce a distinct pattern of activation across the glomeruli of the antennal lobe (Wang et al., 2003).

The larval olfactory system represents a simplified version of the adult system. Rather than over 1000 ORNs belonging to ~50 classes, the larvae have 21 ORNs on each side of the head with their dendrites housed in the dome sensillum. Each of these 21 sensory neurons is thought to belong to a different class and express a different receptor (Fishilevich et al., 2005). Typically, a “tuning” *Or* is singularly expressed along with the obligate co-receptor *Orco* (Larsson et al., 2004). As in adults, different ORNs project their axons to different glomeruli in the larval antennal lobe (LAL) (Fishilevich et al., 2005).

Most odorants naturally activate more than one ORN channel (Hallem and Carlson, 2006; Hallem et al., 2004; Kreher et al., 2005; Kreher et al., 2008; Mathew et al., 2013), however in *Drosophila* it is known that activation of a single glomerulus via a single ORN class is sufficient to drive behavior suggesting that each ORN “channel” is associated with a particular strength and valence (Bellmann et al., 2010; Semmelhack

and Wang, 2009; Suh et al., 2004). For example, adult *Drosophila* find low concentrations of apple cider vinegar (ACV) attractive via activation of DM1 and VA2 glomeruli via Or42a and Or92a, respectively. As concentrations of ACV increase, additional glomeruli are recruited including the repellant channel governed by DM5 via Or85a. Activation of the Or85a channel is sufficient to override Or42a-mediated attraction and cause higher ACV concentrations to become repellant (Semmelhack and Wang, 2009). In this case, a low concentration of an odorant activates a few channels and is perceived as attractive. As odor concentration increases, additional receptors are recruited and the behavioral valence switches from attractive to aversive.

In *Drosophila* larvae, however, most odorants are attractive (Kreher et al., 2008). The use of optogenetic techniques has demonstrated that activation of a single ORN type can effectively drive chemotaxis behavior. While many larval ORNs direct attractive behavior, only a two (Or33b and Or45a) are known to mediate repellency (Bellmann et al., 2010). While powerful, optogenetics relies on the availability of strong promoter-Gal4 drivers, which are not possible for all larval receptors (Couto et al., 2005; Fishilevich et al., 2005). Because of this, the associated valences of all larval ORNs have yet to be determined.

To further investigate the role of individual ORNs in larval olfactory behavior, we have used dilute odorants to selectively activate single receptors (Kreher et al., 2008) and observe the elicited behavior. We identify a novel repellant channel governed by Or7a that is singularly activated by low concentrations of (E)-2-hexenal. Interestingly, as (E)-2-hexenal increases in concentration, it becomes highly attractive due to the activation of additional attractive receptors, which are able to “override” Or7a-mediated

repellency. Our findings suggest that the interaction between attractive and repellant glomeruli may be the opposite in the larval stage compared to what has been observed in the adult (Semmelhack and Wang, 2009).

Results

(E)-2-hexenal avoidance changes to attraction at higher concentrations

Much olfactory behavioral work in larvae has been done with relatively high concentrations of odorants. The disadvantage of this approach is that multiple receptor classes are activated by each odorant and it is difficult to determine the contribution of each channel. However, many of the same odorants at lower concentrations (10^{-4}) activate only one or two receptor types (Kreher et al., 2008; Mathew et al., 2013). Based on the known activation profiles, we tested the larval chemotaxis response to a panel of odorants at low concentrations in order to determine the role of single ORNs (Figure 1.1A). Consistent with what is known thus far about larval olfaction, most odors elicited an attractive or neutral response. However, one odorant, (E)-2-hexenal, caused a robust avoidance response. Previously published data demonstrate that low concentrations of this odorant only activate Or7a in the larvae (Kreher et al., 2008; Mathew et al., 2013), suggesting that Or7a could be a repellant pathway.

In order to test whether a stronger dose of the odorant led to greater repellency, we performed a dose-response analysis. Interestingly, as (E)-2-hexenal increases in concentration, it becomes very attractive (Figure 1.1B). Previously published electrophysiology data show that at least 7 receptors in addition to Or7a are activated at this concentration (Kreher et al., 2008). This attractiveness of the solution suggests that one or more of these channels conveys positive valence and is able to override Or7a-

mediated repellency (Figure 1.1D). This hypothetical dominance of attractive channels over aversive ones is directly opposite to what has previously been observed in adults where increasing concentrations of an odor blend activate an aversive channel that is sufficient to override the attractive response (Semmelhack and Wang, 2009).

Although *Or7a* appears to be a promising candidate for (E)-2-hexenal repellency, larvae could also putatively detect odors via ionotropic (Ir) or gustatory receptors (Gr). To determine whether the behavioral effect was mediated by Or receptors, we tested responses to (E)-2-hexenal 10^{-2} and 10^{-4} dilutions in *Orco* mutant larvae. Both the respective attraction and repulsion are lost, indicating that these behaviors are indeed Or-mediated (Figure 1.1C).

Or7a activation causes avoidance of low concentrations of (E)-2-hexenal

In order to test the role of *Or7a* in the aversion of low concentration of (E)-2-hexenal, we performed RNAi knock down using the pan-neuronal *Elav-Gal4* driver and a *UAS-RNAi Or7a* line from VDRC. This knockdown caused a significant reduction, but not a complete loss, of (E)-2-hexenal repellency (Figure 1.S1A). Due to the lack of available mutants, we created an *Or7a* knockout using the CRISPR system. We recovered 18 putative mutant lines that were isolated and sequenced (Figure 1.2A, 1.S1B). 15 of 18 lines sequenced exhibited indels at the CRISPR cut site, all of which were predicted to result in frame-shift mutations causing non-functional protein products. Three lines had an “intact” *Or7a* sequence identical to the genomic sequence available from Flybase and could be used as controls. Two intact (*Or7a-3* and *Or7a-7*) and two putatively null lines (*Or7a-1* and *Or7a-10*) were selected for further testing.

While it is possible to record neuronal activity from the larval dome sensillum, it is impossible to examine the responses of a single ORN using this technique. Fortunately, *Or7a* is also expressed in the adult antennal neuron type called ab4A. We chose to record from these sensilla in the *Or7a* mutant and control lines to validate that the receptor is non-functional in our putative null lines. Indeed, while the intact lines *Or7a-3* and *Or7a-7* do respond to known ligands (E)-2-hexenal and benzaldehyde, mutant lines *Or7a-1* and *Or7a-10* do not (Figure 1.2B,C). As expected, the ab4A neuron is unresponsive to (E)-2-hexenal and other known activators such as benzaldehyde and bombykol in the mutant lines. The neighboring ab4B neuron expressing *Or56a* retains responses to geosmin in both the mutant and control lines (Figure 1.S1C) (Stensmyr et al., 2012). We also examined responses to E(2)-hexenal in the larval dome sensillum. Responses to E(2)-hexenal at 10^{-4} were substantially reduced in the mutant compared with the control line and a reduction in response was even observed with the 10^{-2} concentration (Figure 1.2E,F).

We next examined larval chemotaxis towards (E)-2-hexenal in the mutant and control lines. Avoidance of (E)-2-hexenal at low concentrations is completely abolished in mutant lines, but all lines maintain attraction to higher concentrations (Figure 1.2D). These results demonstrate that *Or7a* is required for avoidance of low concentrations of the odorant and is consistent with our model in which the higher concentrations of this odor are still attractive through activation of other Ors (Figure 1.1D).

Or42a or Or42b can efficiently override avoidance and cause attraction

In order to elucidate which attractive Or channels are best able to overcome *Or7a* driven repellency, we presented the larvae with (E)-2-hexenal (10^{-4}) alongside

another dilute odorant known to activate an additional receptor (Figure 1.3A). While activation of some receptors was able to reduce Or7a-mediated repellency, activation of Or42a and Or42b was best able to overcome it and cause attraction (Figure 1.3B). We know the tested odorants (ethyl acetate and ethyl butyrate) activate both receptors due to their similar response profiles (Kreher et al., 2008). In order to examine their effects separately, we performed the same experiment in *Or42a*- and *Or42b*- mutants. Activation of either Or42a or Or42b appears sufficient to overcome repellency caused by Or7a (Figure 1.3C) and the degree of the resulting attraction correlates positively to the level of receptor activation to these odorants determined by empty neuron experiments (Hallem and Carlson, 2006; Kreher et al., 2008).

Discussion

Here we demonstrate several novel principles in insect olfaction. First, we identify a previously unreported repellant channel in *Drosophila* larvae mediated by Or7a. This receptor elicits avoidance behavior when activated by low concentrations of E(2)-hexenal. Higher concentrations of the same odorant recruit additional receptors and the Or7a avoidance response is overridden to produce an attractive response. We further demonstrate that activation of single attractive ORNs, in particular Or42a and Or42b, is able to completely reverse Or7a-driven repellency.

In adult flies, it has been shown that activation of a repellant channel in addition to attractive channels results in repulsion, suggesting that repellant glomeruli can override attractive ones. However, what we have observed here suggests that the opposite is true in larvae – certain attractive channels are “stronger” than their repellant counterparts. This is also supported by a previous study showing that activating all Or-

expressing neurons at once using channelrhodopsin results in attraction (Bellmann et al., 2010). This may be advantageous to larvae because they spend much of their time in a high-odor environment where their only navigational tasks involve short-range expeditions to locate and remain near rich food sources. Larva are also most likely to encounter odor gradients rather than the odor plumes which adult flies experience during flight. The interplay between the larval attractive and repellant channels may allow the animal to respond to these kind of stimuli. Given their life-style, what purpose do repellant channels serve in larvae? In the case of Or7a, whose known ligands are mainly food odors (E(2)-hexenal, benzaldehyde and related compounds), this channel may serve to help the animal evaluate and modulate responses to food sources. For example, if the larva loses contact with the food source, Or7a repellency may help drive the animal up an odor gradient back to the food source. Interestingly, Or7a has recently been shown respond to the male-produced pheromone 9-tricosene (Lin et al., 2015). While it is not known if larvae are responsive to 9-tricosene or other cuticular hydrocarbons, it may indicate that Or7a plays a role in social behaviors or detection of other insects.

Materials and Methods

Larval behavioral assay

The larval odor preference assays were performed as previously reported with some modifications (Kreher et al., 2008). 25uL of the odor solution or solvent was presented in inverted 0.2mL PCR tube caps.

Fly lines used

Unless otherwise indicated, wCS was used as the wild-type control line in this study. The *Elav-Gal4/Cyo; UAS-DCR2* stock used for RNAi experiments was made from Bloomington stocks 8765 and 24651. The *Or7a-RNAi* stock is VDRC v107874. CRISPR mutations were created as follows: Targeting oligos (CTTCGTGGTCGGTCGACTGCCATC, AAACGATGGCAGTCGACCGACCAC) were designed using the online tool available at <http://flycrispr.molbio.wisc.edu/>. These oligos were cloned into BbsI-digested pU6-BbsI-chiRNA (Addgene #45946). The U6-chiRNA cassette was then isolated using EcoRI and KpnI digestion and cloned into pattB. We then used the Phi-C31 system to integrate pattB-U6-chiRNA into the attP40 docking site. *attP40{w⁺, pU6-Or7a-chiRNA}/Cyo* individuals were then mated to *vas-Cas9* flies (BL# 51324). *attP40{w⁺, pU6-Or7a-chiRNA}/+; vas-Cas9/+* daughters were then crossed to *FM7* balancer males to capture any *Or7a* CRISPR mutations. 18 isogenic lines were created from *w* progeny and the pertinent segment of the *Or7a* locus was PCR amplified and sequenced (Primers: CACATCATCGCTAGCTT GGT (also used for sequencing reaction), ACTCGTGTGGCCACAAAGCA) from an individual from each line to look for indels at the CRISPR target site. The lines *Or7a-3* and *Or7a-7* may still be segregating *vas-Cas9*. The mutant *Orco*^{-/-} (previously called *Or83b*¹) (Larsson et al., 2004) was obtained from Bloomington stock center.

Electrophysiology

Single unit electrophysiology recordings were performed as previously reported (Dobritsa et al., 2003) using 3-7 day old flies of the indicated genotypes. Diagnostic odorants were used to distinguish individual classes of ORNs in sensilla (ab1-ab7) and

therefore unequivocally identify the target ORN for testing(de Bruyne et al., 2001; Hallem et al., 2004). Odor stimulus flow = 12ml/second. The tested odorants were dissolved in paraffin oil at their respective concentrations and 50uL of the odor solution was applied to cotton in a Pasteur pipette cartridge. Bombykol was provided by Dr. Jocelyn Millar in heptane solution (10mg/mL). 10uL of this solution was applied to a small strip of filter paper and placed in a Pasteur pipette cartridge after the hexanes had evaporated (~5-10 seconds) yielding a final dosage of 0.1mg of bombykol per cartridge. Dried heptane alone did not appear to activate the ab4 neurons (data not shown). Larval dome sensillum recordings were performed similarly to what has been previously reported (Tharadra et al., 2013) with the exception that amplifier settings were optimized to capture action potentials rather than membrane potentials.

Figure 1.1. Low concentrations of E-(2)-hexenal are repellant to larvae via an Or-dependent circuit. **A.** Larval preference indices in a two choice plate assay to lower concentrations (0.01%) of an odor panel selected to activate single or few receptors. Known receptor activities to the odors are represented above the data points. **B.** Dose responses of larvae to E(2)-hexenal. Known receptor activities to particular concentrations are represented above the data points. (N = 5-15) **C.** Preference indices of *wild-type* (*wCS*) and *Orco*- mutant larvae to selected concentrations of E(2)-hexenal. (N = 6-10) **D)** Model explaining the larval response to low or high concentrations of E(2)-hexenal. Error bars represent $\pm$ s.e.m. Mann-Whitney U test: * $p \leq 0.05$.

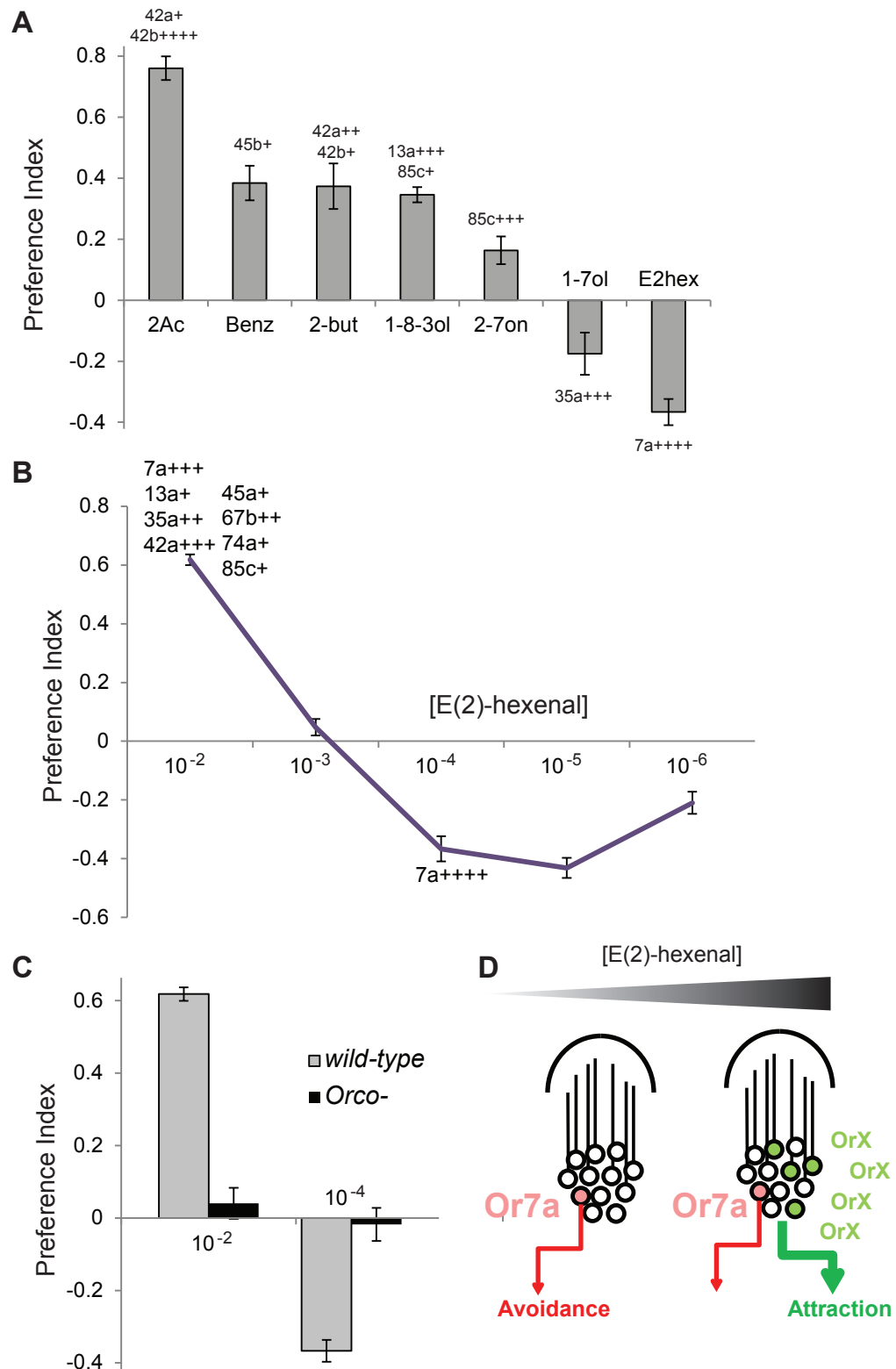


Figure 1.1

Figure 1.2. Behavioral and physiological responses to low concentrations of E(2)-hexenal are Or7a dependent. **A.** Sequences of selected Or7a alleles generated by CRISPR surrounding the predicted Cas9 cut site. Lines 3-7 showed a sequence identical to the wild-type sequence from Flybase. Lines 1 and 10 exhibit indels causing frame-shift mutations and are assumed to be null alleles. **B and C.** Representative traces from single-unit electrophysiological recordings from ab4 sensilla and mean responses. E(2)-hexenal activates Or7a expressed in the A neuron and Geosmin activates the B neuron. Electrophysiology responses from ab4 sensillum (A and B neurons) in response Or7a ligands (E(2)-hexenal, benzaldehyde, bombykol) from wild-type, control and mutant lines. (N = 4-9) Error bars represent $\pm$ s.e.m. **D.** Dose responses of larvae to E(2)-hexenal. Or7a-1 and Or7a-10 are predicted null lines and 3 and 7 are control lines. (N = 8-12) **E and F.** Representative traces from single-unit electrophysiological recordings from larval dome sensilla and mean responses showing the response to E(2)-hexenal at 10^{-4} in control and Or7a mutant animals.

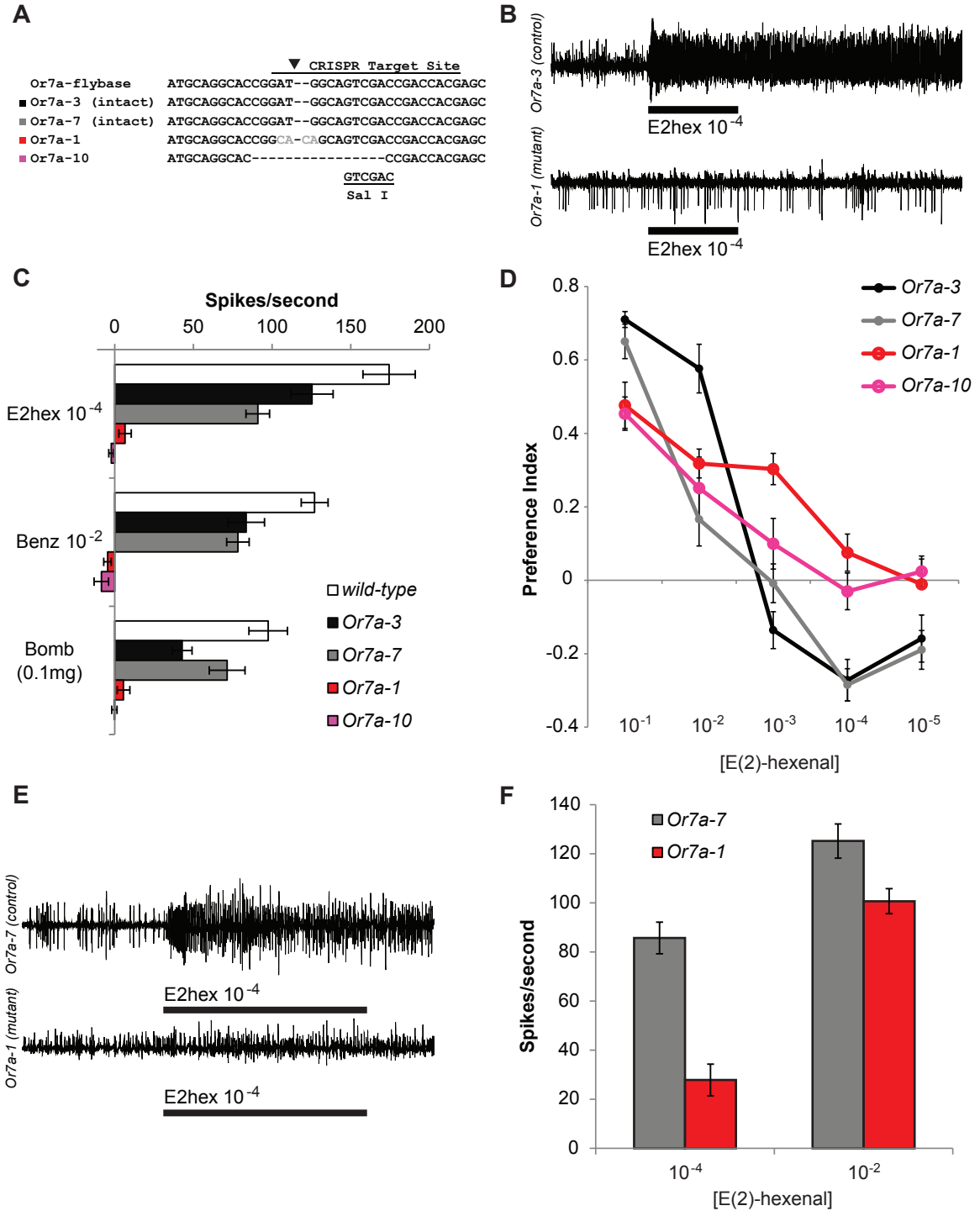


Figure 1.2

Figure 1.3. Or7a-driven repellency can be overridden by Or42a or Or42b. **A.** Schematic showing larval chemotaxis assay for activating Or7a and one or two additional receptors. **B.** Larval preference indices to odors (0.01%) in addition to E(2)-hexenal. Known receptor activities to the odors are represented above the data points. Response to E(2)-hexenal alone is shown with a dotted line. (N = 6-10) **C.** Larval preference indices to odors (0.01%) and odors with E(2)-hexenal in Or42a and Or42b mutants. Known receptor activities to the odors are represented above the data points. (N = 10) Error bars represent $\pm$ s.e.m.

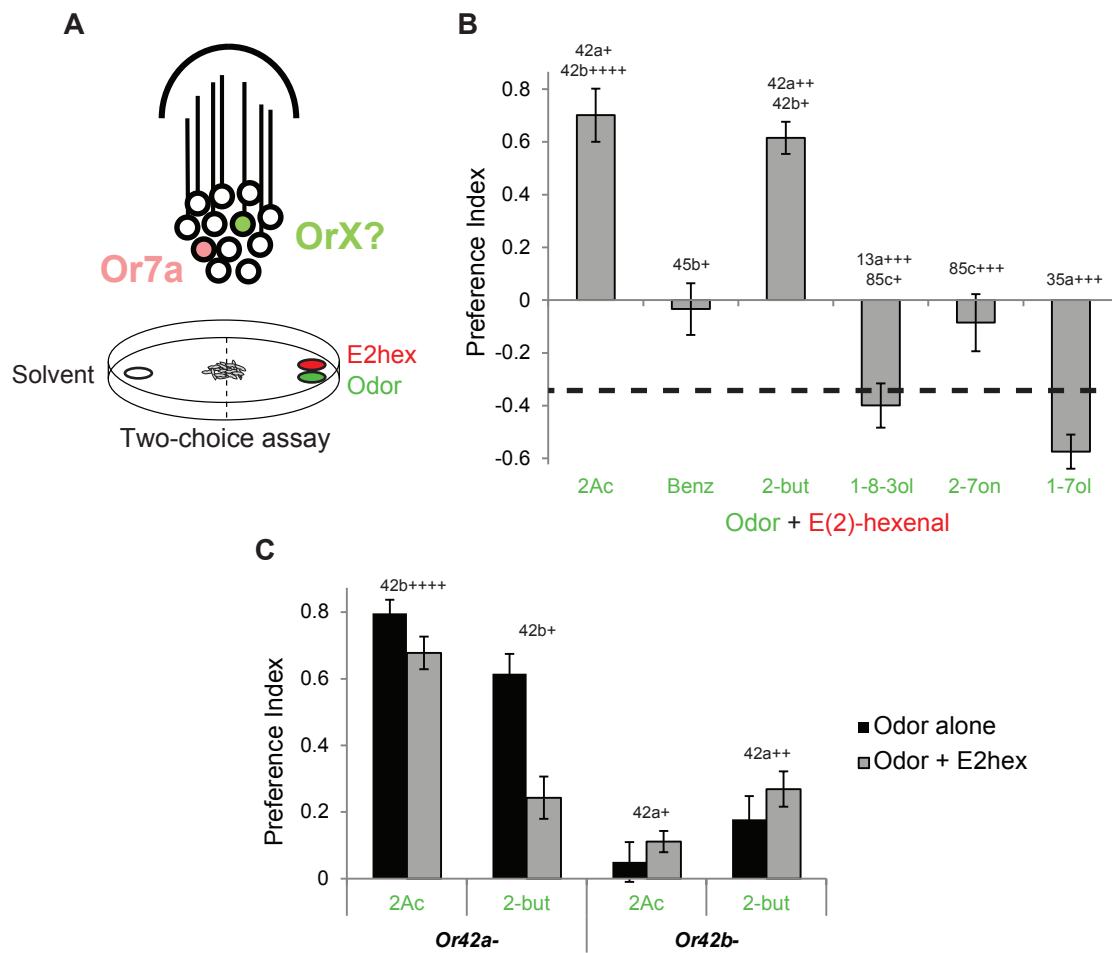
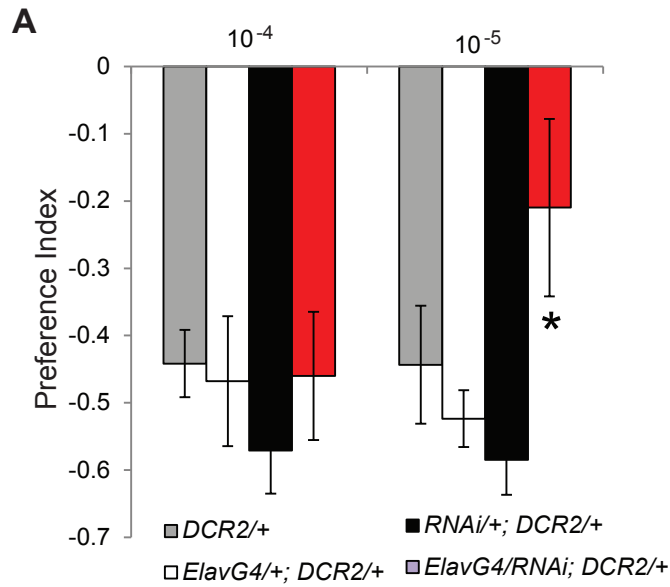


Figure 1.3

Figure 1.S1. Or7a Supplementary information. **A.** Larval preference indices of Or7a-RNAi knock-down animals and selected control genotypes to lower concentrations of E(2)-hexenal. (N = 4-10) **B.** Sequences of Or7a alleles generated by CRISPR surrounding the predicted Cas9 cut site. Lines 3, 6 and 7 showed a sequence identical to the wild-type sequence from Flybase. All the other lines exhibit indels causing frame-shift mutations likely resulting in null alleles. **C.** Example electrophysiological responses of the ab4B neuron to geosmin in control and Or7a mutant animals.



B

▼ CRISPR Target Site

Or7a-flybase	ATGCAGGCACCGGAT--GGCAGTCGACCGACACGAGC
■ Or7a-3 (intact)	ATGCAGGCACCGGAT--GGCAGTCGACCGACACGAGC
Or7a-6 (intact)	ATGCAGGCACCGGAT--GGCAGTCGACCGACACGAGC
■ Or7a-7 (intact)	ATGCAGGCACCGGAT--GGCAGTCGACCGACACGAGC
Or7a-11	ATGCAGGCACCGGCACCGGCAGTCGACCGACACGAGC
■ Or7a-1	ATGCAGGCACCGGCA-CAGCAGTCGACCGACACGAGC
Or7a-14	ATGCAGGCACCGGAT--GCAGTCGACCGACACGAGC
Or7a-17	ATGCAGGCACCGGAT--GCAGTCGACCGACACGAGC
Or7a-19	ATGCAGGCACCGGAT--GCAGTCGACCGACACGAGC
Or7a-13	ATGCAGG-----CAGTCGACCGACACGAGC
Or7a-2	ATG-----GGCAGTCGACCGACACGAGC
Or7a-8	ATGCA-----GTCGACCGACACGAGC
Or7a-9	ATGCA-----GTCGACCGACACGAGC
Or7a-12	ATGCA-----GTCGACCGACACGAGC
Or7a-15	ATGCA-----GTCGACCGACACGAGC
Or7a-16	ATGCA-----GTCGACCGACACGAGC
Or7a-20	ATGCA-----GTCGACCGACACGAGC
■ Or7a-10	ATGCAGGCAC-----CCGACACGAGC
Or7a-18	ATGCAGGCAC-----CCGACACGAGC

GTCGAC
Sal I

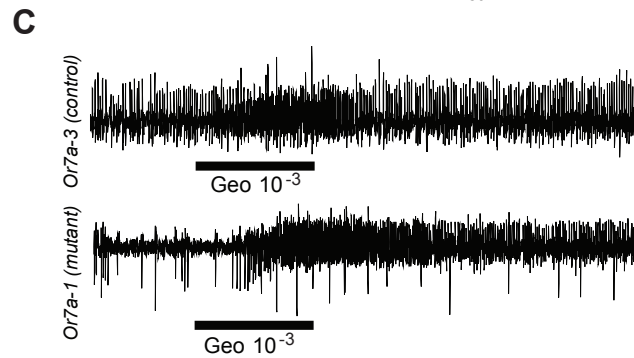


Figure 1.S1

Chapter 2. *Gr21a* is required for neuronal and behavioral responses to carbon dioxide.

Summary

The *Drosophila* carbon dioxide receptor is a heteromer of Gr63a and Gr21a, which is highly conserved across most holometabolous insects. Interestingly, these receptors and their orthologs are the only gustatory receptor family members known to function as odor detectors. Carbon dioxide detection is a critical component of host seeking behavior for many blood feeding insects that transmit human diseases. Understanding the function of this unique class of receptors and how they contribute to insect behavior has the potential to reveal new methods of vector control. Here we use a CRISPR/Cas9 method to generate *Gr21a* mutant fly lines. These lines will be important reagents in further studying carbon dioxide detection and gustatory receptor function in insects.

Introduction

The molecular basis of carbon dioxide detection is highly conserved across most holometabolous insect orders (Robertson and Kent, 2009). In *Drosophila*, two gustatory receptor genes, *Gr21a* and *Gr63a*, are co-expressed in the ab1C neurons of the antenna (Figure 2.1A) and both are required for neuronal responses to carbon dioxide (Jones et al., 2007; Kwon et al., 2007). Activation of these CO₂ sensitive neurons in flies drives a strong avoidance response (Suh et al., 2004). Interestingly, the orthologous receptors in mosquitos, Gr1, Gr2 and Gr3, mediate host-seeking behavior (Tauxe et al., 2013; Turner et al., 2011). It has been shown that *Drosophila* mutants of *Gr63a* or the *Aedes aegypti* mutants of the orthologous *Gr3* completely lose neuronal response and chemotaxis behavior to CO₂ (Jones et al., 2007; McMeniman et al., 2014). While *Gr21a* is also

thought to be necessary for CO₂ detection, this has yet to be demonstrated through mutant analysis.

