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Odors that Modify CO₂ Receptor Activity in Insects and Their Effect on Innate
CO₂-Mediated Behavior and Neuronal Plasticity

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

Doctor of Philosophy

In

Cell, Molecular, and Developmental Biology

by

Stephanie Lynn Turner

August 2010

Dissertation Committee:
Dr. Anandasankar Ray, Chairperson
Dr. Anupama Dahanukar
Dr. Ring Cardé

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The Dissertation of Stephanie Lynn Turner is approved:

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Last but not least, I would like to thank my family for cheering me along. I could not have completed this work without you.

DEDICATIONS:

To my husband, Ryan Chen,

For your inspiration.

To my parents, Steve and Nancy Turner,
and my siblings Mark Turner and Amy Gadd,

for your love and support.

ABSTRACT OF THE DISSERTATION

Odors that Modify CO₂ Receptor Activity in Insects and Their Effect on Innate CO₂-Mediated Behavior and Neuronal Plasticity

by

Stephanie Lynn Turner

Doctor of Philosophy, Graduate Program in
Cell, Molecular, and Developmental Biology
University of California, Riverside, August 2010
Dr. Anandasankar Ray, Chairperson

CO₂ present in exhaled air is considered to be one of the most important olfactory cues for mosquitoes, causing activation of long-distance host-seeking flight behavior, as well as increased sensitivity to other skin odors. Here I show that volatile odorants from fruit can strongly inhibit the CO₂ receptor in *Drosophila melanogaster* and completely abolish CO₂-mediated behavior. Using the 'empty neuron' *in vivo* expression system we establish that the odorants act directly on the CO₂ receptor Gr21a/Gr63a. Following my work on *Drosophila*, I have used electrophysiology assays to perform a comprehensive analysis of structurally related odorants in multiple vector mosquito species that have conserved CO₂ receptor proteins. I have identified three novel classes of odorants that dramatically alter the response of the CO₂-sensitive neuron. Detailed behavioral analyses for some of these odors show a dramatic disruption in the ability of mosquitoes to be attracted to CO₂, offering a powerful approach to develop a

new generation of insect repellents and lures that can reduce the ability of mosquitoes to seek out humans. While looking for inhibitors of the CO₂ response, I have also studied *Drosophila* Stress Odor as well as other activators of the CO₂ neurons across *Drosophila* species to investigate the role of these odors in the ecology of the fruit fly. In addition, I have investigated whether an odor-rich environment shapes the development of the olfactory system. Olfactory stimuli may regulate chemosensory gene expression and neuronal plasticity in invertebrates which could potentially alter the perception of odor stimuli and lead to significant behavioral modifications. I show that when flies are exposed to a CO₂ inhibitory odor immediately after eclosion, a critical period in adult olfactory development, CO₂ receptor expression is severely reduced. This reduced expression is reversed when the fly is removed from odor exposure indicating that neuronal cell death does not occur. In addition, I show that this odor exposure reduces expression of other antenna and palp specific odor receptors.

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CHAPTER I: INTRODUCTION

The olfactory system of *Drosophila*

In *Drosophila melanogaster*, odors are detected by olfactory receptor neurons (ORNs) which are housed in sensory hairs called sensilla. Sensilla are distributed over the surface of the fly's olfactory organs; the third antennal segment, and the maxillary palp (Stocker, 1994) (Figure 1.1). Antennal sensilla are comprised of basiconic, trichoid, and coeloconic morphologies, whereas palp sensilla are only basiconic. Each of these sensilla house between one to four neurons; where two neurons are the most common case. There are approximately 1,200 antennal ORNs of which 38 functional types can be categorized (de Bruyne et al., 2001; Elmore et al., 2003; Hallem et al., 2004). The maxillary palp has approximately 120 ORNs which have been described to follow a pairing rule, where each sensillum combines two particular classes of neurons to form three functional types of sensilla (Clyne et al., 1999b; de Bruyne et al., 1999). The degree of organization observed revealed that there are six palp neurons of different classes which are sorted in this manner. This high degree of stereotypy along with a large research effort, has given rise to a very detailed map of the peripheral olfactory system.

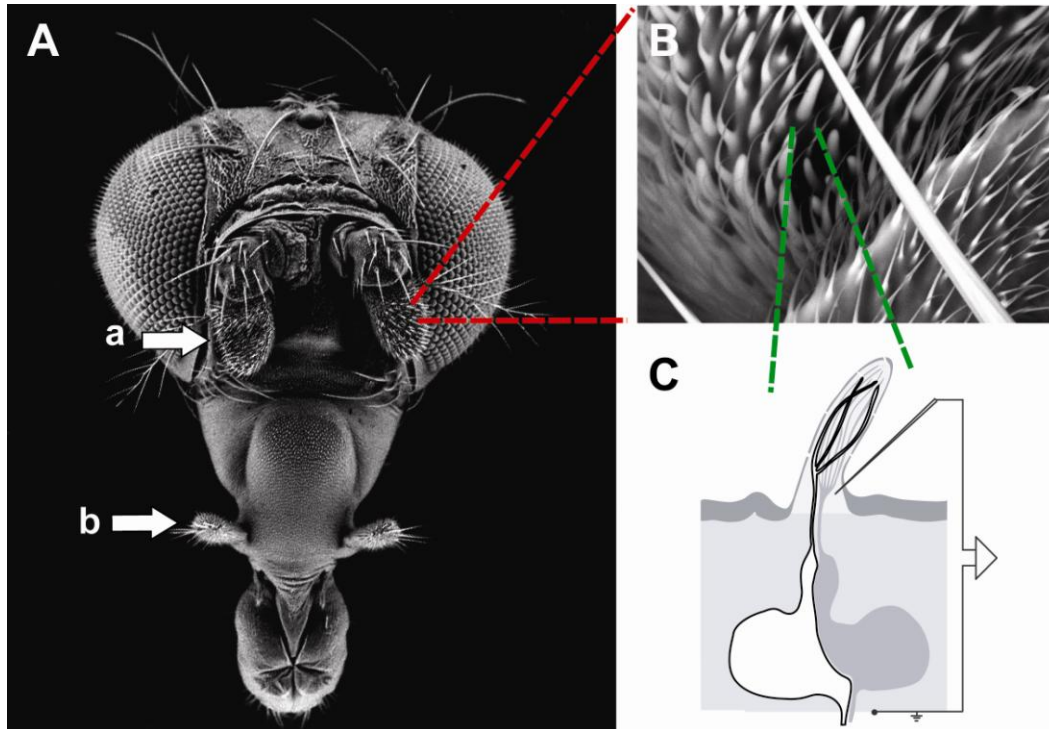


Figure 1.1: Olfactory organs of *Drosophila melanogaster*. **A**, Scanning electron micrograph of the head of *Drosophila* where (a) labels the antenna, and (b) labels the palp. **B**, Scanning electron micrograph of the fly antenna. **C**, Diagram of a sensillum with two neurons, where an electrode is depicted piercing the sensillum to take electrophysiological recordings.

Olfactory neurons are able to respond to odors through the expression of odor receptors (*Or*). After a long research effort, these proteins were identified in the rat to be 7 transmembrane G-protein coupled receptors (GPCR's) (Buck and Axel, 1991). The diversity within the *Drosophila Or* gene family as well as the lack of homology to GPCR's had caused prior efforts in identifying the *Drosophila Or* gene family to fail. The approaches that led to success focused on three key components: *Or* mRNA's must be exclusively expressed in olfactory organs, *Ors*

were most likely seven trans-membrane domain proteins, and *Ors* must be part of a large gene family. A modified difference cloning technique, as well as bioinformatic approaches, led to the identification of the *Drosophila Or* gene family (Clyne et al., 1999b; Vosshall et al., 1999).

The *Drosophila* genome contains a family of 62 *Odor receptor (Or)* genes (Clyne et al., 1999b; Robertson et al., 2003; Vosshall et al., 1999), and 60 *Gustatory receptor (Gr)* genes (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001). Another class of chemosensory receptors expressed in antennal coeloconic sensilla called *Ionotropic receptors (Ir)*, which are related to glutamate receptors, have also been shown to convey olfactory responses (Benton et al., 2009). Interestingly, *Ors* have been shown to have an inverted membrane topology compared to GPCR's (Benton et al., 2006), and are thought to have evolved independently of chemosensory receptors in other animals (Vosshall et al., 1999). Several independent studies have shown that *Drosophila Ors* can function as heteromeric ligand-gated ion channels (Sato et al., 2008; Smart et al., 2008; Wicher et al., 2008), however some debate remains on the involvement of GPCR activity.

In the fruit fly the majority of olfactory neurons also express a second *Or*, the non-canonical receptor *Or83b* (Larsson et al., 2004; Vosshall et al., 1999). This particular *Or* is highly conserved among other insects (Hill et al., 2002; Jones et al., 2005; Krieger et al., 2002), and is paired with nearly all conventional

odor receptors to form a heteromeric pair (Benton et al., 2006; Neuhaus et al., 2004). Or83b has been shown to play an important role in the regulation of localization and function of conventional odor receptors (Larsson et al., 2004). Or83b is necessary and sufficient to mediate both the ciliary targeting and functional expression of *Ors* in any ciliated neuron in vivo (Benton et al., 2006; Larsson et al., 2004). Expression of the conventional odor receptor is what determines the response profile of the ORN (Dobritsa et al., 2003; Hallem et al., 2004). However, flies lacking *Or83b* appear to be anosmic for most odorants, except in the case of carbon dioxide where avoidance to the odor is mediated by carbon dioxide receptors (Jones et al., 2007; Kwon et al., 2007; Suh et al., 2004), which do not require Or83b for function.

CO₂ detection in *Drosophila*

Contrary to the well-established properties of *Drosophila* olfaction, it has been recently shown that CO₂ stimuli are detected through two receptors encoded by members of the gustatory gene family, *Gr21a* and *Gr63a* (Jones et al., 2007; Kwon et al., 2007). These protein products together form the CO₂ receptor, and do not require Or83b to function. The CO₂ receptor is exclusive to the ab1C odor receptor neuron, which resides with three other neurons within the ab1 sensilla, on the fly antenna (de Bruyne et al., 2001). When expressed together in a heterologous system called the “empty neuron” (Dobritsa et al., 2003), *Gr21a* and *Gr63a* confer the CO₂ response (Jones et al., 2007), albeit at a level that is reduced when compared to the endogenous neuron. One possibility for this discrepancy was that there could be a missing component involved in CO₂ signaling needed to confer a full CO₂ response. Unlike the *Gr* proteins, expression of other *Ors* in this heterologous system usually conferred normal responses to odors for a given *Or*. This characteristic of the empty neuron system was crucial to decoding the *Drosophila* palp and antennal *Ors* (Dobritsa et al., 2003; Hallem and Carlson, 2006; Hallem et al., 2004). In a recent study, the role of G-proteins in *Or* and *Gr* signaling has been further elucidated. Including Gαq in the empty neuron along with *Gr21a* and *Gr63a* brings the CO₂ response up to levels comparable to the endogenous ab1C neuron, indicating the Gαq is essential for CO₂ signaling (Yao and Carlson, 2010). In a similar experiment Gγ30A was also shown to enhance CO₂ signaling. Inclusion of Gα

proteins in the empty neuron along with *Ors* only slightly improved the response of the neuron to odorants in certain cases (Yao and Carlson, 2010). Overall, both Gr63a and Gr21a are necessary and sufficient to confer a CO₂ response in both the endogenous ab1C neuron and the empty neuron.

Odor receptor regulation

All *Ors* with the exception of Or83b are expressed in a specific classes of ORNs, creating a huge regulatory undertaking in the fly's peripheral olfactory system. Each *Or* is expressed in a specific zone of the antenna, in a specific sensillum, and finally in a specific neuron (Vosshall and Stocker, 2007). In the case of palp *Ors*, there is organ specification in addition to neuronal specification of expression (de Bruyne et al., 1999; Ray et al., 2007). Of the 62 *Drosophila Or* genes how is only one selected for specific expression in one neuron class?

This regulatory problem is thought to depend largely on a combinatorial code of transcriptional activation and repression. Regulatory elements as well as transcription factors have recently been shown to be involved in *Or* coding (Ray et al., 2007; Ray et al., 2008; Tichy et al., 2008).

In order to uncover features of zonal specification, upstream sequences of all palp *Ors* were compared to those of antennal *Ors* (Ray et al., 2007). This led to the finding of a regulatory motif called Dyad-1, which when mutated caused loss of expression of palp *Ors*. Another motif, Oligo-1, was found to repress expression of palp *Ors* in the antenna (Ray et al., 2007). Although a transcription

factors have not been found which binds these motifs, there are several which have been found to be involved in olfactory coding. In the case of the transcription factor Lozenge, which is required for normal numbers of antennal sensilla (Stocker et al., 1993), maxillary palp *Or* genes that contain binding sites for Lozenge show reduced expression levels in Lozenge mutant flies (Ray et al., 2007). These results indicate the involvement of Lozenge in regulating palp *Or* gene expression. Similarly, POU domain transcription factor Acj6 has been shown to be required for the proper expression of several palp specific *Ors* (Clyne et al., 1999a; Komiyama et al., 2004). Furthermore, it has also been shown that the transcription factor Scalloped plays a role as a repressor. A study by Ray et al. (2008) has shown that of the two odor receptors that have Scalloped binding sites and are co-expressed in the pb3B neuron, Scalloped only represses *Or59c* but not *Or85d*. The authors suggest that the two binding sites are distinct, and that Scalloped binding partners that are specific to each neuron could be what drives specific Scalloped binding to one *Or* gene binding site and not the other (Fuss and Ray, 2009; Ray et al., 2008). These experiments demonstrate the complexity of *Or* gene regulation where combinatorial coding can help select one of many *Ors* to be expressed in one neuron.

Odor processing in *Drosophila*

Axons from the peripheral odor receptor neurons (ORNs) target the antennal lobe (Gao et al., 2000; Vosshall et al., 2000). This structure, which is the functional equivalent of the olfactory bulb in vertebrates, is the first point of olfactory signal processing. Within the antennal lobe, axons of neurons expressing the same *Or* come together and form a spherical structure, called a glomerulus (Boeckh and Tolbert, 1993; Rospars, 1988). As with the fruit fly, this degree of organization has been shown to be conserved in rodents (Mombaerts et al., 1996). Because ORNs that express the same *Or* project axons to the same glomerulus, it is possible to create a stereotypical map of odor representations in the antennal lobe (Couto et al., 2005; Fishilevich and Vosshall, 2005). Both Couto et al. (2005) and Fishilevich et al. (2005) have shown with great detail the assignment of glomerular identity for every antennal basiconic and trichoid ORN and every palp basiconic ORN as well as glomerular identity for every *Or*. These researchers also used a genetic marker for the coeloconic ORNs to infer which eight glomeruli are targeted by these neurons (Figure 1.2). However with the identification of Ionotropic receptors expressed in coeloconic sensilla, and the observation that many of these receptors are expressed in groups of two or three receptors per neuron, it remains to be determined which of these *Ir* expressing neurons (with the exception of *Ir76a*) converge into a glomerulus (Benton et al., 2009).

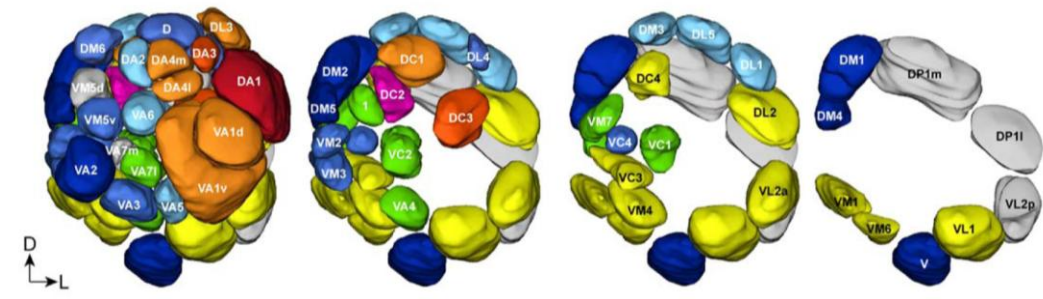


Figure 1.2: 3D reconstruction of the *Drosophila* antennal lobe, showing the positions of 49 glomeruli. The view is anterior, with the labeled glomeruli removed in each successive panel to reveal the underlying glomeruli. Glomeruli are color coded as follows: Blues-antennal basiconic, reds and oranges-antennal trichoid, yellow-antennal celeoconic, green-palp basiconic. (Taken from Couto et. al. 2005)

The glomeruli of the antennal lobe are in turn innervated primarily by two other forms of neurons, local interneurons (IN) and projection neurons (PN) (Figure 1.3). The local INs connect the glomeruli together, and may be of importance in fine tuning the perception of odors as they are transmitted from the periphery (Hansson and Christensen, 1999). The local IN types include inhibitory GABA and excitatory cholinergic neurons (Shang et al., 2007; Wilson et al., 2004), which suggests that the antennal lobe uses “lateral inhibition” in addition to “lateral excitation” to shape odor responses (Keene and Waddell, 2007). The PNs assemble the signal from the glomeruli and transmit the information to higher brain centers, primarily to the mushroom bodies (Lin et al., 2007; Strausfeld, 1976; Turner et al., 2008) and the lateral protocerebrum (Jefferis and Hummel, 2006; Jefferis et al., 2001). Whole-cell electrophysiological recordings revealed that PNs can be more broadly tuned than their afferent odor receptor neurons (Wilson et al., 2004). Some PNs responded to specific odors whereas others are activated, or inhibited, by almost all odors (Wilson and Laurent, 2003).

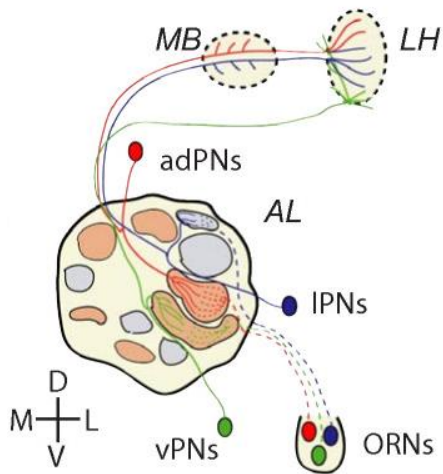


Figure 1.3: Schematic representation of the *Drosophila* antennal lobe and higher brain centers. Olfactory receptor neuron (ORN) axons project to stereotyped glomeruli in the antennal lobe (AL). The anterodorsal (ad), lateral (l) and ventral (v) lineages projection neurons (PNs) send dendrites to specific glomeruli in the AL and axons to specific regions in the mushroom body (MB) calyx and lateral horn (LH). (Spletter et al., 2007).

There exist some cases where an odor can activate only one neuron, which in turn activates only one glomerulus leading to a distinct behavior. For example, the fly pheromone *cis*-vaccinyl acetate activates what is known as a “labeled-line” circuit, which is thought to be important for male flies to decipher between males and females during courtship behavior (Kurtovic, 2007). However, single odorants can typically activate several odor receptors, and therefore elicit a spatial pattern of many activated glomeruli in the antennal lobe. The problem becomes even more complex when using odor mixtures, where activation of many odor receptors leads to integration of these signals in the antennal lobe and behavioral input from individual glomeruli become less clear (Devaud et al., 2003; Fiala et al., 2002; Ng et al., 2002; Wang et al., 2003; Wilson and Laurent, 2005). Although in general, this pattern of activity from the antennal lobe is interpreted by the higher brain centers (Jefferis et al., 2007; Marin et al., 2002; Wong et al., 2002), and is most likely integrated with information from several sensory modalities to generate motor responses. Semmelhack and

Wang (2009) have recently demonstrated this idea, where activation of sets of glomeruli as well as single glomeruli dictate specific behavioral outcomes. When low concentrations of vinegar, a known fly attractant comprised of many individual odor components, was used in a calcium imaging study several glomeruli were activated. These researchers showed that only two of the 6 activated glomeruli, DM1 and VA2 are what contribute to attraction behavior. It is well known that odors which are attractive at low concentrations can become aversive at higher concentrations (Laing et al., 1978; Semmelhack and Wang, 2009). High concentrations of vinegar also lead to an aversive behavior in the fly (Semmelhack and Wang, 2009). How does a once appealing odor become aversive, and how is this information processed? Calcium imaging revealed that only the DM5 glomerulus was activated in addition to DM1 and VA2 with an increased concentration vinegar treatment. When DM5 is activated alone, this results in an aversive behavior. However, when “attraction”-driving DM1 and “aversive”-driving DM5 glomeruli are both activated, this information is integrated as an aversive response. These findings demonstrate that the higher concentration of vinegar recruits an extra glomerulus that independently mediates aversion and acts a behavioral “switch” (Semmelhack and Wang, 2009).

The CO₂ circuit also demonstrates the unique case where activation of one glomerulus drives a behavior. The ab1C neurons of the antenna express the CO₂ receptor and extend their axons to the V glomerulus (Jones et al., 2007),

forming an exclusive circuit for CO₂ detection. Activation of the CO₂ circuit in *Drosophila* evokes an innate avoidance response (Jones et al., 2007; Suh et al., 2007; Suh et al., 2004), which is reminiscent of the observations made in the Semmelhack study, where activation of a single glomerulus by high concentrations of vinegar can also drive an aversion behavior (Semmelhack and Wang, 2009).

Odor-induced neuronal plasticity

Activation of sets of glomeruli contribute to odor-induced behavior, but can odor exposure modify glomeruli to cause a long-term effect on behavior? In the case of the honey bee, glomerular size varies greatly over the course of the insect's life. These changes are correlated to olfactory-driven behaviors such as foraging for nectar and pollen (Winnington et al., 1996). It has only been recently shown, with the nearly complete molecular characterization of the olfactory map, that the field of olfactory-induced neuronal plasticity could be approached with more in-depth studies.

Although the olfactory map in *Drosophila* is highly stable, within the confines of a given glomerulus, olfactory circuits appear to have the ability to change upon contact with olfactory stimuli (Sachse et al., 2007). It has been demonstrated that prolonged odor exposure can cause morphological changes in a 1-day old adult antennal lobe, which can lead to changes in fly behavior (Devaud et al., 2001; Sachse et al., 2007). *Drosophila* pupae have no sensory

input during development suggesting that the olfactory system is hard-wired without odor induced activity or the expression of *Ors* (Dobritsa et al., 2003; Larsson et al., 2004). The flies olfactory system remains plastic during the first few days after eclosion (Berdnik et al., 2006; Sigg et al., 1997) and is subject to experience-dependent structural and functional modifications (Jean-Marc Devaud et al., 2003). Therefore, the time following eclosion could be necessary for the flies olfactory system to sense odorants in their environment. Recent studies suggest that it is only during the first few days of the adult flies life or “critical period” that experience-dependent plasticity can take place (Devaud et al., 2001; Jean-Marc Devaud et al., 2003; Sachse et al., 2007).

Recently, the CO₂ detecting uni-glomerular circuit was used to demonstrate the ability of odors to cause specific odor-induced, activity-dependent neuronal plasticity (Sachse et al., 2007). CO₂ detection is exclusive to the V glomerulus and when activated causes an innate avoidance behavior (Suh et al., 2004). This circuit allows for examination of how a specific odor might effect neuronal modulation in a specific glomerulus. It was shown that constant exposure to 5% CO₂ (166 times above ambient CO₂ levels) can cause a specific volume increase of 38% in the V glomerulus, and not of other glomeruli, over a five day period. Flies exposed to air only, with ambient CO₂ (0.03%), do not experience any volume change in the V glomerulus. Also, activation of the CO₂ neuronal circuit is sufficient to induce neuro-anatomical modulation in the V glomerulus. After long term exposure to above ambient CO₂, flies show a

decreased behavioral response to CO₂ in terms of distance walked, as compared to air exposed flies. Although, this behavioral paradigm does not reveal any information about CO₂ induced avoidance behavior, this experiment does suggest that volume changes in the V glomerulus correlate with an overall motility reduction (Sachse et al., 2007). These experiments demonstrate that prolonged activation of olfactory neuron circuitry can have an effect on neuronal plasticity and can lead to modifications in behavior.

The olfactory system of mosquito

The high degree of molecular characterization of the *Drosophila* olfactory system has given rise to advances in understanding mosquito olfaction; however, the degree of complexity across the three most deadly mosquito species olfactory responses, *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus*, makes decoding their *Ors* a huge but necessary undertaking.

Common to each of these mosquitoes are three structures on the head which serve as the principal chemosensory organs: the antennae, the maxillary palps and the labellum (Figure 1.4). As in *Drosophila*, these organs are capable of detecting a vast number of chemicals originating from host odors, plants, nectar, and oviposition sites via odor receptor neurons housed within the sensilla. *Anopheles gambiae*, *Aedes aegypti* and *Culex quinquefasciatus* have 79, 131, and 102 odor receptor candidates, respectively (Bosch et al., 2000; Hill et al., 2002; Pelletier et al., 2010). Each of these large *Or* gene families, with the

exception of *Anopheles* (Carey et al., 2010; Wang et al., 2010), have yet to be decoded. In a recent study, of the 79 putative *Or* genes in *Anopheles*, 50 have been decoded using the heterologous “empty neuron” system in *Drosophila* and in *Xenopus* oocytes (Carey et al., 2010; Wang et al., 2010). This type of approach makes characterization of the mosquito olfactory system achievable because it does not rely on a highly defined map of *Or* expression in the olfactory organs, which has yet to be elucidated. Rather, odor panels can be tested on fruit flies modified to express the mosquito *Ors* in the empty neuron system, which could reveal important olfactory cues in the mosquito in a more high-throughput fashion.

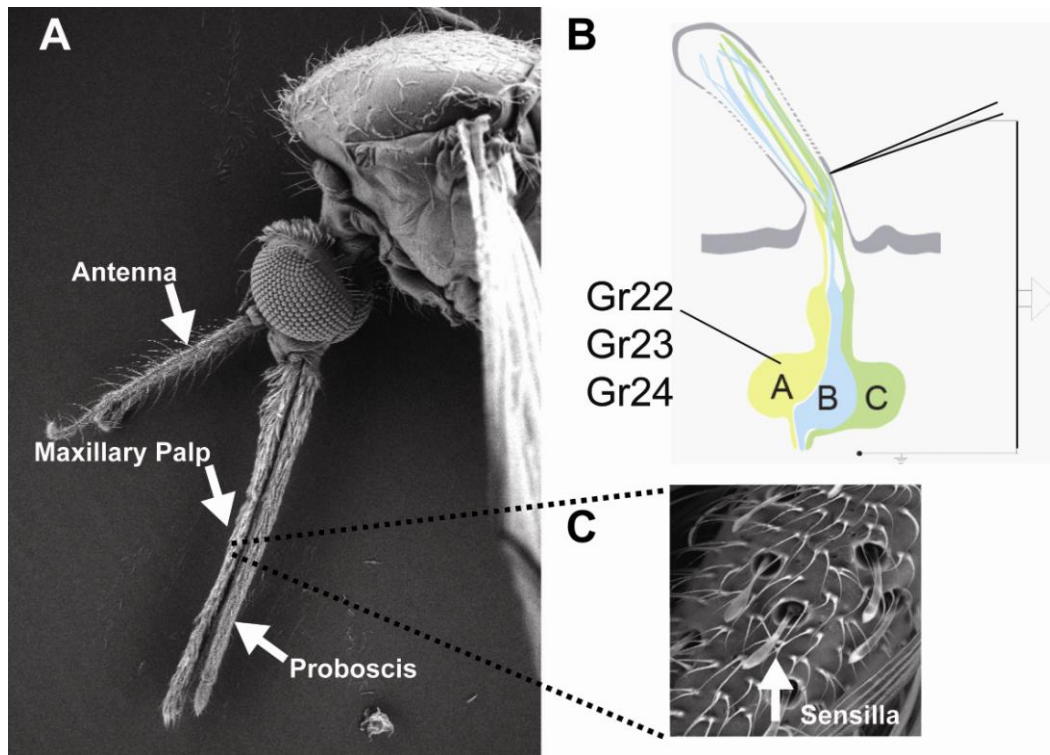


Figure 1.4: Olfactory organs of *Anopheles gambiae*. **A**, Scanning electron micrograph of the head of *Anopheles*. **B**, Diagram of three neurons of the capitae peg sensillum on the maxillary palp, where an electrode is depicted piercing the sensillum to take electrophysiological recordings. The neuron labeled (a) expresses the CO₂ receptor orthologs Gr22, Gr23, and Gr24. **C**, Scanning electron micrograph of the mosquito maxillary palp highlighting the peg sensilla.