Generation of *Gr21a* mutant lines using CRISPR/Cas9

Because the *Drosophila Gr21a* locus has been resistant to traditional forms of targeted mutagenesis such as TALENs, we chose to use the new CRISPR/Cas9 system to create *Gr21a* mutant lines (Cong et al., 2013). In order to ensure higher mutagenic efficiency, we opted for a fully transgenic CRISPR approach. A single chimeric guide RNA cassette with a target site early in *Gr21a*'s first exon was inserted into an *attP* site using the phi-C31 integrase system. The resulting transgenic flies were mated to *vas-Cas9* flies designed to express the Cas9 endonuclease in a germline-specific manner (see Materials and Methods for a more detailed description). Of the 19 isogenic lines recovered using this approach, 19/19 (100%) exhibited an indel at the CRISPR target site. 16/19 (84%) were predicted frame-shift alleles and 3/19 (16%) were predicted in-frame deletions. Seven unique CRISPR events were observed. Three mutant lines were selected for further analysis: *Gr21a[2]* and *Gr21a[3]* contain small deletions at the target site which are predicted to result in frame-shift mutations. *Gr21a[10]* is an in-frame deletion predicted to result in the loss of 4 amino acids near the N-terminal end of the protein (amino acids 45-48, DAME). All three alleles have lost the *NcoI* restriction site found in the *wild-type* sequence (Figure 2.1B). This loss of a molecular marker was intended for generating the recombinants required for downstream applications of the mutant.

Validation of Gr21a mutant lines

In order to verify that the receptor protein was no longer functional in our putative *Gr21a* mutant lines, we performed single unit electrophysiology to record carbon dioxide responses from Gr21a-expressing ab1C neurons on the antenna. Extracellular responses of all four neurons housed in the ab1 class of sensilla can be recorded by inserting an electrode into the sensillar hair and puffing odor stimuli over the antenna. In addition to carbon dioxide, we used several other odors known to activate the three other neurons housed in the ab1 sensilla. The A, B, C and D neurons have distinct spike amplitudes, but it is difficult to sort them confidently so we counted total ab1 sensillar response (spikes/second) for each odor tested. In *wild-type* animals, responses can be observed to CO₂ as well as the other diagnostic odors (Figure 2.2A). However, in *Gr21a[3]* mutant animals, CO₂ response is lost. The other three neurons are observed to respond to their diagnostic odors, indicating that only the C neuron is affected by the loss of Gr21a (Figure 2.2B). As is the case in *Gr63a* mutants, the C neuron appears to lack any spontaneous activity in the absence of Gr21a, although additional testing in an *Orco* mutant background is needed to determine this unambiguously (Turner and Ray, 2009). The other putative null line, *Gr21a[2]*, also exhibits loss of CO₂ response while the in-frame deletion line *Gr21a[10]* maintains some response to CO₂ (Figure 2.2C).

We also evaluated whether or not *Gr21a* mutant lines lost avoidance behavior to CO₂ using a T-maze assay. The control *Gr21a[10]* animals are able to avoid 0.67% CO₂ in a manner comparable to *wild-type*, but *Gr21a[2]* and *Gr21a[3]* mutant animals lose all

avoidance (Figure 2.3A). All lines are able to avoid the control repellant benzaldehyde (Figure 2.3B).

Discussion

Not only does the generation and analysis of *Gr21a* mutants demonstrate that this receptor gene is critical for neuronal and behavioral responses to carbon dioxide, but we believe these lines will be valuable reagents in future studies. All three lines evaluated have lost a *NcoI* restriction site at the CRISPR target site making it possible to recombine these lesions with other second chromosome mutations or transgenes using basic PCR RFLP screening techniques.

The *Gr21a[10]* line still responds to CO₂ and should be useful as a genetic control for the other mutant lines as well as for any CRISPR off-target effects caused by the mutagenesis process. It's also informative that the 4 amino acid deletion (D45 - E48) in the gene product of this mutant occurs before the first predicted transmembrane domain (The online tool available at <http://phobius.sbc.su.se/> predicts the first 72 amino acids to be cytoplasmic). Because this mutant still responds to CO₂, we can surmise that this particular region of the N-terminus is not important for receptor function. Using CRISPR/Cas9 to engineer targeted in-frame deletions has the potential to help us further understand the structure and function of this unusual receptor family. Generation of the first olfactory receptor mutant (*Δhalo*) allowed for the development of the Or “empty neuron” system which was critical for deorphanizing the *Drosophila Or* gene family (Hallem and Carlson, 2006; Hallem et al., 2004). A similar system is being developed for decoding gustatory receptors (Gr's) by ectopically expressing taste Gr's in the unique ab1C neuron (Freeman et al., 2014; Wisotsky et al., 2011). However, creating a truly

“empty” Gr-expressing neuron has been impossible until now. By using a *Gr21a*; *Gr63a* double mutant background, it should now be possible to ectopically express new Gr’s in ab1C neurons without possible interference from the native receptors. Such a strategy was recently employed by our lab to reconstruct the *Aedes aegypti* carbon dioxide receptor in the *Drosophila* system (Tauxe, et al., unpublished data).

Materials and Methods

Generation of Gr21a mutants using CRISPR

CRISPR target site was chosen using the tools available online at <http://flycrispr.molbio.wisc.edu/tools>. Targeting oligos CTTCGGCTCGGGGTGATGCCATGG and AAACCCATGGCATCACCCCGAGCC were ligated directly into BbsI-digested pU6-BbsI-chiRNA (Addgene #45946) and cloned into MM294 cells. Resulting clones were screened for addition of a NcoI restriction site. The U6-Gr21a-chiRNA cassette was removed using KpnI and EcoRI digestion and cloned into similarly cut pattB. The resulting pattB{U6-Gr21a-chiRNA} vector was used to transform *y,w; attP40* embryos using the Phi-C31 integrase system. *Vas-Cas9* (BL# 51324) females were mated with *attP40{Gr21a-chiRNA}* males and the resulting *attP40{Gr21a-chiRNA}/+; vas-Cas9/+* females were mated to balancer males. 19 isogenic lines were created from progeny lacking mini-white. 19/19 lines exhibited indels at the CRISPR target site: 16 frame-shift alleles and 3 in-frame deletion alleles. Two putative null alleles and one in-frame deletion allele were chosen for further validation.

Validation of Gr21a mutant lines

Electrophysiology: Single-unit recordings were performed as previously reported (Turner and Ray, 2009; Turner et al., 2011). Diagnostic ligands for the ab1A (ethyl acetate), B (butanedione) and D (methyl salicylate) neurons were diluted 1:10,000 in Paraffin oil and 50uL of the solution was applied to cotton and placed inside the odor cartridge. 3-7 day old unmated females were used for these recordings.

T-maze assay: T-maze assays were performed using a closed (no air flow) T-maze apparatus with 15 mL unmarked culture tubes as arms. For each trial, 40 flies (20 males, 20 females, 3-7 days old, 24-hour wet starved) were loaded into the elevator and allowed 1 minute to choose between the stimulus arm and the control arm. For carbon dioxide trials, 100 uL of pure CO₂ was injected into the stimulus tube through the cap using a 1mL syringe and needle immediately before attaching the arms to the T-maze for that particular trial. This achieves an estimated 0.67% CO₂ in addition to the ambient CO₂ already present in the room air. Un-augmented room air was used in the control arm in these trials. For benzaldehyde trials, benzaldehyde was diluted 1:10 in Paraffin oil and 10uL of this solution was applied to a filter paper disc and inserted into the arm of the T-maze immediately before the trial. 10uL Paraffin oil was used in the control arm in these trials. Preference Index (PI) is calculated as follows:
$$\frac{(\# \text{ flies in odor arm}) - (\# \text{ flies in control arm})}{(\# \text{ flies in odor arm}) + (\# \text{ flies in control arm})}$$

Figure 2.1. *Gr21a* CRISPR mutant lines. **A.** Schematic showing the ab1 sensillum and the neurons housed inside. **B.** CRISPR target site in the first exon of *Gr21a* and sequences of the mutant alleles compared with the wild type *Gr21a* sequence from FlyBase (black). Putative in-frame deletion line *Gr21a[10]* (grey) and null lines *Gr21a[2]* and *Gr21a[3]* (yellow and orange) were selected for further validation. The NcoI restriction site is indicated below.



Figure 2.1

Figure 2.2. *Gr21a* is required for neuronal response to carbon dioxide. **A.** Example electrophysiological traces showing *wild-type* responses of an ab1 sensillum to diagnostic odors: Ethyl Acetate 10^{-4} (A neuron); Butanedione 10^{-4} (B neuron); CO₂ 1% (C neuron); Methyl Salicylate 10^{-4} (C neuron). **B.** Example electrophysiological traces showing responses in a *Gr21a[3]* mutant ab1 sensillum to the diagnostic odors described in (A). **C.** Average ab1 sensillar responses to the diagnostic odors described in (A) in *wild-type*, *Gr21a[10]*, *Gr21a[2]* and *Gr21a[3]* animals. (N= 3 – 6) Error bars represent $\pm$ s.e.m.

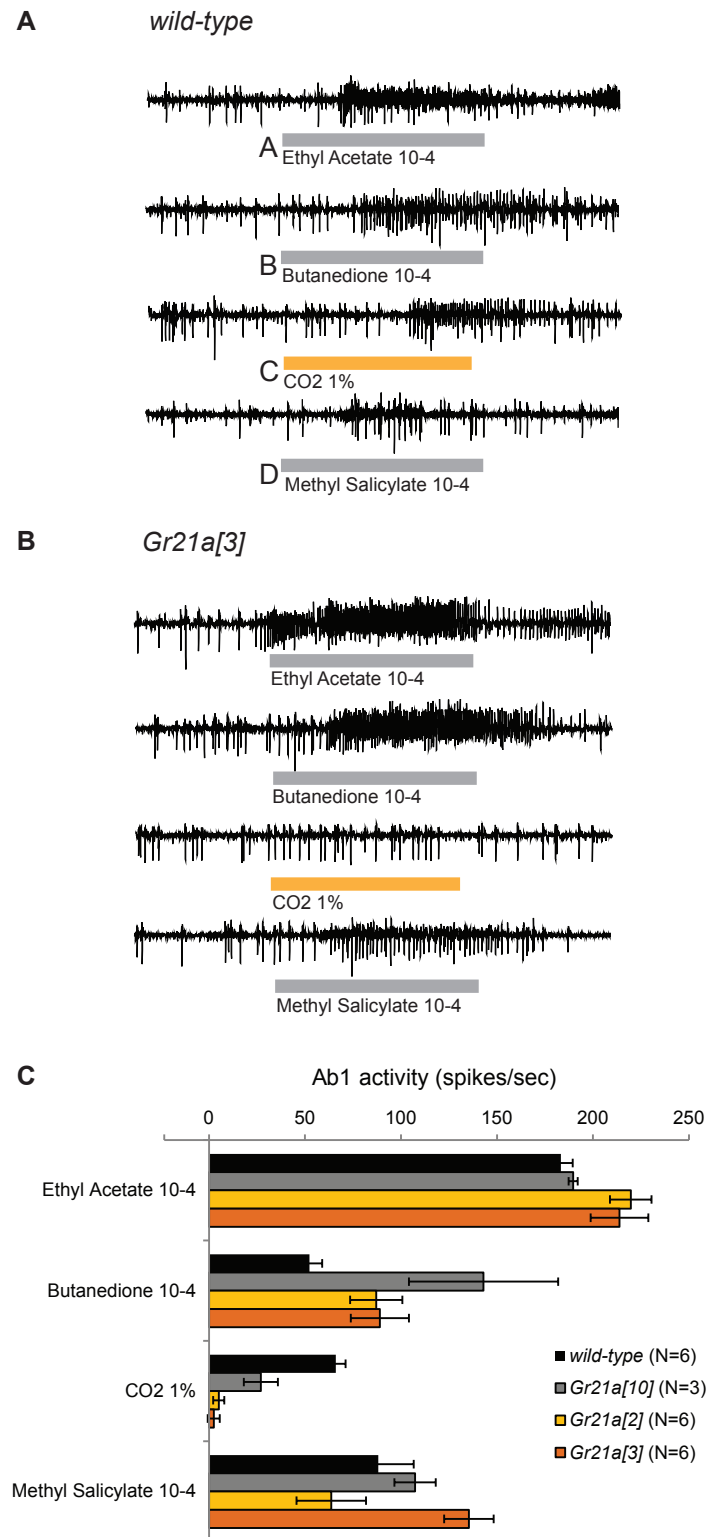


Figure 2.2

Figure 2.3. *Gr21a* is required for avoidance of carbon dioxide. **A.** Behavioral responses to 0.67% CO₂ in a T-maze for *wild-type*, *Gr21a[10]*, *Gr21a[2]* and *Gr21a[3]* animals (N = 10). **B.** Behavioral responses to 10% Benzaldehyde in a T-maze for *wild-type*, *Gr21a[10]*, *Gr21a[2]* and *Gr21a[3]* animals (N = 10). Error bars represent +- s.e.m.

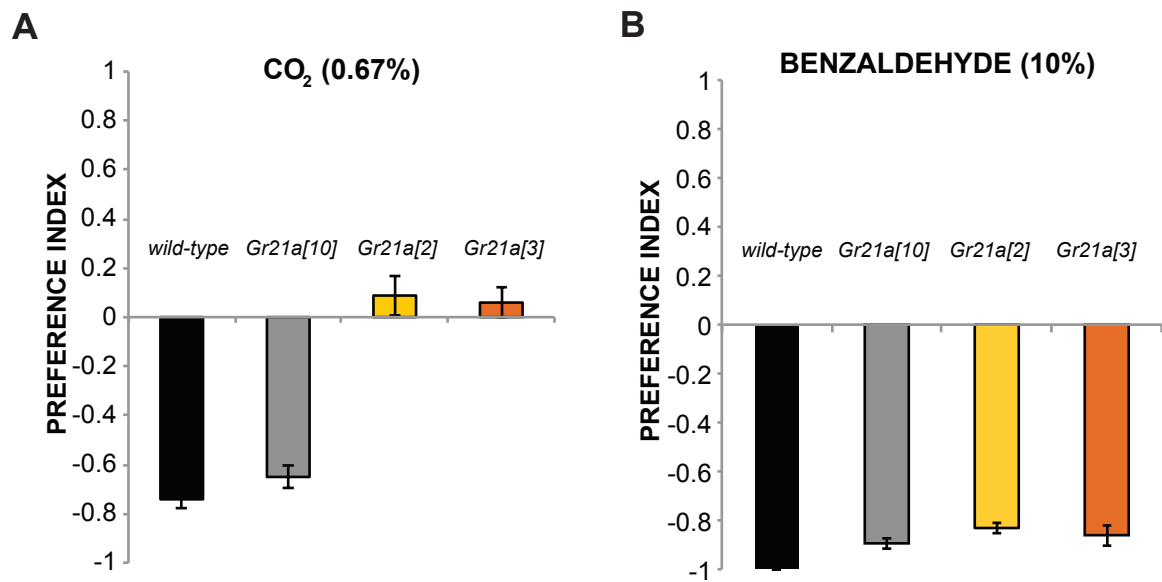


Figure 2.3

II. Epigenetic regulation of olfactory receptor gene choice.

Chapter 3. Epigenetic regulation of carbon dioxide receptor gene expression by members of the MMB/dREAM complex

Summary

An olfactory neuron will express a single receptor or receptor pair from among a large gene family. It is not fully understood how the fly is able to create and maintain such a complex olfactory map. Here we show that epigenetic mechanisms and chromatin structure may play a role in receptor selection. The chromatin modifying complex MMB/dREAM is necessary for proper expression of the carbon dioxide receptor genes *Gr63a* and *Gr21a* in the adult antenna. The presence of Myb in the complex is required for normal expression of *Gr63a/21a*. Additionally, we found that Mip120 and E2F2 prevent aberrant expression of *Gr63a* in tissues other than the antennae. This increase in expression is associated with an increase in activating H3K4me3 histone modifications at the receptor gene locus throughout head tissue. Repressive chromatin in general is important for maintaining singular receptor expression. We find heterochromatic H3K9me2 histone modifications are found at several receptor gene loci in the antennae including *Gr63a*. The histone methyltransferase responsible for these modifications, Su(var)3-9, also influences *Gr63a* expression. Another chromatin modifier, the histone deacetylase RPD3, also participates in control of receptor choice. Finally, we use transcriptome analysis to demonstrate that many other olfactory receptor genes are differentially regulated in several dREAM complex mutants. Together, our findings suggest that chromatin modifiers and ultimately chromatin structure play an

important role in creating and maintaining singular receptor expression in the olfactory system.

Introduction

In both mammals and insects, olfactory neurons (OSN/ORNs) are highly specialized cells that choose to express one or few olfactory receptor genes from among a large gene family (Couto et al., 2005; Fishilevich et al., 2005; Mombaerts, 2006). In *Drosophila*, the third antennal segment and maxillary palps house ~1000 ORNs belonging to over 50 classes each characterized by expression of a specific receptor gene. The system is highly stereotypical and must require sophisticated patterns of transcriptional regulation to establish and maintain. It is known that cis-acting regulatory sequences upstream and downstream of *Or* gene loci are able to recruit combinations of transcription factors in order to achieve organ and cell-type specific expression of receptor genes (Bai and Carlson, 2010; Bai et al., 2009; Jafari et al., 2012; Miller and Carlson, 2010; Ray et al., 2007, 2008; Tichy et al., 2008). However, it is not known if other regulatory mechanisms are involved. In mammals, chromatin state has been shown to play a restrictive role in olfactory gene regulation. During neuronal development, a single receptor allele is selected for expression and all other gene family members are maintained in a stable “OFF” state via repressive histone modifications and compacted chromatin state (Clowney et al., 2012; Lyons et al., 2013; Magklara et al., 2011; McClintock, 2010). We wondered whether a similar mechanism could contribute to receptor gene specification in *Drosophila*.

Chromatin generally exists in one of two states: euchromatin, in which DNA is more loosely packaged around histones allowing access for regulatory factors and

transcription machinery, and heterochromatin, in which histones are tightly packaged and DNA is inaccessible. Euchromatic regions are associated with histone modifications such as methylation of histone 3 lysine 4 (H3K4me3) and acetylation of lysines on H3 and H4. Heterochromatin is characterized by H3K9me2/3, H3K27me3 and H4K20me3 modifications and a lack of acetylation (Filion et al., 2010; Kouzarides, 2007; Margueron and Reinberg, 2010).

The MMB/dREAM complex is a group of associated DNA-binding transcription factors (Myb, Mip130, Mip120, E2F2, RBF1/2), regulators (Mip40, Lin52, DP), chromatin modifiers and histone chaperones (RPD3, L(3)MBT, p55/Caf1) known to regulate cell-cycle related genes and chromatin structure during development (Beall et al., 2002; Cayirlioglu et al., 2001; Clapier and Cairns, 2009; Dimova et al., 2003; Georlette et al., 2007; van den Heuvel and Dyson, 2008; Korenjak et al., 2004; Lewis et al., 2004; Lipsick, 2004; Song et al., 2008; Wen et al., 2008). Interestingly, a ChIP-chip study from cultured cells showed that dREAM complex members associate with numerous promoters throughout the genome, including those of olfactory receptors (Georlette et al., 2007). Of those promoters bound by dREAM complex proteins, *Gr63a* is of particular interest. This gustatory receptor (Gr) family member, along with its co-receptor Gr21a is expressed in adult antenna and is responsible for CO₂ detection and avoidance in flies (Jones et al., 2007; Kwon et al., 2007; Suh et al., 2004). Orthologs of these receptors are found in many other metatrophic insects such as mosquitos and play an important role in host-seeking behavior (Gillies, 2009; Lu et al., 2007). We decided to investigate whether dREAM complex members were required for normal *Gr63a* expression in the *Drosophila* olfactory system.

Results

Myb is required post-developmentally for normal Gr63a expression.

Many dREAM complex proteins are thought to play repressive roles in gene regulation by promoting the formation of heterochromatin. Myb, however, is thought to be the permissive member of the complex. When present in the complex, Myb can promote gene expression such as expression of Polo kinase during cell cycle progression (Wen et al., 2008). Because there is a canonical Myb binding site upstream of *Gr63a* (Georlette et al., 2007), we wanted to first ask whether or not Myb is required for *Gr63a* expression in antennal ab1C neurons. The *myb* mutants are larval lethal, but *mip130,myb* double mutants survive to adulthood (Beall et al., 2004; Manak et al., 2002), so we opted to compare *mip130,myb* double mutants to *mip130* single mutants to determine if loss of Myb resulted in loss of *Gr63a* expression. Using RNA *in situ* hybridization, we can observe *Gr63a*-expressing neurons in whole-mount antenna. *Gr63a* expression is present in *mip130* mutants, but almost completely absent in *mip130,myb* double mutants (Figure 3.1A). Significant reduction of *Gr63a* transcript level in the double mutants was confirmed by qRT-PCR from antennal cDNA. Interestingly, *Gr63a*'s co-receptor *Gr21a* also shows reduced expression in *mip130,myb* mutants. Receptors expressed in several other antennal neuron classes show no change in the two mutants compared with *wild-type* (Figure 3.1B).

We next wondered whether the lack of *Gr63a* expression was due to complete loss of ab1C neurons or simply a reduction in transcription. Using single-unit electrophysiology, we recorded activity from ab1 sensilla and were able to observe spike amplitudes characteristic of the C neuron. While response to carbon dioxide is greatly reduced, it is

not entirely absent, which is consistent with a reduction in receptor levels and not loss of the neuron entirely (Figure 3.1C,D). The other neurons present in the ab1 sensilla responded normally to diagnostic odors indicating that, like the qRT-PCR results suggest, receptor expression in these neurons is largely unaffected in *mip130* and *mip130,myb* mutants (Figure 3.1D).

Because of the greatly reduced neuronal response to CO₂, we next wondered whether *mip130,myb* mutant flies were also deficient in CO₂ avoidance behavior. Using a T-maze assay, we did observe lack of avoidance to low concentrations (0.33%) of CO₂ (Figure 3.1F). Interestingly, the double mutants were still able to avoid higher concentrations (0.67%) of CO₂ (data not shown). Even weak activity in ab1C neurons is likely sufficient to drive avoidance in this situation (Suh et al., 2004).

Myb may act like a molecular “switch” during development: when present, active chromatin state supporting gene expression can be established; when absent, repressive chromatin is established and gene expression is suppressed. Such a situation can be observed with *polo kinase* expression in imaginal discs during pupal development: *polo kinase* shows patchy “ON/OFF” expression in the absence of Myb and Mip130 (Wen et al., 2008). We wondered whether Myb was required to switch *Gr63a* “ON” during development of the neuron and then no longer required after initial expression has been established. Using the pan-neuronal *elav-Gal4* driver, we rescued *myb* post-mitotically and observed a return of *Gr63a* expression in the antenna (Figure 3.2A,B,C). This also resulted in a partial rescue of CO₂ response from ab1C neurons (Figure 3.2D). This indicates that *myb* expression is likely required in the mature neuron itself to promote *Gr63a* expression rather than in the progenitor cells during

development. We next sought to remove Myb activity post-developmentally by use of the temperature sensitive mutant allele *myb*¹ (Katzen and Bishop, 1996; Katzen et al., 1998). At 18C, this mutant form of Myb is thought to have normal activity and we do observe normal *Gr63a* expression when flies are reared and maintained at this temperature. At 25C, however, Myb¹ is thought to be less functional. When flies are shifted to 25C after eclosion, *Gr63a* expression is significantly reduced (Figure 3.2E,F), indicating that Myb activity is required to maintain *Gr63a* expression post-developmentally.

Interestingly, *Gr63a* expression can also be rescued in a *mip130,myb* mutant background by a C-terminal partial construct of Myb lacking the N-terminal DNA binding domain (Peters et al., 1987) (Figure 3.2A,B,C). This version of Myb only contains the conserved protein-protein interaction domain required for its association with the rest of the dREAM complex (Andrejka et al., 2011; Wen et al., 2008). This indicates that Myb is likely activating *Gr63a* by opposing more repressive dREAM complex members rather than as a lone trans-activating factor. It is likely that in the absence of Myb, other dREAM complex members along with other chromatin modifying factors are able to create repressive heterochromatin at the *Gr63a* locus.

Myb is genetically opposed to a repressive chromatin modifier.

In mice, repressive chromatin is used to suppress inappropriate olfactory receptor expression (Magklara et al., 2011; McClintock, 2010). As a result, histone 3 lysine 9 methylation (H3K9me2/3), a marker of heterochromatin, is enriched at OR gene clusters in the olfactory neurons compared with non-olfactory tissue. Because *Gr63a* is regulated by a known chromatin modifying complex, we asked whether a similar

phenomenon could be observed in the *Drosophila* antenna. We performed ChIP-qPCR on antennal tissue and heads lacking antenna using antibodies against H3K9me2, a histone modification associated with heterochromatin. Using this approach, we found enrichment of H3K9 methylation at both *Gr63a* and *Gr21a* in antennal versus head tissue (Figure 3.3A). Interestingly, enrichment was also observed at *Or42b*, another antennal receptor expressed in ab1A neurons. This could suggest that heterochromatin may play a larger role in olfactory receptor gene regulation.

H3K9 is methylated primarily by the methyltransferase Su(var)3-9 (Ebert et al., 2004; Rea et al., 2000). Because H3K9me2 is enriched at *Gr63a* loci in antennal tissue, we speculated that this histone modifier may also play a role in *Gr63a* expression. While loss of one copy of *Su(var)3-9* in a *wild-type* background does not appear to affect *Gr63a* antennal expression, loss of one copy in the *mip130,myb* mutant background is able to partially rescue *Gr63a* expression (Figure 3.3B). This may indicate that in the absence of Myb, the dREAM complex creates a repressive chromatin state at the *Gr63a* locus through interaction with repressive chromatin modifiers such as Su(var)3-9.

Active chromatin, or euchromatin, is also associated with distinct histone modifications, most notably acetylation of lysine residues including H3K9. In order for H3K9-Ac to become methylated, it must first be deacetylated. Interestingly, the dREAM complex is known to associate with the histone deacetylase (HDAC) RPD3. We asked whether or not RPD3 also plays a repressive role in *Gr63a* regulation. Loss of one copy of *rp3* does not appear to affect *Gr63a* antennal expression in either a *wild-type* or *mip130,myb* mutant background (Figure 3.4A,B). However, post-developmental over-expression of

rpd3 using *elav-Gal4* is able to cause a reduction in *Gr63a* expression (Figure 3.4C). This may indicate that RPD3 is able to influence *Gr63a* expression in some situations.

Repressive members of the dREAM complex also regulate *Gr63a* expression.

Because the C-terminal domain of Myb is able to rescue *Gr63a* expression, the other DNA binding factors present in the dREAM complex are likely able to recruit Myb to the *Gr63a* locus. As described earlier, many other members of the dREAM complex serve as transcriptional repressors, especially in the absence of Myb (Korenjak et al., 2004; Lewis et al., 2004; Wen et al., 2008). We hypothesized that some of these repressive members are also required to regulate *Gr63a* in the adult olfactory neurons.

Using mutant analysis, we found that Myb interacting protein 120 (Mip120) is required to repress inappropriate *Gr63a* expression not only in the antenna, but also in other tissues. The *mip120* mutants not only exhibit an increase in *Gr63a*-positive neurons in the antenna (Figure 3.5A,B), but *Gr63a* transcript is also observed in the other olfactory appendage, the maxillary palp (Figure 3.5C). Interestingly, a *Gr63a-Gal4* reporter shows misexpression in other sensory tissues such as the labellum (taste) and antennal second segment (hearing) as well as throughout the brain in *mip120* mutant animals (Figure 3.6A, (Sim et al., 2012)). Because we observed such extensive misexpression we speculated that there may be a detectable change in chromatin structure at the *Gr63a* locus in *mip120* mutant head tissue. Indeed, when we performed ChIP-qPCR examining several histone modifications at the *Gr63a* gene locus, we observe an increase in the active chromatin modification H3K4me3 in *mip120* mutant heads compared with *wild-type*. The repressive chromatin marker H3K9me2 remains unchanged in mutant tissue (Figure 3.6B). This indicates that Mip120's main role may be to prevent the deposition

of active chromatin markers at the *Gr63a* locus in inappropriate cells thus restricting expression to the appropriate neurons in the antenna. Interestingly, we do not observe an increase in H3K4me3 at *Gr21a* or *Or42b* loci in these mutants, suggesting that Mip120 regulation may be *Gr63a*-specific (Figure 3.6B).

We next examined flies mutant for *e2f2*, another repressive dREAM complex member, for abnormalities in *Gr63a* expression. Indeed, we observed an increase in *Gr63a* transcript in both the antenna and maxillary palp using qPCR (Figure 3.7A). This corresponds to an increase in *Gr63a*-positive cells in both the palp and third antennal segments as observed with RNA *in situ* hybridization (Figure 3.7B,C). Interestingly, loss of either one or both copies of *e2f2* results in an increase in *Gr63a*-positive cells in a dosage dependent manner (Figure 3.7C). Loss of one copy in a *mip130,myb* mutant background is also able to partially rescue *Gr63a* expression in the antenna (Figure 3.7C). We wondered whether this increase in *Gr63a* antennal expression was due to misexpression in inappropriate neurons or simply increased transcription in native ab1C neurons. By using single unit electrophysiology, we observed an increase in sensitivity to lower concentrations of CO₂ in *e2f2* mutants (Figure 3.7D,E). We believe this is likely due to the increased levels of *Gr63a* we observed (as well as *Gr21a*, see Figure 3.8C) in ab1C neurons. Interestingly, the dose response curve to CO₂ appears to flatten out in *e2f2* mutants (Figure 3.7E), suggesting that an important aspect of *e2f2* regulation is to maintain appropriate levels of *Gr63a* in CO₂ sensitive neurons so that an appropriate low to high response curve can be maintained for this important odorant.

dREAM complex members co-regulate *Gr21a* and *Gr63a*

Because *Gr21a* is co-expressed in ab1C neurons with *Gr63a* and both are required for neuronal response to CO₂, we hypothesized that *Gr21a* may also be regulated by members of the dREAM complex. A previous ChIP-chip study revealed that the *Gr63a* promoter exhibited a strong Myb binding peak and canonical Myb binding site (Georlette et al., 2007). This promoter region (Figure 8A, black box) shares direct sequence similarities to portions of *Gr21a*'s upstream promoter region (Figure 3.8A, arrows) indicating the possibility of Myb and other dREAM complex members binding there as well. The aforementioned ChIP-chip study did not observe dREAM complex binding upstream of *Gr21a*, however, this may be because of lack of accessibility caused by restrictive heterochromatin in the cell line that was used (Filion et al., 2010). We previously observed a reduction in *Gr21a* transcript in antennal tissue using qPCR (Figure 1B). Using RNA *in situ* hybridization, we do see a marked reduction in *Gr21a*-positive cells in *mip130,myb* mutants (Figure 3.8B). The loss is not as severe as that for *Gr63a*, but it follows the same trend. As with *Gr63a*, *Gr21a* expression can be rescued with pan-neuronal expression of Myb or C-terminal Myb in the double mutant background (Figure 3.8B) indicating that Myb is a co-regulator of both *Gr63a* and *Gr21a*.

As with *Gr63a* expression, loss of *mip130* alone does not affect *Gr21a* expression. However, loss of one or both copies of *e2f2* does cause an increase in *Gr21a*-positive neurons. As discussed previously, we believe this causes an increased level of *Gr21a* receptor in the native ab1C neurons. Because both *Gr21a* and *Gr63a* are up-regulated in *e2f2* mutants, an increase in CO₂ response can be observed (Figure 3.7D,E). Interestingly, loss of *e2f2* in the *mip130,myb* mutant background does not appear to

rescue *Gr21a* expression, at least observed by RNA *in situ* hybridization. Loss of one copy of *Su(var)3-9* also appears to do very little, either in a *wild-type* or double mutant background, although *Gr21a* reporter expression is impacted (Figure 3.8C, (Sim et al., 2012), data not shown). *mip120* mutants exhibited no apparent misregulation of *Gr21a* (data not shown, (Sim et al., 2012)).

Mip130 may regulate expression of other odorant receptors

While *mip130* mutants do not show any irregularities in *Gr63a* or *Gr21a* expression or in ab1 neuronal responses to diagnostic odorants (Figure 3.1), we did observe abnormal responses in another antennal neuronal class, ab3A. In addition to strong pentyl acetate response, ab3A neurons in *mip130* mutant animals also gain strong responses to anisole, ethyl acetate and methyl salicylate (Figure 3.9A). Several other diagnostic odorants were tested (acetophenone, benzaldehyde, and heptanone), but the new response profile does not match that of any currently characterized receptor (Hallem and Carlson, 2006; Hallem et al., 2004; Kreher et al., 2005, 2008). *mip130;orco* double mutants completely lose these responses (data not shown), indicating that they are likely due to the misexpression of a new Or receptor, possibly an uncharacterized one. The ab3A native receptor, Or22a, shows a normal expression pattern in *mip130* mutants (Figure 3.9B). Because these neurons still respond strongly to Or22a's diagnostic ligand pentyl acetate, we believe that a new receptor is likely being expressed in addition to Or22a. This opens up the possibility that dREAM complex members may regulate expression of other olfactory receptors in addition to *Gr63a/Gr21a*.

Differential expression of dREAM complex members may confer regulatory specificity.

Members of the dREAM complex appear to be able to maintain proper expression of *Gr63a* by allowing it to be turned on in appropriate neurons and turned off in inappropriate ones. However, because the complex is required for normal cell cycle progression during development, complex proteins are likely present in all cells. How, then, is it able to have a repressive role in some cell types and an activating role in others? It has previously been shown that the interplay between the activating complex member, Myb, and other repressive members is likely important in determining the regulatory outcome of some target genes (Georlette et al., 2007; Wen et al., 2008). In order to examine differences in expression levels of dREAM complex members between *Gr63a/Gr21a* expressing ab1C neurons and surrounding neurons, we stained whole-mount antennae with antibodies against several of the complex members and measured fluorescence signal intensity to estimate relative levels of these proteins. We found that Myb, the activator of the complex, was consistently higher in ab1C nuclei compared with surrounding neurons. Interestingly, a repressive subunit of the complex, Mip120, was present at lower levels in the ab1C neurons as compared with neighboring neurons. E2F2 levels appeared similar between ab1C and its neighbors (Fig. 3.10A,B). In total, we observed that the Myb:Mip120 ratio in ab1C nuclei is ~45% higher compared with neighboring nuclei. The simplest interpretation of these results is that the ab1C nuclei are able to express the CO₂ receptors by virtue of having higher levels of activating Myb and lower levels of the repressive Mip120 subunit of the dREAM complex, while the neighboring cells with lower Myb and higher Mip120 levels cannot.

Model of MMB/dREAM complex regulation of receptor gene expression.

In summary, Myb is required for normal expression of *Gr63a* and *Gr21a* in antennal ab1C neurons and acts in opposition to histone methyltransferase Su(var)3-9 and possibly histone deacetylase RPD3. Mip120 and E2F2 prevent aberrant expression of *Gr63a* in tissues other than the third antennal segment. Loss of either Mip120 or E2F2 is associated with an increase in H3K4me3 histone modifications across head tissue. E2F2 also suppresses *Gr63a/21a* expression in native ab1C neurons. Mip130 is required for suppression of an unknown *Or* gene in ab3A neurons (Figure 3.10C).

Discussion

Modulation of chromatin state offers another layer of transcriptional regulation.

It has been previously shown in mammals that repressive chromatin plays an important role in singular expression of olfactory receptors (Magklara et al., 2011). Here we demonstrate the first evidence that a similar mechanism may be employed by the *Drosophila* olfactory system to control expression of the carbon dioxide receptors *Gr63a* and *Gr21a*. Chromatin state is an appealing regulatory mechanism because it offers discrete “ON” and “OFF” states, a feature that would be ideal for the specific control needed by these highly specialized neurons – one receptor gene is active while all others are maintained in a stable non-active state. Chromatin state may work in conjunction with known regulatory mechanisms such as combinatorial activation and repression by DNA binding factors to provide the unique transcriptional programs required for each of the ~50 known ORN classes. There is at least some evidence that histone modifications associated with repressive chromatin are found at other olfactory receptor loci in the antenna (Jafari and Alenius, 2015; Sim et al., 2012), suggesting that this strategy for controlling receptor expression may be more wide-spread throughout

the *Or* gene family. If this is the case, other chromatin modifying complexes and associated factors may be involved in *Or* gene regulation.

Receptor specificity may be determined by differential expression of apparently ubiquitous transcriptional regulators.

Previous work has established that combinations of transcription factors are required for expression of particular receptor genes in the antenna and maxillary palp (Bai and Carlson, 2010; Bai et al., 2009; Jafari et al., 2012; Miller and Carlson, 2010; Ray et al., 2007, 2008; Tichy et al., 2008). However, many of these factors are general regulators of gene expression and are likely found in most cell types. This raises the question of how ubiquitously present factors can determine highly specific patterns of receptor gene expression. The same question holds for the dREAM complex, which is likely required in all cells for cell-cycle progression during development. We show here that at least two complex members, Myb and Mip120 show differential expression levels in ab1C neurons compared with neighboring neurons. This would suggest that although present in all nuclei, differences in the levels of activators or repressors may permit gene expression in some cells but restrict it in others. The same principle may be true for other transcription factors known to regulate *Or* gene expression.

A post-mitotic role for canonical cell-cycle regulators.

Epigenetic regulation of gene expression is typically characterized by heritable phenotypic differences in genetically identical cells that are maintained through subsequent cell divisions (Gottschling, 2004). However, there are some emerging theories that post-mitotic cells may also utilize epigenetic-like mechanisms such as chromatin modification to maintain stable states of gene expression (Dulac, 2010). Here

we demonstrate an interesting role for the epigenetic MMB/dREAM complex in mature olfactory neurons. Using a temperature sensitive allele of Myb, the activator of the complex, we show that Myb is required post-developmentally to maintain normal levels of *Gr63a* expression. Post-mitotic rescue of *myb* using the pan-neuronal *elav-Gal4* driver can also rescue *Gr63a* expression. Together, these data imply that Myb is primarily required in the mature neuron to activate and maintain *Gr63a* expression.

Neuronal sensitivity to CO₂ is dependent on the expression levels of Gr63a/Gr21a.

In *mip130,myb* mutants we observe greatly reduced levels (but not a complete absence) of both *Gr63a* and *Gr21a* transcripts. As a result, ab1C neurons exhibit a reduced sensitivity to CO₂, but again, not a complete lack of response. Behavioral response to CO₂ correlates similarly: mutants are still able to avoid higher concentrations of CO₂, but loose avoidance to lower concentrations. Conversely, in the absence of E2F2, we observed an increase in both *Gr63a* and *Gr21a* expression levels. Interestingly, this increase also correlated with alterations in the responsiveness of the neuron to CO₂. In *e2f2* mutants, the ab1C neuron became increasingly sensitive to low concentrations of CO₂ and less sensitive to some of the higher concentrations, resulting in a flat-lined dosage response curve. This raises the possibility that maintaining the correct receptor levels of both *Gr63a* and *Gr21a* is crucial for the fly to be able to properly perceive and navigate concentration gradients of CO₂ and other odorants. It is also possible that sensitivity to CO₂ can be modulated throughout the adult stage of the fly in response to external stimuli, mating state and internal nutritional state. Modulation of receptor expression by dREAM complex members may provide a mechanism for this type of behavioral plasticity. Because both these carbon dioxide receptors and dREAM

complex members are conserved in disease vectors such as mosquitos, this mechanism of regulation may exist in these insects as well.

dREAM complex members may regulate other olfactory receptors.

In *mip130* mutants, we observe aberrant responses in another neuronal class, ab3A, likely due to the de-repression of an *Or* gene. This raises the possibility that dREAM complex members may regulate other olfactory receptors in the adult antenna and palp. As has been previously shown with other transcription factors, a particular TF is usually required for the activation or repression of multiple odorant receptor genes (Jafari et al., 2012). In fact, several DNA binding factors from the dREAM complex are found at or near receptor gene loci in cultured cells (Table 3.S1, (Georlette et al., 2007)). Further work will need to be done to determine if the dREAM complex does indeed play a more wide-spread role in olfactory receptor gene regulation.

Materials and Methods

Drosophila strains. The following fly stocks contain loss-of-function alleles: *myb*^{MH107} (Manak et al. 2002), *mip130*¹⁻³⁶ (Beall et al. 2004), *mip120*⁶⁷⁻²¹⁻⁶ (Beall et al. 2007), *mip120*^{67-9A-9} (Beall et al. 2007), *e2f2*¹⁻¹⁸⁸ (Cayirlioglu et al. 2001), *e2f2*³²⁹ (Cayirlioglu et al. 2001), *Su(var)3-9*¹ (Reuter et al. 1986), and *Su(var)3-9*² (Reuter et al. 1986). *myb*¹ is a temperature-sensitive *myb* allele (Katzen and Bishop 1996). We also used these transgenic flies: *Gr63a promoter-GAL4* (Fishilevich et al. 2005), *Gr63a promoter-GAL4* (Kwon et al. 2007), *Gr21a promoter-GAL4* (K.Scott), *Or42b promoter-GAL4* (Fishilevich et al. 2005), *Or22a promoter-GAL4* (Vosshall et al. 2000), *UAS-mCD8::GFP* (Lee and Luo 1999), *UAS-mCD8::chRFP* (F.Schnorrer), *Gr21a promoter-mCD8::GFP* (Couto et al.

2005), *elav-GAL4* (Luo et al. 1994), *UAS-RFP::myb*⁺ (Wen et al. 2008), *myb promoter-GFP::myb*^{C-terminal} and *myb promoter-GFP::myb* (Andrejka et al. 2011).

Quantitative RT-PCR. Total RNA was isolated from 100 antennae or maxillary palps by Trizol extraction (Invitrogen) and treated with DNase I (Amplification grade, Invitrogen). cDNA was generated using oligo dT and Superscript III reverse transcriptase (Invitrogen). 1µl of the resulting cDNA, fast SYBR green PCR master mix (Applied Biosystems), and gene-specific primers were added in a 20ul reaction on 96-well plates. Primer sequences are provided (Figure S8). Primer efficiencies were checked according to MIQE guidelines (Bustin et al. 2009). The SYBR green reaction was then used for qPCR on StepOnePlus Real-time PCR System (Applied Biosystems). Fold change is calculated by using the $2^{-\Delta\Delta Ct}$ formula to compare expression between mutants and *wild-type*, using *rp49* (for SYBR-green assays) as a normalizer. At least three biological replicates were performed per genotype.

Chromatin Immunoprecipitation was performed with modifications to the protocol from Millipore. 10 fly heads or 100 antennae per sample were harvested in cold Schneider's medium, homogenized and crosslinked in 1% formaldehyde (vol/vol) in Buffer A (10mM HEPES, 1.5mM MgCl₂, 10mM KCl, pH 7.9) and protease inhibitor cocktail (Roche) for 10 mins at room temperature. Crosslinking reaction was stopped by adding glycine (125mM final) for 5 min on ice. Nuclear extract was obtained by adding NP40 (0.5% final), vortexing for 10 seconds, and spinning 13,000 *g* for 3 min at 4 °C to obtain the pellet. To shear chromatin, the pellet was resuspended in cold CHIP Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris, pH 8.1), SDS (1% final) and protease inhibitor cocktail, and then sonicated for 20 cycles (highest energy setting; 30

sec ON, 30 sec OFF per cycle) to obtain 300-500 bp DNA fragments. Dynabeads (Invitrogen) were pre-washed with 0.5% bovine serum albumin (wt/vol) in PBS and incubated with 2 µg antibodies to histones: anti-H3K9me2 (ab1220) and anti-H3K4me3 (ab8580). Both antibodies had been rigorously tested for specificity in (Riddle et al. 2011). Additional testing of anti-H3K9me2 antibody was performed to validate specificity using a peptide array. 1/ 20 volume of the chromatin was kept as input DNA. Immunoprecipitation was done by diluting the sheared chromatin with ChIP Buffer five-fold, and incubating it with the antibody-beads complex overnight at 4 °C. The immunoprecipitated chromatin was washed with ChIP Buffer, then High Salt Buffer (0.1% deoxycholate, 1% Triton X-100, 1mM EDTA, 50mM HEPES, 500mM NaCl, pH7.5), then LiCl Buffer (250mM LiCl, 0.5% NP40, 0.5% deoxycholate, 1mM EDTA, 10mM Tris, pH 8.1), then TE Buffer, each washing step lasting 5 min at room temperature. The chromatin was eluted off the beads by adding TE, SDS (1% final), NaHCO₃ (0.1M final), NaCl (0.2M final), and reverse-crosslinked by overnight incubation at 65 °C. Input DNA was reverse-crosslinked in the same manner. Both ChIP DNA and input DNA were treated with RNase A (Thermo Scientific) and proteinase K (Sigma), extracted by phenol-chloroform and purified by columns (Qiagen). 1 ul of ChIP DNA and input DNA were used for each qPCR reaction on StepOnePlus Real-time PCR System (Applied Biosystems). Primer sequences are provided (Sim et al., 2012). Percentage Input (% Input) represents ChIP enrichment over input; it is calculated by the $2^{-\Delta Ct}$ formula divided by 20 to account for the differences in the amount of ChIP and input DNA loaded in PCR, and subtracting the background from pre-immune serum ChIP control. At least three biological replicates were performed per genotype. For future

studies, we recommend genome-wide ChIP-seq to allow comparison of histone marks at fine resolution, and to allow better normalization of signals across the genome.

Immunofluorescence. Immunostaining was performed on brains as described previously (Lee and Luo, 1999), and on maxillary palps and antennae with 3% Triton X-100 permeabilization instead of 0.3% as stated in the protocol. Primary antibodies were mouse anti-Elav (1:5 dilution; Developmental Studies Hybridoma Bank), mouse anti-nc82 (1:5; Developmental Studies Hybridoma Bank), chicken anti-GFP (1:2000; ab13970), rat anti-CD8 (1:100; Invitrogen), rabbit anti-Myb (1:200)(Manak et al. 2002), rabbit anti-RFP (1:500; gift from S.Heidmann) and rabbit anti-Mip120 (1:500), rabbit anti-E2F2 (1:100). Anti-Mip120 antibody was made by immunizing a rabbit with Mip120::GST fusion protein as antigen at 1.9mg/ml in a buffer containing 20mM Tris pH 8, 250mM NaCl and 3M urea. Anti-E2F2 antibody was raised in a rabbit using E2F2::GST antigen at 4mg/ml in a buffer containing 20mM Tris pH 8.0, 250mM NaCl and 4M urea. Injections were done by Josman LLC using 500ug of antigen for the first injection and 250ug of antigen for the subsequent four booster injections. Secondary antibodies were Alexa Fluor goat anti-mouse/ anti-rabbit/ anti-chicken IgG (1:350; Molecular Probes). Tissues were imaged using a laser scanning confocal microscope in z-stacks of 1-2 μ m intervals.

For intensity quantification, adult antenna were stained with mouse anti-Elav and rat anti-CD8 as stated above and either anti-Myb (1:100), anti-Mip120 (1:200) or anti-E2F2 (1:50). Intensity values were measured using Image J. Measurements were taken from ab1C nuclei and nuclei from 2-3 closest neighbors (labeled N1, N2, N3 in Figure 8C). For example, ab1C/ neighbors (mean) = ab1C (Myb)/ Avg [N1(Myb), N2 (Myb), N3

(Myb)] Neighbor/ neighbors (mean) = $N1 \text{ (Myb)} / \text{Avg [} N1(\text{Myb}), N2 \text{ (Myb)}, N3 \text{ (Myb)}]$repeated for every neighbor N1,2,3 etc.

Single-sensillum electrophysiology. Single-unit recordings were performed as previously reported (Turner and Ray, 2009; Turner et al., 2011).

RNA *in situ* hybridization. Procedures were performed as described previously (Goldman et al., 2005). Antenna whole-mount RNA in situs were performed using ~900-bp antisense RNA–digoxigenin probes in fixed tissue. Probes were detected using an anti-digoxigenin fragment conjugated to an alkaline phosphatase. Signal was developed using Fast Red and imaged using a Zeiss LSM 560 confocal microscope with a 543-nm laser and 560LP filter. A more detailed protocol can be found in Appendix IIA.

Behavior. T-maze assays were performed using a closed (no air flow) T-maze apparatus with 15 mL unmarked culture tubes as arms. For each trial, 40 flies (20 males, 20 females, 3-7 days old, 24-hour wet starved) were loaded into the elevator and allowed 1 minute to choose between the stimulus arm and the control arm. 50 μ L of pure CO₂ was injected into the stimulus tube through the cap using a 1mL syringe and needle immediately before attaching the arms to the T-maze for that particular trial. This achieves an estimated 0.33% CO₂ in addition to the ambient CO₂ already present in the room air. Un-augmented room air was used in the control arm in these trials. Preference Index (PI) is calculated as follows: $((\# \text{ flies in odor arm}) - (\# \text{ flies in control arm})) / ((\# \text{ flies in odor arm}) + (\# \text{ flies in control arm}))$.

Figure 3.1. Myb is required for response to CO₂

A. Whole-mount antennae showing: left, bright field microscopy image; and right, *Gr63a* RNA *in situ* hybridization. Male flies of the following genotypes were used: *wild-type* (*w^{Canton-S}*), *mip130* mutant (*mip130¹⁻³⁶*), and *mip130,myb* double mutant (*mip130¹⁻³⁶,myb^{MH107}*). **B.** qRT-PCR analysis from antennal cDNA of indicated genotypes measuring large basiconic receptor expression levels. An *rp49* control was used to normalize. (N = 3) **C.** Representative single-sensillum electrophysiological traces from the ab1 sensillum representing *wild-type* or *mip130,myb* double mutant responses to 1% CO₂. Characteristic spikes of the four neurons are indicated by their respective letters (**A**, **B**, **C** and **D**). Each spike belonging to the C neuron is denoted by a red dot in the mutant trace. **D.** Average electrophysiological responses of the four ab1 neurons to the indicated diagnostic odors. (N = 10) **E.** Preference index to measure behavioral responses of indicated flies to 0.33% CO₂ in a T-maze. (N = 10) Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

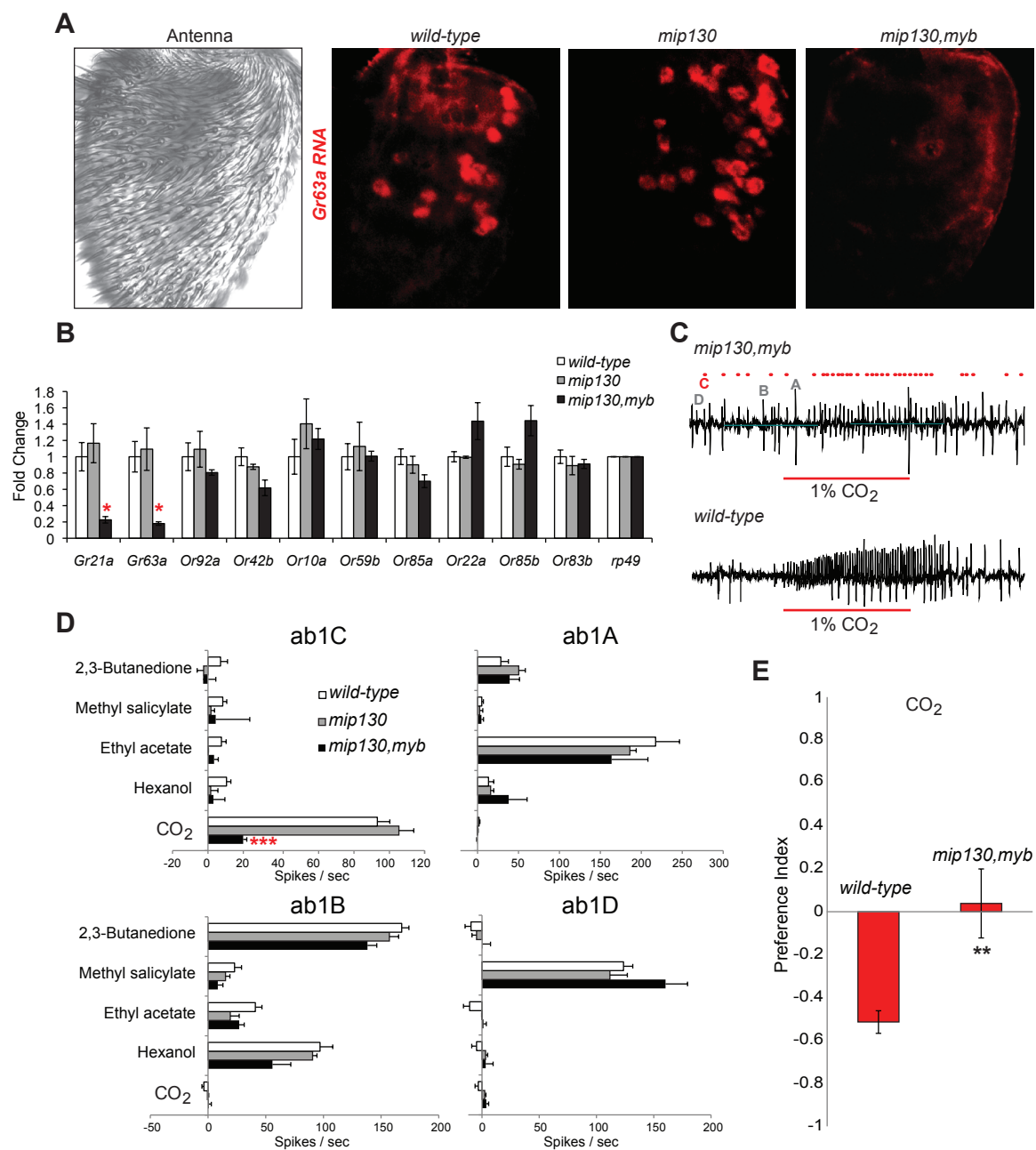


Figure 3.1

Figure 3.2. Myb activates Gr63a expression post-developmentally via the MMB/dREAM complex

A. Whole-mount male antennae showing *Gr63a* RNA *in situ* hybridization in *wild-type*, *mip130,myb* double mutant, *myb* rescue (*mip130¹⁻³⁶,myb^{MH107}; elav-GAL4/+; UAS-RFP::myb⁺/+*), and *myb* C-term rescue (*mip130¹⁻³⁶,myb^{MH107}; myb promoter-GFP::myb^{C-terminal}/+*). **B.** Number of *Gr63a*-expressing cells in genotypes indicated in (A). (N = 9 -13) **C.** qRT-PCR analysis from antennal cDNA measuring *Gr63a* expression in indicated genotypes. (N = 3 biological replicates) **D.** Electrophysiological responses to 1% CO₂ in *wild-type*, double mutant and *myb* rescue genotypes. (N = 8 - 24) **E.** Schematic of culturing temperature-sensitive *myb¹* males at 18°C (permissive) and shifting them to 25°C (restrictive) post-eclosion. Control *myb¹* flies were kept at 18°C throughout the entire period (top panel). **F.** Whole-mount antennae *Gr63a* RNA *in situ* hybridization in *myb¹* flies. (N = 23 - 31) Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. ** $p < 0.01$; *** $p < 0.001$

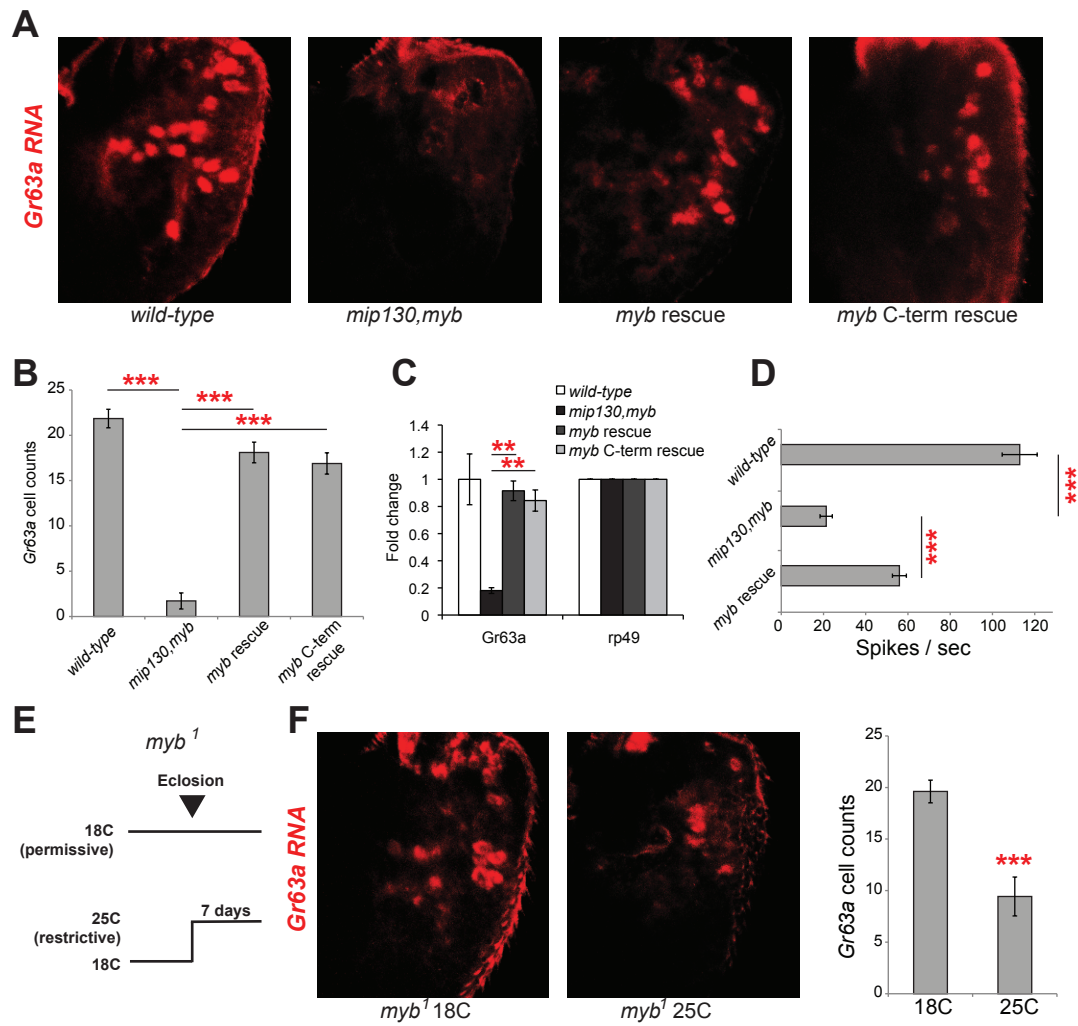


Figure 3.2

Figure 3.3. Myb opposes a repressive chromatin modifier

A. Chromatin immunoprecipitation using antibodies to H3K9me2 on *wild-type* antennae or heads without antennae. qRT-PCR was performed for indicated regions. (N = 3 - 4 biological replicates) **B.** Whole-mount antennae showing *Gr63a* RNA *in situ* hybridization in heterozygous *Su(var)3-9¹/+*, *mip130,myb* double mutant, and heterozygous *mip130,myb; Su(var)3-9¹/+* and associated cell counts. (N = 9 - 21) Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

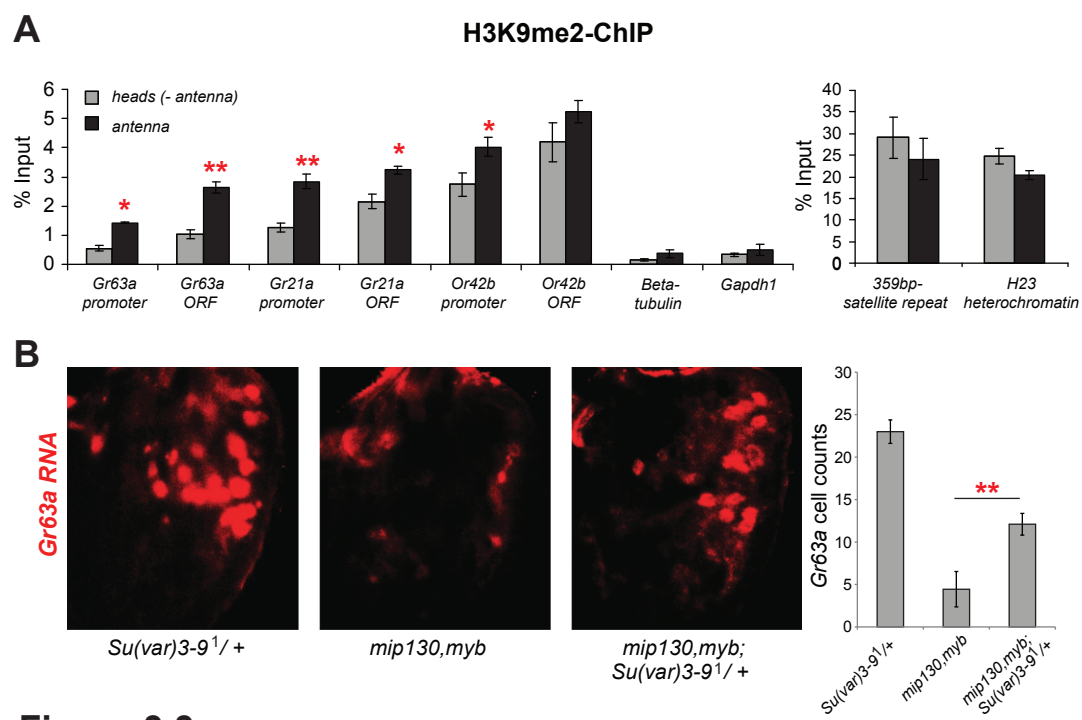


Figure 3.3

Figure 3.4. RPD3 represses *Gr63a* antennal expression.