In the maxillary palp alone, it has been shown in both *An. gambiae* and *Cx. quinquefasciatus* that the capitae peg sensilla are very broadly tuned, and detect the host odors CO₂ and 1-octen-3-ol (Lu et al., 2007; Syed and Leal, 2007). The capitae peg sensilla contain three neurons; cpA, cpB, and cpC (Grant et al., 1995; Lu et al., 2007; Syed and Leal, 2007). The cpA neuron responds to CO₂ and the cpB neuron responds to 1-octen-3-ol (Figure 1.4). Although some odors have been identified as activators of the cpC neuron of peg

sensilla in *An. gambiae* (Lu et al., 2007), a compound which acts as a strong ligand in all three mosquito species has not been found. *Ors* of *Anopheles* have also been shown to be varied in their breadth of tuning (Carey et al., 2010). Of note, the common odor detected in all three mosquitoes is CO₂, as confirmed by electrophysiological data (Grant et al., 1995; Lu et al., 2007; Syed and Leal, 2007). These mosquitoes have three closely related orthologs of the *Drosophila* CO₂ receptors DmGr21a and DmGr63a, all with high amino acid identity and 7 to 8 predicted trans-membrane domains (Robertson 2009). The CO₂ receptor proteins are expressed in the cpA neuron of the capitata peg sensilla of the maxillary palp (Figure 1.4) (Lu et al 2007).

CO₂ mediated behavior in insects

CO₂-induced behaviors vary greatly in the insect world. Typically CO₂ acts as an environmental indicator. For example insects can use plants as an oviposition cue, and CO₂ could indicate the correct state of plant development or type of plant. The moth, *Cactoblastis cactorum*, uses its labial palp organ to probe the surface of a plant before ovipositing (Stange, 1997; Stange et al., 1995). Because this sensory organ is very sensitive to CO₂, it stands to reason that CO₂ plays a role in the oviposition behavior of these moths, which oviposit on *Opuntia stricta*, a CAM photosynthesizing plant which fixes carbon during the night to prevent water loss. It has been argued that the healthiest plants would

process the most CO₂ and therefore this “carbon sink” is what the moth would detect and drive oviposition behavior (Stange et al., 1995).

CO₂ could also be an indicator of a food source for adult insects. Thom et al. (2004) demonstrated foraging behavior in a naive moth offered two choices, one nectar-free surrogate flower and another that emitted levels of CO₂ consistent with the moth’s host flower. Most moths directed their first proboscis extension into the flower emitting the higher level of CO₂, suggesting a role for CO₂ in indicating nectar-laden flowers (Thom et al., 2004). However, the role of CO₂ in this context has more recently been suggested to be a long distance cue for the presence of flowers, rather than a signal for flower probing (Goyret et al., 2008). In the case of social insects, CO₂ could be an indicator of respiration within the nest itself. Honey bees are social insects that are well known to perform various jobs throughout a bee colony. Although honey bees do not have ortholog’s of the CO₂ receptor (Robertson and Wanner, 2006), worker bees in particular beat their wings in order to ventilate their hive. It has been shown that there is a correlation between the rate of wing beating and the level of CO₂ in the hive. (Guerenstein and Hildebrand, 2008; Ohashi et al., 2009; Seeley, 1974).

Various types of fruit flies have been shown to use CO₂ as a means of finding optimal oviposition sites (Stange, 1999). Although, *Drosophila melanogaster* are known to eat and oviposit on various fruits (Faucher et al., 2006; Spieth, 1974), it has been shown that these flies exhibit a robust avoidance

behavior to CO₂ (Suh et al., 2004). This observation was first made when flies were exposed to odor produced from stressed flies, termed *Drosophila* stress odor (dSO). Analysis revealed that CO₂ is a large component of dSO, and flies avoid CO₂ in a concentration-dependent manner. It has also been shown that inhibition of synaptic transmission in *Gr21a*-expressing neurons blocks CO₂ avoidance behavior in *Drosophila*, indicating that ab1C neurons are required for avoidance behavior (Suh et al., 2004). As a confirmation of these findings, *Gr63a*^{-/-} mutant flies did not exhibit avoidance behavior when exposed to CO₂ (Jones et al., 2007). Both of these studies demonstrate that the ab1C neuron, which expresses the CO₂ receptors *Gr21a* and *Gr63a*, are most likely the only CO₂ sensitive neurons in *Drosophila* and confer CO₂-induced avoidance behavior. In addition, recent studies have addressed why flies would avoid CO₂ given that their food sources are known to produce CO₂ during fermentation. Faucher et al. (2006) have proposed that these behavioral responses can be correlated with CO₂ released from fruits as an indicator of fruit ripeness. Unripe bananas emit more CO₂ than ripe ones, suggesting that CO₂ detection depends on the context of the overall odor profile (Faucher et al., 2006). In addition, as will be discussed in CHAPTER II, odor's from fruits have been shown to directly inhibit the CO₂ neuron, and this inhibition leads to the abolishment of avoidance behavior (Turner and Ray, 2009). Once a fly's avoidance behavior to CO₂ is inhibited, it is possible that they could then detect other attractive odors that indicate the ripeness of fruit.

Mosquitoes, which are responsible for transmission of disease, also use CO₂ as an important host-seeking odor cue (Gilles, 1980). Most notably, CO₂ can act synergistically with other host emitted odors, and modify the response to other stimuli. When particular odors such as L-lactic acid are combined with CO₂ their degree of attractiveness is markedly increased as measured by the increased rate of take-off, flight activity, landing, and probing (Dekker et al., 2005; Eiras and Jepson, 1991; Gilles, 1980). More specifically, a brief encounter with a CO₂ filament can instantaneously increase a mosquito's sensitivity to human odors by at least fivefold (Dekker et al., 2005). Dekker et al. (2005) also found that *Ae. aegypti* are several orders of magnitude more sensitive to CO₂ than to human skin odors. Odor plume structure can contribute greatly to the attractiveness of an odor, and therefore affect the range at which the odor can cause behavioral changes in the mosquito (Cardé and Willis, 2008). CO₂ from expired air is most likely detected from a distance where it would be present as an intermittent plume, and cause attraction in the mosquito.

It is thought that because mosquitoes are more attracted to homogeneous mixtures of skin volatiles rather than skin odor plumes (Dekker et al., 2001), these odor cues will serve for short range detection, where they will still be relatively homogeneous (Zwiebel and Takken, 2004). Although human odors do attract mosquitoes, CO₂ is the only odor that increases capture rates of many mosquito species in the field (Cooperband and Cardé, 2006; Dekker et al., 2001; Grant and Oconnell, 1996; Xue et al., 2008), and the CO₂-sensitive cell of these

insects exhibit a similar sensitivity across various species (Grant and Oconnell, 1996). Therefore it can be argued that CO₂ is key in the host orientation of most mosquito species (Dekker et al., 2005).

Despite the high degree of conservation between the CO₂ receptors in fruit fly and mosquito, detection of CO₂ leads to opposite behaviors in these insects. What drove the evolutionary transition from aversion to attraction? One of the key features that differ in detection of CO₂ is the location of the CO₂ neurons. *Drosophila* CO₂ neurons reside in the antenna (de Bruyne et al., 2001; Jones et al., 2007; Suh et al., 2004), whereas mosquito CO₂ neurons reside in the maxillary palp (Grant et al., 1995). Based on these observations one could argue that simply the location of the CO₂ neuron determines the behavioral outcome to CO₂. However, another blood-feeding insect, the tsetse fly, also detects CO₂ through neurons in the antenna ruling out this possibility (Bogner, 1992). What is more likely is that there may be differences in neuronal circuitry in higher brains centers that account for this behavioral difference. For example, in the fruit fly the CO₂-detecting V glomerulus resides on the ventral-most side of the antennal lobe (Jones et al., 2007; Kwon et al., 2007; Scott et al., 2001). In contrast, the mosquito CO₂ glomerulus resides on a medial portion on the antennal lobe (Ignell et al., 2005). Activation of glomeruli at different regions of the antennal lobe could give rise to changes in odor perception resulting in behavior changes (Galizia et al., 1999; Kurtovic, 2007; Wong et al., 2002). Recently, Cayirlioglu (2008) and colleagues have studied this question at the

molecular level. These researchers found that a miRNA called miR-279 could be responsible for suppressing the evolutionary intermediate between CO₂ neurons of the palp and CO₂ neurons of the antenna. Disruption of this single miRNA gene in *Drosophila* uncovered a population of CO₂ neurons that reside in the fruit fly's maxillary palps and are reminiscent of the palp CO₂ neurons of mosquitoes. Perhaps what is most interesting is that these hybrid neurons project to the V glomerulus as well as medial regions of the antennal lobe which is similar to that of mosquito (Cayirlioglu et al., 2008).

In summary, the work presented here focuses on CO₂ detection in *Drosophila melanogaster* as well as mosquitoes. More specifically, I have found that odors can inhibit the avoidance response of *Drosophila* to CO₂, by inhibiting the CO₂ receptor. This work was then directly applied to mosquitoes, where I have found several odors which both activate and inhibit CO₂ sensitive neurons. I have also investigated the role of activators of the CO₂ neuron in the ecology of *Drosophila*. Finally, I have investigated how olfactory stimuli might regulate chemosensory gene expression and neuronal plasticity in *Drosophila* using the CO₂ circuit.

CHAPTER II:
MODIFICATION OF CO₂ AVOIDANCE BEHAVIOR
IN *DROSOPHILA* BY INHIBITORY ODORANTS

Introduction:

Many herbivorous insects use CO₂ to locate leaves, fruits, and flowers as a source of food (Guerenstein and Hildebrand, 2008; Hibbard et al., 1997; Stange et al., 1995; Thom et al., 2004). Although, *Drosophila melanogaster* are known to eat and oviposit on various fruits (Spieth, 1974), it has been recently shown that these flies exhibit a robust avoidance behavior toward CO₂ (Suh et al., 2004). This observation was first made when flies were exposed to odor produced from stressed flies, termed *Drosophila* stress odor (dSO). Later analysis revealed that CO₂ is a large component of dSO, and flies avoid CO₂ in a concentration dependent manner. In *Drosophila*, CO₂ is exclusively detected by a unique heteromeric receptor, encoded by *Gr21a* and *Gr63a* (Jones et al., 2007; Kwon et al., 2007), members of a large *gustatory receptor (Gr)* gene family (Clyne et al., 2000; Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001). *Gr21a* and *Gr63a* are expressed in the ab1C antennal neurons which innervate the ab1 class of large basiconic sensilla (Scott et al., 2001; Suh et al., 2004). It has been shown that inhibition of synaptic transmission in *Gr21a* expressing neurons blocks CO₂ avoidance behavior in *Drosophila*, indicating that ab1C neurons are required for CO₂-mediated avoidance behavior (Suh et al.,

2004). As a confirmation of these findings, *Gr63a*^{-/-} mutant flies did not exhibit avoidance behavior when exposed to CO₂ (Jones et al., 2007). Both of these studies demonstrate that the ab1C neuron, which expresses the CO₂ receptor Gr21a and Gr63a, are likely the only CO₂ sensitive neurons in *Drosophila* and confer CO₂ induced avoidance behavior.

In nature, *Drosophila* would perceive CO₂ in terms of an odor mixture, not as an individual odor in a laboratory setting. Indeed, fruit flies are attracted to fruits and other food sources known to emit CO₂. Although flies have been shown to have an innate avoidance behavior to CO₂ (Suh et al., 2004), a recent study demonstrated that the context in which a female fly perceived CO₂ with food odors can greatly attenuate avoidance behavior (Faucher et al., 2006). For their survival, fruit flies need to overcome the innate CO₂ avoidance response in environments that contain CO₂, such as fruits and plants. Indeed, when flies are exposed to CO₂-rich food sources in a T-maze, they are highly attracted to over-ripe fruits, yeast and beer, while avoiding green fruits (Figure 2.1). It seems reasonable that other odorants in these various micro-environments may counteract the CO₂ mediated avoidance cue by any number of mechanisms. We present two mutually non-exclusive models to address this phenomenon (Figure 2.2). In the first model (Figure 2.2A, top), fruits may be producing attractive odors which counteract any avoidance behavior caused by CO₂. In the second model (Figure 2.2A, bottom), in addition to CO₂ and attractive odors, fruits could also produce inhibitory odors which act directly on the CO₂ detection pathway. In

this study we demonstrate that odorants from fruits inhibit the CO₂ neuron. In addition we use the empty neuron system to show that the CO₂ response inhibiting odors act directly on the CO₂ receptor. Finally, we show that inhibition of the CO₂ neuron by odorants completely abolishes CO₂-mediated avoidance behavior.

Results and Discussion:

To address the latter model, we performed a simple electrophysiological screen using a panel of odors which are representative of the environment in and around fruits. Here we use the *Or83b*² mutant as a means of measuring inhibition to of ab1C neuron to baseline activity from ~0.03% CO₂ in the air. Because the CO₂ receptor Gr63a/Gr21a does not require Or83b for function, we can sort the activity of the CO₂ neuron easily due to the lack of activity of the remaining three neurons of the ab1 sensilla (Larsson et al., 2004). Importantly, ab1C neurons of the *Or83b*² mutant respond strongly to a 1% CO₂ stimulus (Figure 2.2B) (Larsson et al., 2004). Using this method, we have identified two odorants, 1-hexanol and 2,3-butanedione, which inhibit the baseline activity of the CO₂ neuron in *Drosophila* (Figure 2.2B). It has been previously found that as fruits ripen, both odors drastically increase in concentration (Table 2.1), suggesting a role for these odors as a cue representing fruits which are in the correct state for feeding. For example in banana 1-hexanol increases by 777% and 2,3-butanedione by 14,900% (Mayr et al., 2003). 1-hexanol is formed during

ripening by lipid oxidation of unsaturated fatty acids (Galliard et al., 1977) whereas 2,3-butanedione is a natural byproduct of fermentation of carbohydrates through pyruvate by yeasts and bacteria and is therefore also present in fermenting fruit, wine and beer (Hughes, 2001; Martineau et al., 1995; Nykanen and Nykanen, 1991).

We next sought to determine if the odorants 1-hexanol and 2,3-butanedione could inhibit a CO₂ response, and if so whether the order of stimulus delivery affects CO₂ response inhibition. To address this problem we presented the odors in two different contexts. In the first context, a 3-sec stimulus of either paraffin oil or odorants was overlaid on a 1-sec stimulus of 0.33% CO₂ (Figure 2.3A, B top). In the second context, a 1-sec stimulus of either paraffin oil or odorant was overlaid with a 3-sec stimulus of 0.33% CO₂. (Figure 2.3A, B bottom). We found that both odors inhibit a CO₂ response regardless of application before or after the CO₂ stimulus. Although application of 2,3-butanedione was not affected by order of application, 1-hexanol was most effective when applied after the CO₂ stimulus. Overall, both of these odorants are able to inhibit CO₂ response in a dose-dependent fashion, and at a dilution of 10⁻² can completely inhibit the CO₂ response.

A fly approaching an odor source from a distance likely contacts plumes of CO₂, which will vary widely in concentration over baseline atmospheric levels (Cardé and Willis, 2008). We therefore examined how the two inhibitors modulate activity of ab1C neurons at different concentrations of CO₂ over time. The presence of 2,3-butanedione (10⁻¹ dilution) leads to a complete inhibition of CO₂ response across all tested concentrations up to 3.2% CO₂ for the entire duration of the odor stimulus (Figure 2.4). Although 1-hexanol (10⁻¹ dilution) can significantly inhibit the varying concentrations of CO₂ stimuli, the odor shows complete inhibition of CO₂ response only for the lowest concentration of CO₂ tested (Figure 2.4).

The structural features of these odorants could play a role in the inhibition of the CO₂ neurons. Is there a common feature to these odors that confer the inhibitory effect on the CO₂ neuron? We screened compounds that differed in odor carbon chain length and functional group and asked which of these odorants best inhibits baseline activity of the ab1C neuron. Here, a 1-sec stimulus of odorants (10⁻² dilution) was applied to *Or83b²* flies, as above. Using this method, we identified additional structurally-related odorants that also inhibit the CO₂ neuron. Specifically, we identified two aldehydes, butanal and pentanal, that are highly effective inhibitors (Figure 2.5). Similarly, these odors also inhibit a 3-sec 0.33% CO₂ response (Figure 2.6). The inhibitory properties of these odors are specific to the CO₂ neuron. Several previous studies show that all of these odors excite other classes of neurons (de Bruyne et al., 2001; Hallem and

Carlson, 2006), indicating that they are not general inhibitors of odor receptor neurons.

The structural features of the inhibitory odors suggest that they may not bind to the same regions of the receptor as CO₂. The compounds being greatly varied in size and shape suggests that they may act on a different binding site of the CO₂ receptor, and could act in an allosteric manner (Figure 2.7A). It is possible however, that these odors could be acting on other unknown components of the CO₂ detection pathway, such as odorant binding proteins or signaling proteins involved in the olfactory response.

To investigate whether the inhibitory odorants act directly on the CO₂ receptor, we expressed *Gr21a* and *Gr63a* in an *in vivo* decoder system called the “empty neuron” (Dobritsa et al., 2003; Jones et al., 2007; Kwon et al., 2007) (Figure 2.7B). We found that expression of *Gr21a* and *Gr63a* proteins in the empty *ab3A* neuron is sufficient to impart a robust and reproducible dose-dependent CO₂ response, comparable to levels reported previously (Jones et al., 2007). Due to ectopic expression, the *ab3A* neuronal response gave much lower levels than that of the endogenous *ab1C* neuron (Jones et al., 2007; Kwon et al., 2007). Importantly, these odors do not activate the exogenously expressed CO₂ receptor when presented without CO₂. These odors activate the wild-type *ab3A* neuron, but do not activate the *Or22a* null ($\Delta halo$) *ab3A* neuron, meaning that a possible inhibition of the CO₂ response in this system would not be through non-specific activation by the tested odorants (Figure 2.7C).

There was a significant dose-dependent inhibition of CO₂ response upon the simultaneous application of the inhibitory odorants along with CO₂ in the empty neuron system (Figure 2.8). Furthermore, the odorants show differences in the degree of inhibition, as well as in the rate at which inhibition increases with higher concentrations (Figure 2.8). 1-Pentanal and 1-hexanol significantly inhibit the CO₂ response, while 1-butanal and 2,3-butanedione inhibit the CO₂ response completely. The simplest interpretation of these results is that the odorants identified in this study inhibit CO₂ response by direct interaction with the CO₂ receptor Gr21a/Gr63a.

We next asked whether the inhibitory odorants that were identified using electrophysiology could disrupt avoidance behavior of *Drosophila* to CO₂. Using a T-maze choice assay as described earlier (Suh et al., 2004), we found that wild-type *Drosophila* show a robust avoidance behavior to 0.67% CO₂, and inclusion of 2,3-butanedione with CO₂ results in a severe reduction in mean avoidance behavior (Figure 2.9A). Importantly, flies do not show a behavioral response toward 2,3-butanedione in the T-maze, suggesting that the odorant is acting directly on the CO₂ detection pathway rather than causing an attraction response that would cause the fly to overcome CO₂ avoidance behavior. In wild-type *Drosophila*, however, a number of ORN classes are activated by 2,3-butanedione (de Bruyne et al., 2001; Hallem and Carlson, 2006). To ensure that the modification in behavior is not due to attraction to 2,3-butanedione, we tested

the behavior of *Or83b*² mutant flies in which most of the odor receptor neurons are non-functional, but electrophysiological responses to CO₂ are not affected (Larsson et al., 2004). Avoidance is significantly reduced with the addition of 2,3-butanedione with CO₂ (Figure 2.9B). These results suggest that any remaining active neurons, like those of ceoloconic sensilla which are innervated by neurons expressing ionotropic glutamate receptors (Benton et al., 2009), do not play a role in CO₂ avoidance behavior or in detection of 2,3-butanedione. Similar results were found for 1-hexanol in both wild-type and *Or83b*² flies, albeit with reduced effects (Figure 2.9C, D). Taken together these results demonstrate that odorants like 2,3-butanedione can effectively inhibit CO₂-mediated innate avoidance behavior by inhibiting the CO₂ receptor.

We observed that with increasing concentrations of 2,3-butanedione, the CO₂ neuron is silenced well beyond the period of application (Figure 2.10A). To investigate this further, we exposed the fly to a 3-second stimulus of 2,3-butanedione (10⁻¹ dilution) and subsequently tested for the recovery of ab1C neuron responsiveness by applying a 0.5-second stimulus of 0.3% CO₂ every 30-seconds, over a period of 10- minutes (Figure 2.10B). Surprisingly, the inhibitory effect of the initial exposure to 2,3-butanedione persisted for an extended period. We wanted to test whether behavior was also affected in a similar manner. Flies were exposed for one minute to 2,3-butanedione and then transferred to clean air for two minutes before testing for CO₂-mediated avoidance behavior in a T-maze. Remarkably, CO₂ avoidance is almost abolished in pre-treated flies (Figure

2.11A). Prior exposure to another odorant 2-methyl phenol, which does not inhibit the CO₂ response (Figure 2.2B), does not have any effect on behavior (Figure 2.11A). Moreover, pre-exposure to 2,3-butanedione does not have a significant effect on behavioral attraction towards a different odorant, ethyl acetate (Figure 2.11A). Similar observations were made with *Or83b*² mutant flies (Figure 2.11B). Taken together, these observations show that exposure to a long-term CO₂ response inhibitor can exert a profound and specific effect on the behavior of the animal even after it is no longer present in the environment.

Although the peripheral olfactory system in *Drosophila* has been extensively characterized, there remains the possibility that there are unknown factors such as odorant binding proteins, signaling pathways, and perhaps other neurons which could be detecting the inhibitory odorants and lead to an inhibition of CO₂-mediated avoidance behavior. To demonstrate unequivocally that 2,3-butanedione inhibits CO₂ avoidance behavior by inhibition of the CO₂ neuron and not by other mechanisms, we performed the following experiment. We activated the ab1C neuron in a manner that is not inhibited by 2,3-butanedione, and asked whether 2,3-butanedione inhibits avoidance behavior in this context. We identified an odorant, 2-butanone which activates ab1C neurons strongly at 10⁻¹ dilution (Figure 2.12A) in a *Gr63a*-dependent manner (Figure 2.12B). However, electrophysiological response to 2-butanone is not affected by pre-exposure to, or the presence of, 2,3-butanedione (Figure 2.12A, 2.13A, B), unlike what we

observed for CO₂. We found that *Or83b*² mutant flies strongly avoid 2-butanone (10⁻¹ dilution) whereas flies lacking both *Or83b* and *Gr63a* do not (Figure 2.13C), as predicted from electrophysiology data (Figure 2.12, 2.13). In a T-maze behavior assay, 2,3-butanedione has no effect on behavioral avoidance of *Or83b*² mutant flies to 2-butanone, regardless of whether it is used to pre-treat the flies as described above or is included in a mixture with 2-butanone (Figure 2.13C). These results demonstrate that 2,3-butanedione disrupts CO₂ avoidance behavior by directly inhibiting the CO₂ responsiveness of ab1C neurons, rather than by other indirect mechanisms.

Conclusion:

In our study, we have found a paradigm of inhibition which addresses a question that has eluded behavioral entomologists. How do flies approach their CO₂-rich food source despite a strong avoidance to CO₂? Our findings also have wider implications for coding of olfactory behavior: we demonstrate that inhibitory odorants play a critical role in shaping the behavioral response of an insect. Perhaps even more exciting, many insect species particularly crop and vector pests express proteins which are very similar that of the *Drosophila* Gr21a/Gr63a. Indeed, the CO₂ receptor proteins are highly conserved across dipteran species, signifying the importance of this environmental cue across this order (Robertson and Kent, 2009). CO₂ emitted in human breath is a critical component of odor blends used as host-seeking cues by many vector insect species that carry deadly diseases (Zwiebel and Takken, 2004). In the case of blood feeding insects, CO₂ response inhibition could serve as a novel method for repelling or controlling insect populations, helping to reduce human-insect contact and the spread of vector-borne diseases.

Figure Legends:

Figure 2.1: Fruit flies are attracted to fermenting fruits.

Attraction to the T-maze arm containing headspace from complex odor sources quantified as a mean Preference Index (PI, see Methods). n=6 trials (~40 flies each), error bars=s.e.m.

Figure 2.2: Inhibitory odors dramatically reduce response to baseline levels of CO₂.

A, Proposed models for suppression of avoidance behavior to CO₂ in the context of fruits. **B**, Mean odorant responses and representative traces of activity of ab1C neurons using single-sensillum electrophysiology in *Or83b²* flies. Bars indicate a 0.5-s stimulus period. Odorants were tested at 10⁻² dilution in paraffin oil. Bars represent values after subtraction of mean response to paraffin oil (n=3, error bars, s.e.m.)

Figure 2.3: Inhibitory odors dramatically reduce response to CO₂.

A,B, Representative traces and mean responses from single-sensillum electrophysiology of ab1 sensilla in *Or83b*² flies; spikes and bars represent activity of the ab1C neuron. Top, 3-s stimulus of odorant overlaid with a 1-s application of 0.3% CO₂; bottom, 3-s stimulus of 0.3% CO₂ overlaid with a 1-s application of odorant (PO, paraffin oil; d4on, 2,3- butanedione; 6ol, 1-hexanol) (n=6, error bars, s.e.m.). Spikes per second were counted during the 1-s stimulus period, and spontaneous activity subtracted. (For data in **A** and **B**, t-test, ****P,0.001, ***P,0.005, **P,0.01, *P,0.05.)

Figure 2.4: Inhibitory odorants inhibit increasing concentrations of CO₂.

Mean responses of the ab1C neuron to indicated concentrations of CO₂ in the presence of solvent (PO), 1-hexanol (6ol) or 2,3-butanedione (d4on). Odorants were tested at 10⁻¹ dilution. Firing rates were counted in consecutive 0.1-s bins (n=5, error bars, s.e.m.).

Figure 2.5: Baseline CO₂ response inhibition by structurally related compounds.

0.5-s stimuli of odorants were tested on ab1C neurons in *Or83b²* flies at 10⁻² dilution in paraffin oil. Compounds are straight-chained with the functional group present at Carbon 1 (except for ketones where carbonyl group is present at Carbon 2). Bars represent values after subtraction of mean response to paraffin oil. n=3.

Figure 2.6: CO₂ response inhibition by structurally related compounds.

A,B, A 3-sec stimulus of 0.3% CO₂ was delivered in combination with a 1-sec application of indicated odorant (10⁻² dilution). Compounds in **A** are straight-chained with the functional group present at Carbon 1 (except for ketones where carbonyl group is present at Carbon 2). Percent increase or decrease in mean ab1C response was calculated relative to mean response of paraffin oil. n=5.

Figure 2.7: Odorants act specifically on CO₂ receptor in ‘empty neuron’ system.

A, Structures of CO₂ and the four strongest antagonists. **B**, Schematic illustrating „empty neuron“ system used for heterologous expression of *Gr21a* and *Gr63a* in *ab3A* neurons. **C**, Odorants at indicated concentrations were tested on *ab3A* neuron in *wild-type* (*wt*), Δ *Halo* (Δ *H*) mutants that lack *Or22a* and *Or22b*, and *Or22a-Gal4*, *UAS-Gr21a*, *UAS-Gr63a* (*Gr21a* + *Gr63a*). Bars represent values after subtraction of spontaneous activity. *d4on*=2,3-butanedione, *4al*=1-butanal, *6ol*=1-hexanol, *5al*=1-pentanal. *n*=5, error bars=s.e.m.

Figure 2.8: Inhibitory odorants directly affect CO₂ response of Gr21a/Gr63a in ‘empty neuron’ system.

A, Representative traces of recordings from *ab3* sensilla. Top trace, indicates firing of *ab3b* in the Δ *Halo* mutant. Bottom traces, large spikes represent the response of the Δ *ab3A* cell expressing *Gr21a* and *Gr63a*. Bars indicate stimulus periods of 12% CO₂, overlaid with paraffin oil (PO) or 2,3-butanedione (*d4on*) at 10⁻¹ dilution. **B**, Concentration-dependent responses of *ab3A* neuron to CO₂, and binary mixtures of CO₂ with odorants at indicated concentrations: 1-butanal (*4al*), 1-pentanal (*5al*), 1-hexanol (*6ol*). Stimuli were applied as in **A** (*n*=5, error bars=s.e.m.).

Figure 2.9: Avoidance behavior to CO₂ is abolished by inhibitory odorants.

T-maze behavior assay: **A**, mean preference index of wild-type flies, given a choice between room air in a 15-ml tube and either 0.1 ml of pure CO₂ (CO₂), a binary mixture of 0.1ml pure CO₂ and 2,3-butanedione at 10⁻² dilution (CO₂+d4on) or 2,3-butanedione at 10⁻² dilution (d4on) also in 15-ml tubes (see CHAPTER VI: METHODS). **B**, Mean preference index of *Or83b*² mutant flies given choices as in **A**. **C**, Mean preference index of wild-type flies, given a choice between room air in a 15-ml tube and either 0.1 ml of pure CO₂ (CO₂), a binary mixture of 0.1ml pure CO₂ and hexanol at 10⁻² dilution (CO₂+6ol) or hexanol at 10⁻² dilution (6ol) also in 15-ml tubes. **D**, Mean preference index of *Or83b*² mutant flies given choices as in **C**. (n=6–9 trials (approximately 40 flies each); error bars, s.e.m. (t-test, *P,0.0001)).

Figure 2.10: Long-term inhibition of the CO₂ neuron.

A, Representative traces of ab1C neuronal activity. Bars indicate stimulus periods for 0.3% CO₂ overlaid with paraffin oil (PO) or 2,3-butanedione (d4on) at the indicated concentrations. **B**, Recovery of ab1C responsiveness to a 0.5-s, 0.3% CO₂ stimulus applied every 30-s after initial treatment with a 3-s stimulus of either d4on (10⁻¹ dilution) or paraffin oil. (PO) (n=5; error bars, s.e.m. (t-test, ***P,0.005, **,0.01, *P,0.05)).

Figure 2.11: Long-term inhibition of CO₂-mediated avoidance behavior.

A, T-maze behavior assay: mean preference index of wild-type flies, given a choice between room air and CO₂ (CO₂) or ethyl acetate (2ac) (10⁻⁴ dilution). Experiments were performed as in Fig 2.9, or after a 1-min pre-exposure to either 2,3-butanedione (10⁻² dilution) (after d4on), or 2-methyl phenol (10⁻² dilution) (after 2mp) as indicated and a subsequent 2-min hold in clean air (n=6 trials (approximately 40 flies each); error bars, s.e.m. (t-test, *P,0.0001)). **B**, Mean preference index of *Or83b*² mutant flies given choices as indicated, as described for **A** (n=6 trials (approximately 40 flies each); error bars, s.e.m. (t-test, *P,0.0001)).

Figure 2.12: Butanone activates ab1C in *Drosophila*, and is specific to the CO₂ receptor.

A, Electrophysiological response of A, B or C neurons as indicated, from ab1 sensilla of *Or83b*² mutant flies or **B**, *Gr63a*¹ mutant flies to odorants, at the indicated concentrations. CO₂=0.3%. 2-Butanone=4on, 2,3-butanedione=d4on, ethyl acetate=2Ac, pentyl acetate=5Ac, paraffin oil=PO. n=5. Error bars=s.e.m.

Figure 2.13: Butanone activates ab1C in *Drosophila*, and is not inhibited by 2,3-butanedione.

A, Mean responses of ab1C neuron in *Or83b*² mutant flies that were subjected to a 3-sec pre-exposure to 2,3-butanedione (10⁻¹ dilution)(d4on), followed by a 0.5-sec stimulus of 0.3% CO₂ or 2-butanone (10⁻¹ dilution) (4on). n=3-5. **B**, Mean response of the ab1C neuron in *Or83b*² mutant flies to 0.3% CO₂ or 2-butanone (10⁻¹ dilution) after a 1-min pre-exposure to 2,3-butanedione (10⁻² dilution), followed by a 2-min recovery period in clean air. n=3, error bars=s.e.m. **C**, Mean preference index of *Or83b*² or *Gr63a*¹, *Or83b*² flies as indicated, given a choice between room air and 2-butanone (4on) (10⁻¹ dilution); or *Or83b*² flies given the same choice, after a 1-min pre-exposure to d4on (10⁻² dilution), (4on after d4on), or in the presence of 2,3-butanedione (10⁻² dilution) (4on+d4on) (n=6 trials (20 flies each); error bars, s.e.m. 2-Butanone=4on, 2,3-butanedione=d4on. (t-test, *P,0.0001)).

Table 2.1: Odourant quantity in ripe fruit

Fruit	Ripeness	Extraction method	Analysis method	Odourant	Abundance	Abundance rank	Reference
Guava	ripe	CH2cl2 (white flesh)	GC-MS	hexanol	area of peak 1.64%	15th / 79	a
Guava	ripe	CH2cl2 (pink flesh)	GC-MS	hexanol	area of peak 2.52%	6th / 86	a
Guava	ripe	CH2cl2 (puree)	GC-MS	hexanol	area of peak 0.99%	4th / 63	a
Guava	ripe	CH2cl2 (puree)	GC-MS	2,3-butanedione	area of peak 0.1%	27th / 63	a
Mango (Keaw)	ripe	volatile oil	GC-MS	hexanol	0.032 ppm	66th / 92	b
Mango (Keaw)	ripe	volatile oil	GC-MS	2,3-butanedione	0.0039 ppm	87th / 92	b
Mango (Alphonso)	ripe	distilled	capillary GLC-MS	hexanol	1000 ppb	11th/ 84	c
Mango (Baladi)	ripe	distilled	capillary GLC-MS	hexanol	50 ppb	41st/ 89	c
Mango (Jaffna)	ripe	pentane	GC-MS	hexanol	1.5 g/kg	24th / 49	d
Mango (Willard)	ripe	pentane	GC-MS	hexanol	2.1 g/kg	29th /40	d
Mango	ripe	distilled	GC-MS	hexanol	0.25mg/kg	9th/22 †	e
Peach	ripe	headspace	GC-MS	hexanol	area of peak 1.57%	6th / 110	f
Peach	ripe	headspace	GC-MS	2,3-butanedione	area of peak 10.2%	4th / 110	f
Apricot	ripe	headspace	GC-MS	hexanol	area of peak 0.307%	23rd / 83	g
Apricot	ripe	headspace	GC-MS	2,3-butanedione	detected	N.D.	g
Apricot	ripe	vacuum steam distilled	GC-MS	hexanol	740 g/ kg	2nd / 49	g
Apricot	ripe	vacuum steam distilled	GC-MS	2,3-butanedione	220 g/ kg	6th / 49	g
Melon	ripe	dichloromethane	GC-MS	hexanol	0.28mg/kg	8th/10 †	h
Strawberry	ripe	headspace	GC-MS	hexanol	0.03mg/kg	11th/12 †	i
Passion fruit	ripe	distilled	GC-MS	hexanol	1.56 ppm	7th/8 †	j
Musk melon	ripe	distilled	GC-MS	hexanol	1.09 ppm	2nd/9 †	k
Banana	ripe	distilled	GC-MS	hexanol	1.17 ppm	4th/8 †	l

Ripening associated change in odourant quantity

Fruit	Ripeness	Extraction method	Analysis method	Odourant	Abundance	% increase	Reference
Apple (Fuji)	172 days after bloom	headspace	GC-MS	hexanol	not detected		m
Apple (Fuji)	185 days after bloom	headspace	GC-MS	hexanol	1.8 g / kg	nd	m
Apple (Fuji)	192 days after bloom	headspace	GC-MS	hexanol	3.0 g / kg	66%	m
Apple (Fuji)	202 days after bloom	headspace	GC-MS	hexanol	5.0 g / kg	177%	m
Apple (Fuji)	208 days after bloom	headspace	GC-MS	hexanol	8.6 g / kg	377%	m
Apricot	green	vacuum distilled/extraction	GC-MS	hexanol	5.0 g / kg		n
Apricot	tree ripe	vacuum distilled/extraction	GC-MS	hexanol	9.0 g / kg	80%	n
Banana	unripe	headspace	Proton transfer-MS	hexanol *	610 ppb		o
Banana	ripe	headspace	Proton transfer-MS	hexanol *	5350 ppb	777%	o
Banana	unripe	headspace	Proton transfer-MS	2,3-butanedione**	9 ppb		o
Banana	ripe	headspace	Proton transfer-MS	2,3-butanedione**	1350 ppb	14900%	o

†: ranked according to Pelz, D., et al. The Molecular Receptive Range of an Olfactory Receptor in vivo (*Drosophila melanogaster* Or22a). *J. Neurobiol.* **66** 1544-1563 (2006).

* Eluted as a combination of hexanol, 2-propenal, butanol, hexyl acetate, and 2E-hexenal. ** Eluted as a combination of 2,3-butanedione and 2-pentanone.

a. Nishimura, O., Yamaguchi, K., Mihara, S., Shibamoto, T. Volatile constituents of guava fruits (*Psidium guajava* L.) and canned puree. *J. Agric. Food Chem.*, **37**(1), 139-142 (1989).

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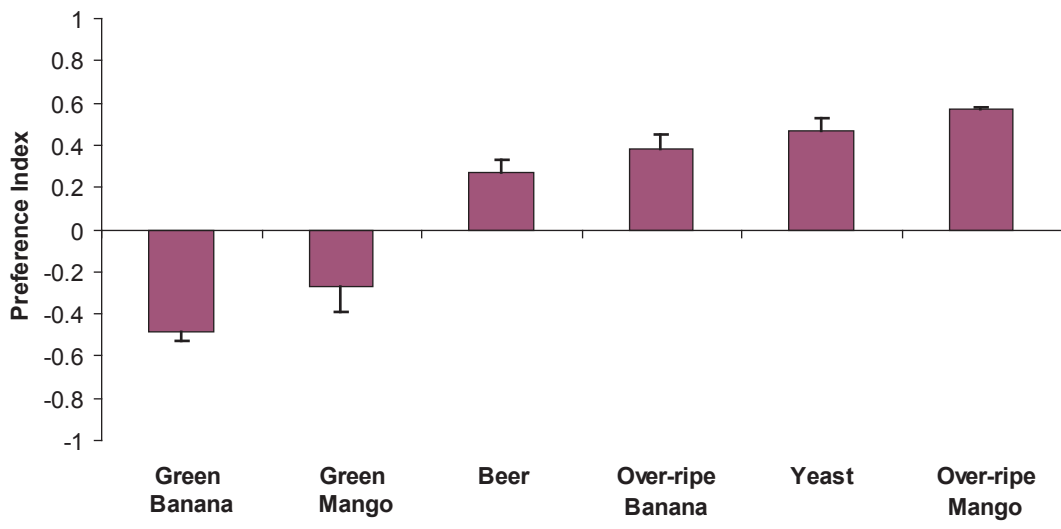


Figure 2.1

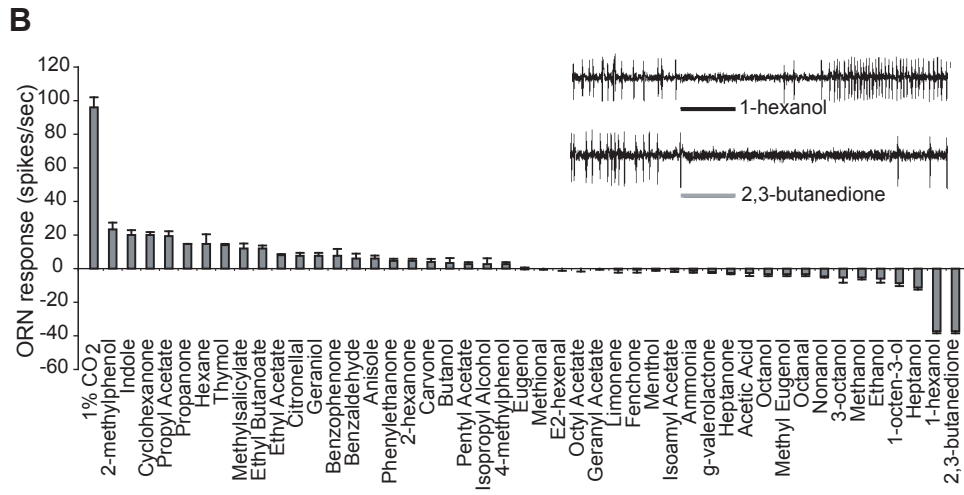
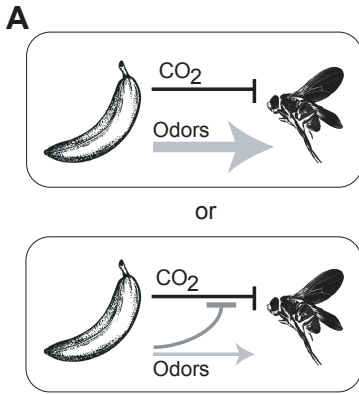


Figure 2.2

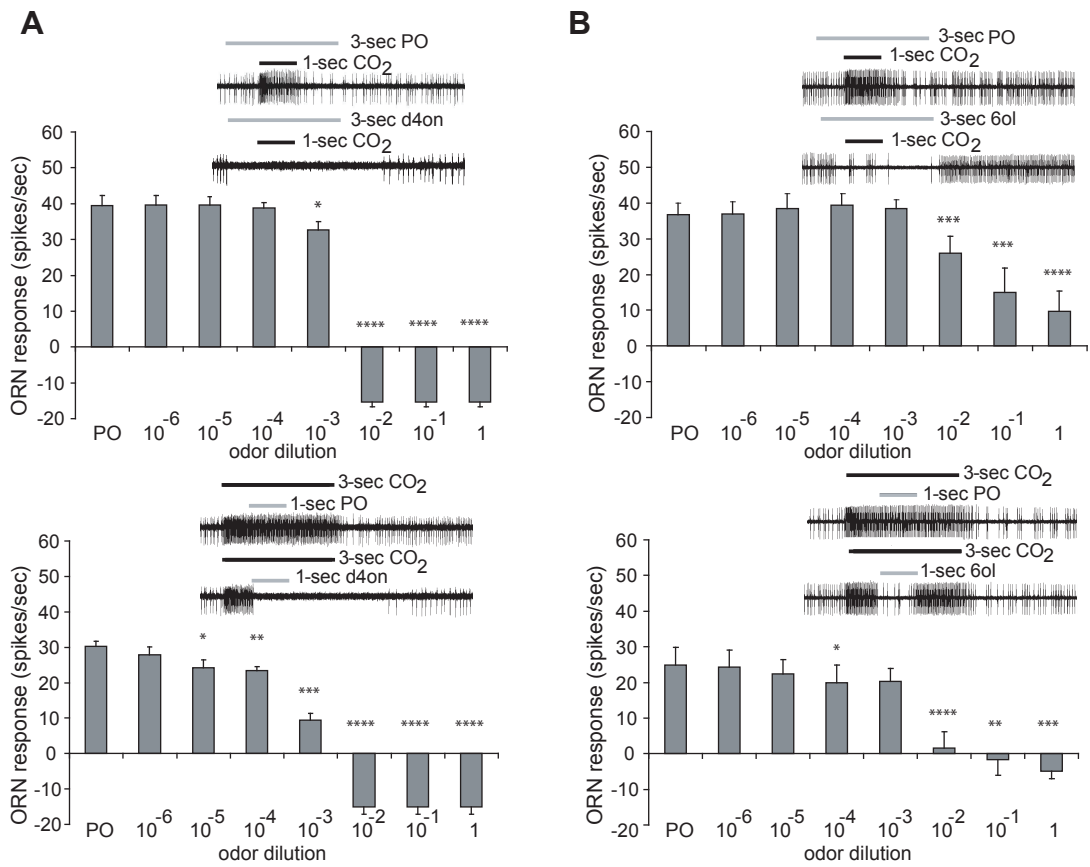


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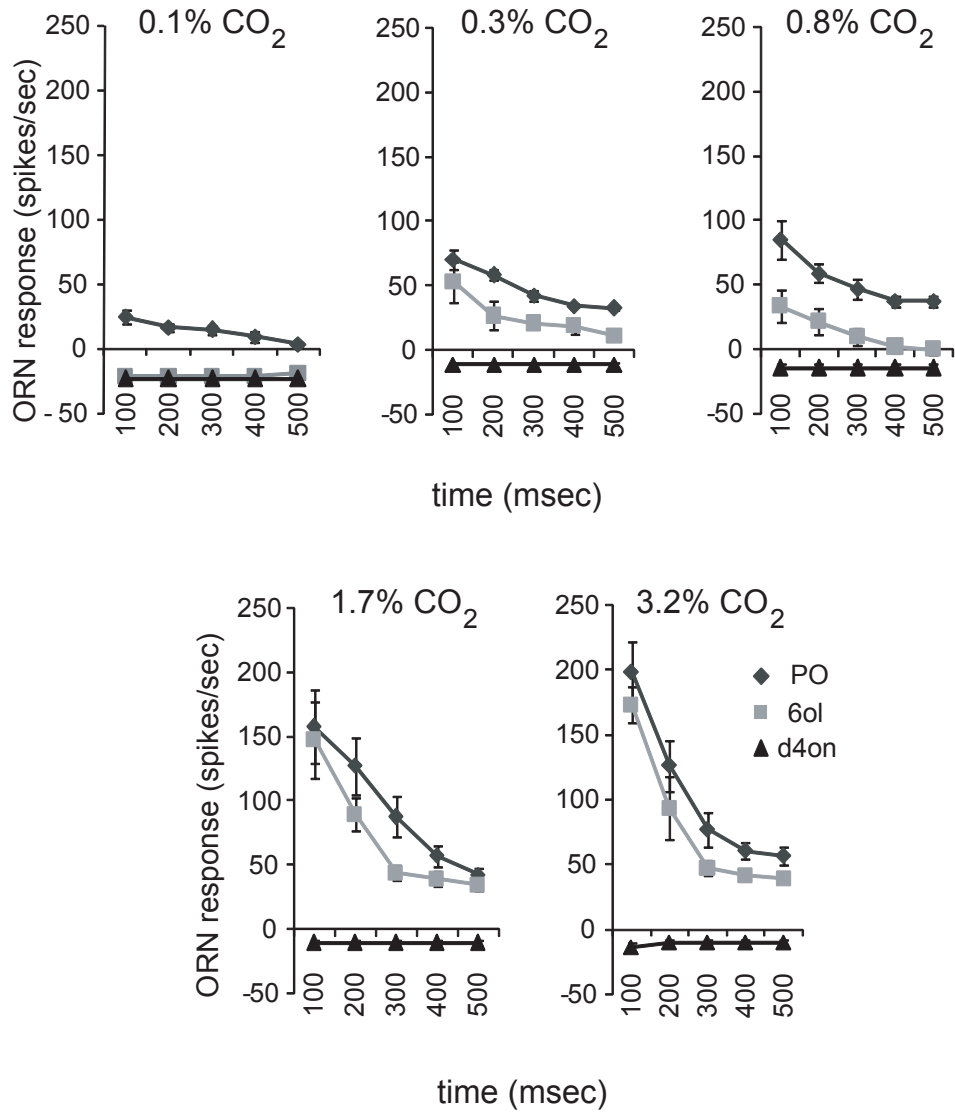


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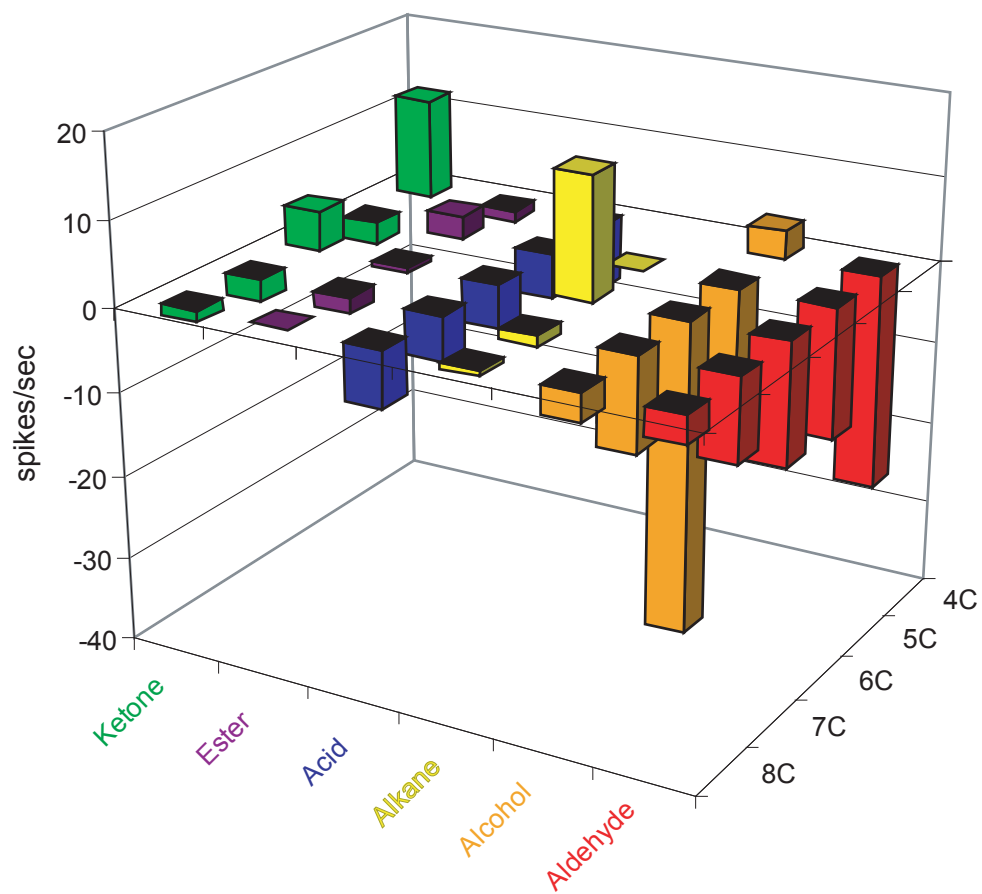
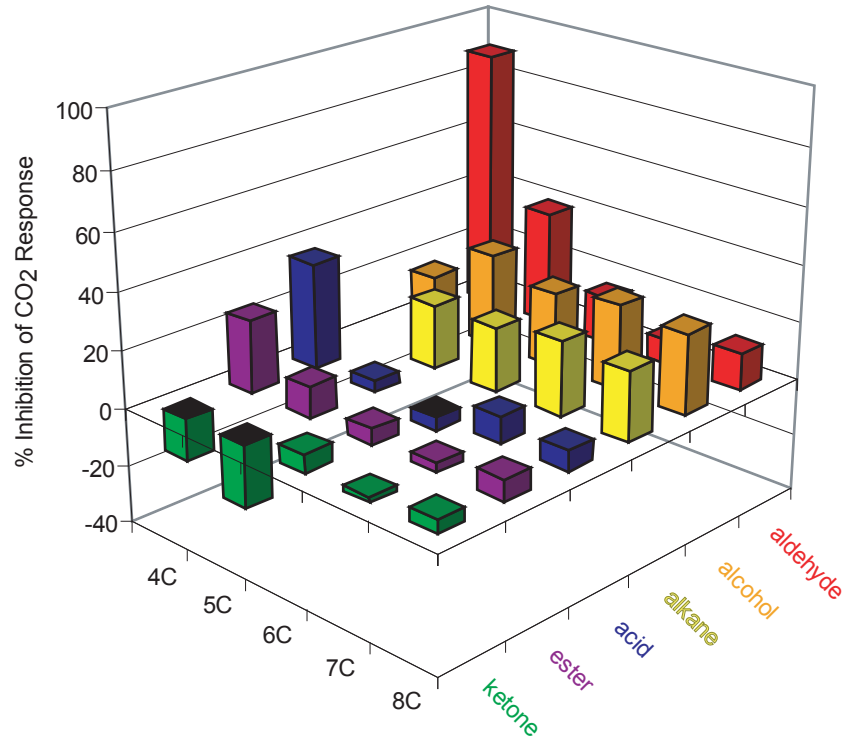
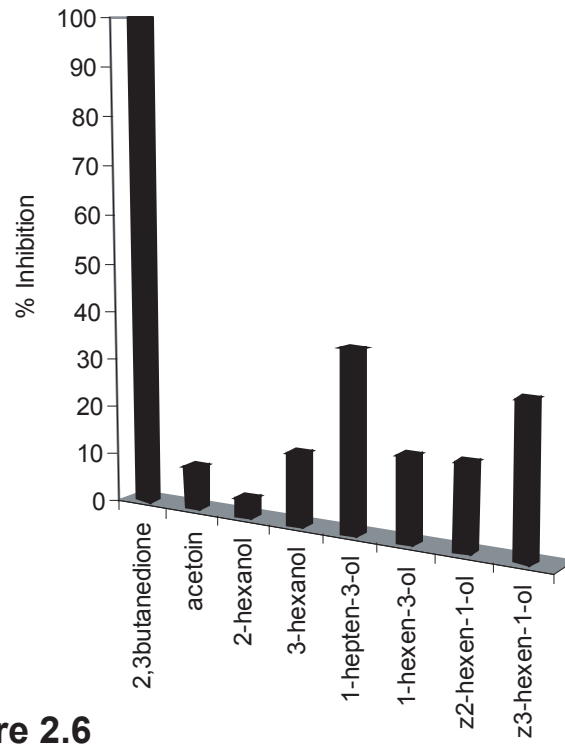


Figure 2.5

A**B****Figure 2.6**

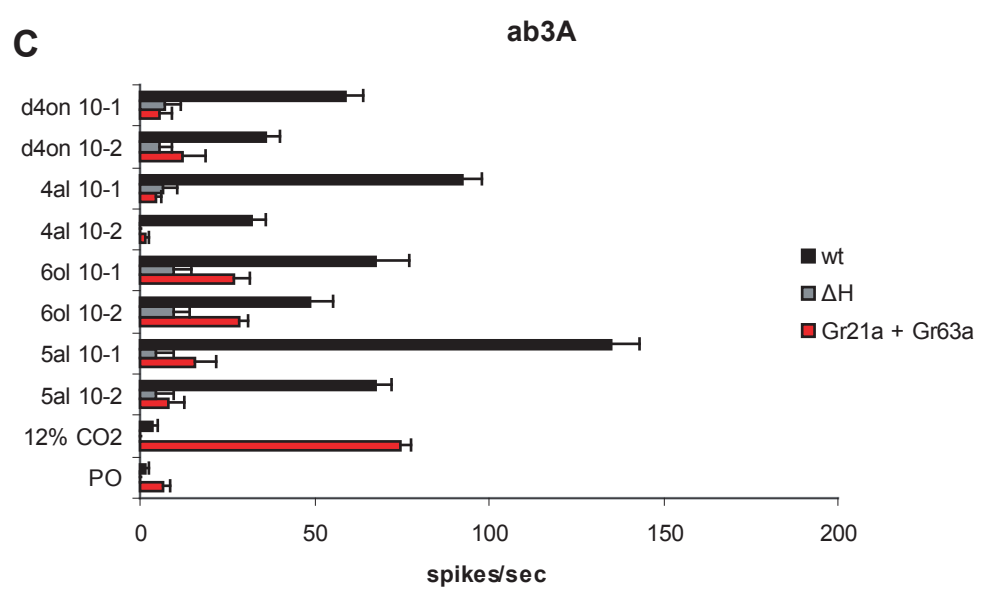
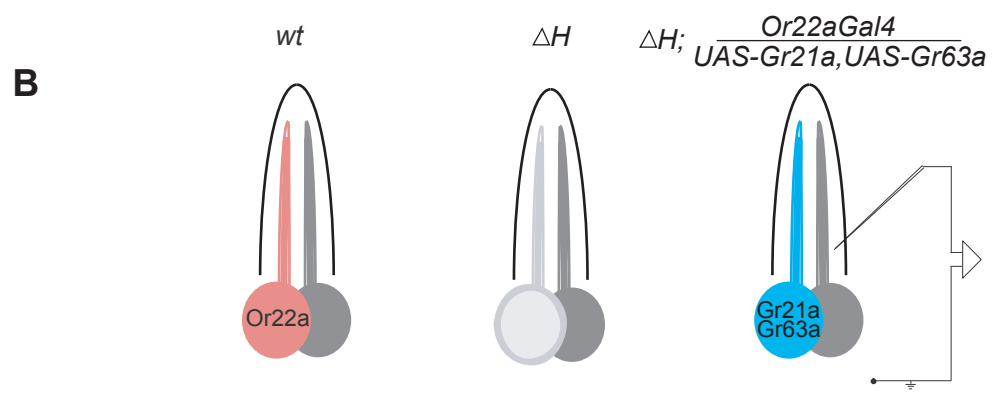
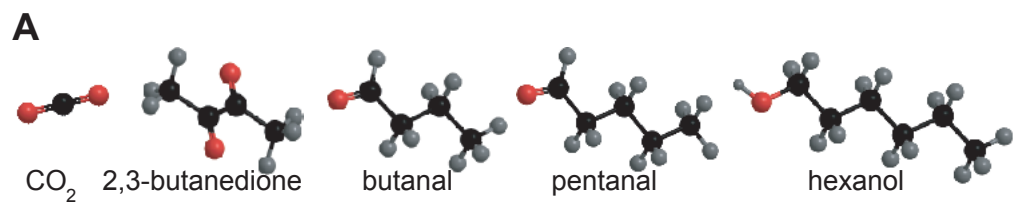