A. Whole-mount antennae showing *Gr63a* RNA *in situ* hybridization in *rpd3*^{-/+} and *mip130,myb; rpd3*^{-/+} mutants. **B.** *Gr63a* cell counts for the genotypes shown in (A) and controls (N = 5 – 11). **C.** Left panels show RNA *in situ* hybridizations of *Gr63a* transcript: Control (+/*Cyo*; *UAS-HDAC11-RNAi/tubP-Gal80[ts]*) and *UAS-RPD3* (*elav-Gal4/+*; *UAS-RPD3/tubP-Gal80[ts]*). Flies were reared at 18°C and shifted to 25°C for 7-8 days post-eclosion. Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. ** $p < 0.01$

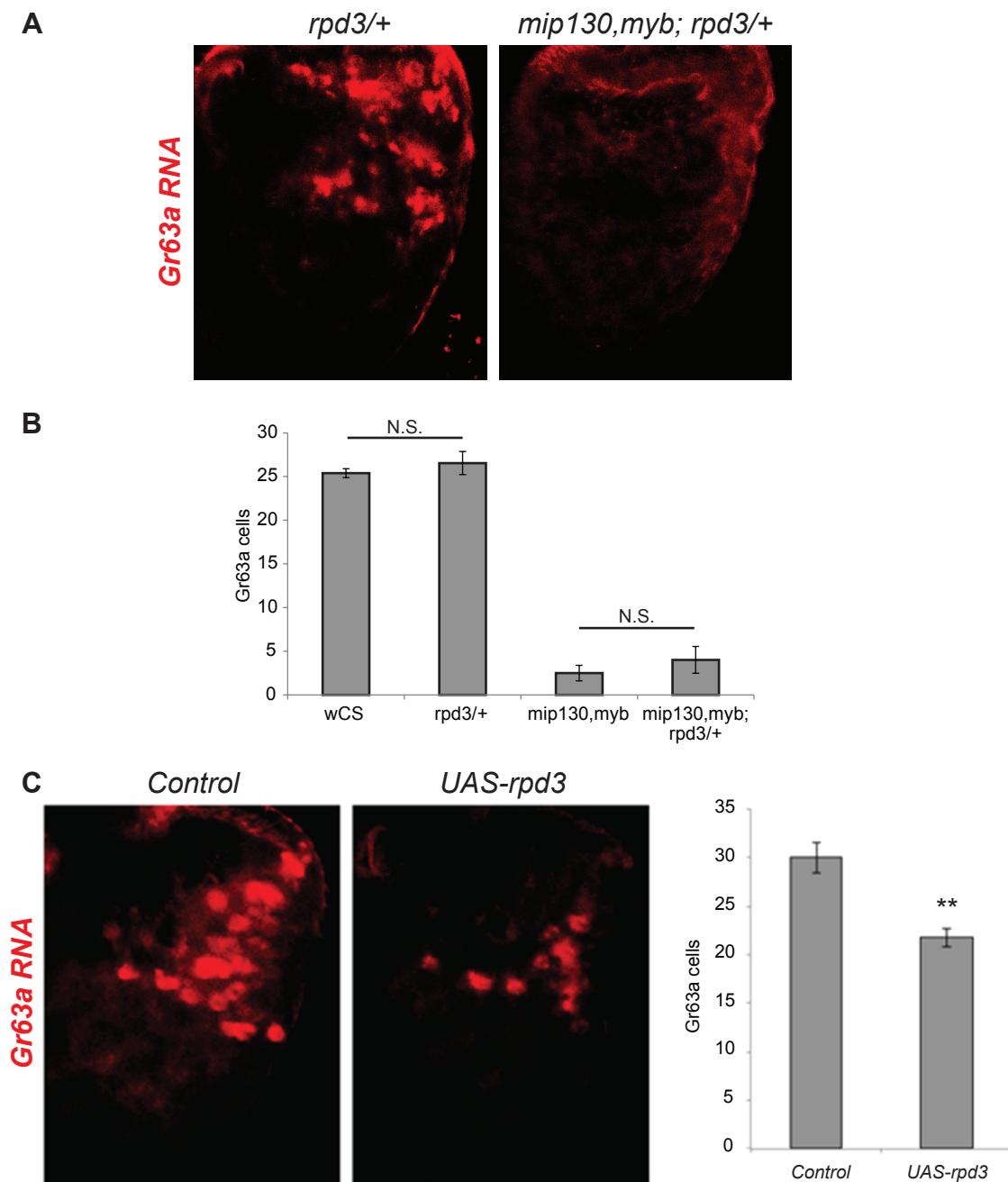


Figure 3.4

Figure 3.5. Mip120 is required to repress *Gr63a* in the antenna and palp.

A. Whole-mount antennae showing *Gr63a* RNA *in situ* hybridization in *wild-type* and *mip120* animals. **B.** *Gr63a* cell counts for the genotypes shown in (A) and controls (N = 9). **C.** Whole-mount maxillary palps showing *Gr63a* RNA *in situ* hybridization in *wild-type* and *mip120* animals. Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. * $p < 0.05$

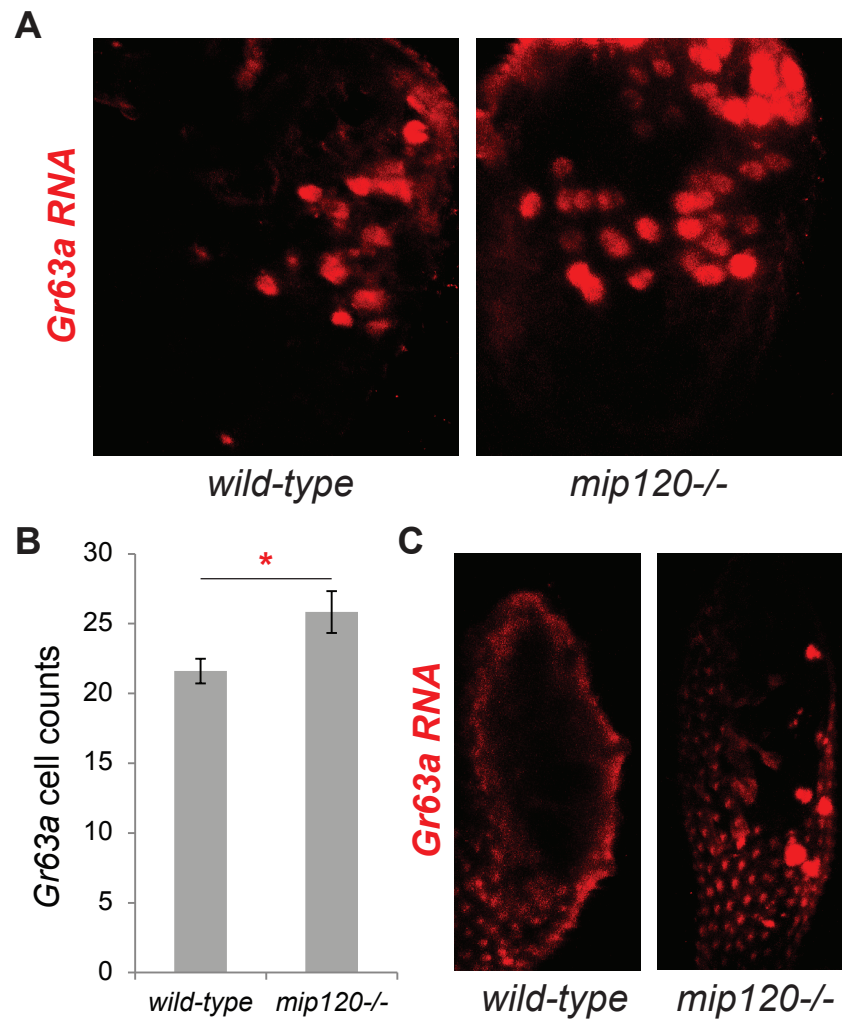


Figure 3.5

Figure 3.6. Mip120 mutants exhibit a change in chromatin structure at the Gr63a locus.

A. Immunofluorescence using antibodies to nc82 (green) and RFP (red) on whole-mount brains of the genotypes indicated in (A). **B.** Chromatin immunoprecipitation using antibodies to H3K4me3 or H3K9me2 on *wild-type* and *mip120* mutant heads. (N = 5 to 6 biological replicates). Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. * $p < 0.05$; *** $p < 0.001$

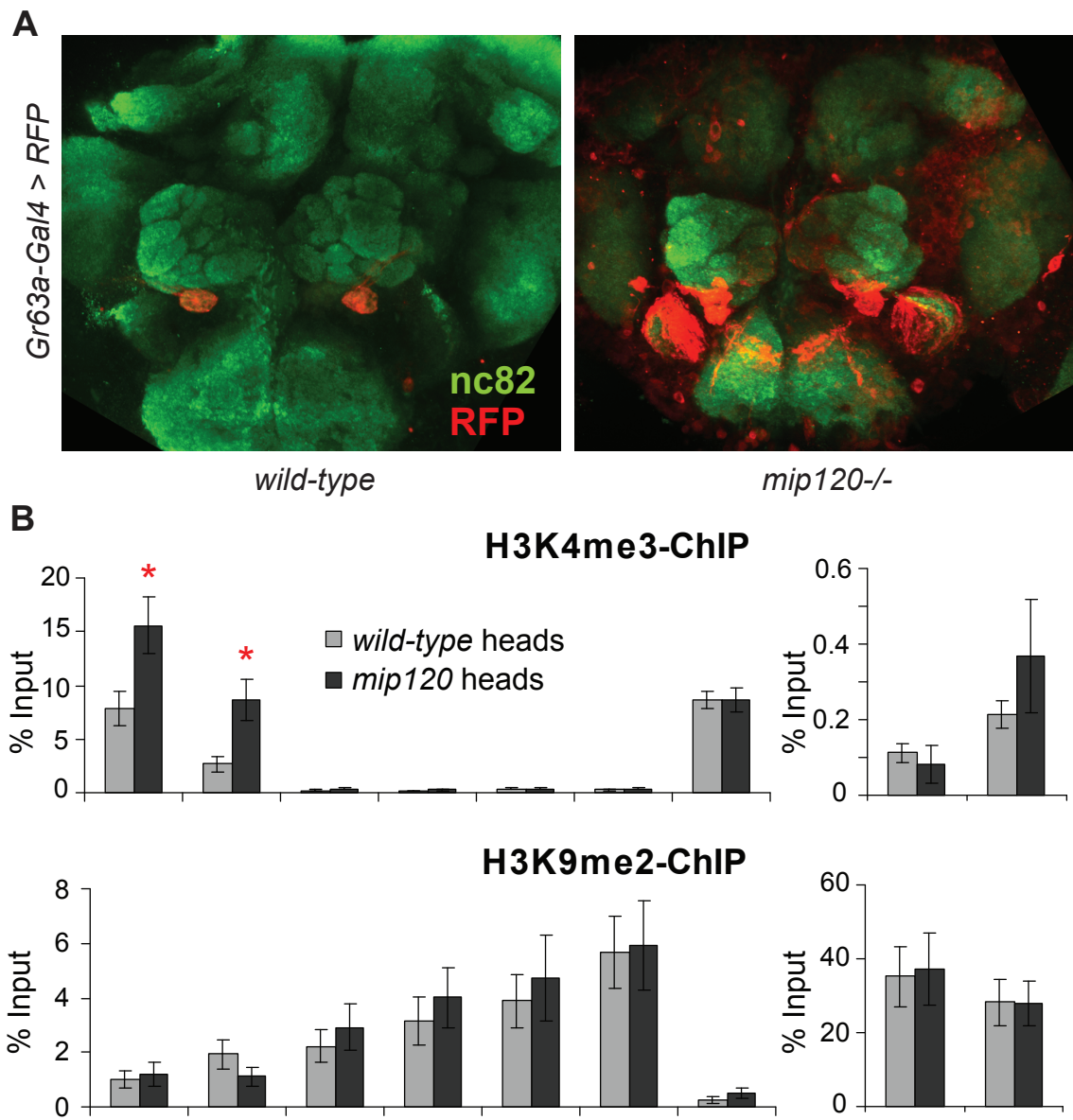


Figure 3.6

Figure 3.7. E2F2 is required to repress *Gr63a* in the antenna and palp.

A. qRT-PCR using antennal (left panel) or maxillary palp (right panel) cDNA of *wild-type* or *e2f2* mutant (*e2f2*¹⁻¹⁸⁸/*e2f2*³²⁹) to measure expression of *Gr63a* or control *Or42b*. (N = 3) **B.** Whole-mount maxillary palp showing *Gr63a* RNA *in situ* hybridization in *wild-type* and *e2f2* mutants. **C.** Whole-mount antennae showing *Gr63a* RNA *in situ* hybridization on heterozygous *e2f2*³²⁹/+ and homozygous *e2f2*¹⁻¹⁸⁸/*e2f2*³²⁹ mutant flies, and *mip130,myb; e2f2*³²⁹/+ flies. Cell counts are shown in the right panel. (N = 9 - 15) **D.** (Top) Representative electrophysiological traces of ab1 sensillum response to 0.3% CO₂ in *wild-type* and *e2f2* mutant flies. (Bottom) Dose response of *wild-type* and *e2f2* mutant ab1C neurons to CO₂. (N = 6). Error bars represent ± s.e.m. A two-tailed student's t-test was used for statistical analysis. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001

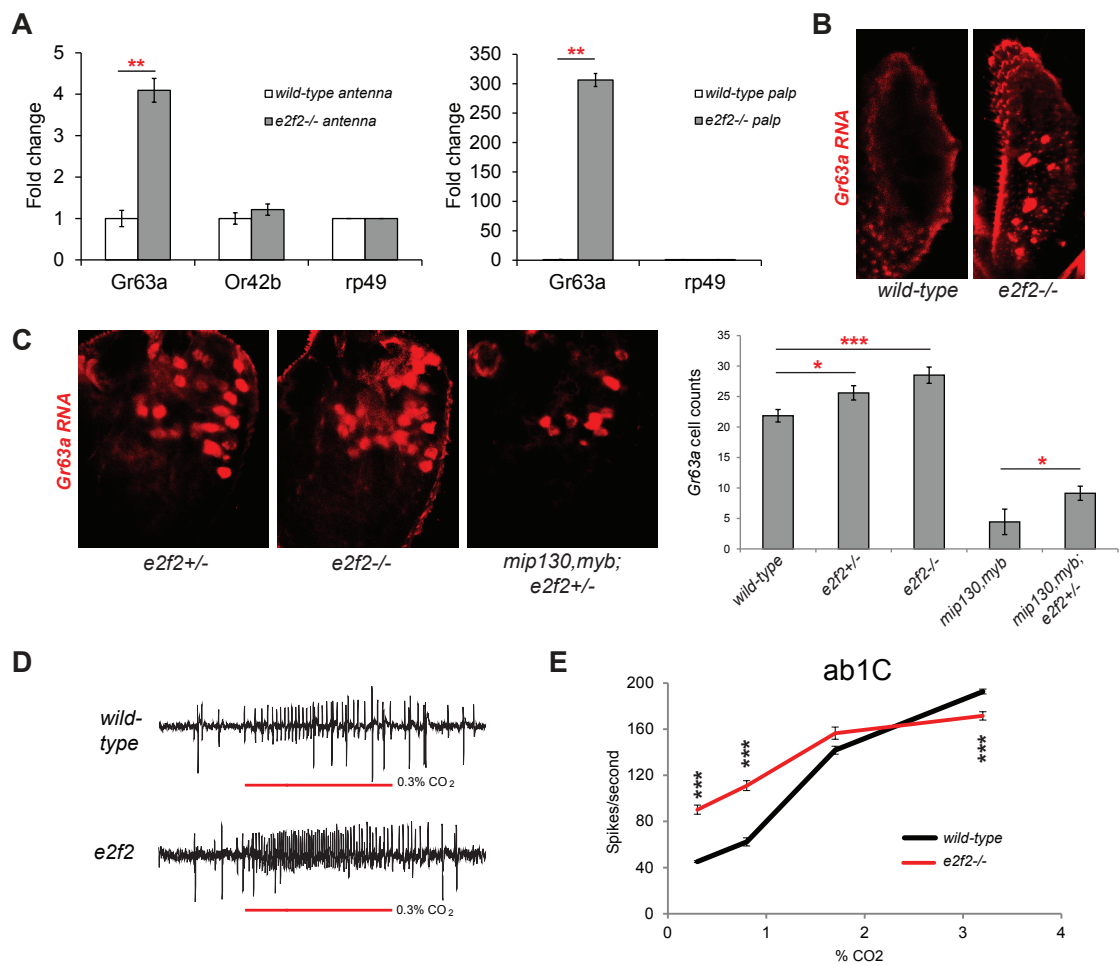
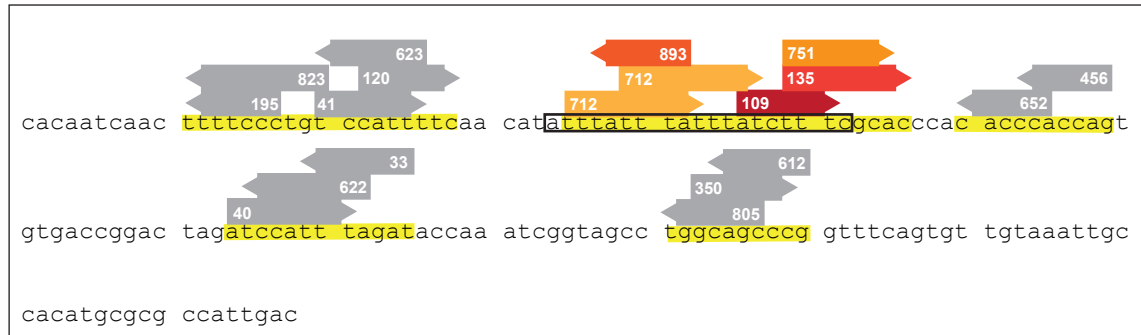


Figure 3.7

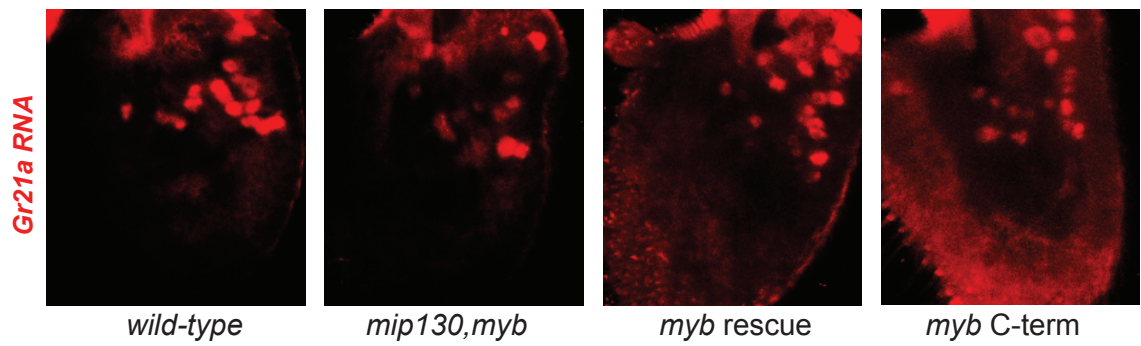
Figure 3.8. The dREAM complex also regulates *Gr21a* expression.

A. Sequence representing the region of the *Gr63a* promoter spanning 3L: 3880344-3880502 reverse strand, which is bound by the MMB/dREAM complex as previously reported (Georlette et al., 2007). Each arrow represents a segment from the *Gr21a* promoter with 100% identity to the underlying *Gr63a* sequence. The number in the arrow indicates its location in the *Gr21a* promoter spanning 2L: 779550-780500. The black box indicates the MMB/dREAM binding site from (Georlette et al., 2007). **B.** Whole-mount antennae showing *Gr21a* RNA *in situ* hybridization on *wild-type*, *mip130,myb* double mutant, *myb* rescue (*mip130,myb; elav-GAL4/+; UAS-RFP::myb⁺/+*), and *myb* C-term rescue (*mip130,myb; myb promoter-GFP::myb^{C-terminal}/+*). **C.** *Gr21a* cell counts for the indicated genotypes determined by whole-mount antenna RNA *in situ* hybridization. (N = 9 – 18) Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

A Region of Gr63a promoter bound by Myb-MuvB complex members:



B



C

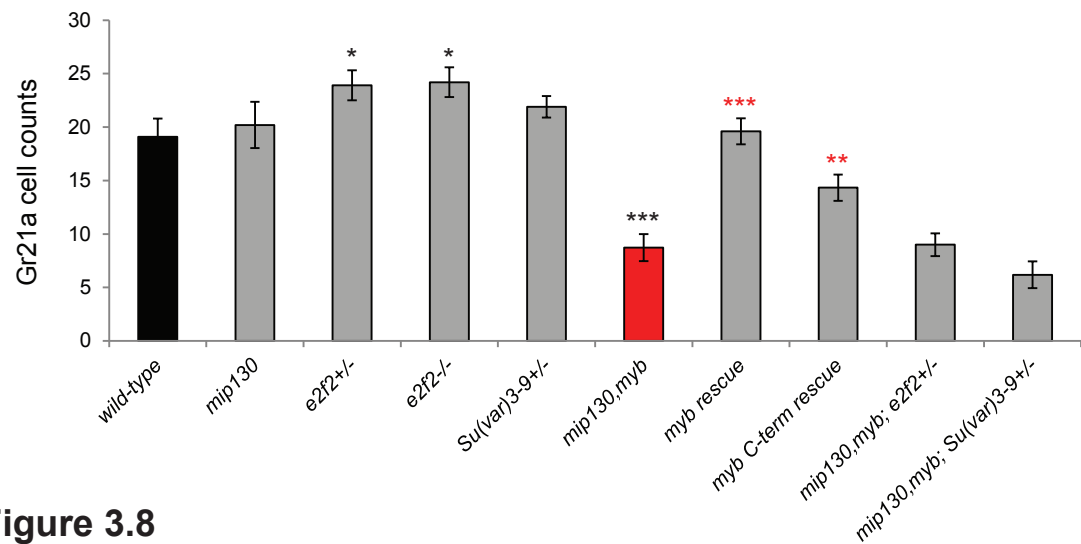
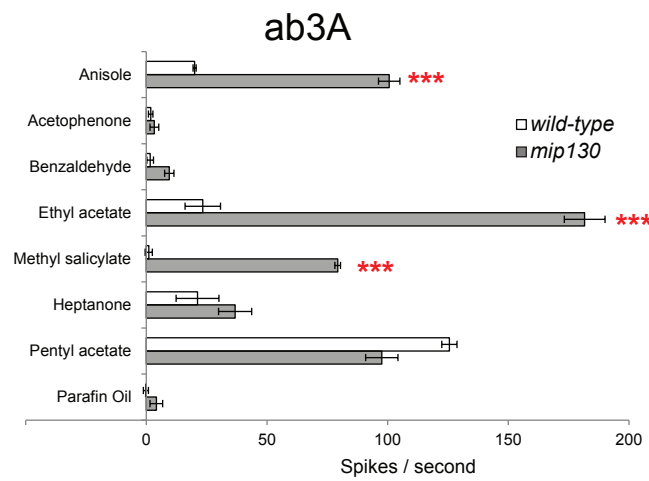


Figure 3.8

Figure 3.9. Mip130 regulates another odorant receptor.

A. Electrophysiological responses of *mip130* mutant ab3A neurons to diagnostic odors. (N = 5) **B.** Whole-mount antennae showing *Or22a* RNA *in situ* hybridization in *wild-type* and *mip130* mutants. Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. *** $p < 0.001$

A



B

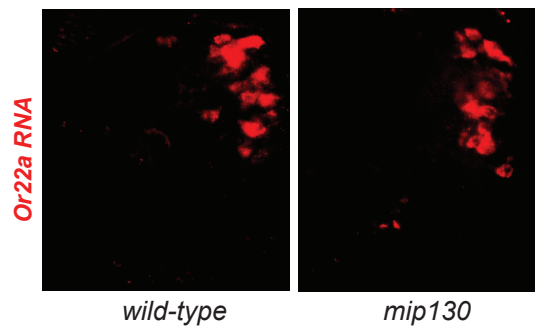


Figure 3.9

Figure 3.10. Differential expression of dREAM complex members and model for olfactory receptor expression.

A. Representative confocal micrograph from *aGr21a promoter-mCD8::GFP* antenna stained with anti-CD8, anti-Elav, and anti-Mip120 antibodies. Overlays of all three (*left*) and of anti-Elav with anti-Mip120 (*right*) are provided. Arrowheads indicate nuclei of *Gr21a*⁺ ab1C neurons. **B.** Schematic showing cells that were selected for analysis of nuclear stain signal intensity and mean Myb, Mip120, or E2F2 signal intensity in ab1C or a neighboring nucleus as a ratio of the mean signal in the neighboring three cells. For Myb signal, $n = 48$ for neighboring cells and $n = 16$ for ab1C cells. For Mip120 signal, $n = 70$ for neighboring cells and $n = 25$ for ab1C cells. For E2F2 signal, $n = 29$ for neighboring cells and $n = 13$ for ab1C cells. Error bars represent $\pm$ s.e.m. A two-tailed student's t-test was used for statistical analysis. * $p < 0.05$; *** $p < 0.001$. **C.** Olfactory receptors have a H3K9me2-repressive chromatin mark (red dashed line). Expression of *Gr63a/Gr21a* receptors in the ab1C neurons of antennae requires Myb (green arrow), and Myb opposes histone methyltransferase Su(var)3-9. Conversely, repression of *Gr63a* in other sensory organs and the brain requires Mip120 and E2F2 (combined red and orange line). The absence of Mip120 or E2F2 causes misexpression and increased H3K4me3 chromatin mark at *Gr63a* gene (green dashed arrow). E2F2 also has a repressive modulatory effect on *Gr63a* expression in ab1C cells (solo red line), while Mip130 represses expression of an unknown olfactory receptor in the antenna (dark-orange line).

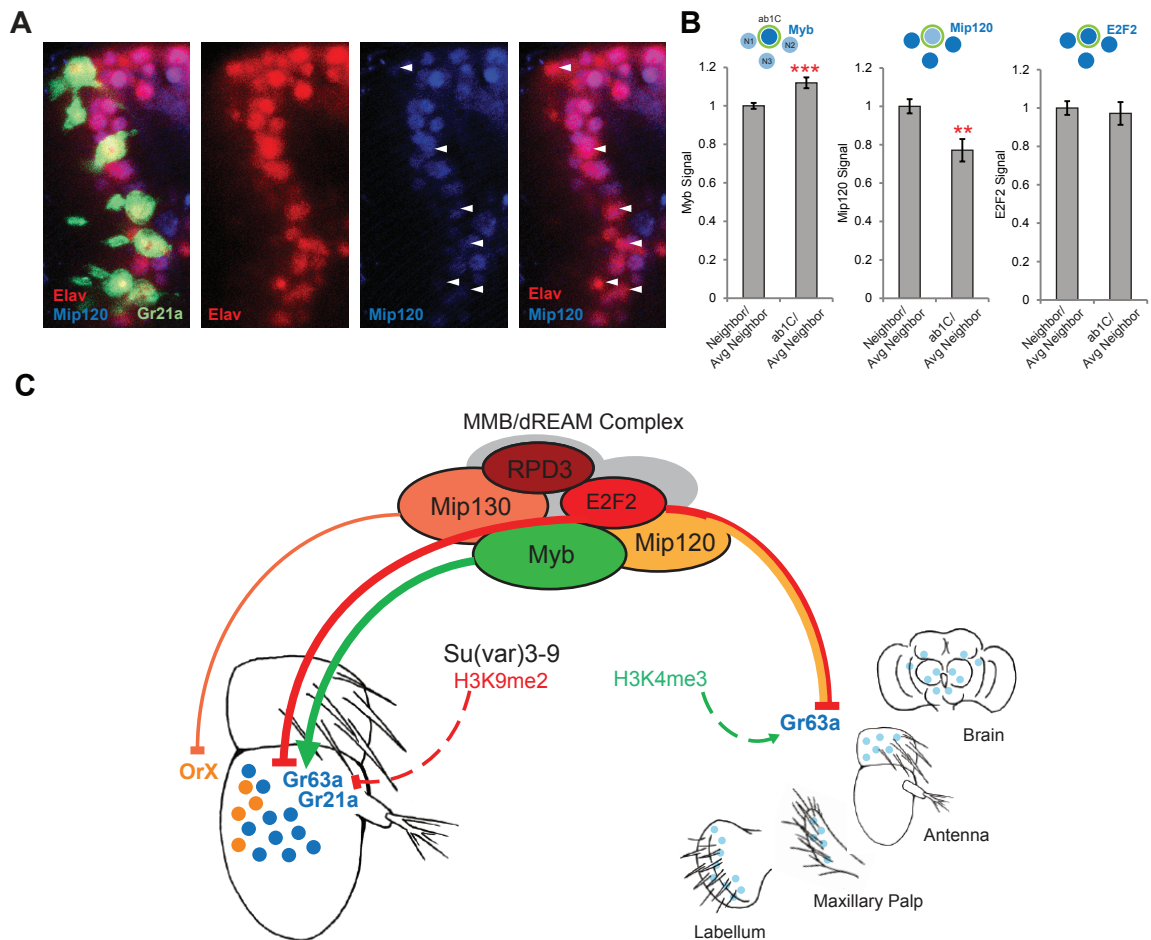


Figure 3.10

Table 3.S1: MMB/dREAM complex members are found at several olfactory receptor loci in Kc cells (Georlette et al., 2007).

Gene	Myb	Mip120	Mip130	E2F2	Lin-52
<i>Or2a</i>	1360	1360	1402	1360	1360
<i>Or9a</i>	-1308	-1345	-1345	-1345	-1345
<i>Or43b</i>	-2486	453	223	5616	262
<i>Or46a</i>	-634	-2735	-2735	-2735	-634
<i>Or67b</i>	-692	-692	-574	4954	-893
<i>Or71a</i>	-935	-935	-935	-1186	779
<i>Or85f</i>	4160	3822	-5744	-5780	-6282

Distance
from TSS:

1 kb

2kb

5 kb

<i>Ir8a</i>	2064	4331	3871	4220	4187
<i>Ir25a</i>	3404	3436	3404	3404	3436
<i>Ir31a</i>	1381	1337	1337	1690	1337

Chapter 4. Antennal transcriptome analysis from dREAM complex mutants.

Introduction

The MMB/dREAM complex is a multi-protein complex comprised of transcription factors and chromatin modifiers typically implicated in regulation of DNA replication and cell-cycle during development (Beall et al., 2002; Cayirlioglu et al., 2001; Clapier and Cairns, 2009; Dimova et al., 2003; Georlette et al., 2007; van den Heuvel and Dyson, 2008; Korenjak et al., 2004; Lewis et al., 2004; Lipsick, 2004; Song et al., 2008; Wen et al., 2008). However, the DNA binding factors in the dREAM complex have numerous targets across the genome and are present at an estimated 1/3 of *Drosophila* annotated gene loci, including several olfactory receptor genes belonging to the *Or* (Olfactory Receptor), *Gr* (Gustatory Receptor) and *IR* (Ionotropic Receptors) families (Georlette et al., 2007). Based on our previous evidence that dREAM complex members regulate the carbon dioxide receptors genes *Gr63a* and *Gr21a* in the adult antenna, we hypothesized the complex may also regulate other antennal receptor genes. In order to interrogate expression levels of the large odorant receptor gene families, we opted to perform Illumina high-throughput sequencing of antennal tissue from *wild-type* and dREAM complex mutant animals.

Results

A complete receptor repertoire of the antenna

At the time we performed this RNA-seq antennal transcriptome analysis, high-throughput sequencing had not yet been performed for olfactory tissues. Previous approaches to build a receptor repertoire included RT-PCR, RNA *in situ* hybridizations and *Promoter-*

Gal4 analysis (Couto et al., 2005; Fishilevich et al., 2005). Using an unbiased RNA-seq approach, we were able to measure mRNA levels of all members from the *Or*, *Gr* and *IR* gene families (Figure 4.1, Tables 4.S1,S2,S3). We observed mapped reads from all previously validated antennal receptors (Figure 4.1, red dots) with an RPKM (a normalized expression score, Reads per Kilobase per Million reads) value of at least 0.5. We selected an RPKM > 0.5 to be indicative of receptor gene expression at putatively functional levels.

Interestingly, a few *Or* family members previously believed not to be expressed in the antenna are expressed at levels above this cut-off: *Or46a*, *Or45b* and *Or63a* (Figure 4.1A, Table 4.S1). Further work will need to be done to determine if these receptors are selectively expressed in a particular neuronal class and whether or not they are functional. *Or46a* is known to be expressed in the maxillary palp (Couto et al., 2005) and *Or45b* has been detected in the larval olfactory system (Fishilevich et al., 2005). Odor response profiles for both of these receptors are known (Goldman et al., 2005; Hallem and Carlson, 2006; Kreher et al., 2008) so it is possible that they mediate responses to odors in the adult antenna.