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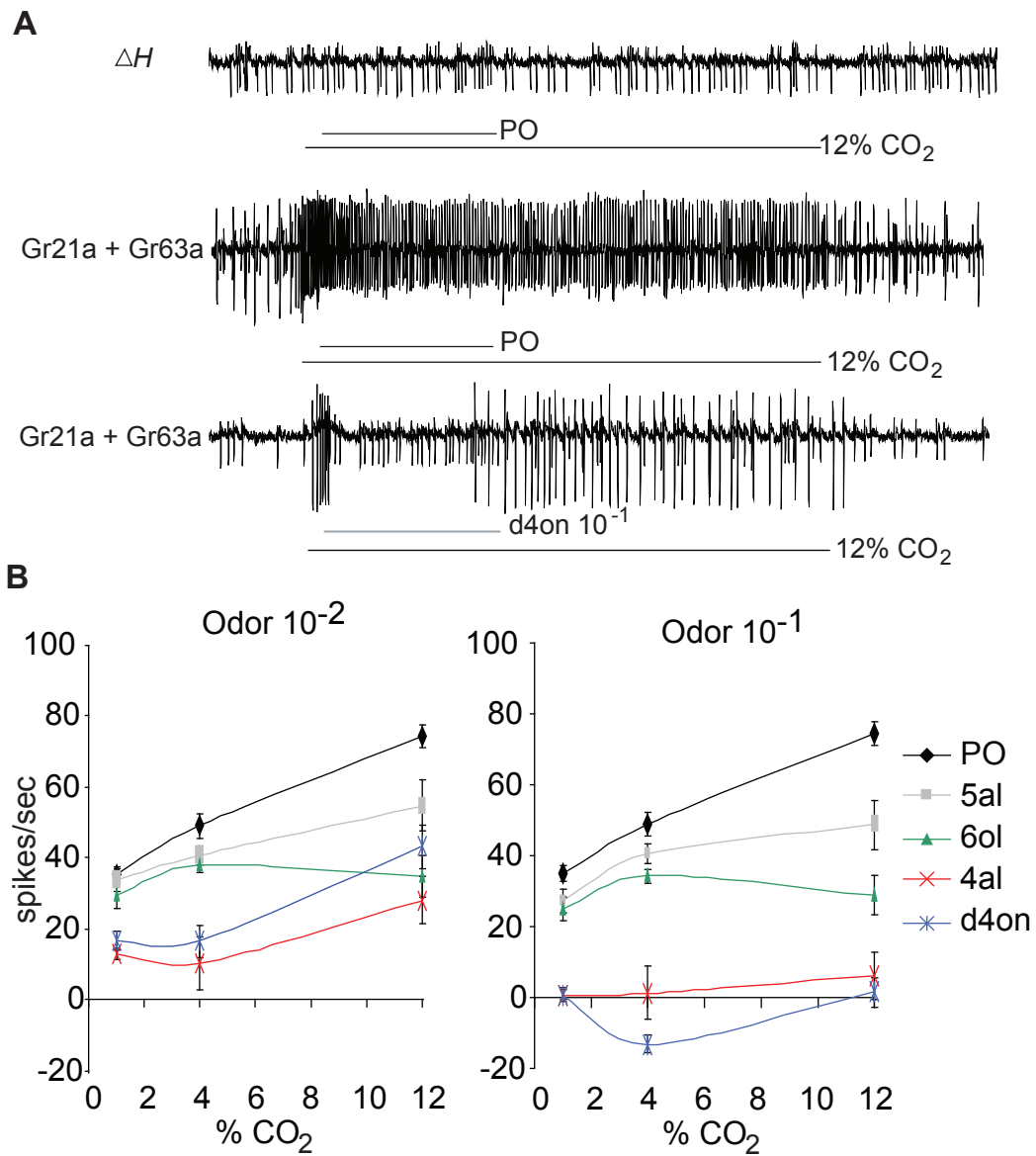


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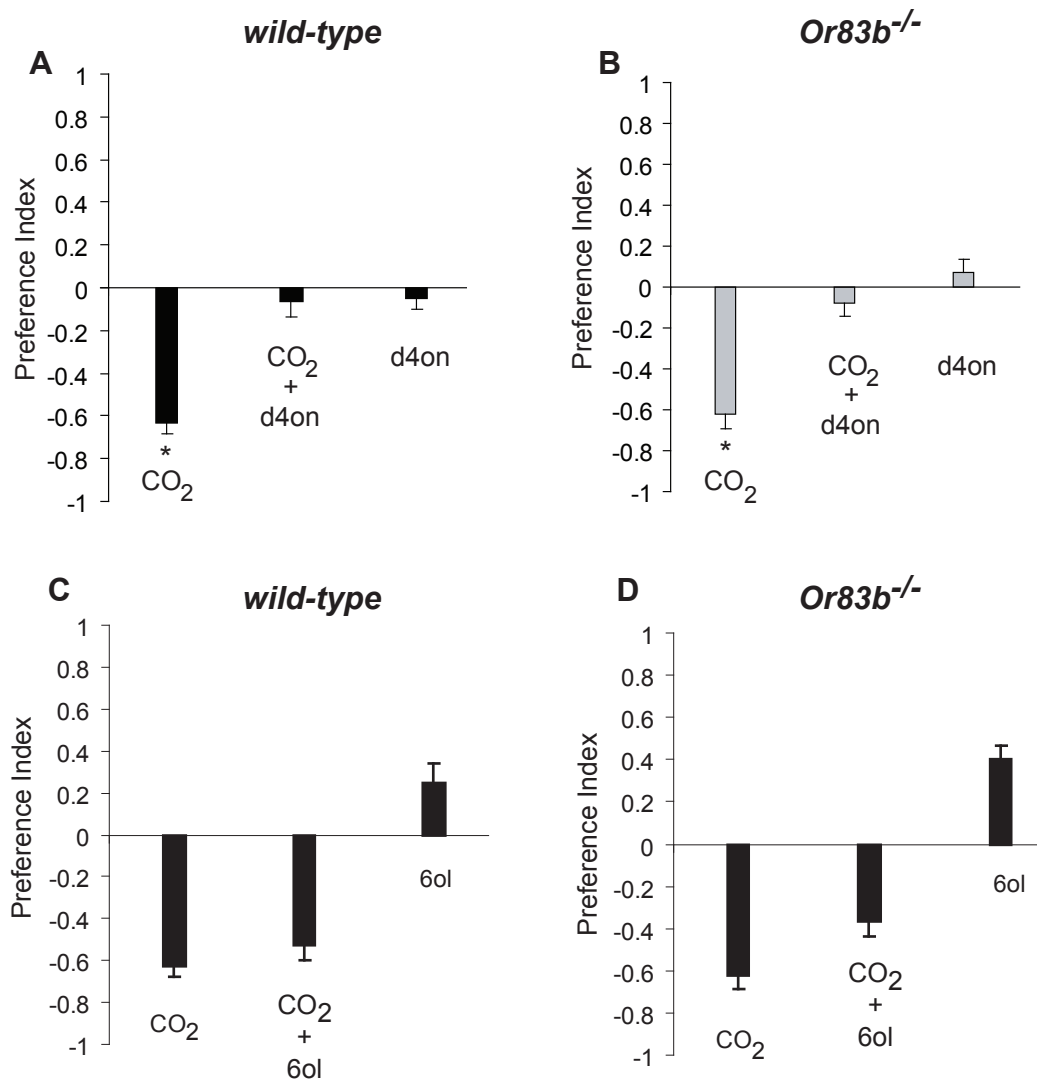


Figure 2.9

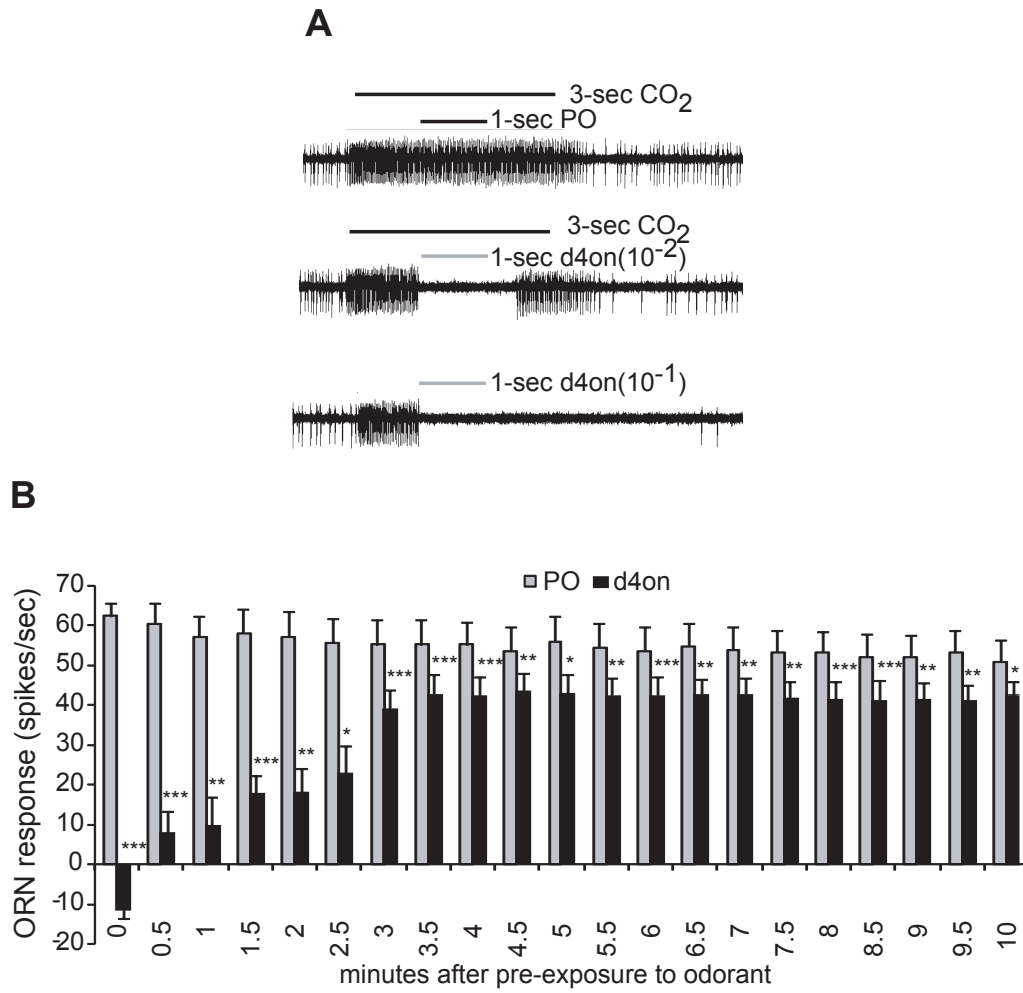


Figure 2.10

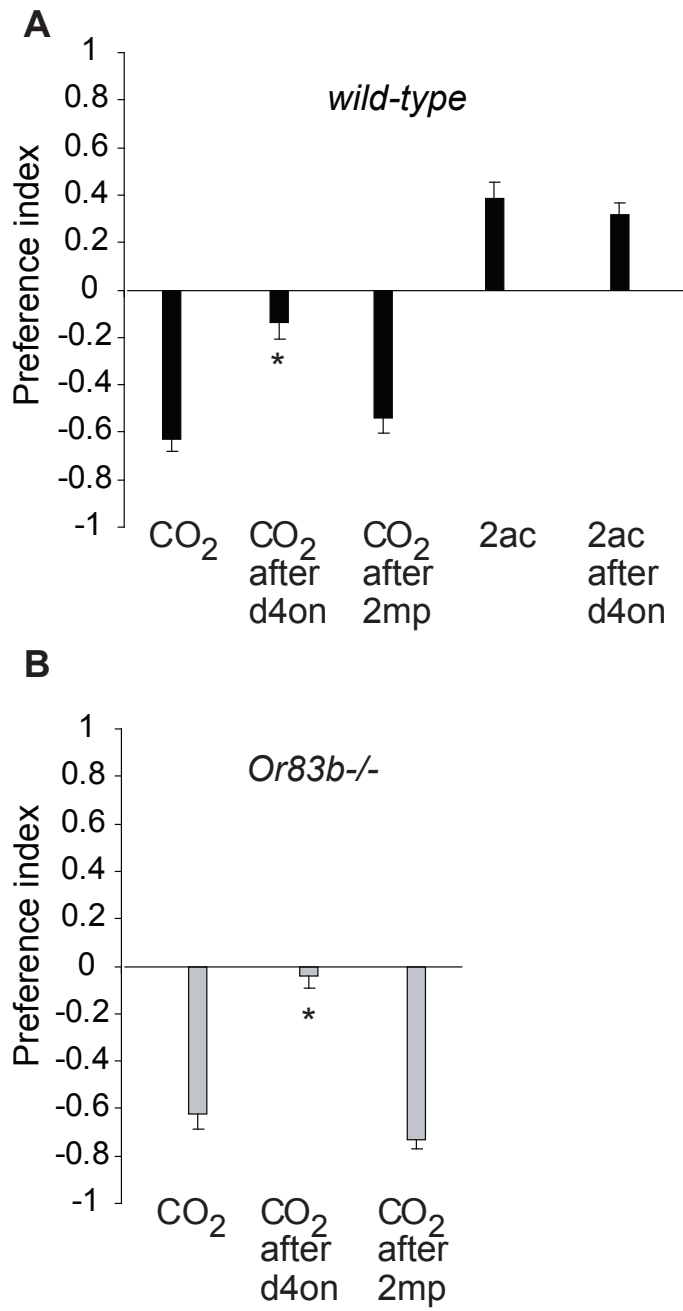


Figure 2.11

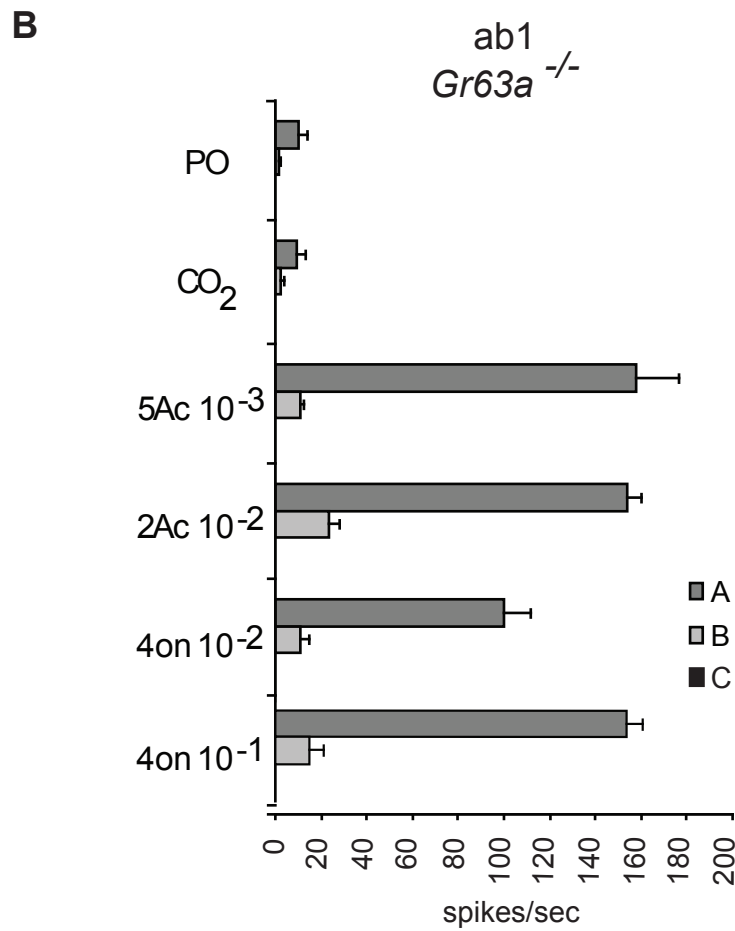
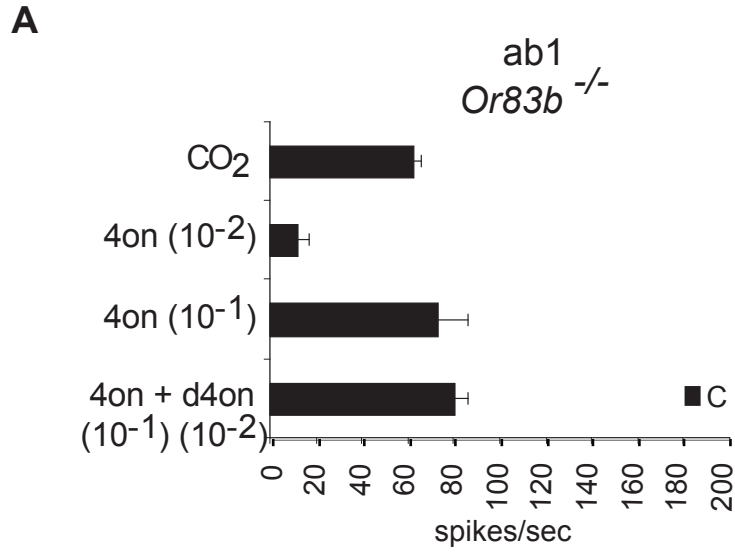


Figure 2.12

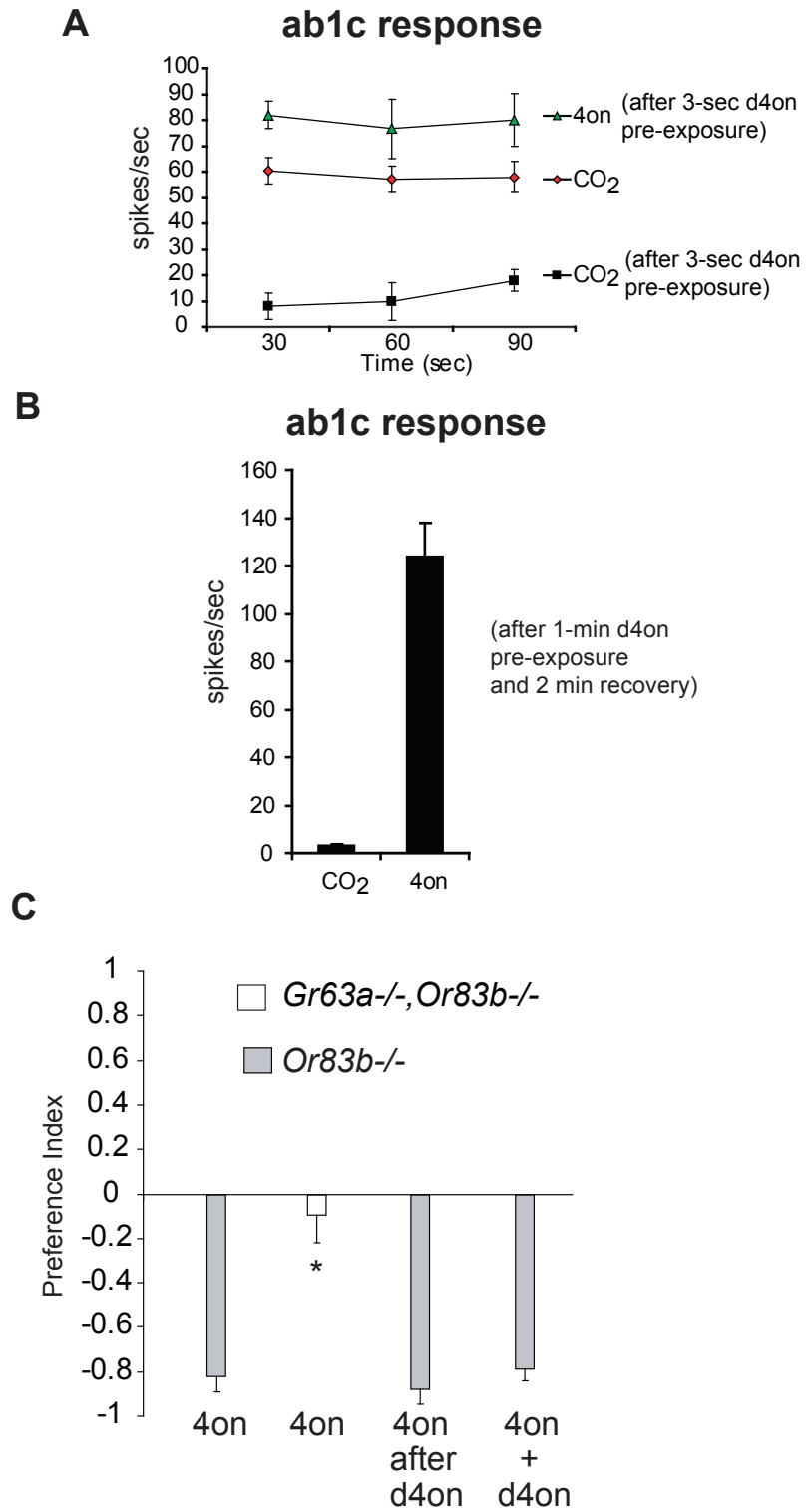


Figure 2.13

CHAPTER III:
VOLATILE ODORANTS AS CO₂ NEURON INHIBITORS, ACTIVATORS AND
ULTRA-PROLONGED ACTIVAOTRS:
NOVEL TOOLS FOR MOSQUITO CONTROL

Introduction:

Carbon dioxide (CO₂) serves as a long-distance orientation and host-seeking cue for most mosquito species (Cardé and Willis, 2008). Human beings generate CO₂ odor plumes through exhaled breath, causing fluctuation in CO₂ between background (0.04%) and expired levels (4%). This intermittency in CO₂ concentration is thought to increase host-seeking behavior in mosquitoes, causing them to fly upwind toward the odor source (Cardé and Willis, 2008; Dekker et al., 2005). Once the mosquito has followed the CO₂ plume back toward its source, it is thought that the insect will then detect other sensory cues such as skin odors and heat (Takken and Knols, 1999). Not surprisingly, mosquito species such as the ornithophilic *Culex quinquefasciatus* and the anthropophilic *Anopheles gambiae* and *Aedes aegypti*, are differentially attracted to host odors such as those from avian, and human sources, respectively (Cooperband et al., 2008; Dekker et al., 2001). However, CO₂ is an odor common to all hosts as it signifies the presence of a vertebrate's exhaled air. When presented in an optimal fashion, CO₂ can readily attract mosquitoes in the field and in the laboratory (Cooperband and Cardé, 2006b; Dekker et al., 2001; Grant and Oconnell, 1996; Xue et al., 2008), as well as increase the sensitivity of

mosquitoes to other human odors (Dekker et al., 2005). Whether for domestic or scientific use, the majority of mosquito traps employ CO₂ as the lure. Although it remains to be determined how host odors affect attraction behavior in mosquitoes, it can be argued that CO₂ is highly influential in host-seeking behavior of many mosquito species. In the three mosquito species presented in this study, the maxillary palp is the primary CO₂ detecting organ, where of the three neurons housed in the club-shaped capitulate peg (cp) sensilla, the cpA neuron expresses the CO₂ receptors Gr22, Gr23, and Gr24 (Hill et al., 2002; Lu et al., 2007; Syed and Leal, 2007) which belong to the gustatory receptor family. These proteins are highly orthologous to the CO₂ receptor of *Drosophila melanogaster*, Gr21a and Gr63a which have been shown to be the required for behavioral response to CO₂ (Jones et al., 2007; Robertson and Kent, 2009).

It has been previously shown that inhibition of the CO₂ response by volatile odorants corresponds to complete loss of innate CO₂ avoidance behavior in *Drosophila* (Turner and Ray, 2009) (CHAPTER II). Given the abolishment of *Drosophila* behavior to CO₂ in the presence of the inhibitory odorants, and that mosquito CO₂ receptors have high amino acid identity with the *Drosophila* ortholog Gr63a and Gr21a (Figure 3.1) (Hill et al., 2002; Kent et al., 2008; Lu et al., 2007; Robertson and Kent, 2009), it is plausible that these identified odorants could have an effect on CO₂-mediated host-seeking behavior in mosquito.

Here we have identified volatile odorants which include: odors that inhibit the CO₂-sensitive neuron and are candidates for use in disruption of host-seeking behavior, odors that activate the neuron and can be a substitute for CO₂ as a lure in trapping devices, and odors that cause strong and prolonged activation of the CO₂ neuron that blocks the ability to detect changes in CO₂ concentration and therefore offers a novel approach for disruption of host-seeking. These compounds could be highly beneficial as tools used for mosquito control as they modify peripheral olfactory responses to arguably the most important host-seeking cue. Modifying a mosquito's response to CO₂ by either inhibiting or activating the neuron with odorants could potentially lower the incidence of human-mosquito contact, and hence lower the spread of vector-borne diseases.

Results and Discussion:

We have used single-sensillum electrophysiology to screen a large number of odorants for their effect on the activity of the CO₂-sensitive neuron in the peg sensilla of the maxillary palp of female *An. gambiae*, *Ae. aegypti*, and *Cx. quinquefasciatus*. The cpA neuronal response to a 1-sec stimulus of 0.15% CO₂ is nearly identical in all three species (Figure 3.2A). When looking for inhibitory odorants, we applied 3-sec of 0.15% CO₂ over 1-sec of odorant and then looked for a decrease in spikes per second from the cpA neuron during the CO₂ stimulus. Interestingly, 1-butanol and 1-hexanol gave differential patterns of CO₂ response inhibition. When counted in 100-msec bins over the 3-sec CO₂ delivery period, 1-butanol inhibited the cpA neuron for the majority of the stimulus while the ability of 1-hexanol to inhibit CO₂ response was apparent only for approximately 0.5-sec (Figures 3.3, 3.4, 3.5). This was the case in all three mosquito species. These odorants also inhibited the CO₂ response in a dose dependent manner, where 1-butanol was generally more effective at inhibiting the CO₂ response than 1-hexanol at lower concentrations (Figures 3.3, 3.4, 3.5). In this initial screen we also found that butyric acid strongly inhibits the CO₂ response in *An. gambiae* and *Ae. aegypti*, (Figure 3.6, 3.7), where the neuron is inhibited for the majority of the stimulus. Again, this inhibition occurs in a dose-dependent manner (Figure 3.6, 3.7).

In a similar manner, a screen for inhibitors of the CO₂ response was performed in the three mosquito species where chemicals varied by carbon number length and functional group. A 3-sec pulse of 0.15% CO₂ was applied over 1-sec of odorant, and percent inhibition of the cpA neuron was compared in all species as well as in *Drosophila*. As was seen above, 1-butanol gave the best inhibition of CO₂ response in all insect species (Figure 3.8). In addition, butyric acid showed the strongest inhibition during the CO₂ stimulus for *An. gambiae* and *Ae. aegypti*, while other odorants acted in a more species specific manner (Figure 3.9). Importantly, the best inhibitors of the CO₂ response do not share common chemical properties such as vapor pressure or solubility (Figure 3.10). Interestingly, neither mosquito species had a significant decrease in CO₂ response by a ketone, which was the functional group that gave the best CO₂ response inhibition in *Drosophila* (Turner and Ray, 2009).

The ability of CO₂ to attract mosquitoes has been exploited for use as a lure in most commercially available mosquito trapping devices (Cooperband and Cardé, 2006a; Costantini et al., 1996; Mboera et al., 1997; Njiru et al., 2006). In order to identify other volatile odorants that have the ability to activate the CO₂-sensitive neuron in mosquitoes, we performed a second electrophysiology screen with a large panel of odorants. Using this approach we have identified an odorant, 2-butanone, which by itself activates the cpA neuron of the peg sensillum in a manner similar to CO₂ and is easily distinguished between the

three neurons of the peg sensilla (Figure 3.2B). 2-Butanone causes a strong dose-dependent activation of the CO₂ sensitive cpA neuron in all three mosquito species (Figure 3.11). When comparing the pattern of activation for both CO₂ and 2-butanone, odor dependent activation is followed by a period of quiescence after which the firing rate returns to a baseline activity level due to CO₂ in the air (Figure 3.12). Because 2-butanone mimics the same pattern of activation as CO₂, it could serve as an ideal candidate to use in place of CO₂ in commonly used trapping devices.

While performing a screen for activators of mosquito cpA neurons we found that 2,3-butanedione causes a novel mode of activation which we term "ultra-prolonged activation". When exposed to a 1-sec stimulus of the odorant, the cpA neuron is activated in a dose dependent manner (Figure 3.13), where the strongest concentration tested causes prolonged firing of the neuron. To further investigate the effect of ultra-prolonged activity on CO₂ responsiveness, we performed the following experiment. A 3-sec stimulus of 2,3-butanedione was applied to the mosquito, followed by repeated 1-sec stimuli of 0.15% CO₂ every 30-sec, to test for the neurons ability to respond to CO₂ after odor treatment (Figure 3.14, 3.15). Then comparing neuronal activity patterns in both *An. gambiae* and *Ae. aegypti* there is clearly a dramatic increase in baseline activity of the cpA neuron (Figure 3.14), where in the case of *An. gambiae* there is little difference between the neurons spike rate before and during a CO₂

stimulus. Astonishingly, the spontaneous activity of the *An. gambiae* cpA neuron remains at 100 spikes/second for the entire assay (Figure 3.15A, left). In addition, the ability of the neuron to respond to CO₂ is completely abolished (Figure 3.15A, right), indicating that the mosquito would be unable to detect changes in CO₂ concentrations, which is an important feature of CO₂ host seeking behavior (Cardé and Willis, 2008). Similar results are seen when *Ae. aegypti* are treated with 2,3-butanedione in the same manner, where the baseline activity of the neuron remains at 60 spikes/sec and the ability of the neuron to respond to CO₂ is significantly reduced (Figure 3.15B). *Culex* are not affected by this treatment (Figure 3.15C). We previously have shown that 2,3-butanedione interacts with the CO₂ receptor in *Drosophila* causing long-term inhibition (Turner and Ray, 2009). Although the mode of action is exactly the opposite in mosquito, given that the CO₂ receptors are highly orthologous to those in *Drosophila*, the simplest interpretation of these results is that 2,3-butanedione is also interacting with the mosquito CO₂ receptor. The prolonged action of 2,3-butanedione and its obvious structural difference to CO₂ suggests that the odor binds to the receptor in an allosteric manner perhaps causing an irreversible conformational change in the receptor. Alternatively, it could be possible that the odor is binding covalently to the receptor.

What is the mechanism of ultra-prolonged activation, and can treatment with other odors augment this activation? To test for modification of the cpA neuron by other odorants we performed the following experiment. The *Aedes* mosquito was treated with ultra-prolonged activating odorant 2,3-butanedione for 3-sec, followed by treatment with either a second odor or solvent after 15-sec, then assessed for CO₂ responsiveness at 30-sec and 60-sec after the initial ultra-prolonged odor treatment (Figure 3.16). Activity of the cpA neuron in *Aedes* mosquitoes was monitored following treatment with a 3-sec stimulus of 2,3-butanedione, and as expected, the baseline activity of the neuron increases while CO₂ responsiveness decreases (Figure 3.16B). In order to see how other odors might affect ultra-prolonged activity, 2-butanone, 1-butanol, and 1-hexanol were applied 15-sec after the ultra-prolonged activating treatment (Figure 3.16C, D, E, 3.17). cpA neuron activator 2-butanone causes increased activation during the initial stimulus, and correspondingly causes subsequent CO₂ stimuli to be inhibited by ~40% (Figure 3.16C, 3.17). Upon further investigation, 1-butanol has a peculiar effect on the cpA neuron. The odorant given as a stimulus alone without any pre-treatment, acts as an activator of the cpA neuron, but when given as a co-stimulus with CO₂ acts as a CO₂ response inhibitor (Figure 3.8). After pre-treatment with 2,3-butanedione, a stimulus with 1-butanol causes further activation of the neuron (Figure 3.17A), but does not have any effect on subsequent CO₂ stimuli (Figure 3.17B, C). cpA neuron inhibitor 1-hexanol caused inhibition of the neuron after pre-treatment with 2,3-butanedione (Figure

3.17A), and caused inhibition of subsequent CO₂ stimuli by ~40% (Figure 3.17B, C). Most interestingly, none of the odor treatments augmented baseline activity of the neuron as caused by ultra-prolonged activator 2,3-butanedione (Figure 3.17B), indicating that ultra-prolonged activation is not reversible.

Previously, it has been shown that specific odorants can cause a prolonged activation of odor receptor neurons, beyond the initial time of odorant application (Hallem and Carlson, 2006). In *Drosophila melanogaster*, 2-heptanone has been shown to cause a prolonged activation of Or85b. In order to demonstrate that 2,3-butanedione can genuinely activate an olfactory neuron beyond what has been previously shown, we performed a similar experiment where *D. melanogaster* was exposed to 2-heptanone to activate the ab3B neuron/Or85b receptor. 2-heptanone was used to activate the ab3B neuron to a level similar to that done by 2,3-butanedione on the cpA neuron of mosquito (~120spikes/sec). We then looked to see how long odorant induced activation persists. Activation of the ab3B neuron quickly diminishes to 40 spikes/sec within 10-sec, and is completely lost 30-sec after the odor stimulus (Figure 3.18A). This is clearly a shortened activation in comparison to cpA neuronal activation by 2,3-butanedione, where in *An. gambiae* an initial activation of the neuron to 120 Spikes/sec caused the neuron to stay activated at levels of ~100spikes/sec for nearly 5.5 minutes (Figure 3.15A). Similarly, activation of the *A. aegypti* neuron to 120 spikes/sec causes the neuron to stay activated at ~60 spikes/sec even 5.5 minutes after the initial odorant application (Figure 3.15B).

2,3-butanedione causes a diminished response to CO₂ in the cpA neuron. Does activation of the fly ab3B neuron by 2-heptanone cause a diminished response to another neuronal activator? After giving a stimulus of 2-heptanone, subsequent 1-sec pulses of 1-hexanol were applied to the fly every 30-sec. Although there is a diminished response to 1-hexanol during the first few minutes following the treatment with 2-heptanone, response to 1-hexanol is completely recovered after 3 min (Figure 3.18B). Although 2-heptanone can modify a response to other ab3B neuronal activators, this modification is short-lived in comparison to 2,3-butanedione induced modification of the CO₂ response in the cpA neuron of *Aedes*.

When performing the electrophysiological odor screens, we observed that butyric acid caused an initial inhibition of the CO₂ response (Figure 3.6, 3.7, 3.8). However, following this brief inhibition, the odorant induced „ultra-prolonged“ activation of the cpA neuron. To investigate if ultra-prolonged activation by butyric acid could cause a reduced response to CO₂, *An. gambiae* and *Ae. aegypti* mosquitoes were exposed to a 3-sec application of the odorant followed by repeated 1-sec stimulus of 0.15% CO₂ applied every 30-sec for a period of approximately 5 minutes. As with 2,3-butanedione, when comparing spike rate in both mosquitoes, there is a profound increase in baseline activity of the cpA neuron (Figure 3.19, 3.20). We found that butyric acid significantly reduced CO₂ response for as long as 5.5 min in *An. gambiae* (Figure 3.20, right), while CO₂ response in *Ae. aegypti* was completely abolished (Figure 3.20, left).

Interestingly, butyric acid is a component of human sweat (Cork and Park, 1996), which has been shown to activate as well as inhibit several sensilla trichodea in *An. gambiae* (Meijerink and van Loon, 1999; van den Broek and den Otter, 1999). Although human sweat is highly attractive to anthropophilic mosquitoes (Braks and Takken, 1999; Healy et al., 2002), it is not clear what role carboxylic acids play in the attractiveness of this host-odor blend. For example, there are several conflicting studies as to the attractiveness of carboxylic acids to mosquitoes where in some cases carboxylic acids are actually unattractive (Healy et al., 2002; Mboera et al., 1997; Smallegange et al., 2005). The varied attractiveness to human skin odors could be attributed to intraspecific preferences for certain human hosts as their emanations differ from individual to individual (Acree et al., 1968; Besansky et al., 2004; Dekker et al., 2001; Qiu et al., 2004; Takken and Knols, 1999). No study, to our knowledge, has looked at the attractiveness of carboxylic acids (or human odors) as it pertains to activation or inhibition of neurons in the maxillary palp. It is unclear from these and other studies if behavioral responses observed result from a direct repellent effect or another mechanism whereby the insects are failing to respond to normally attractive cues such as CO₂. Perhaps levels of butyric acid from person to person can contribute to host preference in the mosquito as a means of CO₂ response modulation. Future behavioral assays will be required to test this hypothesis.

In an effort to increase the effect of ultra-prolonged activation by 2,3-butanedione, we tested several odorant blends. Importantly, combining butyric acid and 2,3-butanedione did not enhance ultra-prolonged activity (data not shown). As a result, we combined the odorants which worked best in *Ae. aegypti* and found that some combinations that included three odorants did reduce the responsiveness of the cpA neuron to CO₂, while keeping baseline activity relatively low (Figure 3.21). When all of the best odors were combined, including 2,3-butanedione, 1-butanal, 1-pentanal, and 1-hexanol, there was a severe reduction in the neuron's ability to respond to CO₂ (Figure 3.22A). The excitatory effect of the "ultra-prolonged" odor blend and subsequent lack of responsiveness is specific to the CO₂-sensitive cpA neuron. The response of the neighbouring cpB neuron to 1-octen-3-ol is not affected (Figure 3.22B, C). Importantly, the individual components of the odor blend are not as effective in lowering the responsiveness of the cpA neuron (Figure 3.23). In fact, the odorant blend is so exceptional at reducing responsiveness to CO₂ that after a 3 min treatment with the blend at 10⁻² concentration the neuron does not respond to CO₂ even after 2 hours of recovery (Figure 3.24). Even 6 hours after treatment the neuron is only able to respond to CO₂ at half normal levels. A full recovery is not seen until 12 hours after the initial treatment. Importantly, a drop in baseline activity of the neuron over time corresponds to a gain in responsiveness to CO₂ (Figure 3.24).

We next sought to find how ultra-prolonged activity of the CO₂ neuron effected CO₂ host-seeking behavior in female *Ae. aegypti* mosquitoes. To test for CO₂ host-seeking behavior, we rely on the reliable and reproducible wind-tunnel assay (Dekker et al., 2005). Briefly, single female mosquitoes are pre-treated with an odorant, and then transferred to the wind tunnel. Air pumped through the wind tunnel passes by a CO₂ emitting ring which forms plumes that reach the pre-treated mosquito. We then record the mosquito's ability to reach the halfway point of the wind tunnel (% upwind) (Figure 3.25A, B). Since CO₂ has been shown to initiate upwind flight behaviour in mosquitoes, we considered the ability of the mosquito to leave the holding cage and fly upwind past the half-way point as a reflection of this step (Carde, 1996; Dekker et al., 2005). We also record the mosquito's ability to reach the CO₂ source at the end of the wind tunnel (% source). All control *Aedes* females that were mock treated with solvent (paraffin oil) left the holding cage and crossed the half-way point of the tunnel (Figure 3.26A,C). Furthermore all the control treated mosquitoes navigated successfully along a turbulent CO₂ plume and found the CO₂ source, as measured by flight through the CO₂-emitting ring (Figure 3.25C, 3.26B, D). We found that pretreatment with the "ultra-prolonged" odor blend significantly impaired CO₂-mediated behavior in a dose-dependent manner, as measured by the ability of the mosquito to fly upwind (Figure 3.26A), as well as to find the CO₂ source (Figure 3.25C, 3.26B). An increase in the duration of pretreatment with

the ultra-prolonged activator blend results in the increased impairment of CO₂-mediated upwind flight and navigation behavior (Figure 3.26C, D).

We wanted to examine in more detail the number of mosquitos performing upwind flight and navigation behavior over time. Mosquitoes were pre-treated for 1 minute so that we could observe variations in timing of behaviors across various pre-treatment groups. We found that all mock-treated control mosquitoes reached the CO₂ source within the first minute upon initiation of the assay (Figure 3.27). The proportion of pretreated mosquitoes that responded increased as the assay progressed through 5 minutes (Figure 3.27), suggesting that in the absence of the ultra-prolonged activators mosquitoes gradually recover their ability to detect CO₂.

Conclusion:

In summary, we have identified odorants that efficiently inhibit CO₂ response in the cpA neurons of the mosquito maxillary palp. CO₂ emitted in human breath is a critical host-seeking cue used by many vector insect species that carry deadly diseases including *Anopheles gambiae* (malaria), *Aedes aegypti* (dengue and yellow fever), *Culex quinquefasciatus* (filarial and West Nile virus) (Cooperband and Cardé, 2006a; Dekker et al., 2005; Zwiebel and Takken, 2004).

We show electrophysiological inhibition of the CO₂ response by several odorants in *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus*. The odorants we have identified can be found in the environment in and around the mosquito, such as components of host odors. Previously, we have demonstrated that inhibitory odorants play a critical role in shaping the behavioral response in *Drosophila*. Based on these findings, we predict that the CO₂ inhibitory odorants identified in this study could cause decreased attraction of mosquitoes to CO₂. Placing these odors in or around a human dwelling could effectively mask CO₂ host-seeking cues. Conversely, we predict that odorants identified as activators of the CO₂ neuron will act as attractants on these mosquito species and could serve well in a trap application, with or without CO₂. In addition, we have identified ultra-prolonged activators of the CO₂ neuron, which severely reduces *Aedes* CO₂ host-seeking behavior, as predicted by electrophysiology. Finally, the structure of these volatile compounds will enable us to identify other volatile

chemicals that are effective for control of mosquitoes in small quantities, can be delivered in multiple forms, are economical, and are environmentally friendly. As mosquito borne diseases such as dengue and malaria cause millions of deaths each year, it is our hope that by developing novel repellants and attractants, and incorporating them along with other disciplines, an all-encompassing approach for mosquito control can be devised to decrease the incidence of disease.

Figure Legends:

Figure 3.1: CO₂ receptors are highly conserved in insects.

A, Alignment of the amino acid sequences of CO₂ receptor orthologs using ClustalW from *D. melanogaster* (Dmel), *An. gambiae* (Agam), *Ae. aegypti* (Aaeg) and *Cx. quinquefasciatus* (Cpip). Recreated using sequences from Robertson et. al. 2009.

Figure 3.2: CO₂ responses are similar across *An. gambiae*, *Ae. aegypti*, and *Cx. quinquefasciatus*.

A, Mean responses of female *An. gambiae* (Ag), *Ae. aegypti* (Ae), and *Cx. quinquefasciatus* (Cx), to a 1-sec stimulus of paraffin oil (solvent) overlaid with a 3-sec stimulus of 0.15% CO₂. Data represented as mean spikes per second of cpA neuron counted in 100-msec bins. n=5. Error bars=s.e.m. **B**, Representative close up view of electrophysiological recording from the peg-sensillum on the maxillary palp of female *An. gambiae* showing distinct spike amplitudes of cpA (large), cpB (medium), and cpC (small). (top) Stimulus of a diagnostic odorant for cpC 2,4,5-trimethylthiazole (10⁻³ dilution), and 2-butanone (10⁻² dilution). (bottom) Stimulus of a diagnostic odorant for cpB 1-octen-3-ol (10⁻³ dilution), and 2-butanone (10⁻² dilution).

Figure 3.3: Inhibitory odorants dramatically reduce responses of the CO₂ sensitive neuron in *An. gambiae*.

A, Scanning electron micrograph of maxillary palp from female *An. gambiae*.

B,C, Representative traces and mean responses from single sensillum electrophysiology of peg sensilla. The largest spike amplitude represents activity of the CO₂-sensitive cpA neuron. A 1-sec stimulus of either paraffin oil solvent (PO), 1-butanal (4al) **B**, or 1-hexanol (6ol) **C** is overlaid with a 3-sec stimulus of 0.15% CO₂. Odorant diluted 10⁻¹ applied on filter paper of odor cartridge (See Methods). Mean spikes per second of cpA neuron counted in 100-msec bins, n=5, error bars=s.e.m. **D, E**, Mean CO₂ response using stimuli as in B and C. using indicated odorants. n=5, error bars=s.e.m.

Figure 3.4: Inhibitory odorants dramatically reduce responses of the CO₂ sensitive neuron in *Ae. aegypti*.

A, Scanning electron micrograph of maxillary palp from female *Ae. aegypti*.

B,C, Representative traces and mean responses from single sensillum electrophysiology of peg sensilla. The largest spike amplitude represents activity of the CO₂-sensitive cpA neuron. A 1-sec stimulus of either paraffin oil solvent (PO), 1-butanal (4al) **B**, or 1-hexanol (6ol) **C** is overlaid with a 3-sec stimulus of 0.15% CO₂. Odorant diluted 10⁻¹ applied on filter paper of odor cartridge (See Methods). Mean spikes per second of cpA neuron counted in 100-msec bins, n=5, error bars=s.e.m. **D, E**, Mean CO₂ response using stimuli as in B and C. using indicated odorants. n=5, error bars=s.e.m.

Figure 3.5: Inhibitory odorants dramatically reduce responses of the CO₂ sensitive neuron in *C. quinquefasciatus*.

A, Scanning electron micrograph of maxillary palp from female *C. quinquefasciatus*. **B,C**, Representative traces and mean responses from single sensillum electrophysiology of peg sensilla. The largest spike amplitude represents activity of the CO₂-sensitive cpA neuron. A 1-sec stimulus of either paraffin oil solvent (PO), 1-butanol (4al) **B**, or 1-hexanol (6ol) **C** is overlaid with a 3-sec stimulus of 0.15% CO₂. Odorant diluted 10⁻¹ applied on filter paper of odor cartridge (See Methods). Mean spikes per second of cpA neuron counted in 100-msec bins, n=5, error bars=s.e.m. **D, E**, Mean CO₂ response using stimuli as in B and C. using indicated odorants. n=5, error bars=s.e.m.

Figure 3.6: Butyric acid dramatically reduces response of the CO₂ sensitive neuron in *An. gambiae*.

A, Representative traces and mean responses from single sensillum electrophysiology of peg sensilla. The largest spike amplitude represents activity of the CO₂-sensitive cpA neuron. A 1-sec stimulus of either paraffin oil solvent (PO), or butyric acid (4ac) is overlaid with a 3-sec stimulus of 0.15% CO₂. Odorant diluted 10⁻¹ applied on filter paper of odor cartridge (See Methods). Mean spikes per second of cpA neuron counted in 100-msec bins, n=5, error bars=s.e.m. **B**, Dose-response of butyric acid. n=5, error bars=s.e.m.

Figure 3.7: Butyric acid dramatically reduces response of the CO₂ sensitive neuron in *Ae. aegypti*.

A, Representative traces and mean responses from single sensillum electrophysiology of peg sensilla. The largest spike amplitude represents activity of the CO₂-sensitive cpA neuron. A 1-sec stimulus of either paraffin oil solvent (PO), or butyric acid (4ac) is overlaid with a 3-sec stimulus of 0.15% CO₂. Odorant diluted 10⁻¹ applied on filter paper of odor cartridge. Mean spikes per second of cpA neuron counted in 100-msec bins, n=5, error bars=s.e.m. **B**, Dose-response of butyric acid. n=5, error bars=s.e.m.

Figure 3.8: Structurally related odorants inhibit CO₂ sensitive neuron of mosquitoes.

Comparison of percentage of CO₂ response inhibition in the cpA neuron of *An. gambiae* (Ag), *Ae. aegypti* (Ae), *Cx. quinquefascitus* (Cx), compared to *D. melanogaster* (Dm). Functional group is on the primary carbon atom except for ketones on C2, and the length of the carbon chain (carbon number) is indicated. A 1-sec stimulus of odorant was applied over a 3-sec stimulus of CO₂. Odorants were diluted 10⁻², 0.15% CO₂ and 0.33% CO₂ was used for mosquito and fly respectively. n=3. Dm data taken from (Turner and Ray, 2009).

Figure 3.9: Inhibition of CO₂ response by multiple classes of odorants.

Representative traces from *An. gambiae* (Ag), *Ae. aegypti* (Ae), *Cx.*

quinquefascitus (Cx), where CO₂ response is inhibited by 40% or greater. A 1-sec stimulus of either paraffin oil solvent, 1-butanol, 1-hexanol, butyric acid, pentanone, or pentanoic acid is overlaid with a 3-sec stimulus of 0.15% CO₂. Odorant diluted 10⁻².

Figure 3.10: Inhibition of CO₂ response is independent of specific chemical properties.

% Inhibition of the CO₂ response as a function of **A, B**, vapor pressure, or **C,D**, solubility. Differing shapes indicate insect; *An. gambiae* (□), *Ae. aegypti* (▲), *Cx. quinquefascitus* (—), *D. melanogaster* (◇). Differing colors indicate chemical class; alcohol (red), aldehyde (orange), ester (pink), alkane (blue), ketone (green), acid (purple). Arrows indicate the best inhibiting odors overall, 1-butanol and 1-hexanol. n=3.

Figure 3.11: 2-Butanone activates the cpA neuron in mosquito.

A, Representative trace from *An. gambiae* peg sensillum to a 1-sec stimulus of solvent (PO) or 2-butanone (10^{-1} dilution). **B**, Mean responses of the cpA neuron to 1-sec 2-butanone at indicated dilutions on *An. gambiae* (Ag), *Ae. aegypti* (Ae), and *Cx. quinquefasciatus* (Cx). n=5, error bars=s.e.m.

Figure 3.12: 2-Butanone can activate the cpA neuron in a manner similar to CO₂.

A, B, C, Representative long-term recordings from the cpA neuron in response to repeated pulses of 1-sec 0.15% CO₂ (black square) or 1-sec (40n) 2-butanone (10^{-1} dilution) stimulus (green square) from *An. gambiae*, *Ae. aegypti*, and *Cx. quinquefasciatus*. **D**, Expanded view of the traces for each species during the stimulus period indicated in the rectangular box in **A, B** and **C**.

Figure 3.13: Ultra-prolonged activator of mosquito CO₂ neuron.

A, Representative trace from *An. gambiae* peg sensillum to a 1-sec stimulus of solvent (PO) or 2,3-butanedione(10^{-1}). **B**, Mean responses of the cpA neuron to 1-sec 2,3-butanedione at indicated dilutions on *An. gambiae* (Ag), *Ae. aegypti* (Ae), and *Cx. quinquefasciatus* (Cx). n=5, error bars=s.e.m.

Figure 3.14: Ultra-prolonged activation reduces responsiveness of cpA neuron to CO₂.

Long-term recordings from the cpA neuron of *An. gambiae* and *Ae. aegypti* to a 3-sec stimulus of 2,3-butandione (10^{-1} dilution) followed by repeated 1-sec pulses of 0.15% CO₂. Expanded 1-sec views of the indicated regions of the traces are shown.

Figure 3.15: Ultra-prolonged activation reduces responsiveness of cpA neuron to CO₂.

A, *An. gambiae* **B**, *Ae. aegypti* **C**, *Cx. quinquefasciatus* showing **(Left panels)** mean activity of the cpA neuron counted every 30-sec interval after pre-exposure to a 3-sec stimulus of 2,3-butanedione (10^{-1} dilution) or paraffin oil (untreated). **(Right panels)** Mean increase in frequency of response of the cpA neuron to stimulus of 1-sec 0.15% CO₂ applied approx. every 30-sec, following a 3-sec pre-exposure to 2,3-butanedione (10^{-1} dilution) or paraffin oil (untreated). n=5, error bars=s.e.m.

Figure 3.16: The effect of inhibiting or activating odorants on ultra-prolonged activation.

Mean responses from the cpA neuron following treatment with either 3-sec ultra-prolonged activator (2,3-butanedione 10^{-1} dilution) or paraffin oil **A**, followed by activating or inhibiting odorants to test for reduction of the CO₂ response or baseline activity. 15-sec following the ultra-prolonged odor treatment, a 1-sec stimulus of either paraffin oil (PO) **B**, 2-butanone (4on) **C**, 1-butanal (4al) **D**, or 1-hexanol (6ol) **E** is given. A 1-sec 0.15% CO₂ stimulus is given at the 30-sec and 60-sec time points. Spikes/sec are counted every 3-sec following treatment with 2,3-butanedione. Odorants diluted 10^{-1} . n=5, error bars=s.e.m.

Figure 3.17: The effect of inhibiting or activating odorants on ultra-prolonged activation.

Quantification of mean responses from Figure 3.16.

A, Mean responses from the cpA neuron following pre-treatment with either 3-sec ultra-prolonged activator (2,3-butanedione) or paraffin oil (PO) to 2-butanone (4on), 1-butanal (4al), or 1-hexanol (6ol). **B**, Mean response from the cpA neuron to 1-sec 0.15% CO₂ prior to (no treatment) or following ultra-prolonged activator 2,3-butanedione and either PO, 4on, 4al, or 6ol. Spikes/sec are counted during the 1-sec CO₂ stimulus at 30-sec and 60-sec after the odor treatments. **C**, Table of either % inhibition or % excitation of the cpA neuron during odor delivery period, and CO₂ stimulus periods at 30-sec and 60-sec following indicated odor treatments. Negative values indicate % inhibition. Odorants diluted 10⁻¹. n=5, error bars=s.e.m.

Figure 3.18: Prolonged neuronal activation in *D. melanogaster*.

A, Mean response from the ab3B neuron to a 3-sec stimulus of 2-heptanone (10⁻³ dilution) given at time=0. Spontaneous activity is counted at 1.5, 10, 20, and 30-sec after the initial stimulus and every 60-sec thereafter. **B**, Mean responses of the ab3B neuron to repeated 1-sec stimuli of 1-hexanol (10⁻² dilution) every 30-sec following a 3-sec treatment with 2-heptanone as in **A** (6ol after 7on) or solvent (no treatment). n=5, error bars=s.e.m.

Figure 3.19: Butyric acid is an ultra-prolonged activator of the CO₂ sensitive neuron in *An. gambiae* and *Ae. aegypti*.

A,B, Long-term traces from the cpA neuron of *An. gambiae* and *Ae. aegypti*, respectively. A 3-sec stimulus paraffin oil **top** or butyric acid (4ac) **bottom** is given followed by 1-sec pulses of 0.15% CO₂ every 30-sec. Odor diluted 10⁻¹.

Figure 3.20: Butyric acid is an ultra-prolonged activator of the CO₂ sensitive neuron in *An. gambiae* and *Ae. aegypti*.

Mean responses from the cpA neuron as in figure 3.19.

(Top panels) mean activity of the cpA neuron counted every 30-sec interval after pre-exposure to a 3-sec stimulus of butyric acid (10⁻¹ dilution) or paraffin oil (Solvent). **(Bottom panels)** Mean increase in frequency of response of the cpA neuron to stimulus of 1-sec 0.15% CO₂ applied approx. every 30-sec, following a 3-sec pre-exposure to butyric acid (10⁻¹ dilution) or paraffin oil (Solvent). n=5, error bars=s.e.m.

Figure 3.21: Certain combinations of odorants reduce responsiveness to CO₂ in *A. aegypti*.

(Left panels) mean activity of the cpA neuron counted every 30-sec after pre-exposure to a 3-sec stimulus of odorant mixtures (10⁻² dilution) (d4on, 4al, 6ol) **A**, (d4on, 5al, 6ol) **B**, (d4on, 4al, 5al) **C**, (4al, 5al, 6ol) **D** or paraffin oil (PO). **(Right panels)** Mean increase in frequency of response of the cpA neuron to stimulus of 1-sec 0.15% CO₂ applied approx. every 30-sec, following a 3-sec pre-exposure to odor mixtures as in left panel or paraffin oil (PO). d4on=2,3-butanedione, 4al=1-butanal, 5al=1-pentanal, 6ol=1-hexanol. n=5, error bars=s.e.m.

Figure 3.22: Four odorant mixture increases ultra-prolonged activity of 2,3-butanedione.

A, Baseline activity of cpA neuron (**left**) and mean increase in responses (**right**) to 1-sec 0.15% CO₂ pulses applied approx. every 30-sec following a 3-sec pre-exposure to odor mixture of 2,3-butanedione, 1-butanal, 1-pentanal, and 1-hexanol (10⁻² dilution). n=5, error bars=s.e.m. **B**, Representative traces from peg sensillum from the cpA neuron to 0.15% CO₂ and the cpB neuron to 1-octen-3-ol (10⁻³ dilution) after a pretreatment to 3-min of paraffin oil, or odor mixture from **A** (10⁻² dilution) (see CHAPTER VI: METHODS). **C**, mean responses from the cpA neuron to 0.15% and 0.33% CO₂ and the cpB neuron to 1-octen-3-ol (10⁻³ dilution) before and after a pretreatment to 3-min of paraffin oil or odor mixture (10⁻³ dilution). n=5, error bars=s.e.m.

Figure 3.23: Effect of individual odor mixture components on ultra-prolonged activation in *Ae. aegypti*.

Representative traces from peg sensillum and mean responses from the cpA neurons to 0.15% and 0.33% CO₂ and the cpB neuron to 1-octen-3-ol (10⁻³ dilution) before (untreated) and after a pretreatment to 3-min of paraffin oil, or individual odor mixture components 2,3-butanedione (**A, E**), 1-butanal (**B, F**), 1-pentanal (**C, G**), and 1-hexanol (**D, H**) (10⁻¹ dilution). n=5, error bars=s.e.m.

Figure 3.24: The persistence of ultra-prolonged activity.

Mean responses **top**, and mean baseline activity **bottom**, of the cpA neuron to 0.15% CO₂ and the cpB neuron to 1-octen-3-ol (10⁻³ dilution) after a 3-min exposure to odor mixture of 2,3-butanedione, 1-butanal, 1-pentanal, and 1-hexanol (10⁻² dilution). n=5. error bars=s.e.m.

Figure 3.25: CO₂ host-seeking behavior of *Ae. aegypti*.

A, Schematic of the experimental strategy and **B**, the wind-tunnel apparatus. **C**, Percentage of female mosquitoes flying through the CO₂ emitting glass ring after pre-exposure to 3 minutes of paraffin oil (control), indicated odorants (10⁻² dilution) or ultra-prolonged activating blend (Mixture) (10⁻² dilution). N=26 individuals for each condition. Pearson's χ^2 test, compared to controls, a= P<0.05, b=P<0.01, c=P<0.001.

Figure 3.26: Exposure to ultra-prolonged activator gives long-term disruption of CO₂-mediated attraction behavior of female *Aedes* mosquitoes

A, B, Flying upwind to half-way point and **B**, flying through the CO₂ emitting glass ring after pre-exposure for 3 minutes to paraffin oil (control) or ultra-prolonged activating blend at indicated concentrations. **C, D**, Similar experiment as above except three pre-exposure times were tested to the ultra-prolonged blend at indicated concentrations. N=26 individuals for each condition. Pearson's χ^2 test, compared to controls, a= P<0.05, b=P<0.01, c=P<0.001.

Figure 3.27: Recovery of CO₂-host seeking behavior after ultra-prolonged activating odor treatment in *Ae. aegypti*.

A, B, Time-course of percentages of female mosquitoes getting to half-way point and reaching the CO₂ source after a 1 minute pre-exposure to paraffin oil or ultra-prolonged blend at the indicated concentrations. N=26 individuals for each condition. Pearson's χ^2 test, compared to controls, a= P<0.05, b=P<0.01, c=P<0.001.