For the *Gustatory receptor (Gr)* family in addition to the expected *Gr63a* and *Gr21a*, we also observe other *Gr* family members with reasonable expression levels: *Gr10a*, *Gr64a-f*, *Gr43a*, *Gr28b*, *Gr94a*, *Gr66a* and *Gr39a* (Figure 4.1B, Table 4.S2). *Gr10a* has previously been reported to be co-expressed with *Or10a* in ab1D neurons, although its functionality has not yet been determined (Jones et al., 2007). Other *Gr promoter-Gal4* reporters are known to drive expression in the third antennal segment, but it is not known if their expression is restricted to particular neuronal classes or what their function

might be, if any (Dahanukar lab, unpublished data). The expression level of several of these genes is comparable to *Gr63a/21a* and it will be interesting to discover if these taste receptors are able to participate in odor detection.

Several *IRs* are also detected in addition to known *IR* receptors previously reported (Benton et al., 2009) such as: *IR62a*, *IR60a*, *IR51b*, *IR94b*, *IR100a*, *IR94d* and *IR41a* (Figure 4.1C, Table 4.S3). Again, additional experimentation is required to determine the expression patterns and functionality of these receptors. Antennal second segments were included in the tissue collection for RNA-seq library preparation, so it is possible that some of these *IRs* are expressed in the Johnston's organ along with other *IRs* known to be involved in hearing (Senthilan et al., 2012).

Differentially expressed genes in dREAM complex mutants

In addition to *wild-type* antennae, we also collected antennal tissue from three dREAM complex mutants (*mip130*, *mip130,myb* and *e2f2*) in order to isolate mRNA and profile the antennal transcriptome. We performed differential expression analysis by comparing transcriptome data from these mutants to *wild-type*. We used two computational methods to identify differentially expressed genes (DEGs): EdgeR and DEseq. EdgeR ($P < 0.1$) appeared to be the more conservative analysis of DEGs: *mip130* mutants exhibited 221 up-regulated and 139 down-regulated genes compared with wild-type; *mip130,myb* double mutants had 259 up-regulated and 151 down-regulated genes; *e2f2* mutants had 280 up and 1176 down-regulated genes. 27 genes were up-regulated and 54 were down-regulated in all three mutant genotypes (Figure 4.2A). In contrast, the DEseq analysis ($P < 0.1$) gave a less conservative estimate of DEGs: 1124, 916 and 1286 up-regulated genes in *mip130*, *mip130,myb* and *e2f2* mutants respectively, with an

overlap of 240 genes; 1303, 942 and 1921 down-regulated genes in *mip130*, *mip130,myb* and *e2f2* mutants respectively, with an overlap of 756 genes (Figure 4.2B). By comparing the two analysis methods, we can see a fair amount of overlap between the two DEG sets: *mip130*, 90 UP and 125 DOWN; *mip130,myb*, 191 UP and 119 DOWN; *e2f2*, 256 UP and 1040 DOWN (Figure 4.2C).

Differentially expressed receptor genes in dREAM complex mutants

Given the conservative nature of the analysis and possibly the low expression level of some genes, very few *Or*, *Gr* or *IR* family members were determined to be differentially expressed in the EdgeR analysis. The DEseq analysis, however, did identify several antennal receptor genes as being putatively differentially regulated (Figure 4.3, black dots). Many of these genes as well as a few others show a fold-change reduction or increase in one or more of the dREAM complex mutants ($FC < 0.58$ or > 1.5 , red dots). These fold-change cut-offs were chosen based on *Gr21a* and *Gr63a* expression levels, which are known to be regulated by Myb and E2F2 (Sim et al., 2012). This is a fairly arbitrary guide-line, but based on what we've observed for *Gr63a* and *Gr21a* expression, genes falling into these up or down-regulated categories may be interesting for further investigation. Overall, we identify 13 known antennal receptor genes that exhibit DEseq differential regulation and whose $FC < 0.58$ or > 1.5 in one or more of the mutants, 4 genes based on DEseq DEG alone and 5 genes based on FC alone for a total of 22 receptor genes that are candidates for regulation by one or more member of the dREAM complex.

Discussion

Combinatorial control of receptor gene expression by members of the MMB/dREAM complex

Similar to what has been observed for transcriptional activators and repressors (Jafari et al., 2012), loss of a single dREAM complex member results in expression changes of multiple receptor genes (Table 4.1). Interestingly, all three mutants exhibit both up and down-regulated receptors even though Myb is considered to be an activator and Mip130 and E2F2 are considered to be repressive. Some of these genes may be direct targets of the dREAM complex and others may be exhibiting changes in expression due to aberrant dREAM complex activity at other gene loci. Of particular interest are genes that are down-regulated in *mip130,myb* double mutants, but not *mip130* single mutants (such as *IR75a*) or up-regulated in *mip130* or *e2f2* mutants (such as *Or7a*, *Or56a*, *Or19a*, *IR8a* and *IR76a*). These are more likely to be direct targets of the dREAM complex. However, further experiments will need to be done to determine if this is indeed the case.

The impact of changes in receptor levels

Interestingly, none of the dREAM complex mutants exhibited complete loss of expression of any receptor gene. Rather, it appears that members of the complex may be responsible for modulation of receptor gene expression. There is evidence that the amount of receptor present in the dendrites of ORNs can greatly influence the responsiveness of the neurons to odors (Tanoue et al., 2008). This certainly appears to be true for Gr63a and Gr21a, although these olfactory-functioning gustatory receptors may be an exceptional case. In our previous study, we did not observe any abnormal responses in ab1A, B or D neurons to standard diagnostic odorants, although this

transcriptome data suggests that *Or42b*, expressed in ab1A neurons, may be down-regulated in *mip130* and *mip130,myb* mutants. The primary diagnostic odorant for Or42b (1% ethyl acetate) saturates neuronal response and it may be worthwhile to interrogate ab1A neurons with lower concentrations of this and other activating odorants. A more direct analysis of *Or42b* expression patterns using RNA *in situ* hybridization would also be useful in this instance. This same strategy could be applied to further investigate more subtle changes in receptor expression levels for some of these dREAM complex candidate target genes.

In general, we believe this data set will provide a good foundation for future work on receptor gene regulation. This is also the first transcriptome analysis looking at loss of dREAM complex members in post-mitotic tissue. Because MMB/dREAM is typically associated with cell-cycle regulation, this may provide insight into the role of this complex in cells not undergoing DNA replication and cell division. As we have seen with *Gr63a* and *Gr21a* expression, canonical epigenetic regulators can be re-purposed for post-developmental roles.

Materials and Methods

Fly Stocks

The fly stocks used for sequencing experiments were *wild-type* ($w^{Canton-S}$), *mip130* mutants ($mip130^{1-36}$), *mip130,myb* double mutants ($mip130^{1-36},myb^{MH107}$) and *e2f2* mutants ($e2f2^{1-188}/e2f2^{329}$).

RNA Isolation And Sequencing

The second and third antennal segments from 40-60 male flies, 3-7 days old, of a particular genotype were collected in 1.5 ml microfuge tubes on liquid nitrogen. Antennae were mechanically crushed with disposable RNase-free plastic pestles, and total RNA was isolated using a Trizol based protocol. cDNA libraries were prepared from total RNA (300-1000 ng/sample) using the Illumina TruSeq RNA Library Preparation Kit (Illumina Catalog #RS-122-2001). Samples were submitted to the UCR IIGB sequencing core for high-throughput sequencing using the Illumina HiSeq2500 platform. Raw reads were aligned to the drosophila melanogaster reference genome *Drosophila* (dmel-all-chromosome-r5.48.fasta) with Tophat2 and generated a count table of reads overlapping with exonic regions of genes by the UCR bioinformatics core. Two biological replicates were used for each analysis. RPKM values and differential expression analysis were performed using EdgeR ($p < 0.1$). For additional analysis, the read count table for all the genes were then imported into R and analyzed using the DESeq package (1.22.0). We considered genes to be differentially expressed if they had a padj < 0.1 .

Figure 4.1. Antennal expression profiles for major receptor families.

RPKM values for all annotated Odorant Receptor (*Or*) genes (**A**), Gustatory Receptor (*Gr*) genes (**B**), and Ionotropic Receptor (*IR*) genes (**C**) from *wild-type* (*wCS*) antennal RNA-seq. Red dots below the gene names indicate that these receptors had been shown to be expressed in the antenna previous to high-throughput sequencing methods. For exact RPKM values see Tables S1, S2 and S3.

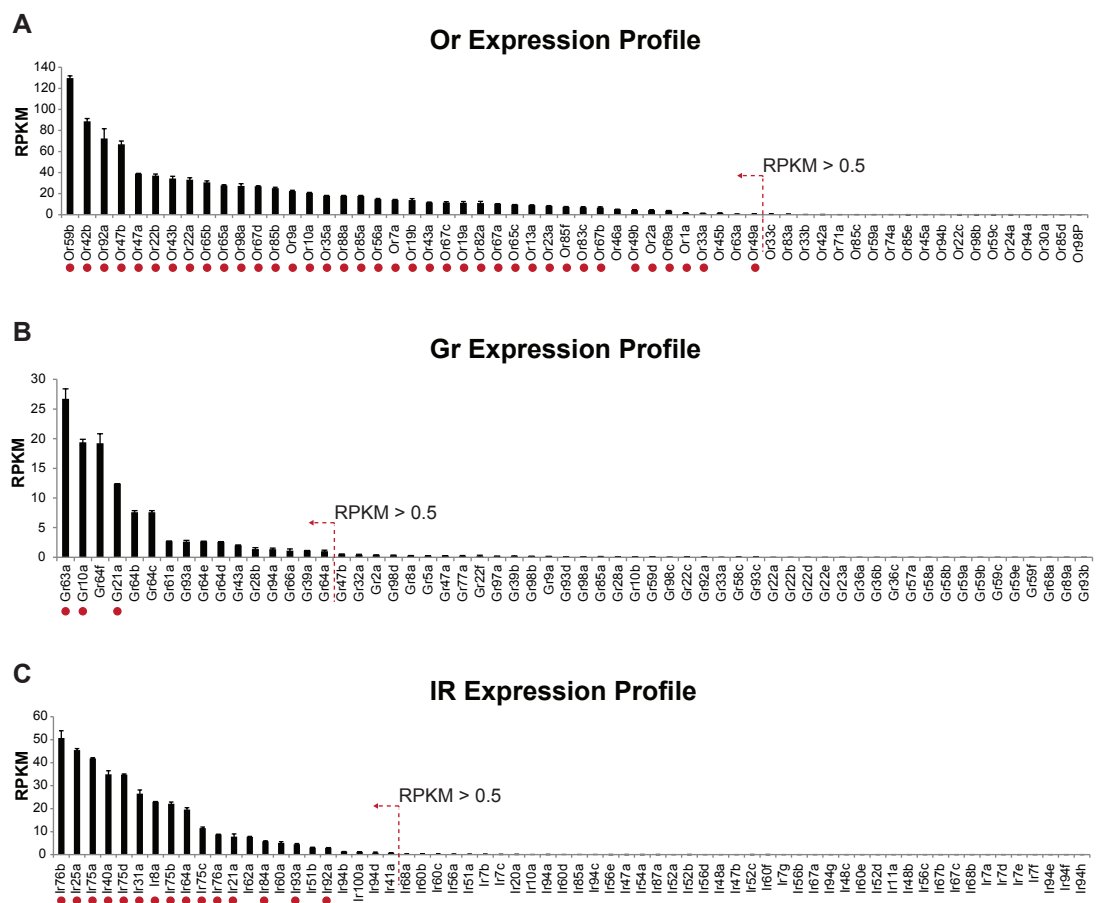


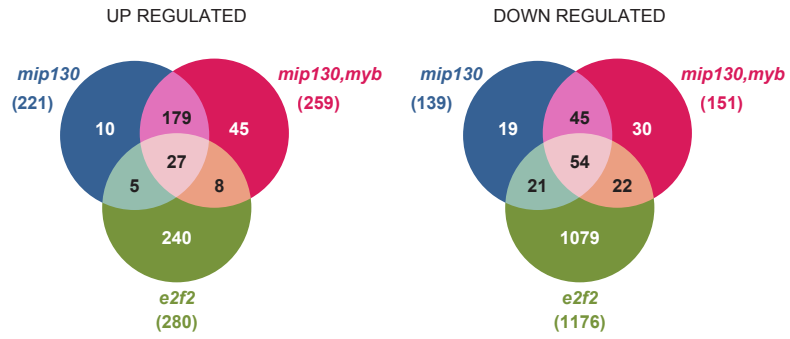
Figure 4.1

Figure 4.2. Differential gene expression in *mip130*, *mip130,myb* and *e2f2* mutant antennae.

A. Venn Diagrams showing numbers of up-regulated (left) and down-regulated (right) genes in the indicated mutants when compared with *wild-type* based on an EdgeR analysis of antennal RNA-seq data. **B.** Venn Diagrams showing numbers of up-regulated (left) and down-regulated (right) genes in the indicated mutants when compared with *wild-type* based on a DEseq analysis of the same antennal RNA-seq data shown in (A). **C.** Comparison of differentially regulated genes found by EdgeR and DEseq analysis of RNA-seq data from the indicated mutants.

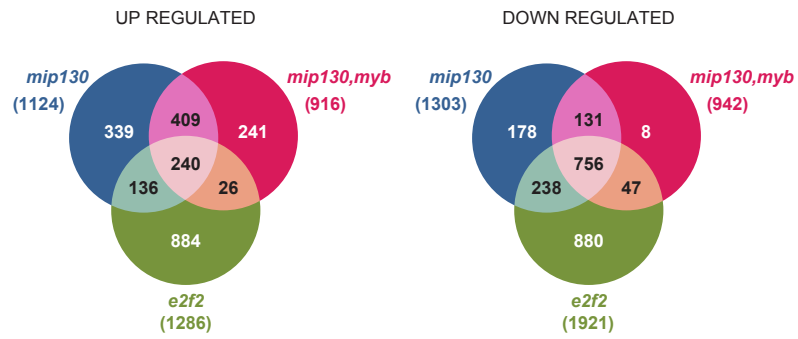
A

EdgeR Differential Expression



B

DEseq Differential Expression



C

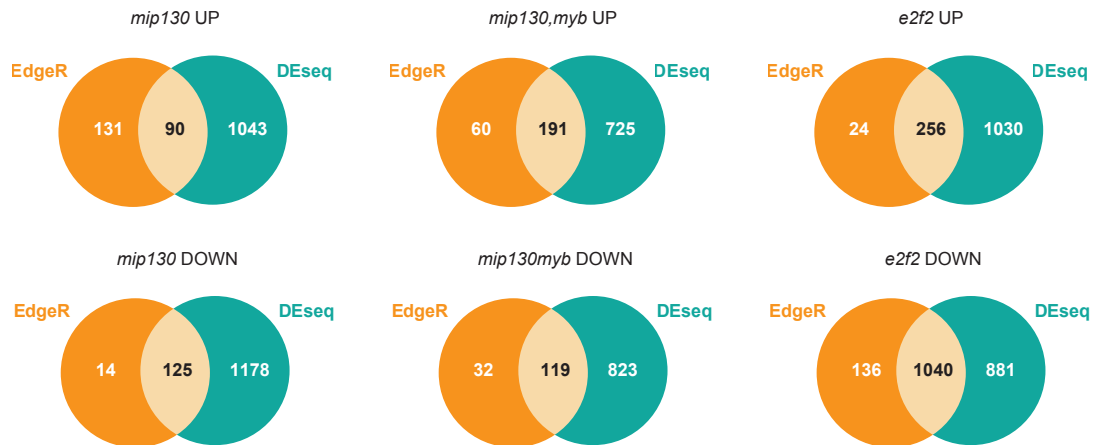


Figure 4.2

Figure 4.3. Antennal receptor genes are differentially regulated in dREAM complex mutants.

Average RPKM values for known antennal receptor genes grouped according to sensillar class for the indicated genotypes. Red dots indicate genes that are potentially up or down-regulated in one or more of the mutants based on an arbitrary fold-change cut-off ($FC < 0.54$ or > 1.5) when compared with *wild-type*. Black dots indicate differentially expressed genes (DEG) determined by DEseq analysis ($P < 0.1$).

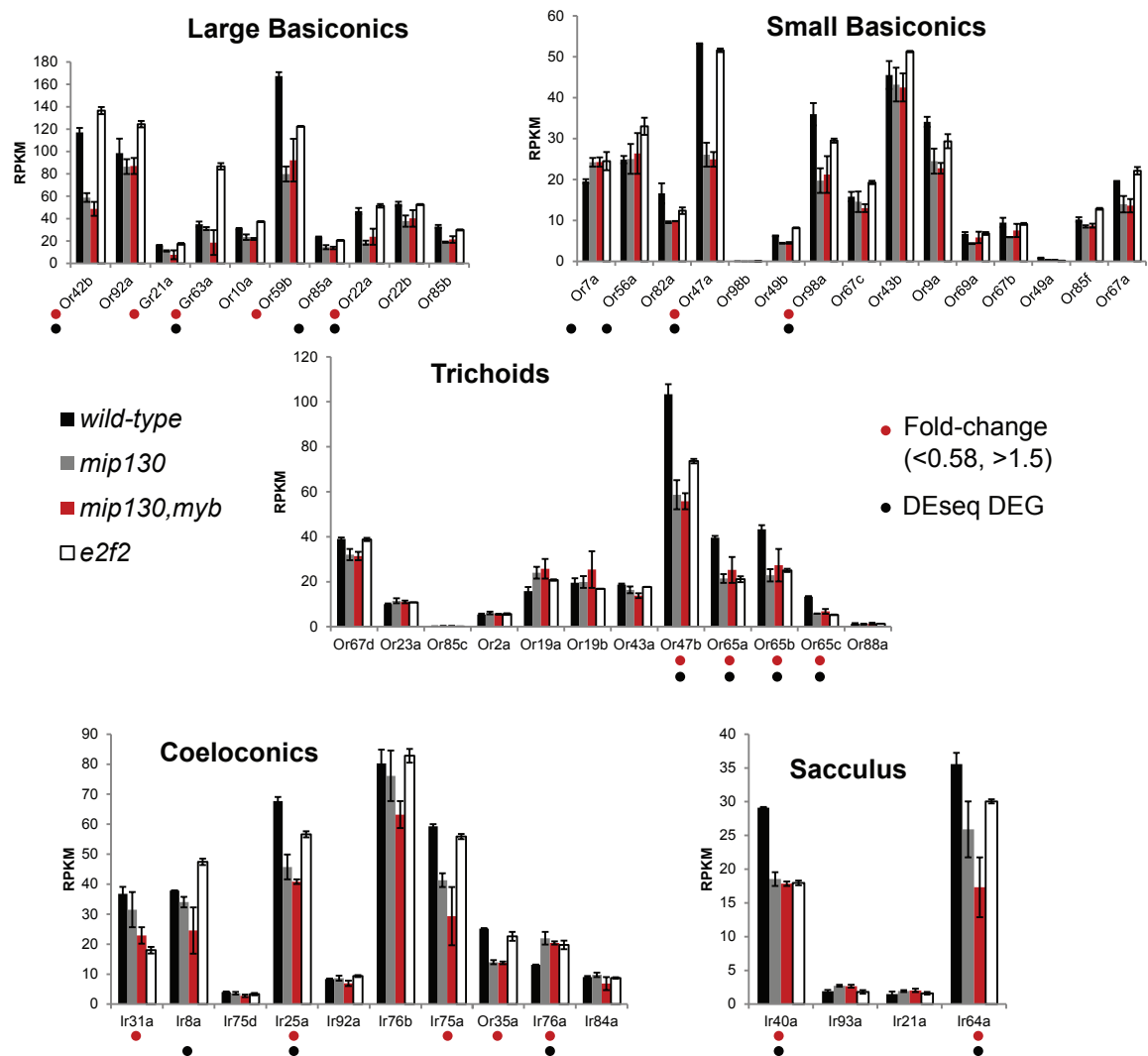


Figure 4.3

Table 4.1. Combinatorial control of receptor gene expression by members of the MMB/dREAM complex

Large Basiconics										
	Or42b	Or92a	Gr21a	Gr63a	Or10a	Or59b	Or85a	Or22a	Or22b	Or85b
<i>mip130</i>	↓					↓	↓	↓		↓
<i>mip130,myb</i>	↓		↓	↓		↓	↓	↓		
<i>e2f2</i>				↑						

Small Basiconics													
	Or7a	Or56a	Or82a	Or47a	Or49b	Or98a	Or67c	Or43b	Or9a	Or69a	Or67b	Or49a	Or85f
<i>mip130</i>	↑		↓	↓		↓						↓	
<i>mip130,myb</i>			↓	↓		↓						↓	
<i>e2f2</i>	↑	↑										↓	

Trichods											
	Or67d	Or23a	Or83c	Or2a	Or19a	Or19b	Or43a	Or47b	Or65a	Or65b	Or65c
<i>mip130</i>					↑			↓	↓	↓	↓
<i>mip130,myb</i>					↑			↓			↓
<i>e2f2</i>									↓	↓	↓

Coeloconics									
	Ir31a	Ir8a	Ir75d	Ir25a	Ir92a	Ir76b	Ir75a	Or35a	Ir76a
<i>mip130</i>								↓	↑
<i>mip130,myb</i>							↓	↓	↑
<i>e2f2</i>	↓	↑							↑

Table 4.S1. RPKM values for Or genes

Flybase ID	SYMBOL	wCS_1	wCS_2	Mip130_1	Mip130_2	Mip130Myb_1	Mip130Myb_2	E2F2_1	E2F2_2
FBgn0029521	Or1a	2.242441267	2.323317063	0.886110888	1.581312296	1.26308457	1.581312296	1.104672279	1.382428842
FBgn0030204	Or9a	35.32341111	32.86153269	27.54999307	21.41439784	24.07290591	21.41439784	27.61680699	31.0885742
FBgn0030298	Or10a	31.59813356	30.4368916	25.90226621	20.97213033	22.74264182	20.97213033	37.26691325	37.43861546
FBgn0030715	Or13a	19.30189361	19.80092499	12.75022054	11.81189404	16.70204584	11.81189404	22.75156926	22.85714922
FBgn0041626	Or19a	13.9898384	17.6267979	26.61991921	21.42019199	30.12770746	21.42019199	21.09607315	20.46635191
FBgn0062565	Or19b	17.6280004	21.51872247	22.5402381	17.27123588	33.58950898	17.27123588	16.85354794	16.86805138
FBgn0026398	Or22a	49.47739629	43.90964547	20.25152552	16.62934037	30.90507607	16.62934037	49.7606858	53.02863373
FBgn0026397	Or22b	51.31793985	55.15231007	42.78629991	32.79350843	47.66859185	32.79350843	52.11390414	53.01633434
FBgn0026396	Or22c	0.038704382	0.065355765	0	0.055737606	0	0.055737606	0	0
FBgn0026395	Or23a	9.750096601	10.37689393	12.6819117	10.43184714	11.61126373	10.43184714	10.84942371	10.85958723
FBgn0026394	Or24a	0	0.044007307	0	0	0	0	0	0
FBgn0023523	Or2a	5.060749564	5.820863052	6.698861951	5.527545603	5.583611725	5.527545603	5.252333603	6.015371984
FBgn0032096	Or30a	0	0	0	0	0	0	0	0.06850564
FBgn0026392	Or33a	1.934299981	1.922678082	2.547701646	1.975572153	1.977453679	1.975572153	1.813673683	1.650185892
FBgn0026391	Or33b	0.266805599	0.30034987	0.874769517	1.241335166	2.561363446	1.241335166	1.261470335	0.980856107
FBgn0026390	Or33c	0.7089939	0.570094659	0.452261791	0.175030561	0.935396625	0.175030561	0.798735444	0.853256292
FBgn0028946	Or35a	24.9375635	25.3319476	14.65162741	13.31299526	14.21994018	13.31299526	24.06720531	21.12469675
FBgn0033041	Or42a	0.127951243	0.329229666	0.1483984	0.17548637	0.06843643	0.17548637	0.148366398	0.325739819
FBgn0033043	Or42b	121.0199413	113.0355185	62.7824165	54.88277731	42.41233662	54.88277731	133.4290495	139.7719952
FBgn0026389	Or43a	18.26203426	19.14736082	17.91249141	14.89169772	12.77837622	14.89169772	17.63412495	17.73900024
FBgn0026393	Or43b	48.93112365	42.1190133	47.30251555	39.08623334	45.90863038	39.08623334	51.4422195	51.09828451
FBgn0033404	Or45a	0.040560761	0.05327033	0.041162226	0.006490106	0	0.006490106	0.148956008	0.202608782
FBgn0033422	Or45b	1.984111394	2.056648792	1.116414497	0.92413981	1.667144369	0.92413981	3.622358141	2.800651343
FBgn0026388	Or46a	0	0	0	0	0	0	0	0
FBgn0026386	Or47a	53.22298957	53.27666535	28.97748628	23.13892876	26.73227842	23.13892876	51.11706612	51.99652599
FBgn0026385	Or47b	98.98790866	107.8193939	65.15635972	52.21250645	59.36533457	52.21250645	72.66262767	74.64601979
FBgn0033727	Or49a	0.923299361	0.77400761	0.179423758	0.396059919	0.24516829	0.396059919	0.182257013	0.175040709
FBgn0028963	Or49b	6.090680398	6.312752302	4.520024666	4.314710753	4.721485783	4.314710753	8.27696608	8.08508509
FBgn0034473	Or56a	23.97243423	25.75307606	28.68093525	21.37479699	31.33157343	21.37479699	35.07975592	30.91002961
FBgn0026384	Or59a	0.041047015	0.092415345	0	0	0.025613634	0	0	0
FBgn0034865	Or59b	170.8481379	163.8636884	86.56408346	73.18589412	111.2855175	73.18589412	122.1279595	122.6262554
FBgn0034866	Or59c	0.050171834	0	0	0.016055937	0.04174345	0.016055937	0.038789868	0
FBgn0035382	Or63a	1.375481181	1.22646674	0.760131503	0.614508733	1.597646722	0.614508733	1.310874021	0.915615367
FBgn0041625	Or65a	38.73345624	40.38970634	23.36505593	19.52460795	31.01578282	19.52460795	22.45979773	19.81359562
FBgn0041624	Or65b	41.54321016	45.16998654	25.61057403	20.16267969	34.60420895	20.16267969	25.7335036	24.3226826
FBgn0041623	Or65c	13.16900106	13.62845266	5.892608007	5.738532397	7.909689746	5.738532397	5.215444079	5.395133305
FBgn0036009	Or67a	19.64580894	19.42389049	15.98777153	11.9738676	15.23369626	11.9738676	21.2246784	23.02867967
FBgn0036019	Or67b	8.348637425	10.63590696	5.892353511	5.953652124	9.175774248	5.953652124	8.885129763	9.39257789
FBgn0036078	Or67c	14.65429121	16.97361831	17.07986196	12.05381935	13.96290918	12.05381935	19.69692045	18.84294569
FBgn0036080	Or67d	38.3381217	39.66433589	34.58591286	29.54856214	33.29641798	29.54856214	39.52356083	38.11377478
FBgn0041622	Or69a	6.312905354	7.155441224	4.215973094	4.457044647	7.255890218	4.457044647	7.157611878	6.449173411
FBgn0036474	Or71a	0.163271449	0.147039069	0.149123305	0.438899617	0.081506052	0.438899617	0.340825702	0.383539367
FBgn0036709	Or74a	0.038513249	0.086710694	0	0	0.048065092	0	0.111660547	0.12478772
FBgn0030016	Or7a	19.04522021	20.08247944	25.27313173	23.14950635	25.4144173	23.14950635	22.28356129	26.73441258
FBgn0041621	Or82a	14.16334706	19.0600663	9.349868115	9.756920947	9.85925857	9.756920947	13.1918547	11.58731566
FBgn0037322	Or83a	0.498169723	0.599478392	0.749618938	0.907066113	0.900426445	0.907066113	1.394527093	1.029704835
FBgn0037399	Or83c	9.601701313	11.2941768	11.01677905	9.312241552	8.803882397	9.312241552	9.680801072	8.301479544
FBgn0037576	Or85a	22.90691599	23.97806676	16.30642387	12.60444816	14.84432393	12.60444816	20.72509525	20.34894221
FBgn0037590	Or85b	34.36716469	31.57012172	19.51318776	18.38343151	24.32050621	18.38343151	29.30789497	30.27817637
FBgn0037591	Or85c	0.059991792	0.180091441	0.081175193	0.383970177	0.149741248	0.383970177	0.092764147	0.129587247
FBgn0037594	Or85d	0	0	0	0	0	0	0	0.030592631
FBgn0026399	Or85e	0	0.112557151	0.067645994	0.06399503	0.249568746	0.06399503	0.077303455	0.161984059
FBgn0037685	Or85f	10.79546946	9.673041233	8.820831115	8.26854863	9.213087448	8.26854863	12.61167519	13.06877499
FBgn0038203	Or88a	1.067018183	1.616119088	1.299408877	1.005772629	1.767468209	1.005772629	1.282429713	1.304334329
FBgn0038798	Or92a	111.5613968	85.46097768	93.07008903	79.89208984	94.20047149	79.89208984	121.5071249	127.2741834
FBgn0039033	Or94a	0	0.022627468	0.040796811	0.077189881	0.075256555	0.077189881	0	0.032563806
FBgn0039034	Or94b	0.040619442	0.045726342	0	0	0.025346826	0	0	0
FBgn0039551	Or98a	33.23364373	38.66932018	22.74944989	16.70559697	25.70244555	16.70559697	29.99684839	28.99962357
FBgn0039582	Or98b	0.020274522	0.045647094	0	0	0	0	0.047025152	0
FBgn0067407	Or98P	0	0	2.487439851	0.106963121	0	0.106963121	0	0