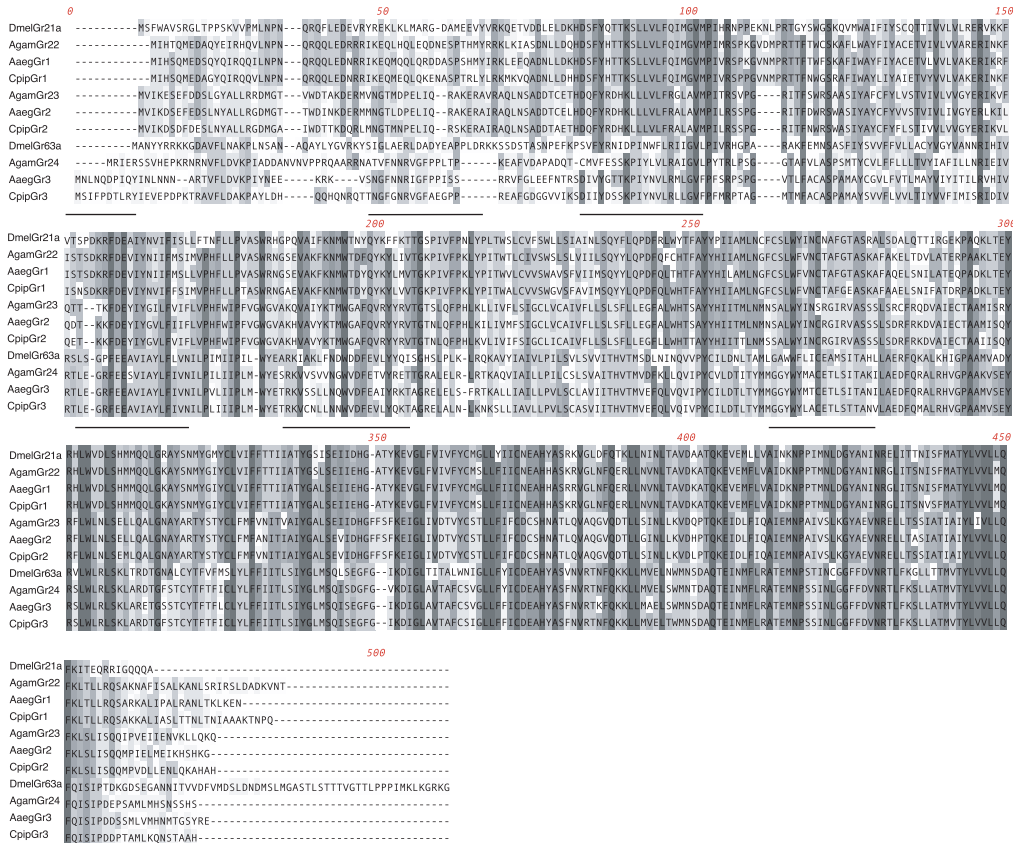


Figure 3.1

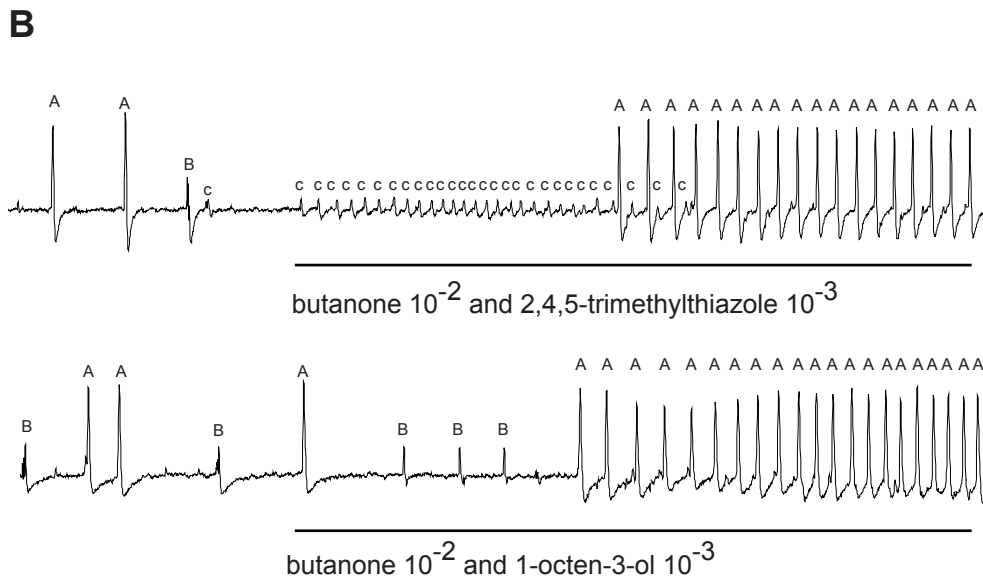
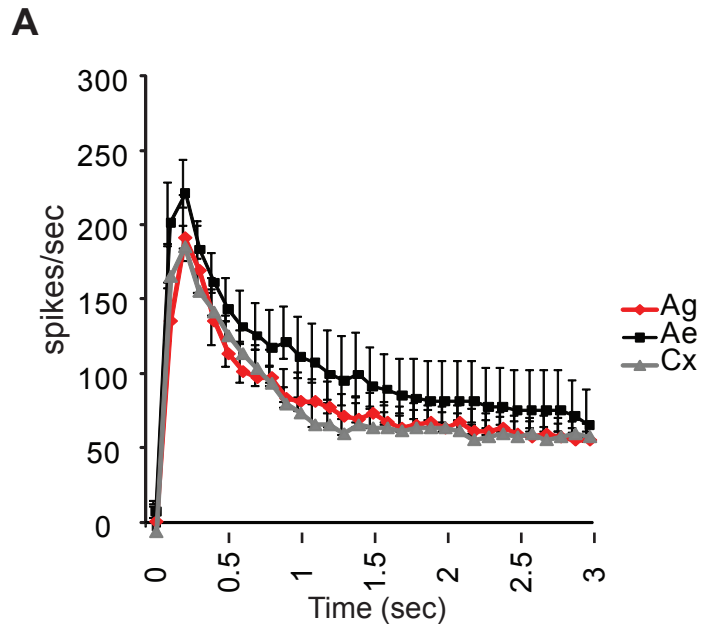


Figure 3.2

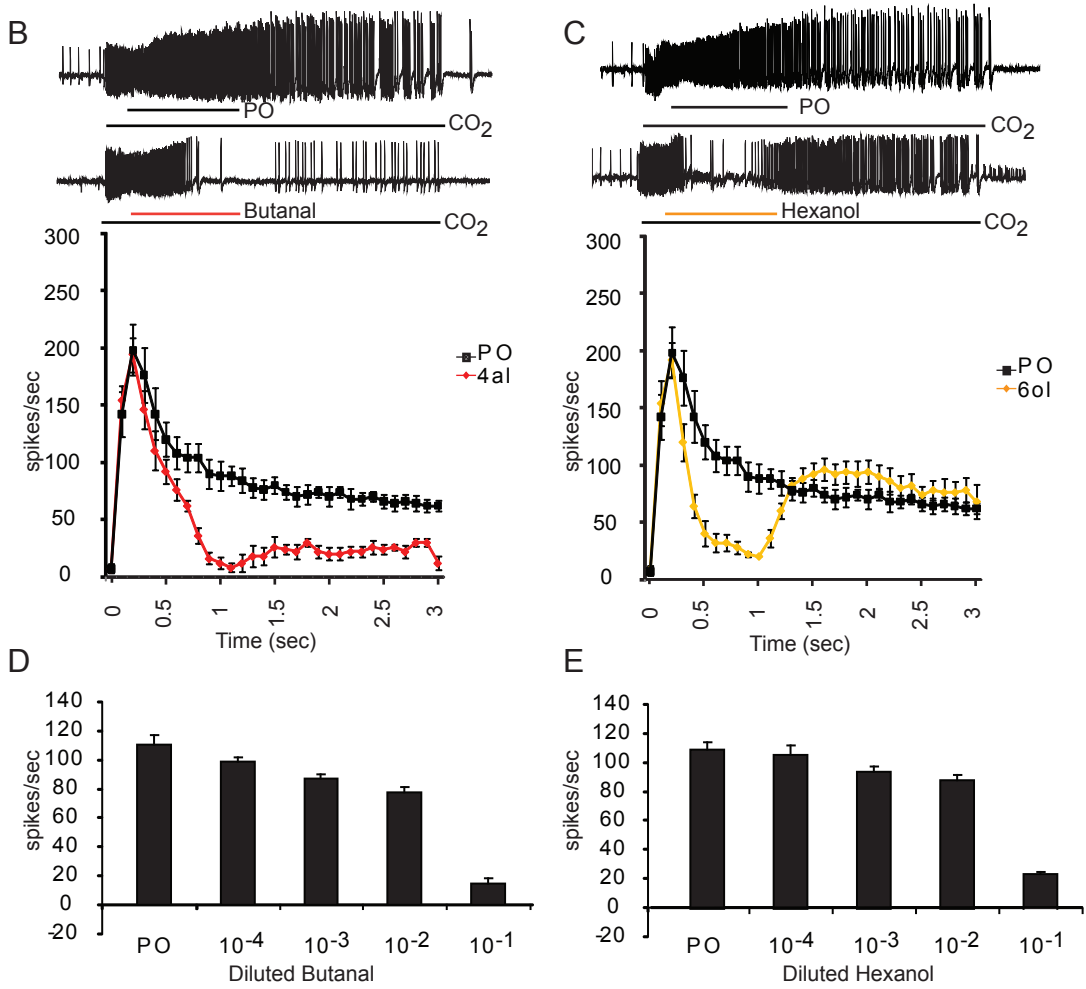
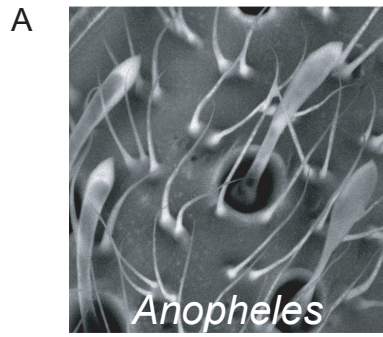


Figure 3.3

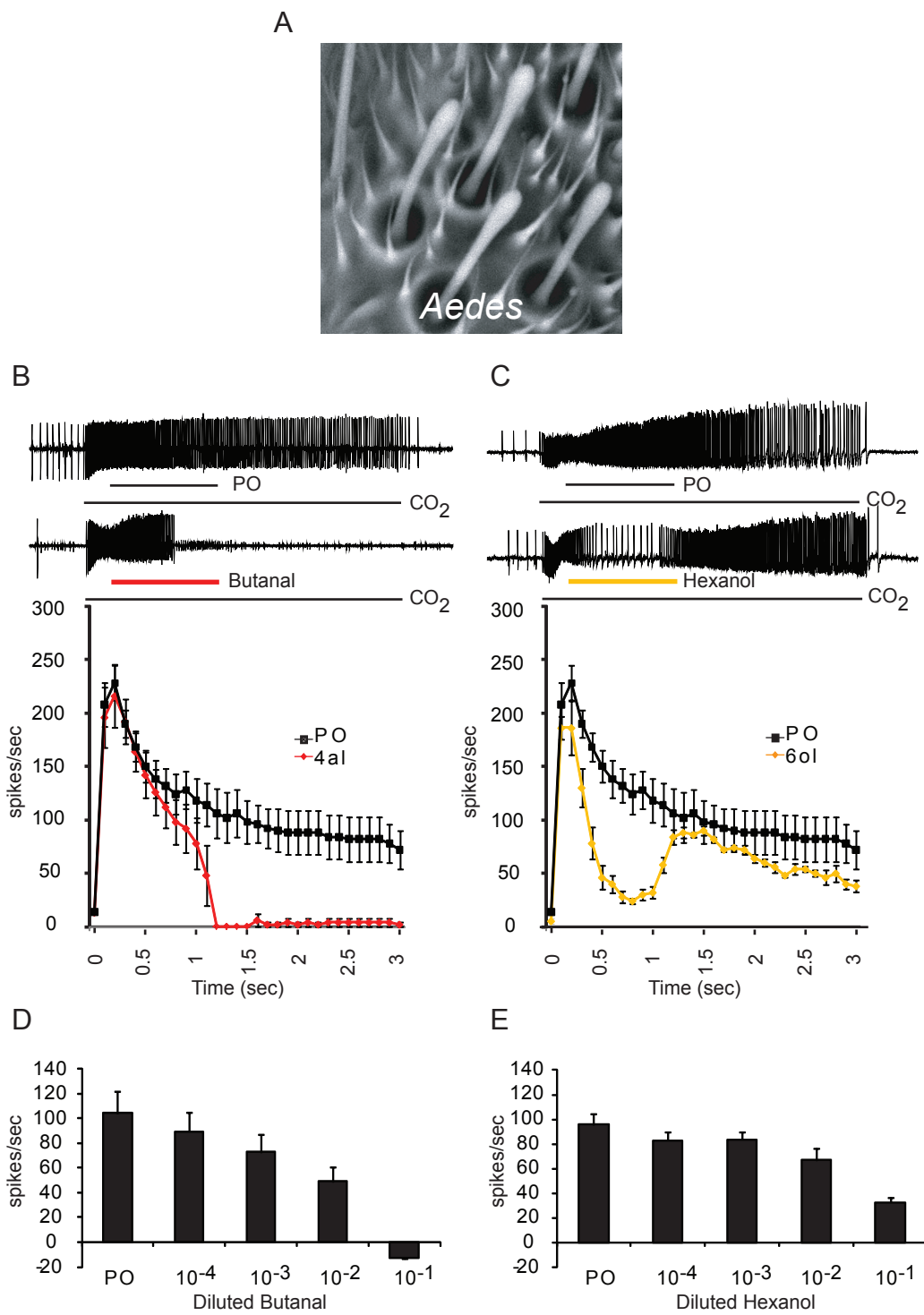


Figure 3.4

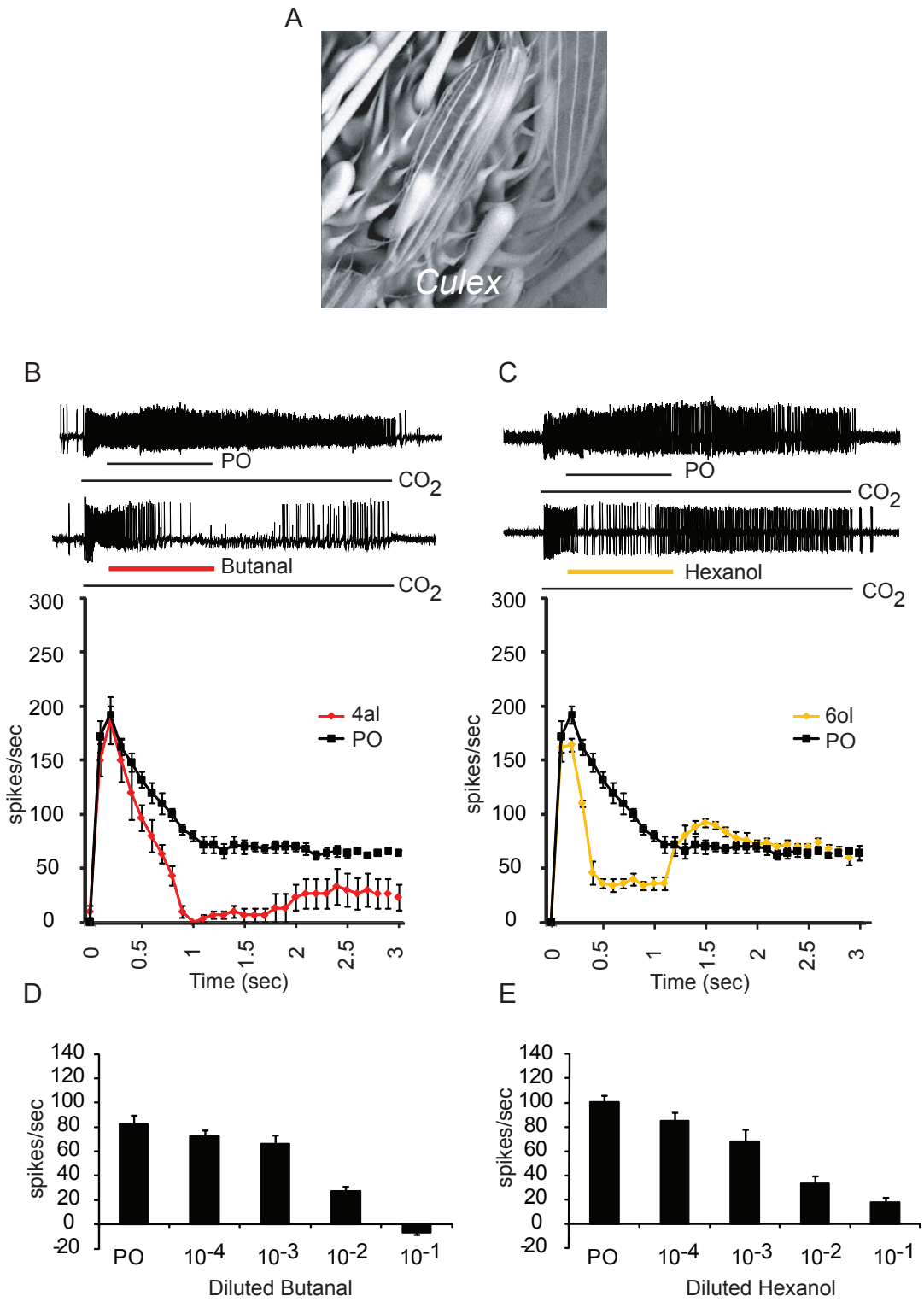


Figure 3.5

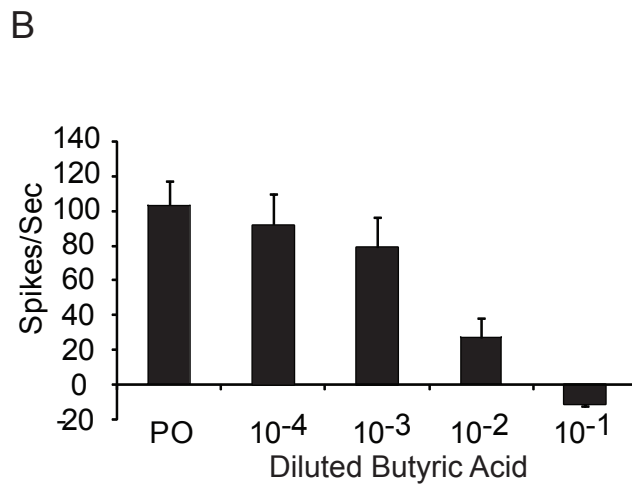
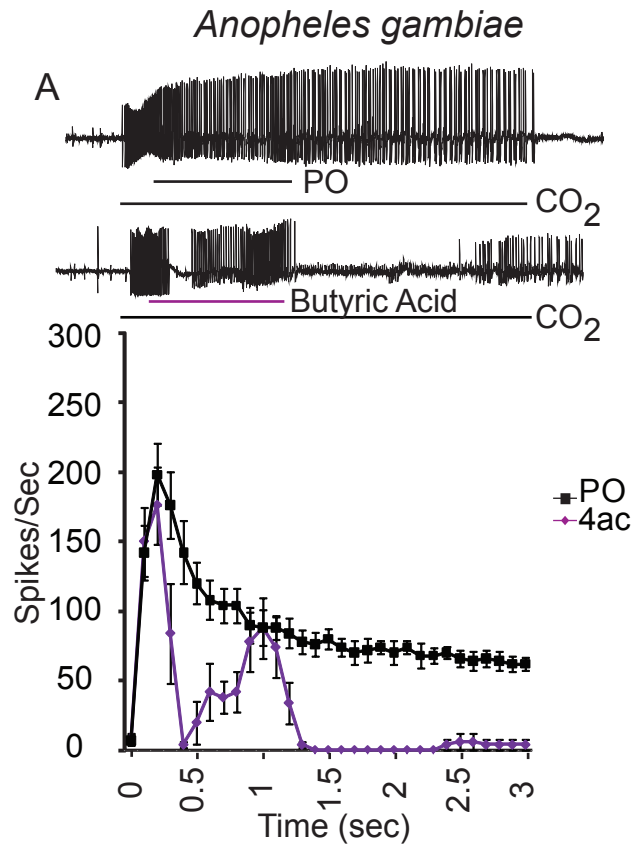


Figure 3.6

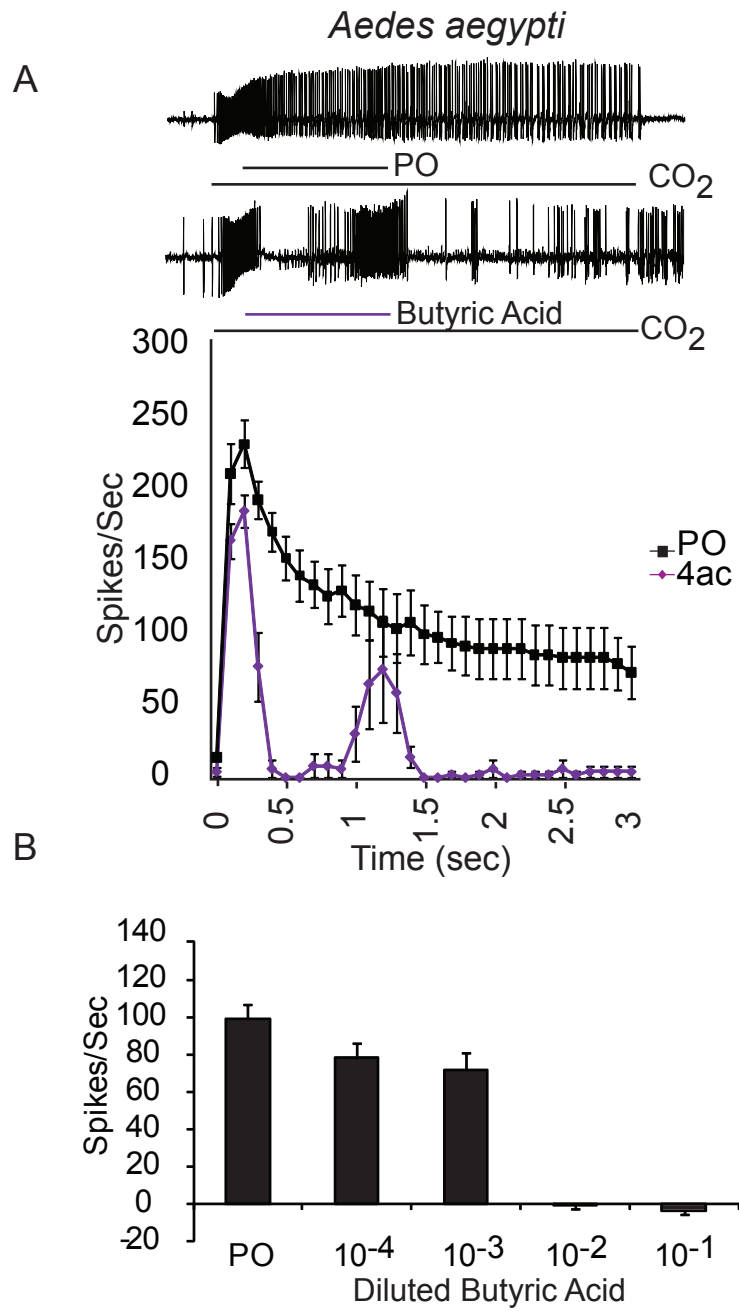


Figure 3.7

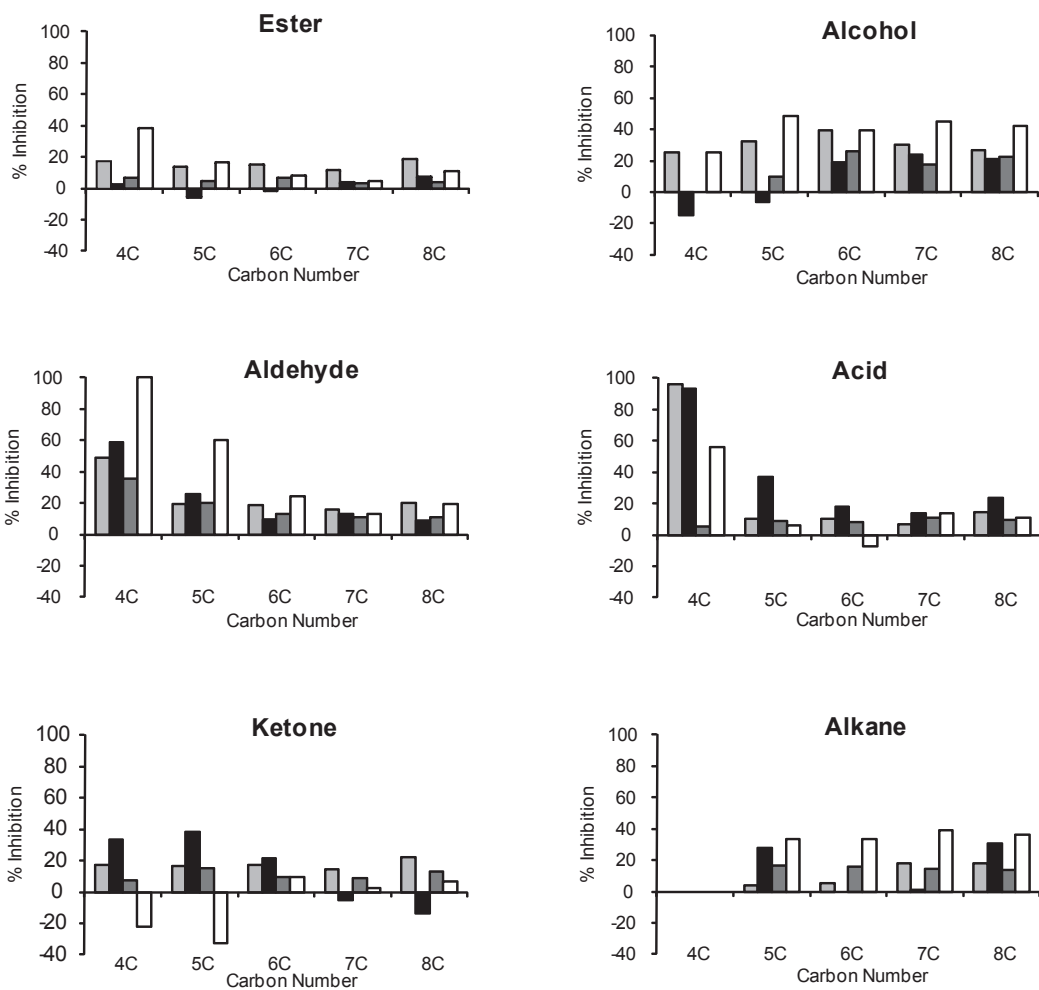
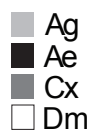


Figure 3.8

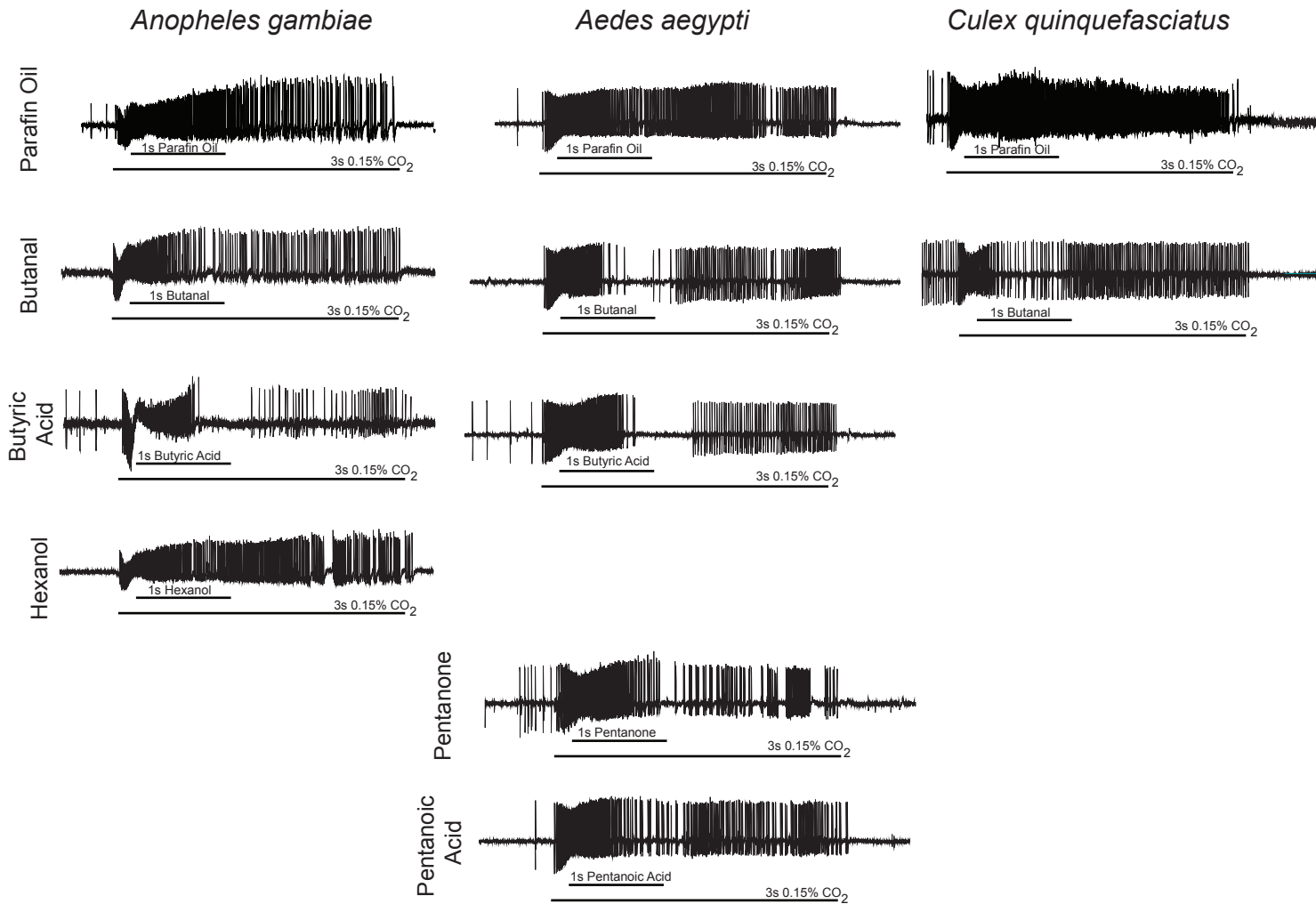


Figure 3.9

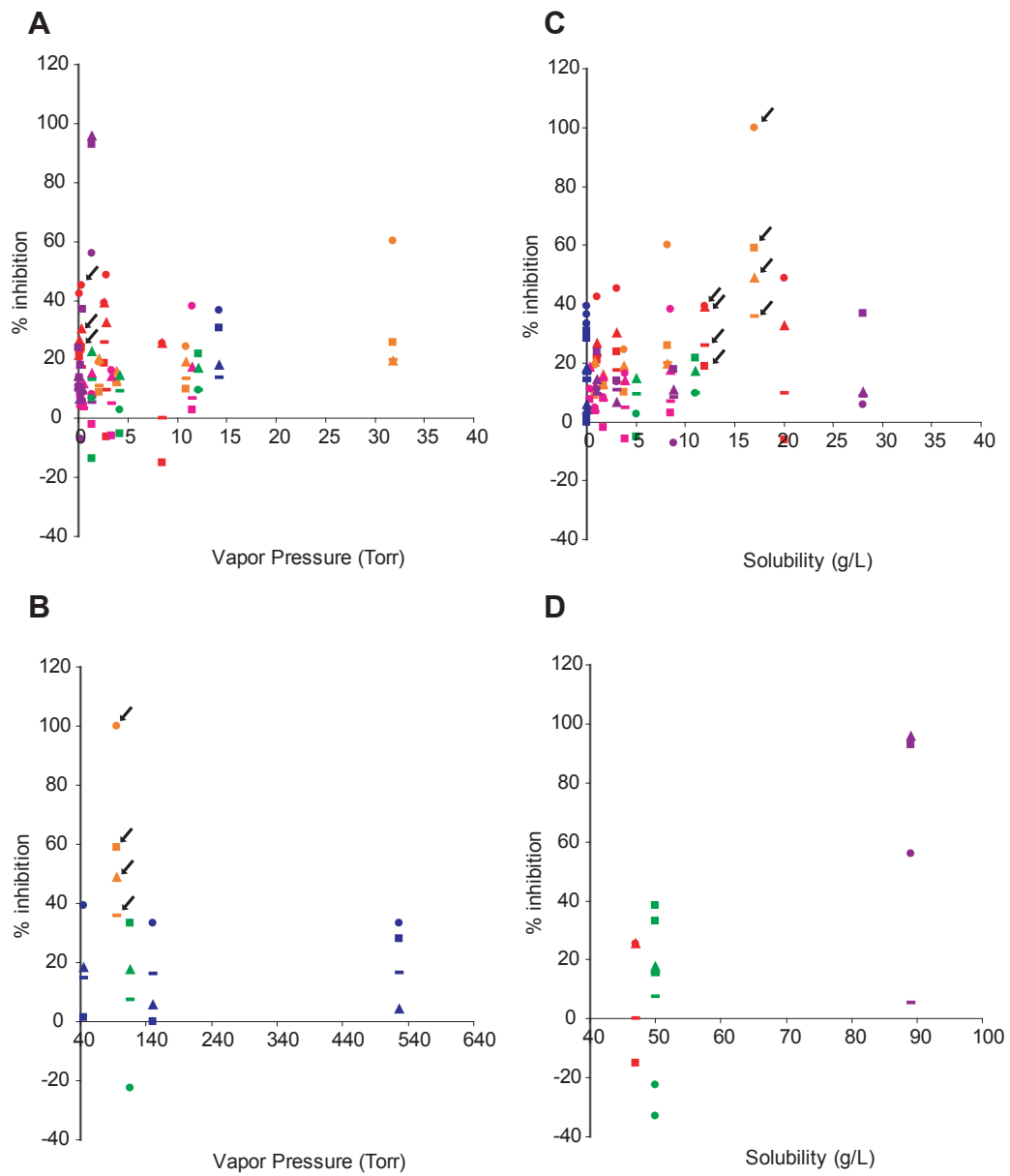
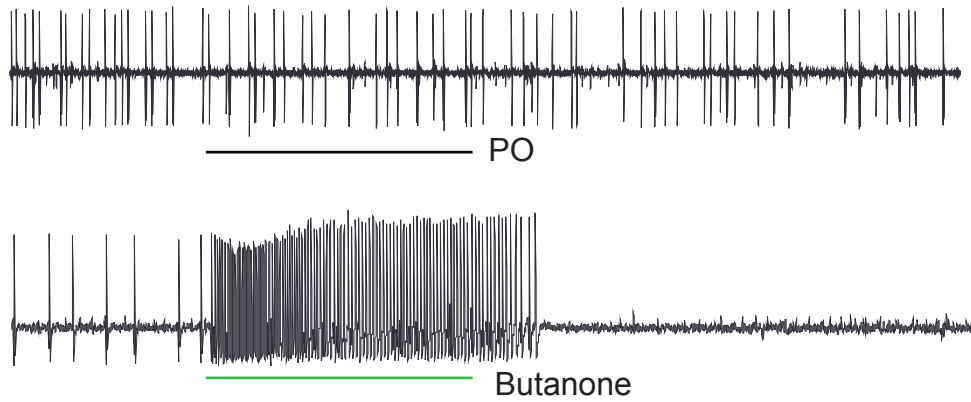


Figure 3.10

A



B

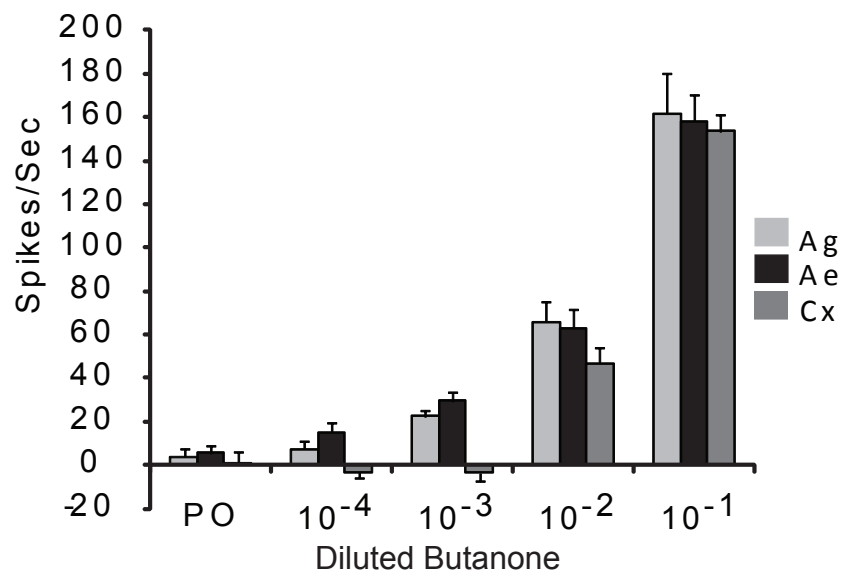


Figure 3.11

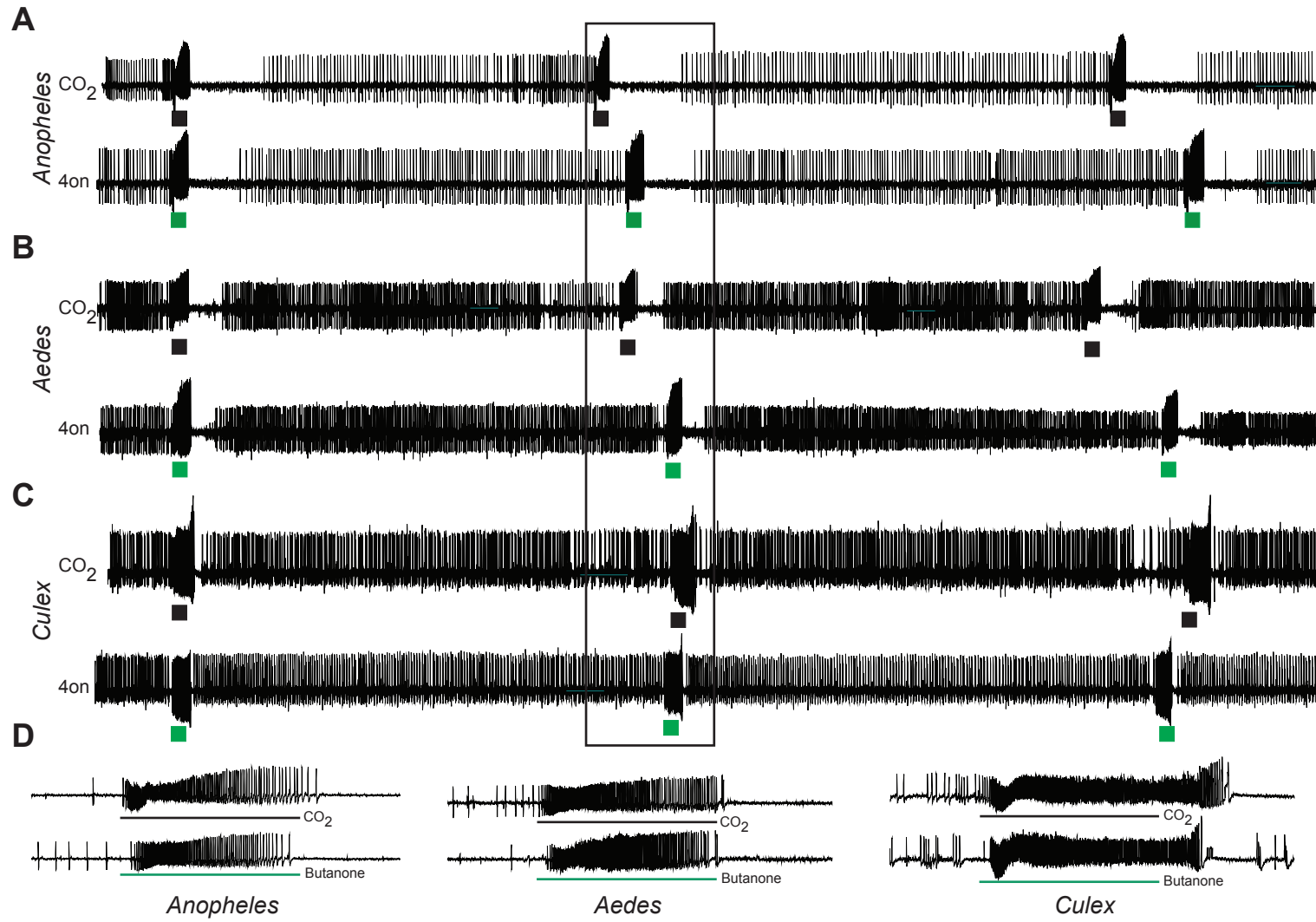
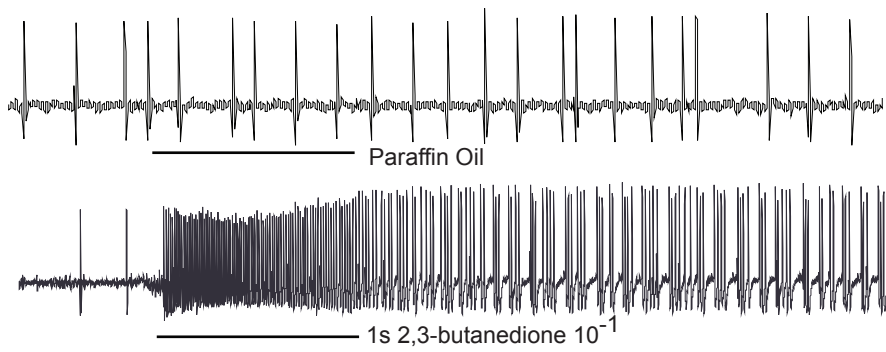


Figure 3.12

A



B

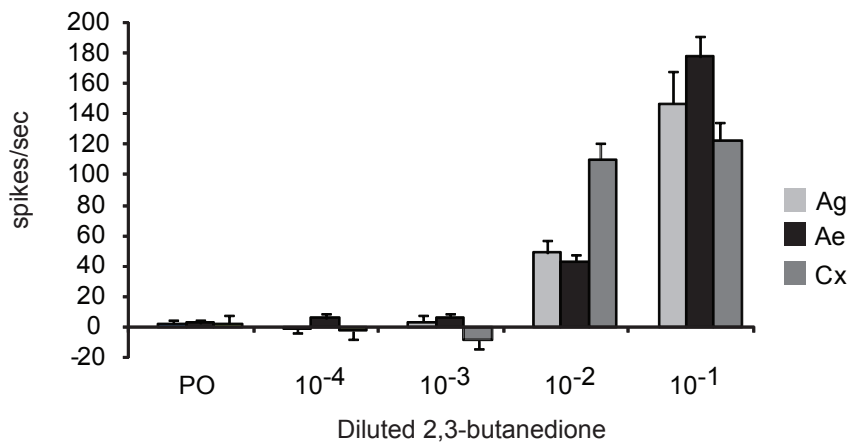


Figure 3.13

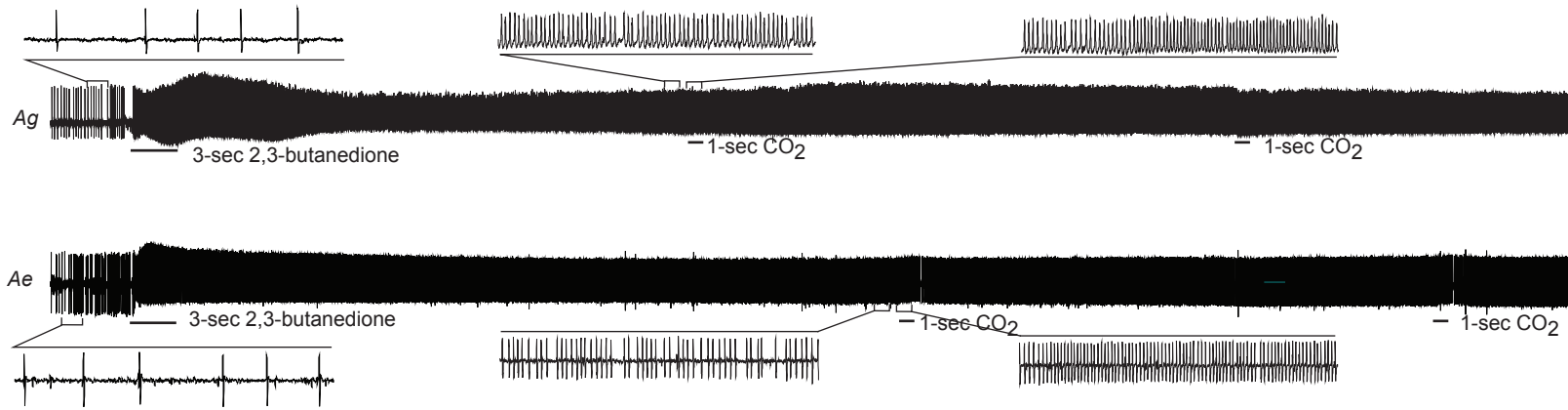


Figure 3.14

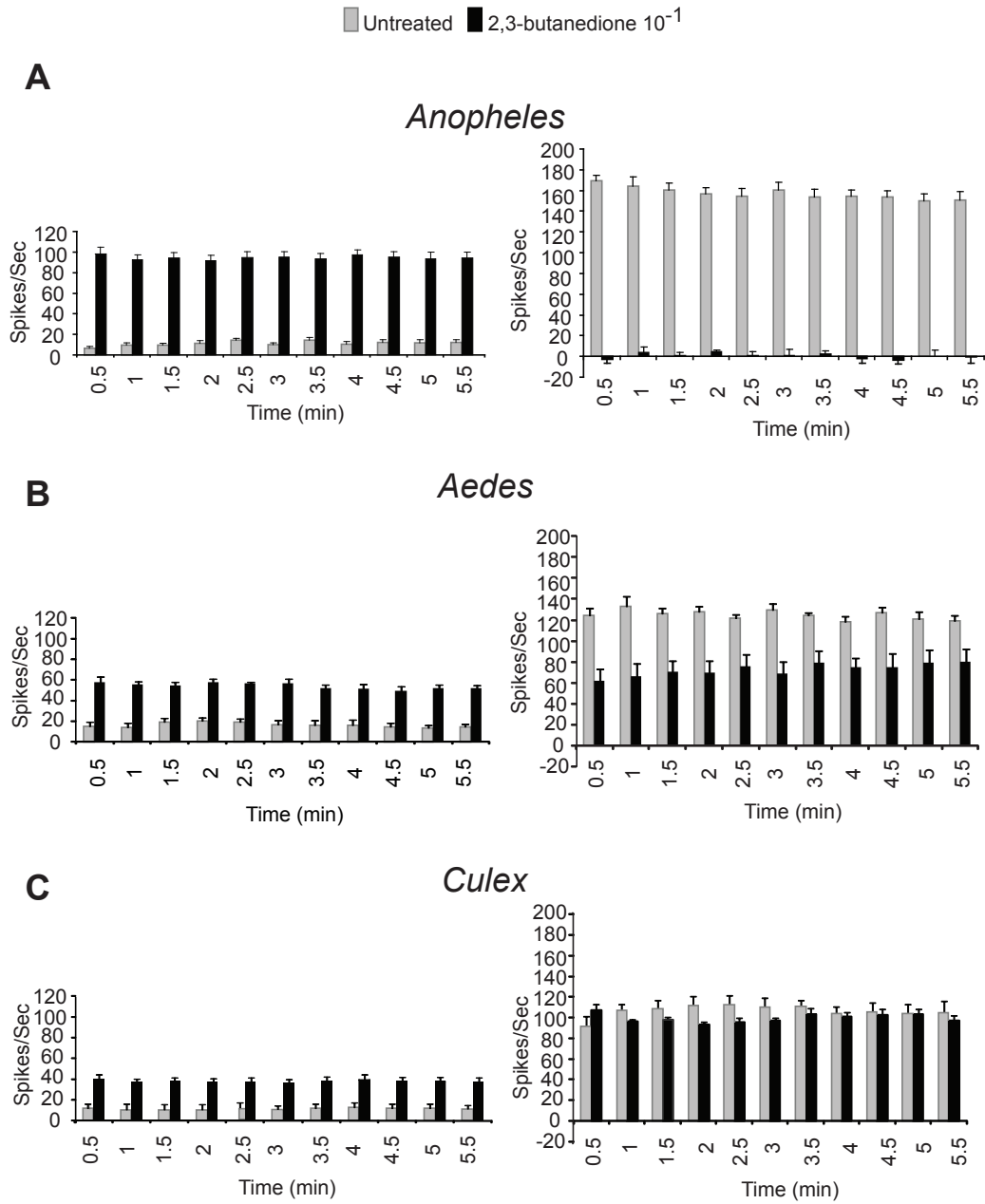


Figure 3.15

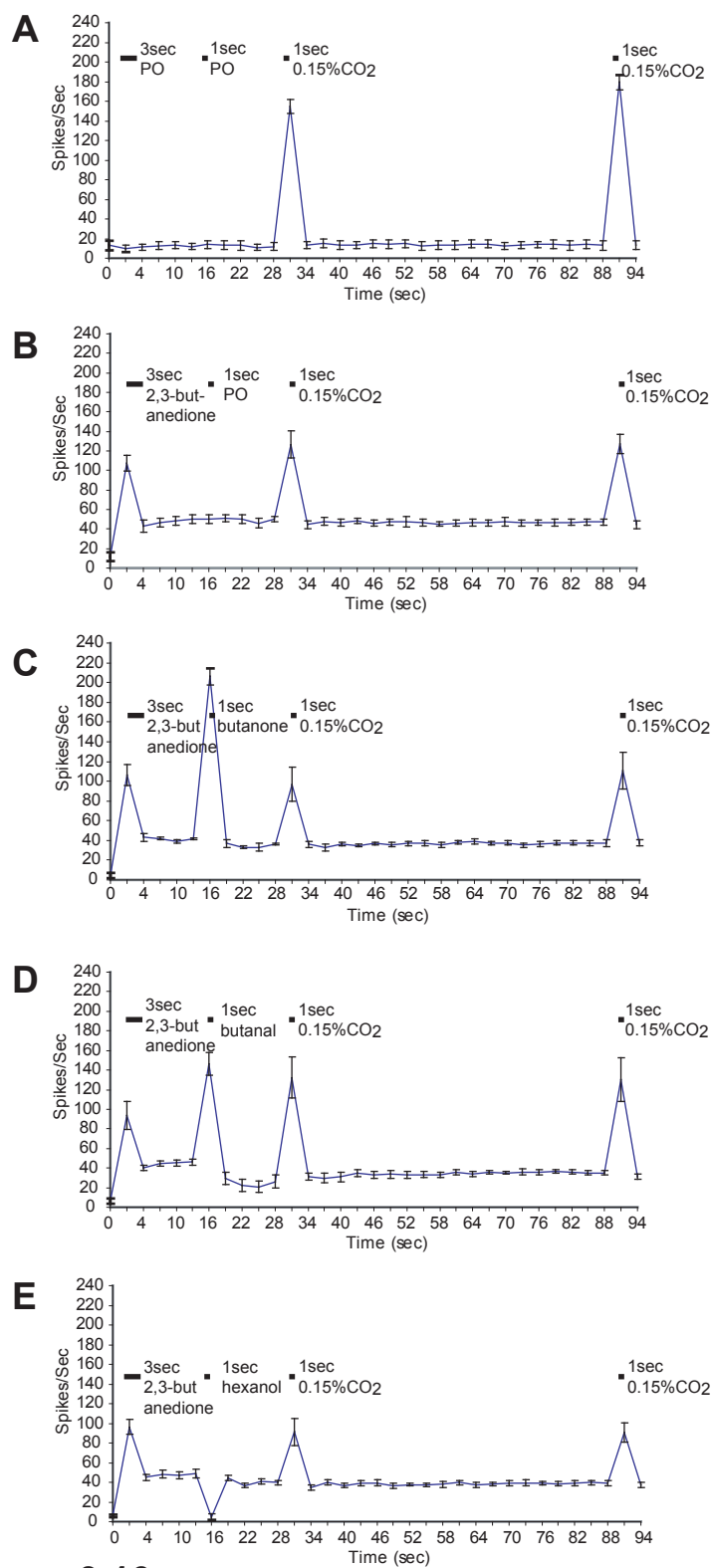
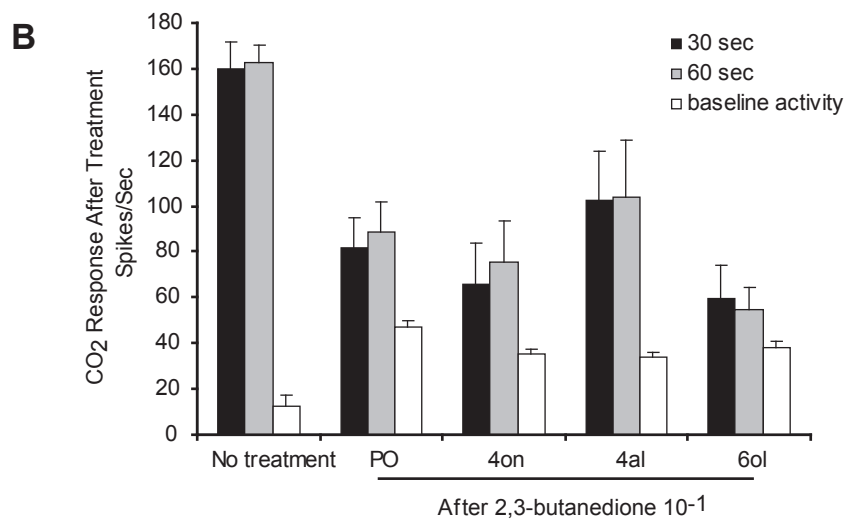
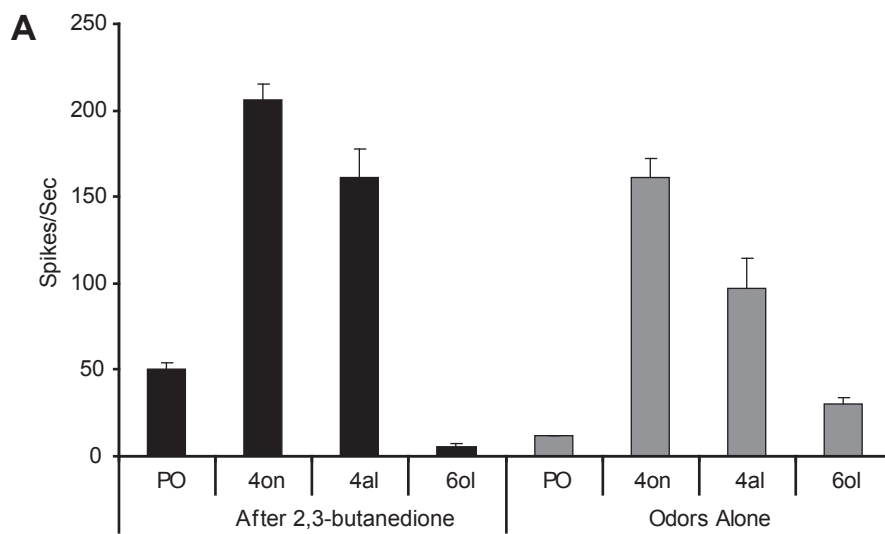


Figure 3.16



C

% inhibition or % excitation at point of:	PO	4on	4al	6ol
Odor delivery	257.1429	1368.571	1054.286	-62.85714
CO ₂ at 30 Sec	-24.48037	-42.26328	-23.09469	-43.41801
CO ₂ at 60 Sec	-22.60274	-36.75799	-21.46119	-46.80365

Figure 3.17

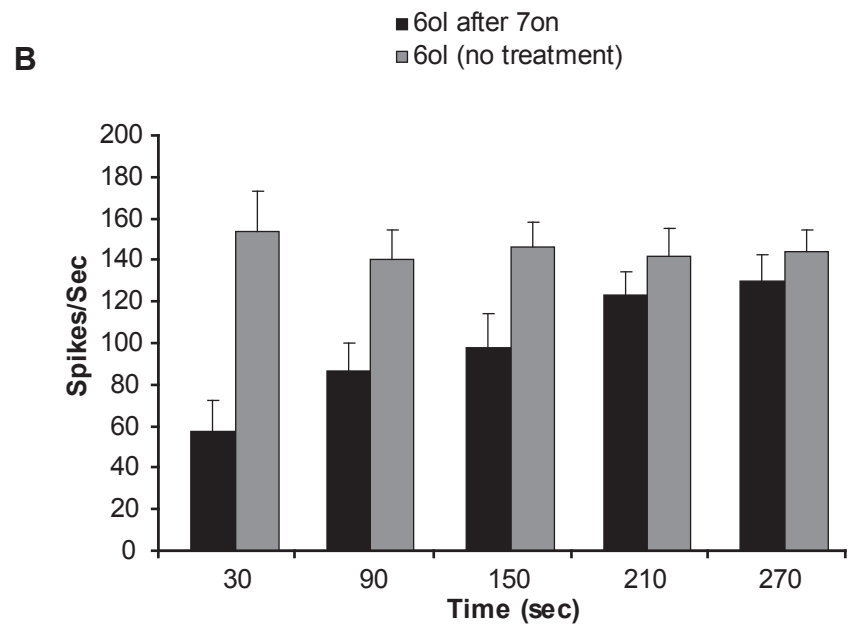
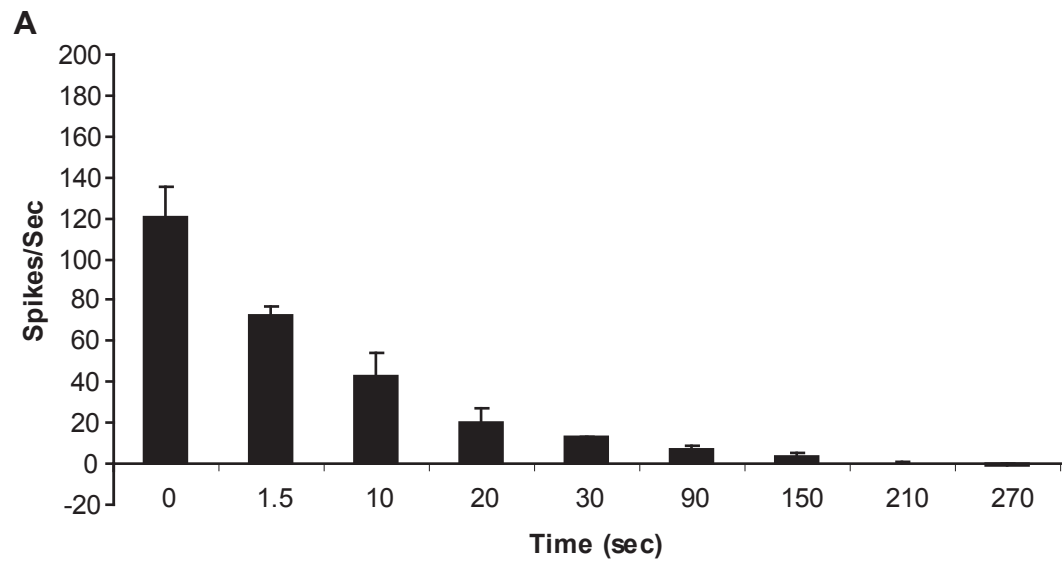