Table 4.S2. RPKM values for Gr genes

Flybase ID	SYMBOL	wCS_1	wCS_2	Mip130_1	Mip130_2	Mip130Myb_1	Mip130Myb_2	E2F2_1	E2F2_2
FBgn0052693	Gr9a	0.228038974	0.154025575	0.115710254	0.153251255	0.056919188	0.153251255	0.026445919	0.092359332
FBgn0045502	Gr10a	38.27006928	39.92614037	31.3487085	25.15333243	31.00815395	25.15333243	49.84428525	50.49268878
FBgn0030297	Gr10b	0.093512385	0.063161567	0	0	0.046681924	0	0.043378917	0
FBgn0041250	Gr21a	16.07212524	15.99285006	10.47077721	11.62573488	3.709131174	11.62573488	16.67122142	18.14965716
FBgn0045501	Gr22a	0	0	0	0	0	0	0	0
FBgn0045500	Gr22b	0	0	0	0	0	0	0	0
FBgn0265138	Gr22c	0	0.114315856	0	0	0	0	0	0
FBgn0045498	Gr22d	0	0	0	0	0	0	0	0
FBgn0045497	Gr22e	0	0	0	0	0	0	0	0
FBgn0041249	Gr22f	0	0.440131127	0.041765601	0	0	0	0	0
FBgn0041248	Gr23a	0	0	0	0	0	0	0	0
FBgn0041247	Gr28a	0.099069014	0.074349677	0.134050778	0.142667818	0.329705499	0.142667818	0.689348245	0.588492546
FBgn0045495	Gr28b	2.436252023	3.34171199	2.839894337	2.702424252	2.547427322	2.702424252	2.042649538	1.857878144
FBgn0041246	Gr32a	0.303854529	0.494082038	0.256966927	0.291717602	0.126404949	0.291717602	0.606882322	0.218783665
FBgn0032416	Gr33a	0.028637453	0.032237911	0.02906211	0.123721113	0	0.123721113	0.365322695	0.208775048
FBgn0045487	Gr36a	0	0	0	0	0	0	0	0
FBgn0045486	Gr36b	0	0	0.04007383	0	0	0	0	0.031986726
FBgn0045485	Gr36c	0	0	0	0	0	0	0	0
FBgn0264556	Gr39a	1.819136438	1.591233477	0.667344526	0.890937894	1.058454682	0.890937894	0.292218027	0.413193381
FBgn0041245	Gr39b	0.337251153	0.142369585	0.042781521	0.020236266	0.05261179	0.020236266	0.048889212	0.017073995
FBgn0041243	Gr43a	1.961315799	2.207903236	0.438828272	0.37066428	0.674576908	0.37066428	4.781945832	4.703632171
FBgn0041242	Gr47a	0.32980562	0.283912813	0.039376027	0.130377933	0.048423787	0.130377933	0.112493834	0.125718971
FBgn0041241	Gr47b	0.526193063	0.698125556	0.820065055	0.487133249	1.172672421	0.487133249	1.569167009	1.172140073
FBgn0041240	Gr57a	0	0	0	0	0	0	0	0
FBgn0041239	Gr58a	0	0	0	0	0.04915748	0	0.045679315	0
FBgn0041238	Gr58b	0	0	0	0	0	0	0	0
FBgn0041237	Gr58c	0	0.042515534	0.03832727	0	0	0	0	0
FBgn0045483	Gr59a	0	0	0	0.040692492	0	0.040692492	0.147464744	0.051500367
FBgn0045482	Gr59b	0	0	0	0.04080337	0	0.04080337	0	0
FBgn0041235	Gr59c	0	0	0	0	0	0	0	0
FBgn0041236	Gr59d	0.03989224	0.089815425	0	0	0	0	0	0
FBgn0041233	Gr59e	0	0	0.079145813	0	0	0	0.610504039	0.694911615
FBgn0041234	Gr59f	0	0	0	0.073586422	0.0478289	0.073586422	1.022228986	0.776090702
FBgn0003747	Gr5a	0.329753539	0.353535211	0.302772575	0.316582123	0.293955134	0.316582123	0.382418638	0.228951966
FBgn0035167	Gr61a	3.907816487	3.806938317	1.296499942	1.382847628	0.828466699	1.382847628	3.021290512	3.662591389
FBgn0035468	Gr63a	37.38712937	32.62733175	32.48208792	29.65934537	7.448863083	29.65934537	89.59596697	83.87931288
FBgn0045479	Gr64a	1.116379508	1.681549367	0.893581764	1.192552537	1.530621221	1.192552537	1.677609666	1.999654023
FBgn0045478	Gr64b	0	0	0	0	0	0	0	0
FBgn0045477	Gr64c	0	0	0	0	0	0	0	0
FBgn0035486	Gr64d	0.007901654	0	0.008018826	0	0	0	0	0.038403516
FBgn0045476	Gr64e	0	0	0	0	0	0	0	0
FBgn0052255	Gr64f	30.04692614	25.0815629	14.74542333	13.66997561	12.47947286	13.66997561	27.69644882	27.01269026
FBgn0035870	Gr66a	1.004408025	1.745914893	1.259137941	0.822481573	0.884834645	0.822481573	1.404638924	1.531485652
FBgn0041231	Gr68a	0	0	0	0.038397018	0.049913749	0.038397018	0.092764147	0.064793624
FBgn0045474	Gr77a	0.418997112	0.150936236	0.204100952	0.176994849	0.271915518	0.176994849	0.40816889	0.271520558
FBgn0045473	Gr85a	0	0.154412573	0.099429414	0.056437828	0.073365687	0.056437828	0.090899541	0.063491239
FBgn0038440	Gr89a	0	0	0	0.041252994	0.053626342	0.041252994	0	0.017403246
FBgn0030108	Gr8a	0.231957687	0.400385053	0.313863107	0.252384892	0.270187446	0.252384892	0.322804515	0.125261963
FBgn0045471	Gr92a	0	0.045371875	0.061353344	0.019347335	0	0.019347335	0	0
FBgn0041229	Gr93a	3.582130561	2.997125231	2.603624345	2.246225538	2.899816712	2.246225538	3.162457706	3.698933923
FBgn0045470	Gr93b	0	0	0	0	0	0	0	0.031905951
FBgn0045469	Gr93c	0.039190618	0	0.039771766	0.037625218	0	0.037625218	0.022724885	0.079364049
FBgn0045468	Gr93d	0.081664219	0.137897242	0.062156398	0.156804575	0.101918127	0.156804575	0.260443841	0.214989314
FBgn0041225	Gr94a	1.848635947	1.387371101	0.801229222	1.035297367	0.672911286	1.035297367	0.714627499	1.513051102
FBgn0041224	Gr97a	0.147149677	0.248475219	0.055999396	0.21190807	0.045911232	0.21190807	0.383964805	0.134095294
FBgn0039520	Gr98a	0.073153097	0.10980041	0.024745955	0.023410376	0.030432041	0.023410376	0.028278804	0.019752095
FBgn0046887	Gr98b	0.212347183	0.19558198	0.078362192	0.129732498	0.09636813	0.129732498	0.917882861	0.656757151
FBgn0046886	Gr98c	0.076273182	0.064397001	0.096755273	0.036613293	0.142785053	0.036613293	0.375932941	0.200796866
FBgn0046885	Gr98d	0.245486992	0.48892864	0.210799987	0.199422768	0.047134049	0.199422768	0.240894787	0.58125999

Table 4.S3. RPKM values for IR genes

Flybase ID	SYMBOL	wCS_1	wCS_2	Mip130_1	Mip130_2	Mip130Myb_1	Mip130Myb_2	E2F2_1	E2F2_2
FBgn0039879	Ir100a	1.510720446	1.380874977	0.484833625	0.68180135	0.692925144	0.68180135	1.093127174	0.93087197
FBgn0083979	Ir10a	0.143059914	0.146405632	0.039594904	0.012485967	0.146078871	0.012485967	0.120660424	0.115882982
FBgn0030385	Ir11a	0.011816565	0	0.02398358	0	0.029494488	0	0	0
FBgn0031181	Ir20a	0.055311581	0.155664144	0	0	0.069029653	0	0.128290841	0.022402051
FBgn0031209	Ir21a	1.148036148	1.853403975	2.086270396	1.77715828	2.287982471	1.77715828	1.81646948	1.405730815
FBgn0031634	Ir25a	69.04240712	66.37093832	49.87046766	41.61810685	40.07509105	41.61810685	55.58323182	57.62252124
FBgn0051718	Ir31a	39.1221769	34.48546174	37.38354396	25.61868367	20.14162044	25.61868367	19.10017057	16.90868778
FBgn0259683	Ir40a	29.01318248	29.22403006	19.56398769	17.51263243	18.17632629	17.51263243	18.31539953	17.60674334
FBgn0040849	Ir41a	2.374600466	2.324965143	1.083403246	0.919563547	0.423362248	0.919563547	1.249645795	1.268863619
FBgn0033515	Ir47a	0.086306099	0	0.047774133	0.007532614	0	0.007532614	0.045495494	0.069910625
FBgn0265141	Ir47b	0.035912201	0.02021364	0	0	0.044818946	0	0.145767145	0.05818
FBgn0053471	Ir48a	0.038167698	0.021483176	0.096834193	0.036643157	0.047633839	0.036643157	0	0.061834045
FBgn0033648	Ir48b	0	0.014608083	0.131690205	0.049833068	0	0.049833068	0.030098184	0.02102289
FBgn0033651	Ir48c	0.02775421	0	0	0	0.017318828	0	0.032186848	0.022481773
FBgn0033997	Ir51a	0.208775717	0.172351044	0.08474864	0.080174618	0.034740682	0.080174618	0.387391022	0.574990412
FBgn0050081	Ir51b	3.873383064	3.479484091	2.130848823	1.752907332	2.783234059	1.752907332	3.7508981	3.137560802
FBgn0034023	Ir52a	0.038994665	0.029264859	0	0	0.032443937	0	0	0
FBgn0050469	Ir52b	0.052254157	0.014705959	0	0	0.032606972	0	0	0
FBgn0050468	Ir52c	0.051992886	0	0	0	0.097331811	0	0	0
FBgn0050464	Ir52d	0.026214901	0	0	0.025167793	0	0.025167793	0	0
FBgn0034272	Ir54a	0.053144347	0.029912974	0	0	0	0	0.046224042	0.193718585
FBgn0050125	Ir56a	0.271154952	0.130819735	0.628973352	0.54544108	0.257832612	0.54544108	0.509127725	0.543880252
FBgn0034456	Ir56b	0	0.042931334	0	0	0	0	0	0.030891825
FBgn0034457	Ir56c	0	0	0	0	0	0	0	0.011122145
FBgn0034458	Ir56d	0.036961767	0.027739203	0.025006576	0.02365693	0.092257641	0.02365693	0.100018215	0.049900303
FBgn0265142	Ir56e	0	0	0	0	0	0	0	0
FBgn0034994	Ir60a	7.113671016	5.742769433	6.236734252	5.941898332	5.18559997	5.941898332	7.619080321	7.189652307
FBgn0259185	Ir60b	0.350817051	0.243029972	0.219088757	0.077724067	0.202072964	0.077724067	0.281662763	0.306032167
FBgn0259186	Ir60c	0.316886664	0.21800008	0.125061105	0.118311353	0.043942127	0.118311353	0.081665953	0.085562686
FBgn0259187	Ir60d	0.054253466	0.106880355	0	0.052086389	0.016927271	0.052086389	0.251673163	0.076907214
FBgn0035019	Ir60e	0.027221406	0	0	0	0	0	0	0
FBgn0265154	Ir60f	0.050752275	0	0	0	0	0	0	0
FBgn0053971	Ir62a	9.584849993	8.996413046	8.08408638	7.055689224	8.225900991	7.055689224	9.506414721	10.05368608
FBgn0035604	Ir64a	37.23537035	33.8825817	30.0478001	21.73963244	12.89080612	21.73963244	30.35167369	29.73575281
FBgn0036010	Ir67a	0	0.03001524	0	0	0	0	0	0
FBgn0036083	Ir67b	0	0	0	0	0	0	0	0
FBgn0052058	Ir67c	0	0	0	0	0	0	0	0
FBgn0036150	Ir68a	0.442492647	0.523031525	0.348017052	0.350474907	0.345148266	0.350474907	0.436188859	0.537649218
FBgn0036250	Ir68b	0	0	0.049777241	0.070636023	5.876637642	0.070636023	0	0.01986597
FBgn0036757	Ir75a	60.0394041	58.51578267	43.60557435	39.0653087	19.60540763	39.0653087	55.0279126	56.72604623
FBgn0036829	Ir75d	3.997290437	4.05562662	4.046295479	3.157540224	2.361727766	3.157540224	2.957459015	3.746161621
FBgn0060874	Ir76a	13.02410701	13.20040802	24.07023458	19.85284212	20.9062638	19.85284212	21.18961389	18.33207932
FBgn0036937	Ir76b	75.66451714	84.88077131	84.53307828	67.71766592	58.67741844	67.71766592	80.5070607	85.10395431
FBgn0029961	Ir7a	0	0	0	0	0	0	0	0.02054432
FBgn0029965	Ir7b	0.118886172	0.200749796	0.446401692	0.353826177	0.40060349	0.353826177	1.047838911	1.165248134
FBgn0029966	Ir7c	0.089300758	0.15637711	0.231597164	0.057155866	0.247663641	0.057155866	0.63288516	0.530466881
FBgn0259190	Ir7d	0	0	0	0.025167793	0	0.025167793	0	0.021234885
FBgn0259189	Ir7e	0	0	0	0.012294612	0	0.012294612	0.029702806	0
FBgn0259188	Ir7f	0	0	0.095789184	0.067964464	0.088349601	0.067964464	0.301027374	0.124244959
FBgn0029968	Ir7g	0.02775421	0.031243622	0.154911734	0.079936852	0.173188276	0.079936852	0.128747392	0.078686207
FBgn0037501	Ir84a	9.382902222	8.711399936	10.51596312	9.042944156	4.6477826	9.042944156	8.46958231	8.96186226
FBgn0037630	Ir85a	0.10295621	0.057950216	0.078362192	0.135910236	0.06424542	0.135910236	0.253723718	0.1250966
FBgn0038153	Ir87a	0.019376231	0.076343111	0.078654225	0.027903423	0.048363633	0.027903423	0.078647863	0.054933724
FBgn0052704	Ir8a	37.8294024	37.92875663	35.76343373	32.26701787	16.81752422	32.26701787	46.3518779	48.50964458
FBgn0038789	Ir92a	8.166482024	8.520858108	9.499828858	7.818232236	6.020524388	7.818232236	9.031183958	9.666798334
FBgn0259215	Ir93a	1.715082619	2.113217401	2.580465252	2.867173371	2.438619771	2.867173371	2.087954491	1.555153305
FBgn0051164	Ir94a	0.130854579	0.044191901	0	0.050251131	0	0.050251131	0.060701371	0.021199256
FBgn0051424	Ir94b	1.578198901	1.643372361	0.600600608	0.378790141	0.032826918	0.378790141	0.03050423	0.085226014
FBgn0051423	Ir94c	0.051563193	0.058046002	0.039245858	0.061879491	0.064351611	0.061879491	0	0.020883895
FBgn0259193	Ir94d	0.0787771	0.029560464	0	0.012605082	0	0.012605082	0	0
FBgn0259194	Ir94e	0	0	0	0	0	0	0	0
FBgn0051225	Ir94f	0	0	0	0	0	0	0	0
FBgn0039079	Ir94g	0.02856752	0	0	0	0.035652678	0	0	0
FBgn0039080	Ir94h	0	0	0.028266362	0	0	0	0	0

III. Chapter 5. The Role of Histone Deacetylase 6 in Synaptic Plasticity and Memory

Summary

Histone deacetylases (HDACs) have been extensively studied as drug targets in neurodegenerative disease models in connection with their role as chromatin modifiers. However, HDACs also have many non-histone targets and relatively little is known about the role of these proteins in healthy neurons. HDAC6 in particular is atypical because it contains two deacetylase (DAC) domains and is thought to reside primarily in the cytoplasm where it affects non-histone targets. In order to study the role of HDAC6 in the brain, we used a larval appetitive olfactory conditioning assay to test learning and memory and we find that HDAC6 is required for normal appetitive memory. Interestingly, *HDAC6* mutants acquire associative conditioning normally, but the memory is lost within 20 minutes of training, much more rapidly than wild-type animals. A similar phenomenon is observed in an adult appetitive-conditioning assay, which could suggest a shared biochemical deficit. Neuronal expression of *HDAC6* using a *UAS* construct rescued the ability to retain memory. A similar neuronal rescue using catalytically inactive constructs of HDAC6 revealed that only the first DAC domain is required for 20-minute memory. This suggests that deacetylation of synaptic targets associated with the first DAC domain such as Bruchpilot or cortactin, rather than tubulin, which is targeted by the second DAC domain, may be required for retention of appetitive memory. We also observe that HDAC6 mutants exhibit a partial block of homeostatic plasticity, suggesting that HDAC6 may be a regulator of synaptic plasticity in general and the learning deficit in HDAC6 mutants could be the behavioral result of these synaptic defects.

Introduction

Neurodegenerative diseases are complex health issues that greatly affect the quality of human life. Among emerging treatments, drugs that serve as HDAC inhibitors (HDACi) have been found to be quite effective (Fischer et al., 2010; Sleiman et al., 2009; Vecsey et al., 2007). Histone deacetylases (HDACs) are a highly conserved class of chromatin modifiers with the ability to dramatically impact gene expression. Deacetylation of lysine residues on histone tails promotes compacted chromatin structure and results in transcriptional repression of large regions of the genome. Treatment by HDAC inhibitors is thought to relieve this repression by promoting a more relaxed chromatin state in these regions. This results in a shift in gene expression that can ameliorate neuronal senescence and improve the inclusion and breakdown of harmful protein aggregates associated with many neurodegenerative disorders (Babenko et al., 2012; Qureshi and Mehler, 2011).

While many of these studies suggest a promising role for HDAC inhibitors in diseased brains, it is not clear how the loss of HDAC function affects healthy neurons. Commonly used HDAC inhibitors, such as sodium butyrate and vorinostat (SAHA), target all zinc-dependent HDACs (up to 11 family members in mammals) regardless of their diverse roles in cellular processes (Bertrand, 2010). Despite their name, HDACs are able to target and regulate many non-histone proteins in both the nucleus and the cytoplasm and participate in a variety of cellular pathways (Bertrand, 2010; Cho et al., 2005). It is not fully understood how inhibition of HDACs impacts these aspects of cellular function.

The *Drosophila* model offers a relatively simple and effective approach to examine complex neuronal functions such as those driving learning and memory. To examine the role of individual HDAC family members in complex neuronal function, we performed RNAi knock-down of individual *Drosophila* HDACs and observed the effect on larval olfactory learning and memory. We find that pan-neuronal knock-down of *RPD3* or *HDAC6* causes a deficit in immediate-term memory. While *RPD3* has been previously reported to be involved in courtship memory (Fitzsimons and Scott, 2011), *HDAC6* has not yet been characterized as being involved in *Drosophila* learning and memory.

HDAC6 is thought to reside predominantly in the cytoplasm, where it is involved in numerous cellular functions. It is known to deacetylate multiple targets including tubulin, cortactin, Hsp90 (DmHsp83) and Bruchpilot (Hubbert et al., 2002; Kovacs et al., 2005; Miskiewicz et al., 2014; Zhang et al., 2007). It is also able to bind ubiquitin and regulate protein degradation as well as serving as a molecular scaffold for many additional protein complexes (Valenzuela-Fernández et al., 2008). *HDAC6* is both conserved and highly expressed in neurons in both flies and mammals, but is apparently not required for normal development (Du et al., 2010; Govindarajan et al., 2013). This suggests that it may have a role in higher functions in the mature brain.

In terms of neurodegenerative diseases, *HDAC6* appears to have diverse roles depending on the disease model. It can be neuroprotective in some contexts, such as promoting alpha-synuclein inclusion in a Parkinson's disease model (Du et al., 2010). Conversely, loss of *HDAC6* can slow tau-induced axon degeneration and rescue cognitive deficits in Alzheimer's disease models (Govindarajan et al., 2013; Xiong et al., 2013). These seemingly contradictory roles are likely due to *HDAC6*'s involvement in

multiple cellular pathways, and the effect of loss of function can vary greatly depending on the disease context.

While a great deal of work has been examining HDAC6 function in diseased neurons, relatively little has been done to look how it participates in healthy neuronal function. Given its conservation across animal taxa and high expression level in the mammalian mid-brain structures, HDAC6 is likely indispensable for some important neuronal functions. Using the genetically tractable *Drosophila* larval model, we demonstrate that deacetylase activity of HDAC6's first domain is required for proper memory retention. The mutants also show defects in homeostatic plasticity at the level of the synapse.

Results

In order to investigate the role of individual HDACs in a complex neuronal function, we chose to employ a *Drosophila* larval olfactory learning and memory model developed by (Gerber and Hendel, 2006; Gerber and Stocker, 2007; Hendel et al., 2005; Scherer et al., 2003). In this associative conditioning assay, one of two odors is presented to animals on a sucrose plate and a second odor on a plain agarose plate. This training is repeated for a total of three repetitions before animals are tested for their preference between the two odors. The reciprocal training is also performed and the resulting preference indexes are used to calculate a learning index (Figure 5.S1A, (Scherer et al., 2003)). Because some HDAC mutants (such as *RPD3* and *HDAC3*) are embryonic lethal, we chose an RNAi approach to knock down individual HDACs in post-mitotic neurons using the *Elav-Gal4* driver and transgenic RNAi constructs from the TRiP collection (Table 5.S1). Using this approach, we found that knock down of *RPD3* and

HDAC6 resulted in a reduction, but not a complete loss of larval immediate-term memory (Figure 5.1A). *RPD3* has previously been implicated in long-term courtship suppression memory (Fitzsimons and Scott, 2011), but *HDAC6* has not yet been characterized for memory deficits in *Drosophila*, so we chose to pursue it further.

Interestingly, *HDAC6* null (*HDAC6(KO)*) animals are adult-viable and apparently healthy (Du et al., 2010) so we examined them for memory deficits. We find that *HDAC6(KO)* larvae have immediate-term learning comparable to that of *wild-type*. However, while *wild-type* animals can retain some learning 10 and 20 minutes after training, *HDAC6* mutants perform poorly in 10 and 20 minute retention tests (Figure 5.1B). In the 20 minute memory test, *HDAC6(KO)* larvae show a clear deficit compared with wild-type (Figure 5.1C). Feeding wild-type animals with the zinc-dependent HDAC inhibitor SAHA also caused a similar effect: Immediate-term memory appeared unaffected, but memory assayed after 20 minutes was significantly reduced (Figure 5.1D).

We speculated that the poor memory retention observed in *HDAC6* mutants may be due to a consolidation deficit. In *Drosophila* adult olfactory associative conditioning, observed learning is supported by genetically distinct memory phases: Short-term memory is consolidated to more intermediate-term phases via two pathways: *amnesiac*-dependent anesthesia-sensitive memory (ASM) and *radish*-dependent anesthesia-resistant memory (ARM) (Folkers et al., 1993; Krashes and Waddell, 2008; Margulies et al., 2005; McGuire et al., 2005). These previous studies show that applying cold anesthesia to the animals at a particular point after training and prior to testing is disrupts the ASM portion of the memory trace, leaving only ARM observable. In order to test ARM in larvae, we anesthetized the animals for 2 minutes in ice water directly after

training and allowed them to recover on a plain agarose plate for 3 minutes before testing their odor preference. *Wild-type* animals retain some memory after cold anesthesia, but *HDAC6* mutant larvae do not (Figure 5.1G). We believe this larval cold-shock resistant memory is similar to the ARM memory that has been observed in adult aversive and appetitive assays (Folkers et al., 1993; Krashes and Waddell, 2008). These results indicate that *HDAC6* mutants are likely deficient in consolidation to anesthesia resistant memory.

To further understand its role in neurons, we wanted to characterize where *HDAC6* was expressed in the larval nervous system as well as what its potential targets might be. While many members of the HDAC family are known to regulate gene expression through deacetylation of lysine residues on histone tails, HDACs have many non-histone targets as well. This is especially relevant since *HDAC6* is thought to be primarily cytoplasmic, as observed in mammalian neurons and *Drosophila* cell lines (Cho et al., 2005; Govindarajan et al., 2013). However, its sub-cellular localization pattern has not been characterized *in vivo* in flies. To examine this, we used a transgenic line expressing a C-terminally GFP-tagged *HDAC6* under control of endogenous promoter sequences (Sarav et al., 2015) and looked at GFP expression in the larval central nervous system (CNS). *HDAC6*-GFP is expressed ubiquitously throughout the larval CNS to some degree. It appears to be highly expressed in the ring gland (Figure 5.2A) as well as the imaginal discs (not shown). Most GFP signal is observed in the soma outside of the nucleus labeled by anti-elav (Figure 5.2B, bottom arrow) as well as non-neuronal cells (Figure 5.2B, middle arrow). In a few cells, it can be observed localizing to the nucleus (Figure 5.2B, top arrow). Signal is also observable in the neuropil regions labeled by NC82 (anti-Bruchpilot), although it is more diffuse (Figure 5.2C). While

HDAC6-GFP is observable in most cells, it appears to be more abundant in larger non-*elav* labeled cells which are likely neuroblasts (Figure 5.2D). HDAC6-GFP can also be visualized in the adult CNS. While it appears to be fairly ubiquitous, it is most visible in axonal tracts surrounding neuropil regions (Figure 5.2E).

Although HDAC6 can deacetylate histone peptides in vitro (Cho et al., 2005), it appears primarily to be localizing to the cytoplasm in neurons, indicating that non-histone targets may be important during learning and memory. HDAC6 is the only HDAC family member with two deacetylase (DAC) domains as well as a ubiquitin binding domain (Figure 5.3A, (Du et al., 2010), reviewed in (Boyault et al., 2007)). The second DAC domain is thought to be devoted exclusively to tubulin deacetylation (Hubbert et al., 2002) while the first DAC domain is associated with all other targets such as Bruchpilot (flies), cortactin (mammals) and potentially histones (Figure 5.3A, (Kovacs et al., 2005; Liu et al., 2012; Miskiewicz et al., 2014; Zhang et al., 2007)). In order to understand which DAC domains are important for memory retention, we performed pan-neuronal (*elav-Gal4*) rescue of *HDAC6* in an *HDAC6* mutant background using a fully functional *HDAC6* construct as well as constructs lacking DAC activity in the first domain (*H237A*), second domain (*H664A*) or both domains (*H237A.H664A*). *HDAC6* is located on the first chromosome, so *HDAC6(KO)* females crossed to *wild-type* males produce a larval population comprised of heterozygous females and *HDAC6* null males. This population performed poorly in the 20 minute memory assay, so we expressed the various *HDAC6* transgenes in this *HDAC6* “reduced” background by crossing *HDAC6(KO)* females to *elav-Gal4>UAS-HDAC6* males and testing the resulting progeny.

Overexpressing the various *HDAC6* constructs in a *wild-type* background did not produce any 20 minute memory deficits (Figure 5.3B). When overexpressed in the *HDAC6* “reduced” background, only *wild-type HDAC6* and *HDAC6.H664A* (the first DAC domain intact) were able to rescue the 20 minute memory deficit (Figure 5.3C-D). Rescue using constructs lacking activity in the first DAC domain (*H237A* constructs) did not rescue 20 minute learning and still showed a learning deficit compared with overexpression controls (Fig 5.3C-D). This indicates that activity in the first DAC domain is most critical for 20 minute memory retention suggesting that HDAC6’s tubulin deacetylase activity is dispensable in this context and other first domain targets are most important.

In *Drosophila*, the only confirmed target of HDAC6’s first domain is Bruchpilot, an important member of the presynaptic active zone in neurons. Multiple Bruchpilot fibers form umbrella-shaped T-bar structures at active zones with their N-termini anchored to Cacophony calcium channels and the C-termini fanning into the cytoplasm where they can contact and tether vesicles (Fouquet et al., 2009; Kittel et al., 2006; Wagh et al., 2006). In *HDAC6* mutants, the C-terminal region of Bruchpilot becomes hyperacetylated, presumably by its acetyltransferase ELP3, resulting in narrower T-bar structures which contact further vesicles. Overexpression of HDAC6 results in expanded T-bar tops and increased vesicle tethering (Miskiewicz et al., 2014; Miśkiewicz et al., 2011). Because gain or loss of HDAC6 function is known to correlate with increased or decreased synaptic transmission at the neuromuscular junction (Miskiewicz et al., 2014), we speculated that HDAC6 may modulate synaptic transmission during synaptic plasticity processes such as those occurring during learning and memory. In order to investigate whether or not HDAC6 is involved in modulating synaptic function,

we chose to examine its role in homeostatic plasticity using the neuromuscular junction (NMJ) as a model synapse.

Reducing sensitivity of the post-synaptic muscle to glutamate released by the motor neuron by genetic loss of one of the glutamate receptor subunits is known to induce a compensatory increase in presynaptic release in motor neurons, restoring synaptic strength (Davis et al., 1998; DiAntonio et al., 1999; Petersen et al., 1997). This type of homeostatic plasticity can also be observed with pharmacological treatment by philanthotoxin (PhTx), which acutely blocks postsynaptic glutamate receptors and dramatically reduces the postsynaptic sensitivity to glutamate release (Frank et al., 2006). Following PhTx treatment, evoked responses (EJP) in wild-type animals is able to recover to baseline levels after approximately 10 minutes because of a homeostatic increase in presynaptic release (quantal content) (Figure 5.4A,C,D). However, in *HDAC6(KO)* animals, EJP amplitudes do not fully recover following PhTx application, indicating that these mutants are not able to respond normally to the homeostatic challenge (Figure 5.4B-D). The miniature excitatory junctional potentials (mEJP) and EJP amplitudes can be used to estimate quantal content (the number of vesicles released per stimulus) before and after PhTx treatment. In *wild-type* animals, PhTx causes an approximate doubling of the quantal content, which restores evoked response to baseline levels. In contrast, *HDAC6(KO)* animals show a small increase in quantal content that fails to restore EJP amplitudes to baseline levels (Figure 5.4E,F). This indicates that *HDAC6(KO)* mutants do not have the capacity to fully express homeostatic plasticity. While we cannot rule out HDAC6's interaction with other targets such as cortactin or even histones, our findings are consistent with previously published data.

Discussion

The understanding of HDACs in complex neuronal functions has thus far been mainly limited to their role as chromatin modifiers and modulators of gene expression. However, we are beginning to discover that many HDAC family members have non-histone targets and are likely involved in many diverse cellular processes. Here we find that HDAC6, a primarily cytoplasmic deacetylase is involved in retention and consolidation of associative olfactory memory. *HDAC6* mutants appear to have normal immediate-term memory but lose the learned response more rapidly than *wild-type* animals, over the course of 10 to 20 minutes. Furthermore, appetitive memory in *HDAC6* mutant larvae is highly sensitive to cold anesthesia, suggesting that HDAC6 is involved in anesthesia resistant consolidation of memory (ARM). While outside the scope of this study, it will be interesting to further understand the genetic interactions between *HDAC6* and other known *Drosophila* memory pathways.

Relatively little is known about the molecular mechanisms underlying anesthesia resistant memory. The only known classical mutation deficient in ARM is *radish* (Folkers et al., 1993), but more recently, knock-down of *Bruchpilot* in the adult mushroom bodies has also been observed to result in an ARM deficit suggesting that consolidation to ARM may be directly related to changes at active zones (Knapek et al., 2011). HDAC6 is thought to be *Bruchpilot*'s (BRP) only deacetylase and loss or gain of HDAC6 function is known to alter *Bruchpilot*'s acetylation levels and ability to tether vesicles and affect synaptic transmission (Miskiewicz et al., 2014). In the context of what we observe behaviorally, it's possible that HDAC6 is required biochemically during memory consolidation to deacetylate BRP and strengthen key synapses. It has not escaped our

attention that HDAC6 may be required for the ability of the active zone to homeostatically modulate presynaptic release. Indeed, modulation of BRP via acetylation has recently been implicated in the mechanisms driving synaptic homeostasis at the fly NMJ (Tsurudome et al., 2010; Weyhersmüller et al., 2011) (Figure 5.5).

Furthermore, when we employed a homeostatic model of presynaptic strengthening to examine rapid, PhTx-induced plasticity using electrophysiology, we observe that *HDAC6* mutants are unable to fully compensate for the homeostatic challenge. The molecular events underlying plasticity at the NMJ could be similar to those required to effect plasticity in other synapses, such as those involved in learning and memory. Our findings could also provide a connection between synaptic plasticity and behavioral plasticity.

HDAC6 is highly conserved from flies to mammals and it is thought to interact with similar targets across taxa. While mammals do not have a direct ortholog of Bruchpilot, it is conceivable that HDAC6 may have other synaptic targets in mammals and participate in modulation of synaptic plasticity. In addition, HDAC6 has other substrates and many additional interaction partners. It has the potential to regulate cytoskeleton dynamics, affect gene expression and influence protein degradation. All of these interactions could also be important for healthy neuronal function.

Materials and Methods

Fly stocks

Unless otherwise stated, all *Drosophila* used in this study were reared on standard cornmeal-dextrose media at 25C. A *white*¹¹¹⁸ backcrossed to *Canton-S* line (*wCS*) line was used as the *wild-type* control for all behavior experiments. A complete list of mutant and transgenic lines can be found in Table 5.S1. Naïve responses to odors and sucrose for selected genotypes can be found in Table 5.S2.

SAHA treatment

SAHA (Sigma-Aldrich) was dissolved at 50mM in DMSO and diluted 1:1000 into molten fly food for a final concentration of 50uM. Larvae were grown in treated media from egg to third instar (about 5 days) before being used for behavior experiments. DMSO at 1:1000 in food was used as a control.

Larval appetitive olfactory learning assay

See Figure S1A. The larval learning assay in this study was adapted from a well-established protocol (Scherer et al., 2003). Approximately 30-50 third instar larvae were used for each plate test. 2M Sucrose agarose plates were used for the unconditioned stimulus. Odors Isoamyl acetate (AM, 2% in Paraffin oil, Fluka) and 1-octanol (OCT, pure, Acros Organics) were presented in inverted 200uL PCR tube caps containing 10uL solution each. Larvae were maintained on each training and test plate for 3 minutes. For 10 and 20 minute memory tests, larvae were housed in an empty petri dish containing a small amount of water for the indicated amount of time between training and testing. All larval behavior experiments were carried out at 25C in $\geq 30\%$ humidity.

Preference index (PI) is calculated as: (number of larvae on AM side – number of larvae on OCT side) / (total number of larvae on both sides) and ranges from 1 (perfect attraction to AM) to –1 (perfect attraction to OCT). Normalized AM learning index is calculated as: (AM PI – Average OCT PI)/2. Normalized OCT learning index is calculated as: (Average AM PI – OCT PI)/2. Normalized AM and OCT learning indexes are averaged for the learning index.

Adult appetitive olfactory learning assay

See Figure S1B. The adult learning assay in this study was adapted from a well-established protocol (Krashes and Waddell, 2008). For each trial, 50 flies (25 males and 25 females, 3–7 days old) were starved with access to water (40 hours for *wild-type*, 36 hours for *HDAC6(KO)*). Flies were trained in 50mL culture vials. 10uL of the odor solution was presented on filter paper and sucrose was presented dried on filter paper. After training, flies were tested for their odor preference in a T-maze apparatus. The arms of the T-maze were 15 ml culture tubes, one containing 4-methylcyclohexanol (MCH, Sigma-Aldrich) and the other containing 3-octanol (OCT, Sigma-Aldrich). No air flow was used. Both odors were diluted to 0.2% in paraffin oil and 10uL of the solution was applied to a small disc of filter paper inserted into the test arm of the T-maze 5 minutes before each trial. For 20 minute memory tests, 3 rounds of training were used and then flies were returned to their starvation vials for 20 minutes between prior to testing. All adult behavior experiments were carried out at 25C in $\geq 30\%$ humidity. Preference index (PI) is calculated as: (number of flies in MCH arm – number of flies in OCT arm) / (total number of flies in both arms) and ranges from 1 (perfect attraction to MCH) to –1 (perfect attraction to OCT). Normalized MCH learning index is calculated

as: $(\text{MCH PI} - \text{Average OCT PI})/2$. Normalized OCT learning index is calculated as: $(\text{Average MCH PI} - \text{OCT PI})/2$. Normalized MCH and OCT learning indexes are averaged for the learning index.

Immunohistochemistry

Brain tissue antibody staining was performed as previously described (Kain et al., 2013). The primary antibodies used were mouse anti-nc82 (1:10, DSHB), rat anti-elav-7E8A10 (1:5, DSHB) and rabbit anti-GFP (1:500, Invitrogen). The secondary antibodies used were goat anti-rabbit-488, goat anti-rat-568 and goat anti-mouse-647 (1:200, Invitrogen). Images were taken using a Leica SP-5 inverted confocal microscope and processed using Image J.

Electrophysiology

NMJ electrophysiology was performed as previously reported (Dickman and Davis, 2009; Frank et al., 2006). All dissections and recordings were performed in modified HL3 saline containing (in mM): 70 NaCl, 5 KCl, 10 MgCl₂, 10 NaHCO₃, 115 Sucrose, 5 Trehelose, 5 HEPES, and 0.4 CaCl₂ (unless otherwise specified), pH 7.2. Neuromuscular junction sharp electrode (electrode resistance between 10-35 MΩ) recordings were performed on muscles 6 and 7 of abdominal segments A2 and A3 in wandering third-instar larvae. Larvae were dissected and loosely pinned; the guts, trachea, and ventral nerve cord were removed from the larval body walls with the motor nerve cut, and the preparation was perfused several times with HL3 saline. Recordings were performed on an Olympus BX51 WI microscope using a 40x/0.80 water dipping objective. Recordings were acquired using an Axoclamp 900A amplifier, Digidata 1440A acquisition system and pClamp 10.5 software (Molecular Devices). Electrophysiological

sweeps were digitized at 10kHz, and filtered at 1kHz. Data were analyzed using Clampfit (Molecular devices), MiniAnalysis (Synaptosoft) and Excel (Microsoft) software.

Miniature excitatory junctional potentials (mEJPs) were recorded in the absence of any stimulation, and cut motor axons were stimulated for 3 msec to elicit excitatory junctional potentials (EJPs). An ISO-Flex stimulus isolator was used (A.M.P.I.) to modulate stimulatory currents. Intensity was adjusted for each cell, set to consistently elicit action potentials in both axons innervating the muscle segment, but avoiding overstimulation. Average mEJP, EJP, and quantal content were calculated for each genotype with corrections for nonlinear summation (Martin, 1955). Muscle input resistance (R_{in}) and resting membrane (V_{rest}) potential were monitored during each experiment. Recordings were rejected if the V_{rest} was above -60 mV, if the R_{in} was less than 5M Ω , or if either measurement deviated by more than 20% during the experiment. Larvae were incubated with or without philanthotoxin-433 (Sigma; 20 μ M) resuspended in HL3 for 10 mins, similar to previously described (Frank et al., 2006).

Figure 5.1. HDAC6 is required for retention of olfactory memory. **A.** Larval learning indexes for control and HDAC-RNAi animals after training. All larvae tested are *UAS-DCR2/+; Elav-Gal4/{UAS-RNAi}attP2* with RNAi constructs from the TRiP collection. Control larvae have an empty attP2 site derived from the TRiP donor line. (N=12-14) . **B.** Larval preference for AM vs OCT (1 = all prefer AM; -1 = all prefer OCT) in *wild-type* and *HDAC6(KO)* larva 0 minutes, 10 minutes and 20 minutes after training. (N=10-12) (Box and whisker plots represent minimum, 1st quartile, median, 3rd quartile and maximum) **C.** Larval learning Indexes calculated from the AM+ and OCT+ preferences shown in (A). **D.** Immediate and 20 minute learning indexes of larvae treated with either solvent (DMSO) or 50 uM HDAC inhibitor (SAHA). (N = 12,12) **E.** Adult preference for MCH vs OCT (1 = all prefer MCH; -1 = all prefer OCT) in *wild-type* and *HDAC6(KO)* adult flies in the immediate and 20 minute tests. (N=9-12) **F.** Adult learning Indexes calculated from the MCH+ and OCT+ preferences shown in (B). **G.** (left) Preference for AM vs OCT in *wild-type* and *HDAC6(KO)* larva after 2 minute cold anesthesia applied directly after training. (N=12) (right) Larval learning Indexes calculated using these AM+ and OCT+ preferences. Error bars represent +- s.e.m. 2-tailed Mann-Whitney U test was used for statistical analysis. * p < 0.05; ** p < 0.01; *** p < 0.001; ****p < 0.0001

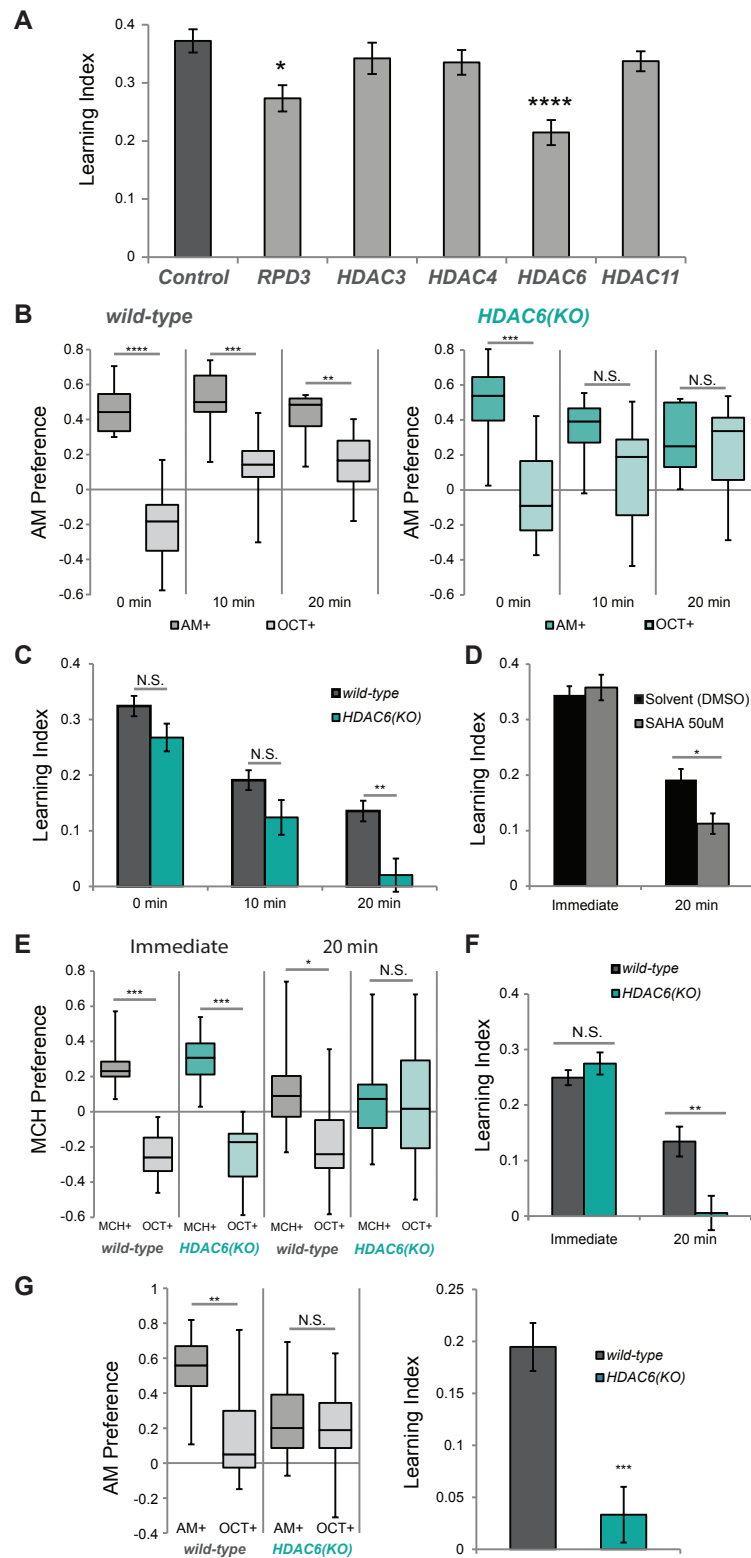


Figure 5.1

Figure 5.2. HDAC6 localizes largely to the cytoplasm in the nervous system. **A.** Images showing whole mount antibody staining of the larval central nervous system (CNS) from an *HDAC6-GFP* transgenic animal (*VK00033{HDAC6-2XTY1-SGFP-V5-preTEV-BLRP-3XFLAG}*). The Z-stack is shown in three portions: Dorsal (left), medial (middle) and ventral (right). Anti-Bruchpilot (nc82) is shown in red; anti-GFP (labeling HDAC6-GFP) is shown in green. **B.** Images showing whole mount antibody staining of larval ventral nerve cord (VNC) from an *HDAC6-GFP* transgenic animal. Anti-GFP (labeling HDAC6-GFP) is shown in green; anti-Elav (labeling neuronal nuclei) is shown in blue. White arrows indicate example cell bodies: Neuron with nuclear HDAC6 signal (top); non-neuronal cell with cytoplasmic HDAC6 (middle); Neuron with cytoplasmic HDAC6 (bottom). **C.** Images showing whole mount antibody staining of the larval central brain neuropil region from an *HDAC6-GFP* transgenic animal. Anti-Bruchpilot (nc82) is shown in red; anti-GFP (labeling HDAC6-GFP) is shown in green. **D.** Images showing whole mount antibody staining of the larval optic lobe from an *HDAC6-GFP* transgenic animal. Anti-GFP (labeling HDAC6-GFP) is shown in green; anti-Elav (labeling neuronal nuclei) is shown in blue. White arrows indicate example neuroblasts. **E.** Images showing whole mount antibody staining of the adult central nervous system (CNS) from an *HDAC6-GFP* transgenic animal. Anti-Bruchpilot (nc82) is shown in red; anti-GFP (labeling HDAC6-GFP) is shown in green.

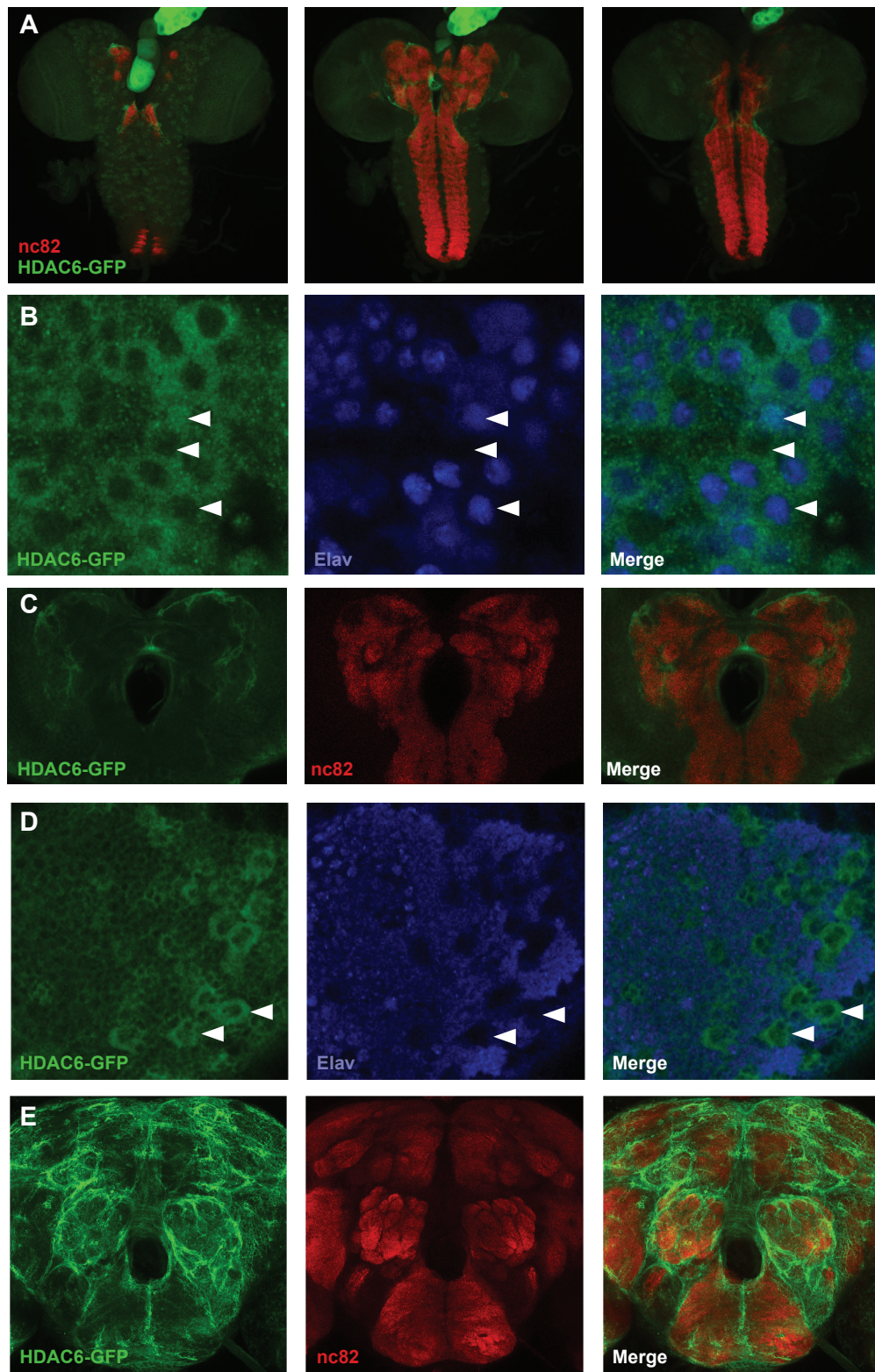


Figure 5.2

Figure 5.3. The deacetylase activity of HDAC6's first domain is required for normal memory retention. **A.** Schematic showing the functional domains of HDAC6. Critical active site residues for the deacetylase (DAC) domains are shown above and known targets of HDAC6 are shown below. Adapted from (Du et al., 2010), reviewed in (Boyault et al., 2007) . **B.** Larval preference for AM vs OCT (1 = all prefer AM; -1 = all prefer OCT) after 20 minutes in *wild-type* and *HDAC6* overexpression controls. (N = 10 - 12) (Box and whisker plots represent minimum, 1st quartile, median, 3rd quartile and maximum) **C.** Larval preference for AM vs OCT for *HDAC6* overexpression in the *HDAC6* reduced background after 20 minutes. (N= 12 – 16) **C.** Larval 20 minute learning Indexes calculated from the AM+ and OCT+ preferences shown in (B,C). Error bars represent +/- s.e.m. 2-tailed Mann-Whitney U test was used for statistical analysis. * p < 0.05; ** p < 0.01

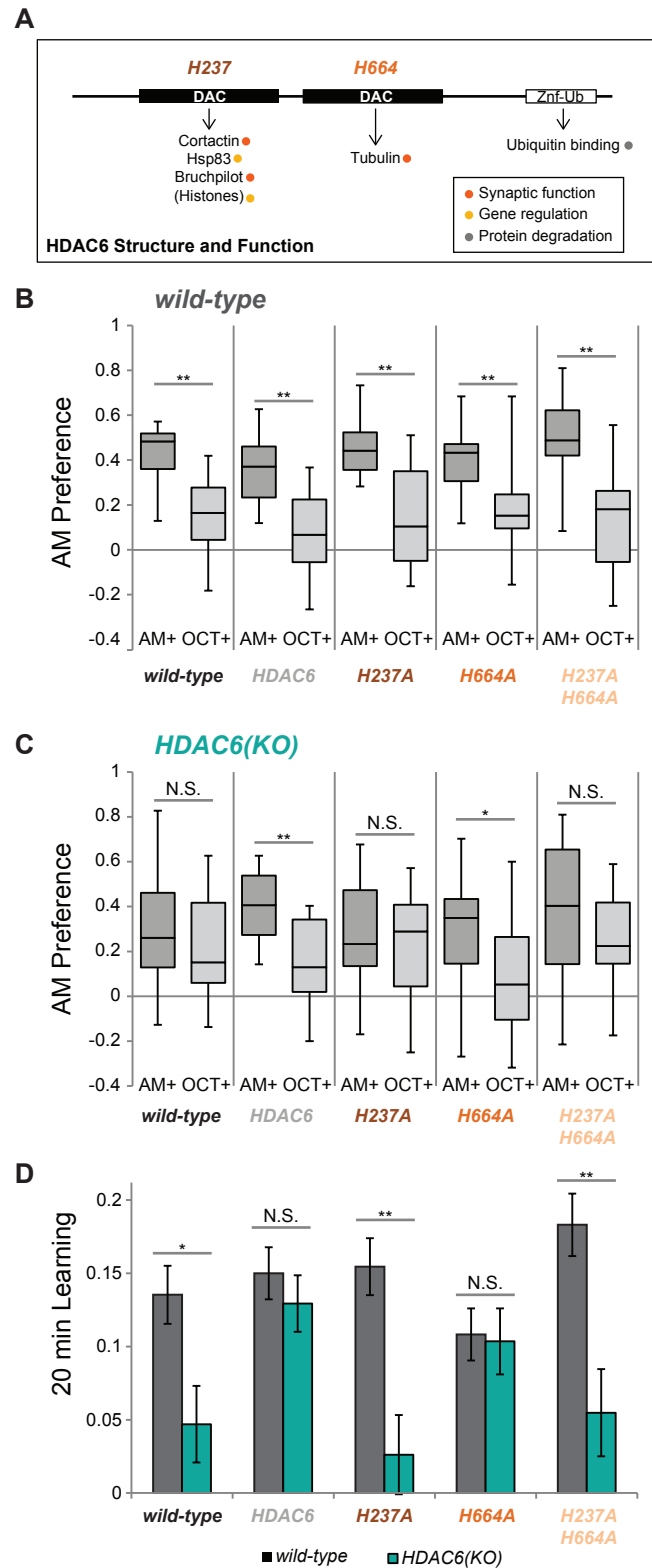


Figure 5.3

Figure 5.4. HDAC6 mutants exhibit a partial block in homeostatic plasticity at the larval neuromuscular junction. **A.** Representative electrophysiological traces showing evoked excitatory junctional current (EJC) and mini excitatory junctional currents (mEJCs) from *wild-type* larva before and after PhTx treatment. **B.** Representative traces from *HDAC6(KO)* larva before and after PhTx treatment. **C.** Average mEJC amplitudes in *wild-type* and *HDAC6(KO)* larva with and without PhTx treatment. **D.** Average EJC amplitudes in *wild-type* and *HDAC6(KO)* larva with and without PhTx treatment. **E.** Estimated quantal content in *wild-type* and *HDAC6(KO)* larva with and without PhTx treatment. **F.** mEJC amplitude and quantal content in *wild-type* and *HDAC6(KO)* larva following PhTx treatment shown as percent base line. (N = 10-14) Error bars represent $\pm$ s.e.m.

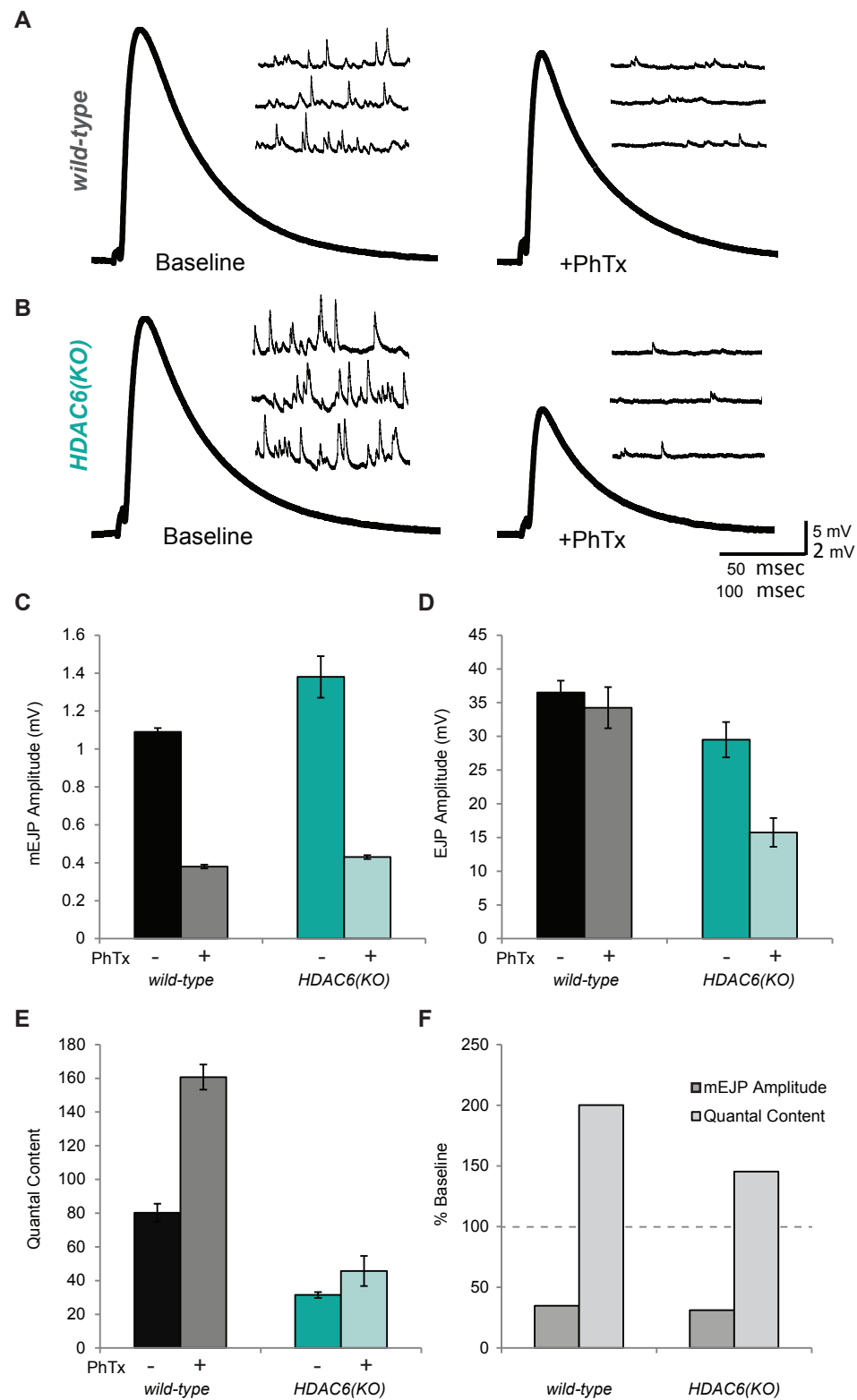
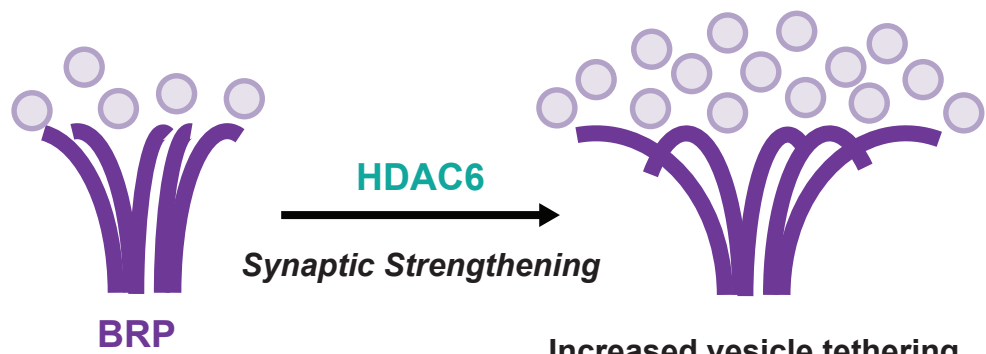


Figure 5.4

Figure 5.5. Model for HDAC6 activity at active zones during synaptic strengthening. **A.** In *wild-type* animals, HDAC6 may deacetylate BRP C-terminal domains to increase vesicle tethering during synaptic strengthening. This would result in increased quantal content and increased transmission at these synapses. **B.** In *HDAC6* mutant animals, BRP would not be deacetylated during synaptic strengthening. This may result in a poor increase in quantal content and a deficit in plasticity.

A *wild-type*



B *HDAC6-*

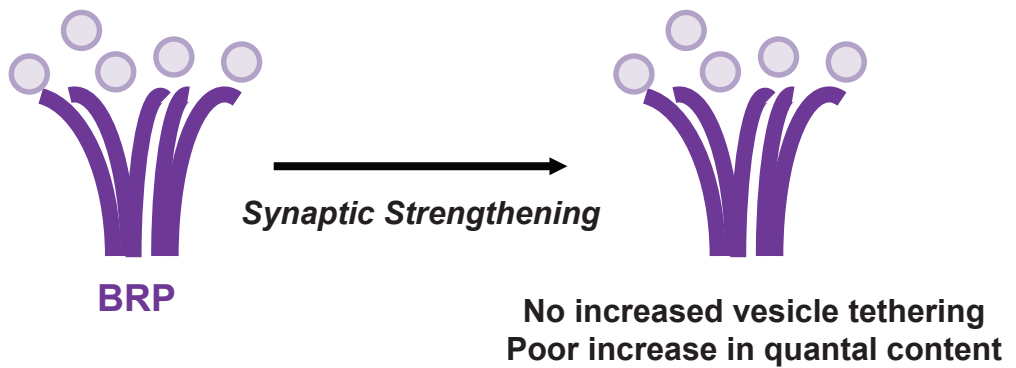


Figure 5.5

Figure 5.S1. Learning assay schematics and supplemental data. **A.** Schematic showing larval appetitive olfactory conditioning assay. One of two odors AM (isoamyl acetate) or OCT (1-octanol) is paired with sucrose. Larval are tested for their preference for AM vs OCT immediately after training. This assay has been adapted from (Scherer et al., 2003). **B.** Schematic showing adult appetitive olfactory conditioning assay. One of two odors MCH (4-Methylcyclohexanol) or OCT (3-octanol) is paired with sucrose. Adults are either tested for their preference for MCH vs OCT immediately after training, or housed in starvation vials until 20 minutes after training. This assay has been adapted from (Keene et al., 2006) **C.** Immediate larval learning indexes of the genotypes shown in Figure 3C. Error bars represent $\pm$ s.e.m.

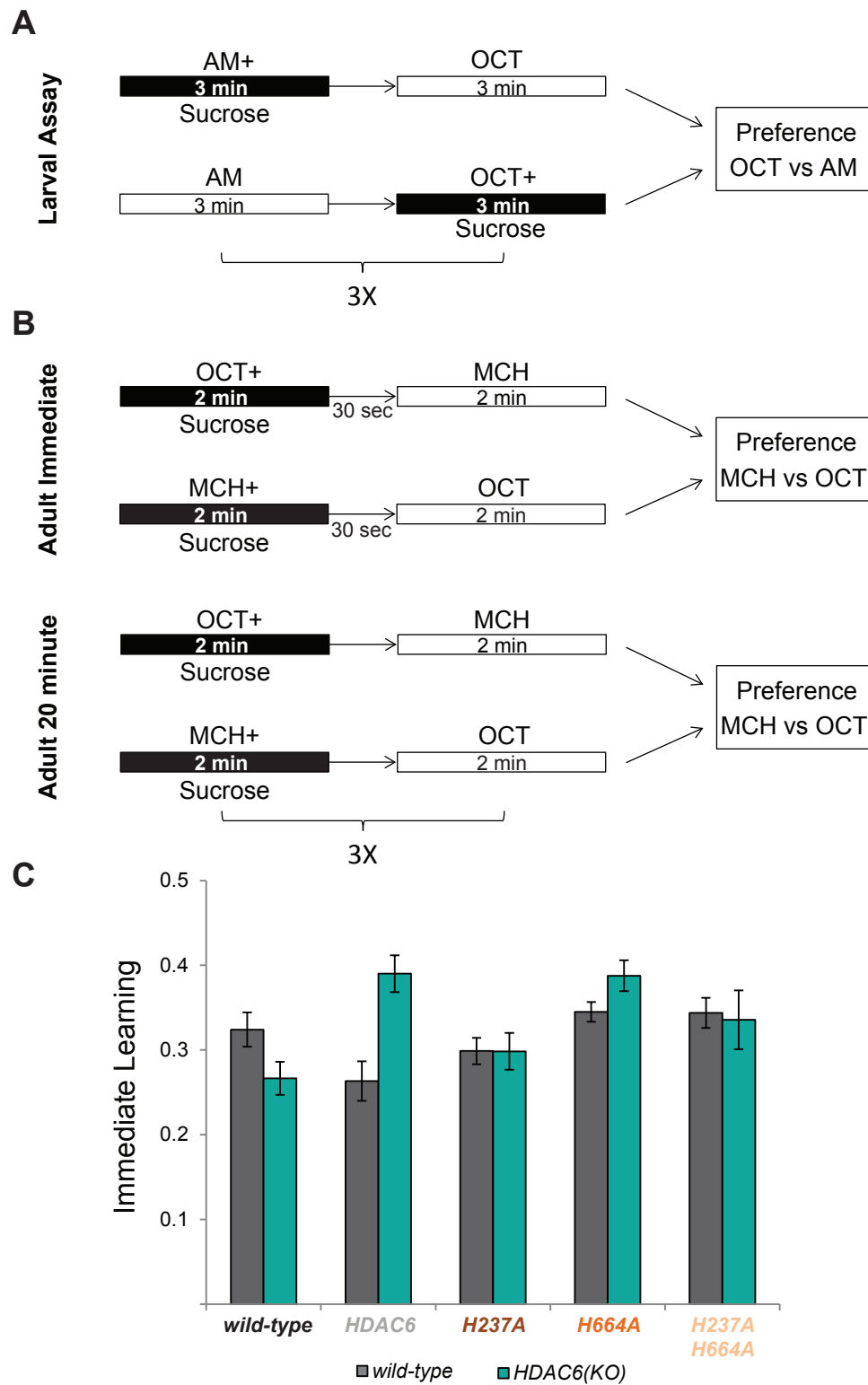


Figure 5.S1

Table 5.S1. Fly stocks used

Genotype	Source	Stock Number
wild-type (w Canton S)	lab stock	N/A
HDAC6(KO)	Bloomington	51182
UAS-HDAC6	Bloomington	51181
UAS-HDAC6.H237A	Bloomington	51183
UAS-HDAC6.H664A	Bloomington	51184
UAS-HDAC6.H237A.H664A	Bloomington	51185
Elav-Gal4	Bloomington	8760
UAS-DCR2	Bloomington	24650
TRiP attP2 background line	Bloomington	36303
{RPD3-RNAi}attP2	Bloomington	31616
{HDAC3-RNAi}attP2	Bloomington	31633
{HDAC4-RNAi}attP2	Bloomington	28549
{HDAC6-RNAi}attP2	Bloomington	31053
{HDAC11-RNAi}attP2	Bloomington	32480
HDAC6-GFP	VDRC	318243

Table 5.S2 Odor and sucrose acuity tests for selected genotypes

Genotype	Learning Index		AM Preference		OCT preference		Sucrose Preference	
	AVG	SEM	AVG	SEM	AVG	SEM	AVG	SEM
wCS	0.32415	0.01836	0.2229	0.12389	0.15559	0.05598	0.34735	0.07766912
HDAC6(KO)	0.26767	0.02486	0.24388	0.06373	0.37035	0.03083	0.18831	0.10764921
HDAC6(KO) X wCS	0.26664	0.01961	0.2559	0.09015	-0.0183	0.07348	0.39557	0.07997843
wCS X ElavG4, UAS-HDAC6	0.26335	0.02341	0.55472	0.07245	0.05013	0.07497	0.59129	0.01661686
HDAC6(KO) X ElavG4, UAS-HDAC6	0.39014	0.02174	0.35157	0.06959	0.29076	0.08842	0.6185	0.08980798
DCR2; ElavG4 X TRiP control	0.37226	0.01988	0.37177	0.13693	-0.0799	0.15601	0.29827	0.07956409
DCR2; ElavG4 X HDAC6-RNAi	0.21455	0.02155	0.34432	0.05932	-0.0958	0.11645	0.22819	0.03484299

Appendix I. Additional data related to HDAC6

Summary

This section contains data related to HDAC6 that we chose not to include in the main chapter. It may, however, be useful as preliminary data for future studies.

Figure AI.1 summarizes the adult long-term memory data acquired using the assay described in Appendix IIB. Because this assay has not been completely validated with known memory mutants, we do not believe it to be publication-ready. However, it appears to work reasonably well for the fly lines shown here and we were not able to effectively use the long-term memory assays already established in the field (Krashes and Waddell, 2008) so this provides an interesting look into the role of HDAC6 in adult long-term memory. The *HDAC6* null line (*HDAC6(KO)*), as well as two transgene disruption lines (*HDAC6^{MB06564}* and *HDAC6^{EY09607}*) show disrupted appetitive long-term memory in this assay, which could be consistent with its role in an anesthesia-resistant memory pathway (Krashes and Waddell, 2008).

Figure AI.2 summarizes larval experiments in which we investigated whether or not loss of *ELP3* function could rescue the larval memory deficits caused by loss of *HDAC6*. *ELP3* is the acetyltransferase which opposes HDAC6's deacetylation of Bruchpilot (BRP) at presynaptic active zones (Miskiewicz et al., 2014; Miśkiewicz et al., 2011). We hypothesized that if the level of BRP acetylation is important in synaptic and behavioral plasticity, reducing *ELP3* in the *HDAC6*-reduced background may rescue some of the larval memory deficits. This does not appear to be the case, at least using this genetic interaction approach. It may be that only sufficient dosage HDAC6's catalytic activity is required to achieve the molecular events needed for memory retention, regardless of

BRP's initial acetylation level. Because this is a negative result, we cannot conclude anything concretely but this information may be useful if we choose to further investigate the interaction between *HDAC6* and *ELP3*.

Figure AI.1. HDAC6 disruption lines exhibit a long-term memory deficit.

A. RPKM values for the *Drosophila* zinc-dependent HDACs from adult brain RNA-seq transcriptome analysis (Ray lab, unpublished data). *HDAC6* shows robust expression levels. **B.** RT-PCR from male and female head cDNA of *wild-type* (*wCS*), *HDAC6* gene disruption lines *HDAC6*^{MB06564} (BL#27744) and *HDAC6*^{EY09607} (BL#17585), and *HDAC6* null line *HDAC6(KO)* (BL#51182). *Syt-1* was used as a house-keeping control. The *HDAC6*^{MB06564} line shows low to no *HDAC6* expression (likely hypomorphic). *HDAC6*^{EY09607} shows irregular expression, possibly as a result of the transgene insertion directly in front of the coding region. *HDAC6(KO)* shows no expression, as previously reported (Du et al., 2010; Miskiewicz et al., 2014). **C.** Benzaldehyde preference for the indicated genotypes 24 hours after sucrose-only (control) or benzaldehyde+sucrose (paired) training (See Appendix IIB for assay details). *Wild-type* lines *wCS* and *w*¹¹¹⁸ show increased benzaldehyde preference after paired training indicating 24 hour memory formation. All three *HDAC6* mutant lines show no significant difference between control and paired trainings indicating a possible long-term memory deficit. 0.4% benzaldehyde was used for *HDAC6(KO)* flies (0.1% and 0.2% were also tested and no training was observed). 0.1% was used for all other genotypes. (N = 10 – 18) **D.** Learning indexes calculated from the preference index data shown in (C). LI = (benz+suc PI)/(average suc-only PI). Error bars represent $\pm$ s.e.m. A two-tailed Mann-Whitney U test was used for statistical analysis. ** $p < 0.01$

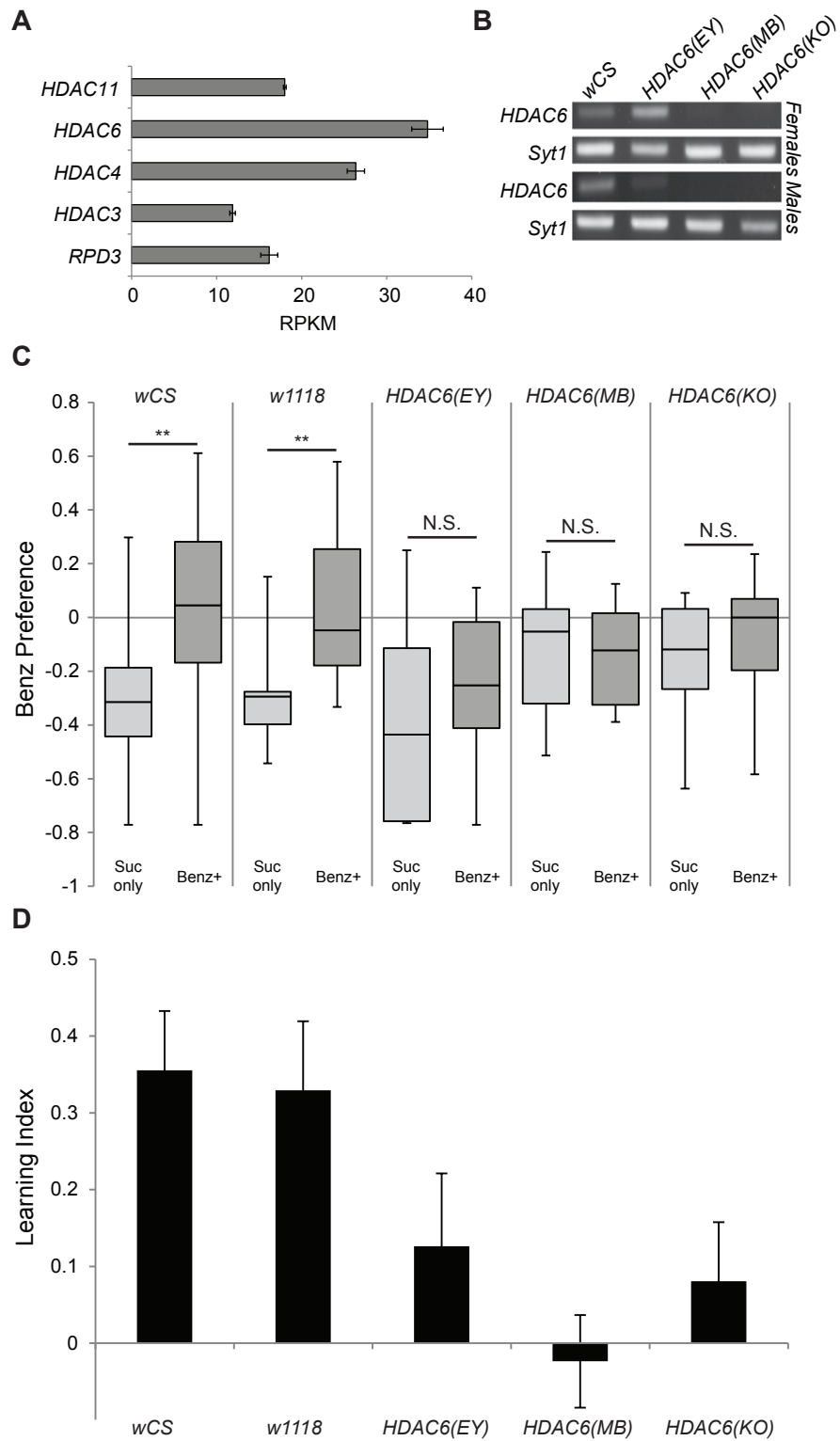


Figure AI.1

Figure Al.2. Loss of one copy of ELP3 does not rescue larval memory deficits in the HDAC6(KO) mutant background.

A. Immediate-term larval memory for larval progeny from the following crosses: “*wild-type*, +/+” (*wCS*); “*wild-type*, *ELP3*-/+” (*wCS* females x *ELP3*^{Δ4}/*Cyo,Tb* males, non-tubby larvae only); “*HDAC6*(*KO*), +/+” (*HDAC6*(*KO*) females x *wCS* males, female larvae are *KO*/+ and males are *KO*); “*HDAC6*(*KO*), *ELP3*-/+” (*HDAC6*(*KO*) females x *ELP3*^{Δ4}/*Cyo,Tb* males, non-tubby larvae only). Loss of one copy of *ELP3* in the *HDAC6*-reduced background does not affect immediate-term memory performance. (N = 10 – 12) **B.** 20-minute larval memory for the same crosses described in (A). Loss of one copy of *ELP3* in the *HDAC6*-reduced background does not rescue the 20-minute memory deficit. Loss of one copy in a *wild-type* background may cause a deficit. (N = 10 – 12) **C.** Larval memory following cold-shock. Loss of one copy of *ELP3* in the *HDAC6*-reduced background does not rescue the cold-shock memory deficit. (N = 10 – 12) Error bars represent ± s.e.m. *ELP3*^{Δ4}/*Cyo,Tb* flies were provided by Dion Dickman by permission from Patrik Verstrekin (Miśkiewicz et al., 2011).

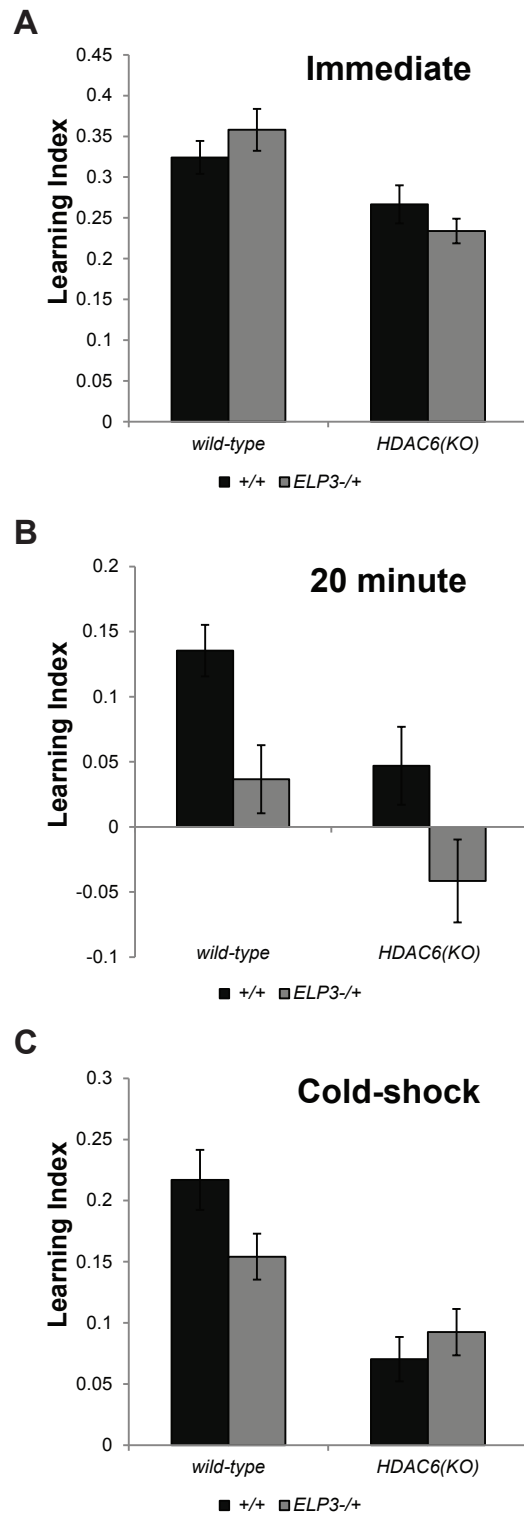


Figure AI.2

Appendix IIA. RNA *in situ* hybridization for whole-mount antenna

- Use DIG-RNA probes, ~900bp complementary to your transcript (It is not necessary to hydrolyze or fragment them).

Fixative

10 mL 16% paraformaldehyde
4 mL 10X PBS
26 mL RNase-free water
20 µL Tween 20

Hybridization Buffer

250mL formamide
125mL 20X SSC
833uL Heparin (30mg/mL)
5mL Tween 20 (10%)
Yeast tRNA to 100ug/mL
DEPC water to 500mL

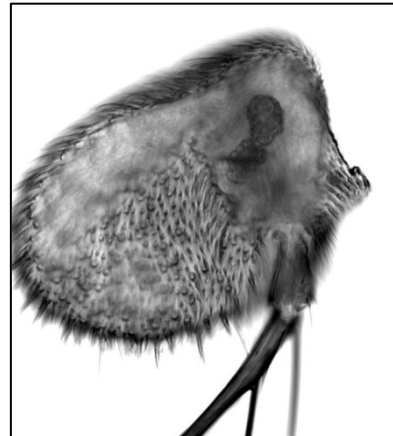
PBST

4 mL 10X PBS
36 mL RNase-free water
40 µL Tween 20

PTX

4 mL 10X PBS
36 mL RNase-free water
400 µL Triton

Anti-Dig-AP: Roche #11093274910
FAST RED tablets: Roche #11496549001



Day 1

1. Anesthetize flies on ice. Dissect off heads into cold fixative. 20 heads/sample works well. (I usually use 1.2 mL screw-cap tubes)
2. Fix 1 hour at room temperature on the nutator (end-over-end rotation)
3. Wash 3X 10 min in PBST at RT. (I usually do 500 µL washes)
4. Dissect off 3rd segment of the antennae into cold fix.
 - a. Leave heads in final PBST wash and place on ice
 - b. Use a P1000 to put ~5 heads and some liquid onto a glass slide
 - c. Use a sharpened pair of forceps to grip the second segment of the antennae. You can do this while it is on the head or you can take the antennae off first.
 - d. While gripping the second segment using your non-dominant hand, use as brush (or another pair of forceps) in your other hand to wiggle the third

segment off without damaging it. Ideally, it should just tear or break at the “neck” allowing an opening for staining (See picture).

- e. Place the third segment into a fresh tube of cold fix. Check to make sure antennae is not still stuck to brush.
- You can take the third segment off however you want, but it needs to be completely detached from the second segment and undamaged.
5. Fix 30 minutes at RT on nurator.
6. Wash 3X 10 minutes in PTX. (This can be done at RT or 4C. All washes should be done with end-over-end rotation. To remove a wash, pulse tubes in microfuge to push antennae to the bottom. Use a P200 to carefully pipette off wash. I then dispense the wash into a petri dish under the dissecting scope and replace any antennae that get sucked off.)
7. Remove the last wash and add pre-warmed Hybridization Buffer (HB). Pre-hyb 1-2 hours in 55C oven with end-over-end rotation.
8. Prepare probe by diluting 1:25 in HB (The dilution factor depends on how well the DIG has been incorporated during probe synthesis. This can be estimated by doing a spot test after synthesis. In some cases, probes can be used at 1:50 or 1:100. It's helpful to do a control staining with a new probe batch to check its efficiency). Heat 5 minutes at 80C. vortex. Cool on ice 5 minutes.
9. Remove pre-hyb HB and add probe solution. Incubate overnight in 55C oven.

Day 2

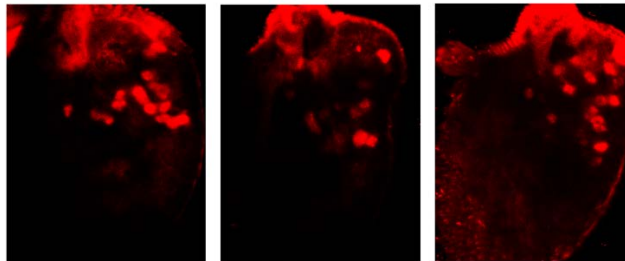
1. Wash 4-5X in HB at 55C. Leave in last wash overnight.

Day 3

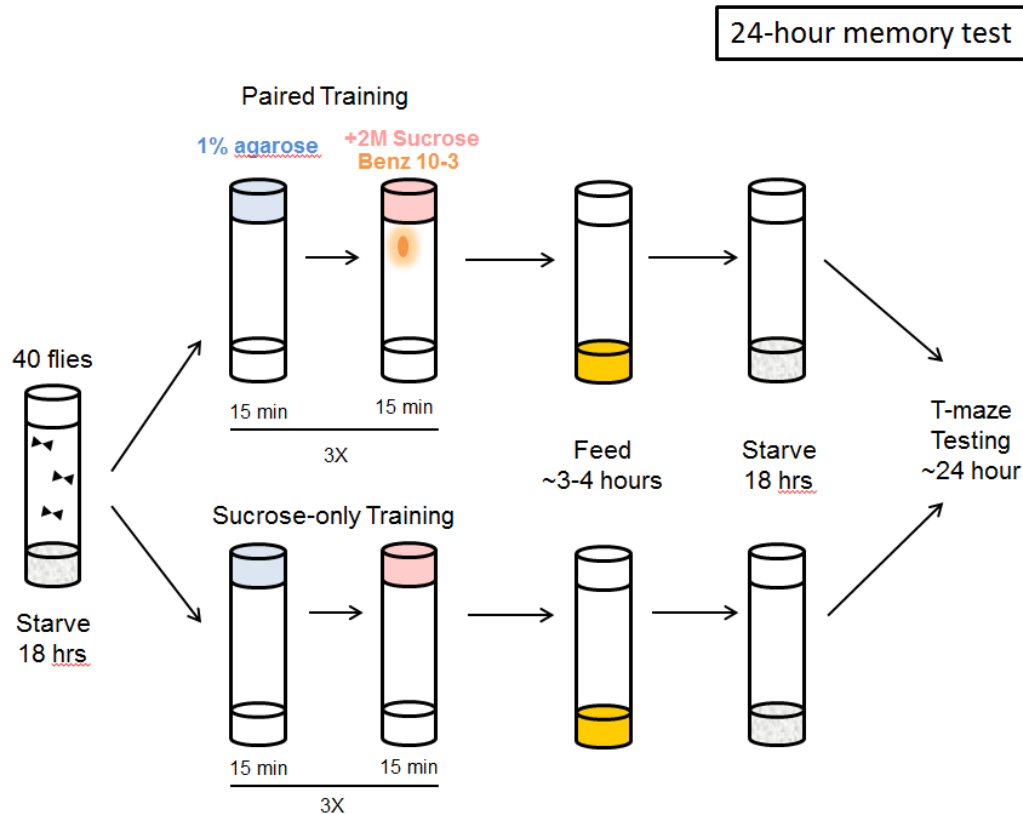
1. Wash 1X 20 min in HB at 55C.
2. Wash 3X 10 min in PBST at RT.
3. Prepare Anti-DIG 1:500 in PBST + 1X BSA.
4. Incubate in Anti-DIG 3 hours at RT.
5. Wash 3X 10 min in PBST at RT.
6. Dissolve 1 FAST RED tablet in ~2.5-3 mL 0.1M Tris-HCL pH 8.2. (wrap tube in foil)
7. Rinse sample in 0.1 M Tris
8. Incubate in FAST RED solution 30 min at RT on nurator. (wrap tubes in foil)
9. Repeat 6-8 with fresh FAST RED.
10. Rinse 3X 10 minutes in PBST at RT. (wrap tubes in foil)
11. Resuspend in 80% glycerol. Store in the dark at 4C. (I like to let my samples sit in glycerol overnight instead of mounting them right away. It makes them swell up a little and I get better pictures. Samples can be stored like this for several months)
12. To mount, pipette antennae in glycerol onto microscope slide (about 20 µL makes a good mount). Put tiny clay “feet” on a coverslip and gently tap onto slide until glycerol touches the glass. Be careful not to squish antennae. You do

not need to position samples with forceps because they lay down flat on their own. Imaging from the bottom or the top does not seem to make a difference.

13. Image using the 543 laser and 560 LP filter (This is for a Zeiss LSM 560, but most confocals will work). The cuticle will have high background, but turn up the gain and look for little round cell bodies inside. This works fantastically well for probes that stain a subset of neurons like probes against *Or* transcripts. If you're staining for something with a more diffuse expression pattern, I would suggest using sense probe to prepare a negative control. Stain the two sample sets concurrently and image them using the same confocal settings to look for a difference in staining intensity. Take 2 micron slices.



Appendix IIB. 24-hour benzaldehyde appetitive learning assay



For each genotype or condition to be tested, you should have $N = 10$ for paired conditioning and $N = 10$ for control training. These N need not be done all at once – it's advisable that all the N for a particular condition be done over at least 2 training sessions (for example, 4 paired and 4 control one session; 6 paired and 6 control in a second session).

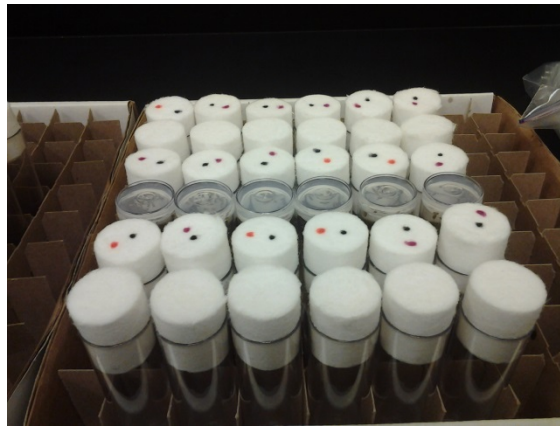
For each N , sort 40 flies into each vial at least 24 hours before you plan to starve them.

Flies should be starved at 25°C for 18 hours before the beginning of training (18 hours is ideal, but up to 20 hours is permissible). I typically perform training between 7 and 11 am, so flies should be starved the afternoon before. Flies should be wet starved in clean vials with two crumpled up Kim wipes and 5 mL of Milli-Q or DI water.

Training vials should be prepared either the night before or the morning of (it's possible to make them further ahead of time and store them in plastic bags at 4°C, but I typically don't do this). For each N , you will need to make 3 agarose vials (1% agarose in Milli-Q water) and 3 sucrose vials (1% agarose + 2M sucrose in Milli-Q water). It's helpful to make these gel solutions in large batches ahead of time and re-melt them to pipette

them into the vials the morning of training. I use a large serological pipette to add the molten agarose solution to the vials, enough to cover the bottom (about 1-2 mL). It's okay if some gets on the inside walls of the vial. When the agarose has solidified, plug the vials with clean flugs. For example, if planning to do 4 paired and 4 control trainings, prepare 24 agarose and 24 sucrose training vials. It's helpful to mark the top of the flugs for what will go into the vials. Leave agarose-only blank, mark half the sucrose vials with a black dot (sucrose only) and the other half with a black and orange dot (sucrose + odor). Arrange the plugged vials in a tray like this:

Paired training:



Training should be done in a quiet room that maintains 25C and at least 30% humidity. Humidity can be higher; 50-60% is ideal. A humidifier can be kept on during training as long as it's not too loud.

Benzaldehyde diluted 1:1000 (10⁻³, 0.1%) in Paraffin oil will work for most flies. If your flies seem unresponsive to this odor concentration, it can be increased to 0.2% or 0.4%. I usually prepare 5mL of odor solution and store it frozen (-20C) until right before I use it. I will use the same tube of odor several times before making it again (usually 3 trainings and testings). Odor solution is applied to the training vials by pipetting 10 uL on a filter paper disc (these discs are made ahead of time by a binder hole-puncher) and sticking that disc on the inside of the vial close to the bottom.

Training:

1. Flip the first vial of flies into their first agarose vial and place it flug-side down in the tray (so that flies will climb upwards and encounter the substrate). Start the 15 minute timer. Continue to flip the other vials in order (you will flip the flies in the same order every time so that they are in each vial for close to 15 minutes).
2. A few minutes before the timer is up, vortex odor and add odor discs to the next row of paired tubes (the ones marked with an orange dot). Ideally, you should finish adding discs right before the timer is done.

3. Flip the flies into the next row of tubes. As the flies settle down, you should be able to observe them eating. If you are concerned that the flies are not eating or are unaware of the sucrose, you may need to test them for sugar acuity. The flies will do most of their eating in the first sucrose tube.
4. Repeat steps 1-3 for the remaining 2 repetitions (see diagram). After the last sucrose tube, flies should be flipped into a clean food vial and put in the incubator to rest.

Flies will need to be re-starved for the t-maze testing so that they are motivated. They should be starved 18 hours as before so that the t-maze is run 23-25 hours after the start of the training protocol. Again, 18 hours is ideal, but up to 20 hours starvation is okay. It's possible that starve times may need to be further optimized for some fly lines.

T-maze:

T-maze should be done in the same room and under the same conditions as the training protocol. Be aware that some flies are extremely distracted by loud noises such as the sound of the humidifier fan. Try some trials with and without the fan on to determine if this is going to be a problem for your flies.

1. Mark t-maze tubes 5cm up from the bottom (half-way). This is where you will be sticking the odor disc.
2. Add Paraffin oil (PO) discs to all the paraffin oil tubes and cap them.
3. Add the odor disc to the first tube and cap. Immediately load the flies into the loading tube and tap into the elevator. Uncap and attach the Paraffin oil and odor tubes to the t-maze apparatus. Make sure they are level and lower the elevator. Start the 1 minute timer.
4. When the timer is up, raise the elevator and collect the flies into the odor, paraffin oil and loading (no choice) tubes. Set these aside for counting.
5. Repeat steps 3-4 for the remaining vials of flies.

When performing T-maze, the side you place the odor on should alternate. It is also important to alternate sucrose-only trained flies with paired trained flies. An example run would look like this:

Flies	Odor side
Sucrose-only 1	R
Paired 1	R
Sucrose-only 2	L
Paired 2	L

Sometimes there's a lot of side-bias (when the flies prefer a particular side of the room regardless of where the odor is). If there is too much, your data might not be useable. To help reduce side-bias, make sure the room is quiet and that the t-maze is directly below the light source (no uneven lighting).

When you are finished with your trials, the tubes of flies can be freeze-killed at -20C for 45 minutes or -80C for 10 minutes before counting. You can also anesthetize them for counting if desired. Preference index is calculated as such:

Preference Index (PI) = (# in odor tube) - (# in PO tube) / (# in both odor and PO tubes)

Participation = (# in both odor and paraffin oil tubes) / (# in odor + PO + no-choice tubes)

If your participation is consistently lower than 50%, your flies may be too weak for the assay or may have a motor-deficit.

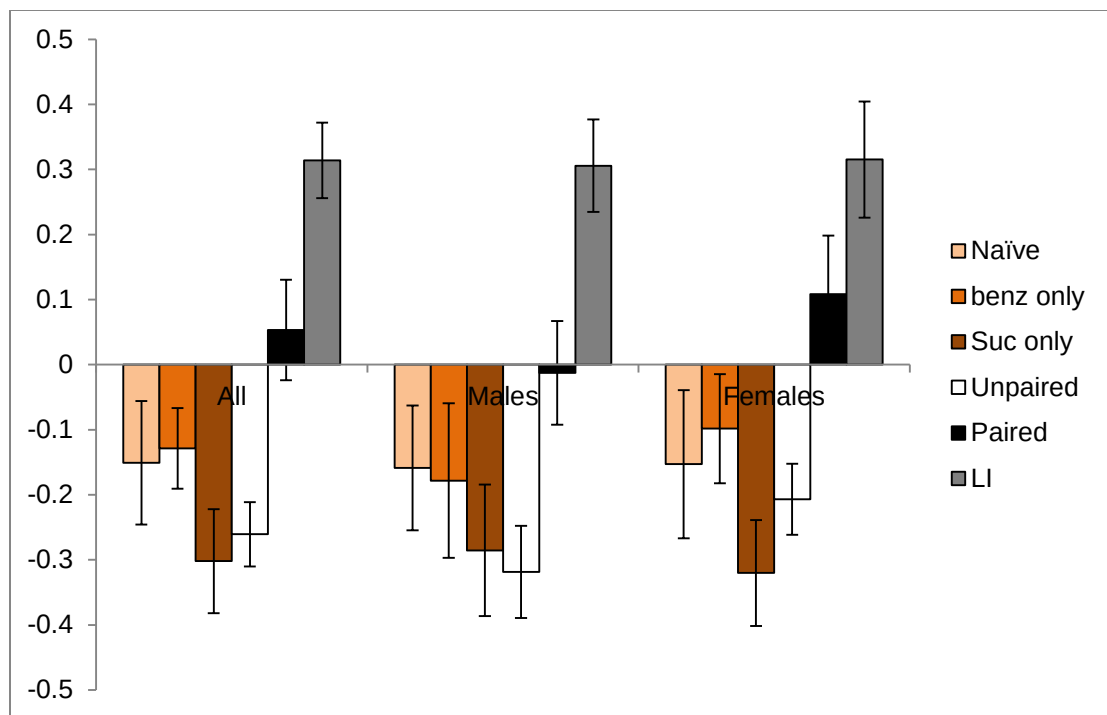
Learning index (LI) = (Paired PI) - (Sucrose-only PI)

If your flies are not responding to the odor after either the paired or sucrose-only training (the PI-s are close to 0), you may need to increase the odor concentration or try testing the odor with naïve flies (starved but untrained) to make sure that they don't have a chemotaxis defect.

This training protocol seems to work reasonably well for most white-eyed fly lines tested, but I've had difficulty with red-eyed flies and some transgenic lines. It's not clear if this is an eye-color issue or a genetic background issue, but the assay may need to be further optimized so that it works consistently for all lines. It may be helpful to backcross the desired mutation or transgene to *wCS* to be confident of the results.

This assay has yet to be tested with known learning mutants. Further work will need to be done if it is to be used in a publication. However, because it's fairly easy to use, it may still prove useful in generating preliminary data, especially if you're working with *wCS* or *w1118* lines.

If the flies are learning, the data should look something like the *wCS* data (additional controls are shown here):



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