Figure 3.18

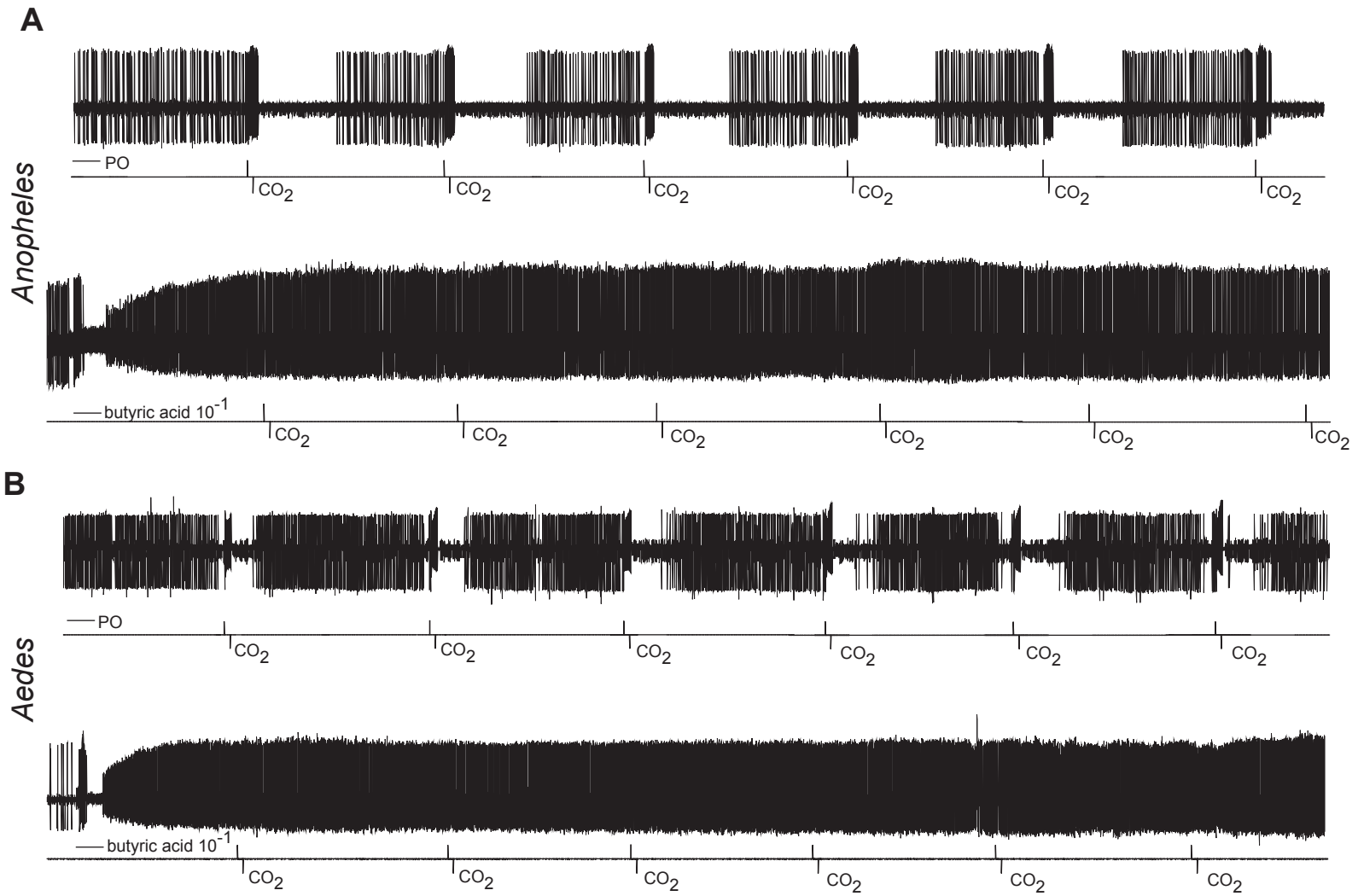


Figure 3.19

Aedes

Anopheles

■ Butyric Acid 10^{-1} □ Solvent

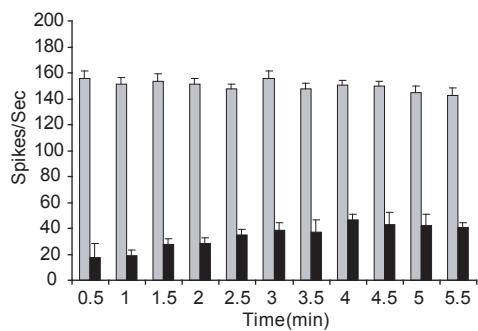
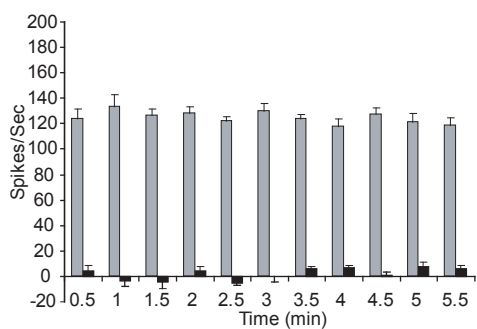
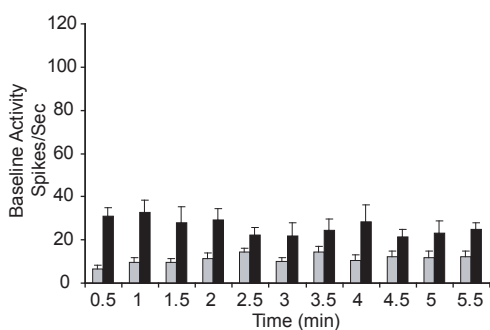
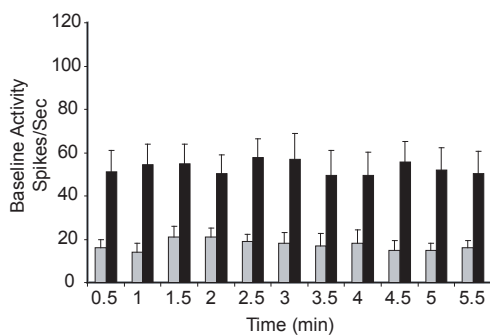


Figure 3.20

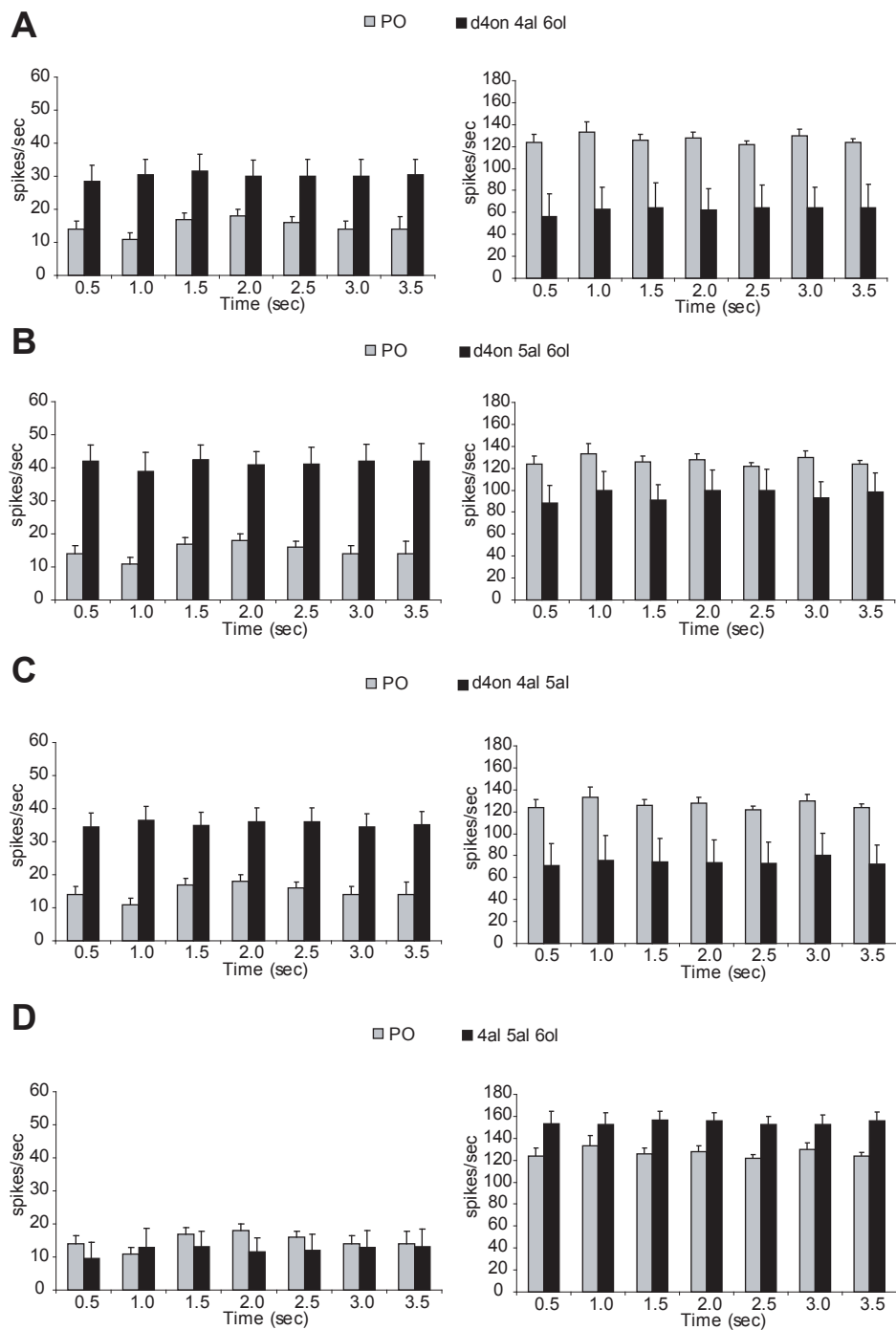


Figure 3.21

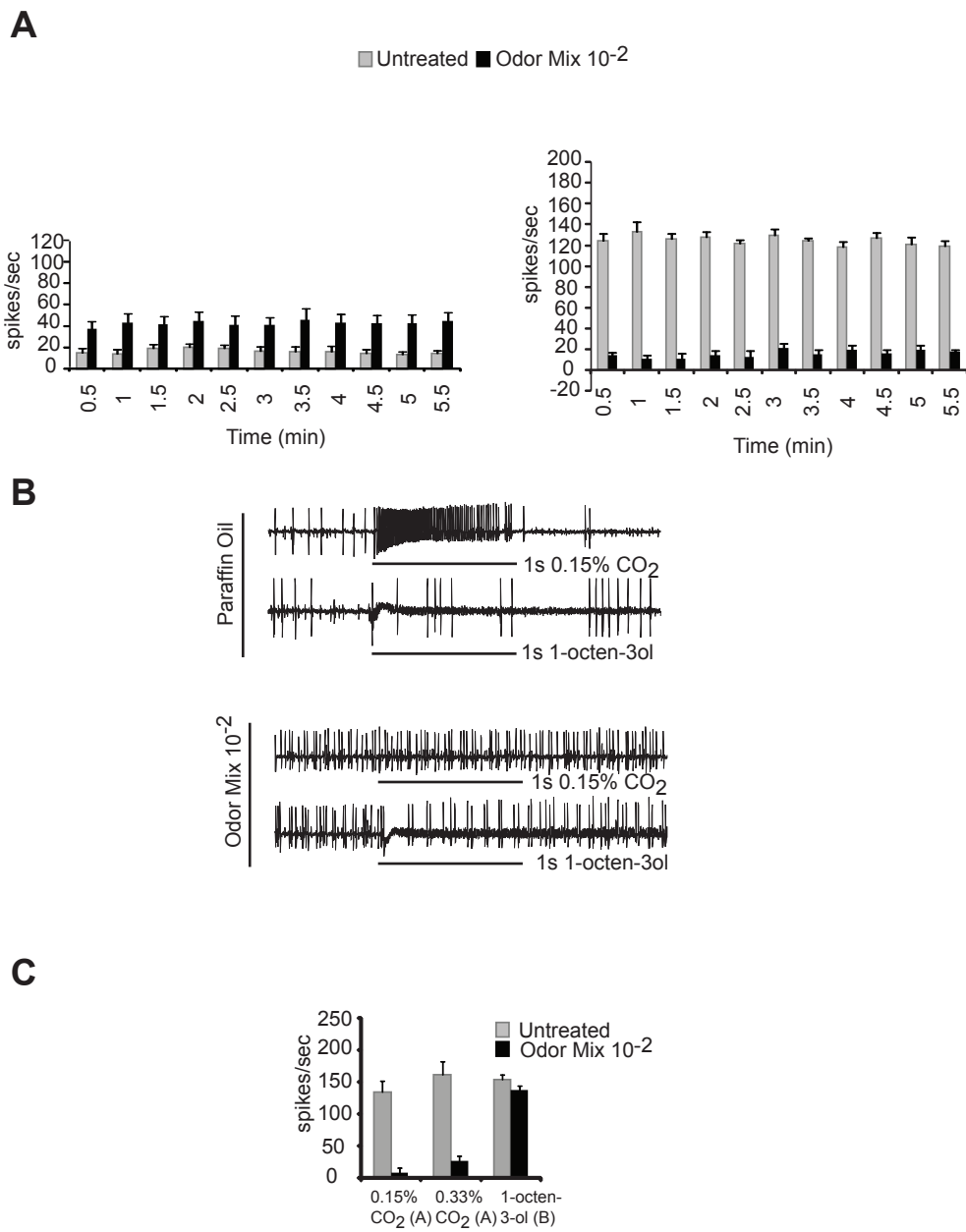


Figure 3.22

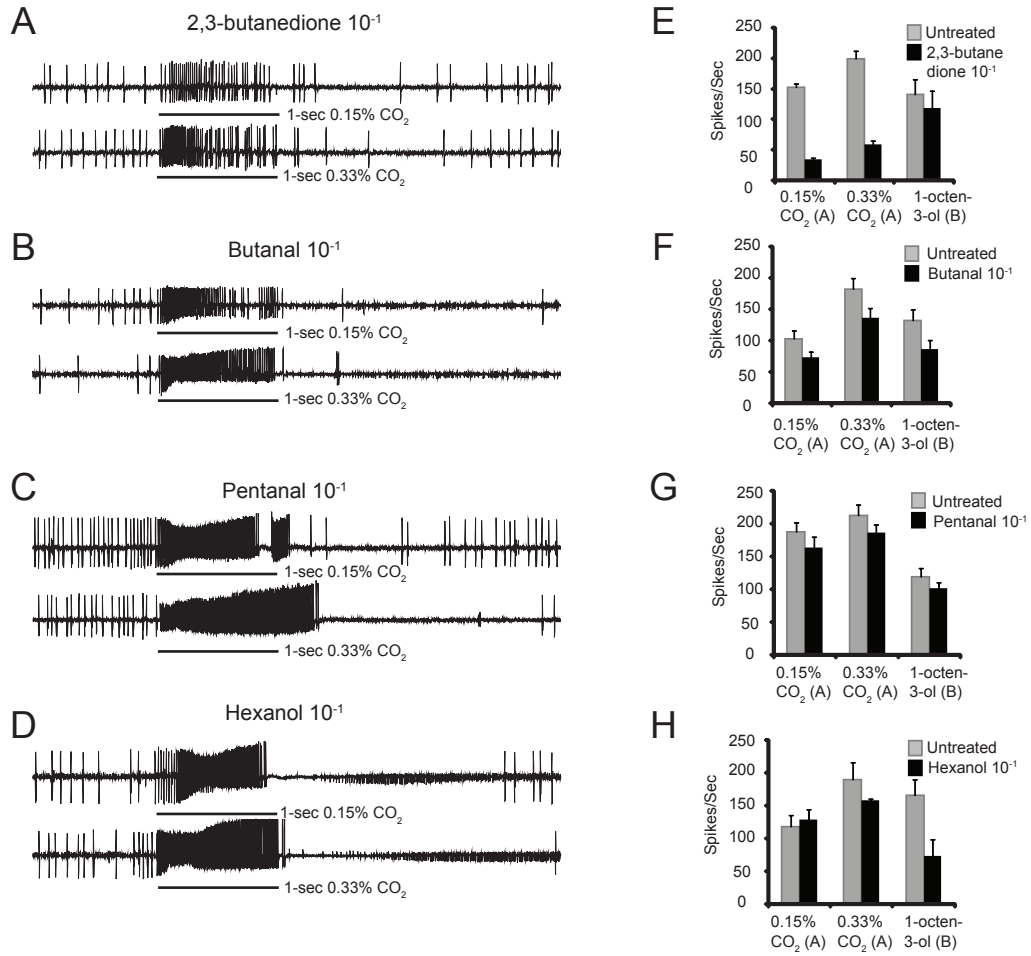


Figure 3.23

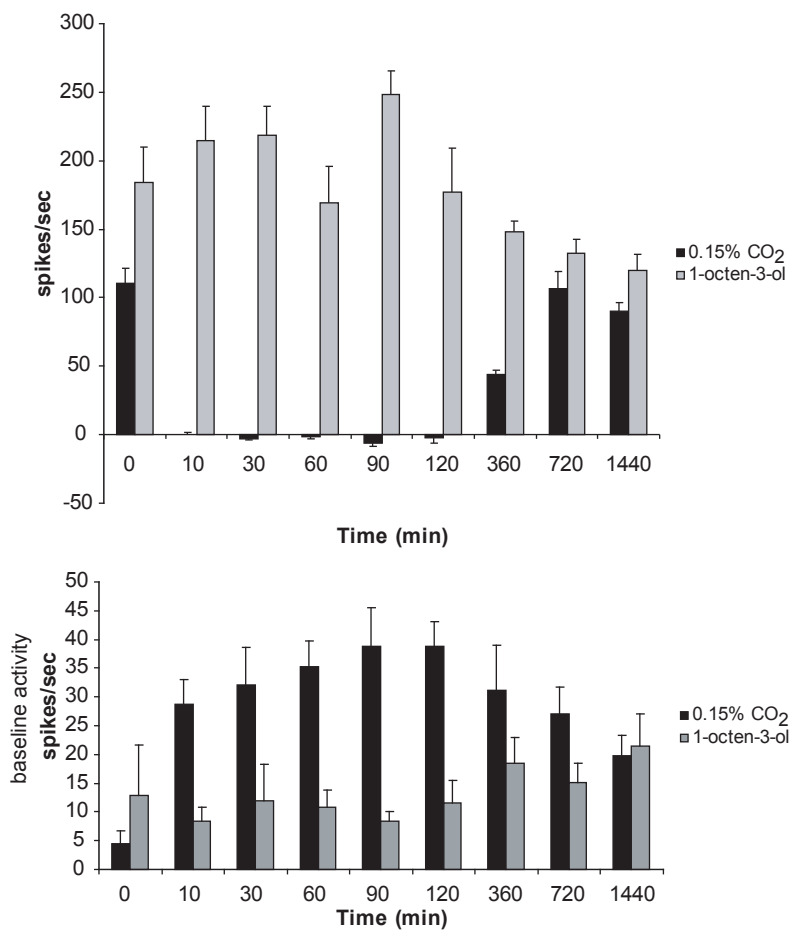
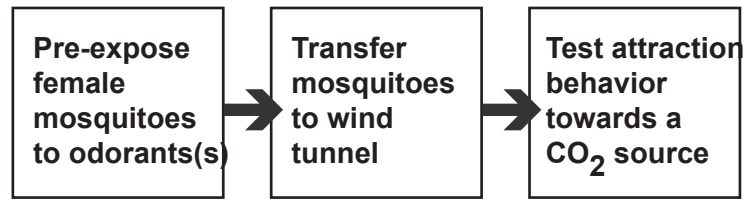
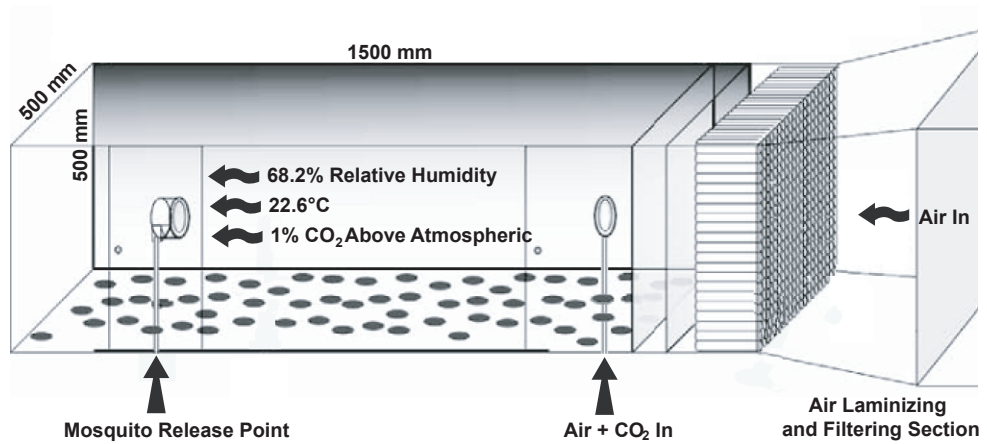


Figure 3.24

A



B



C

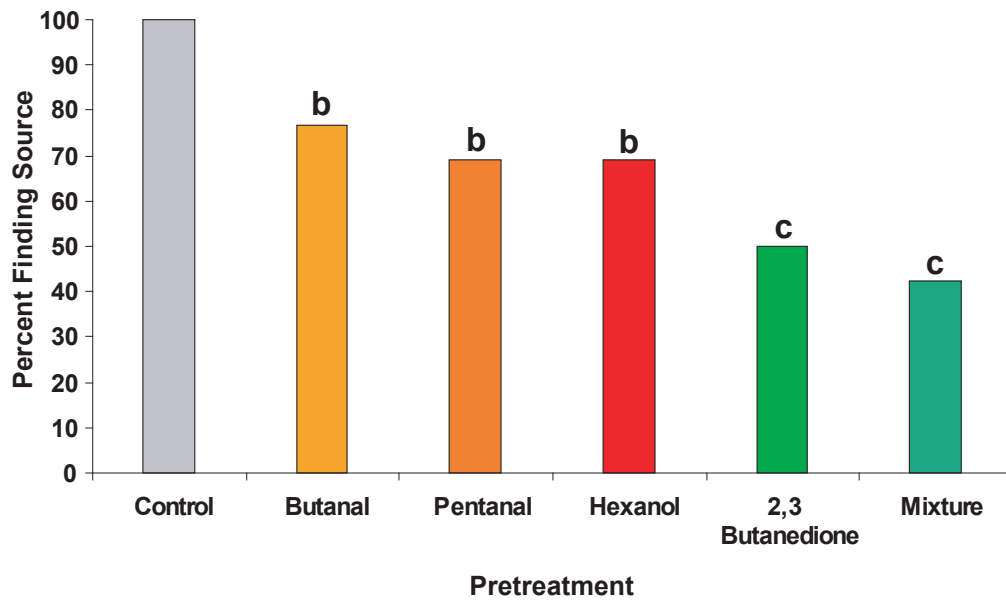


Figure 3.25

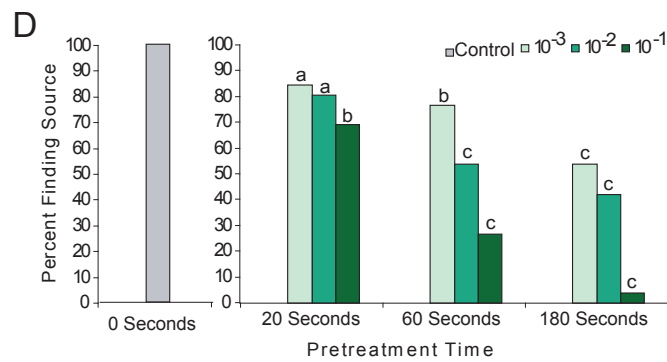
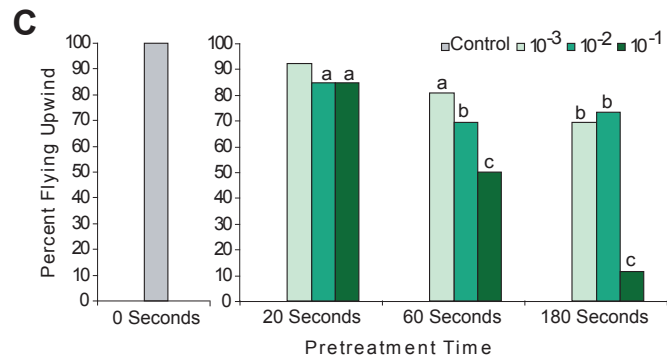
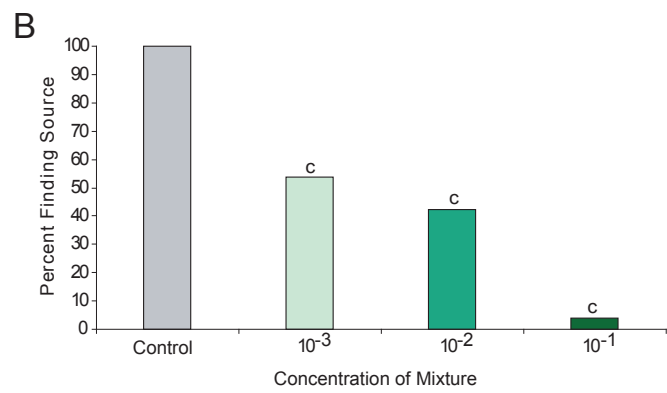
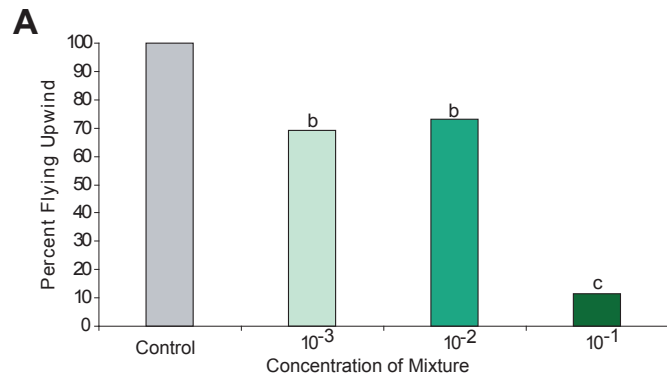


Figure 3.26

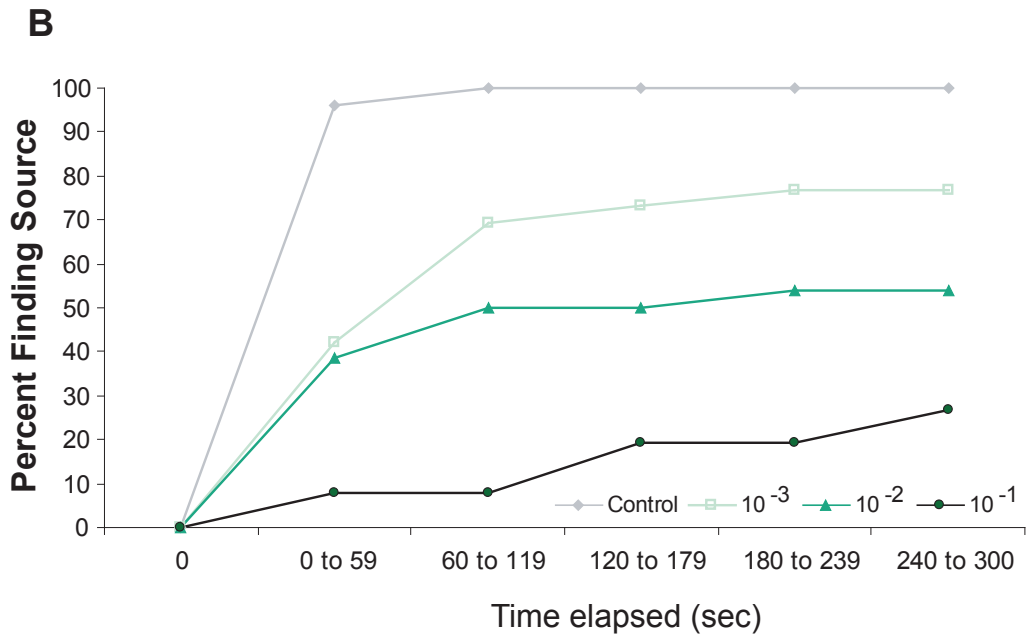
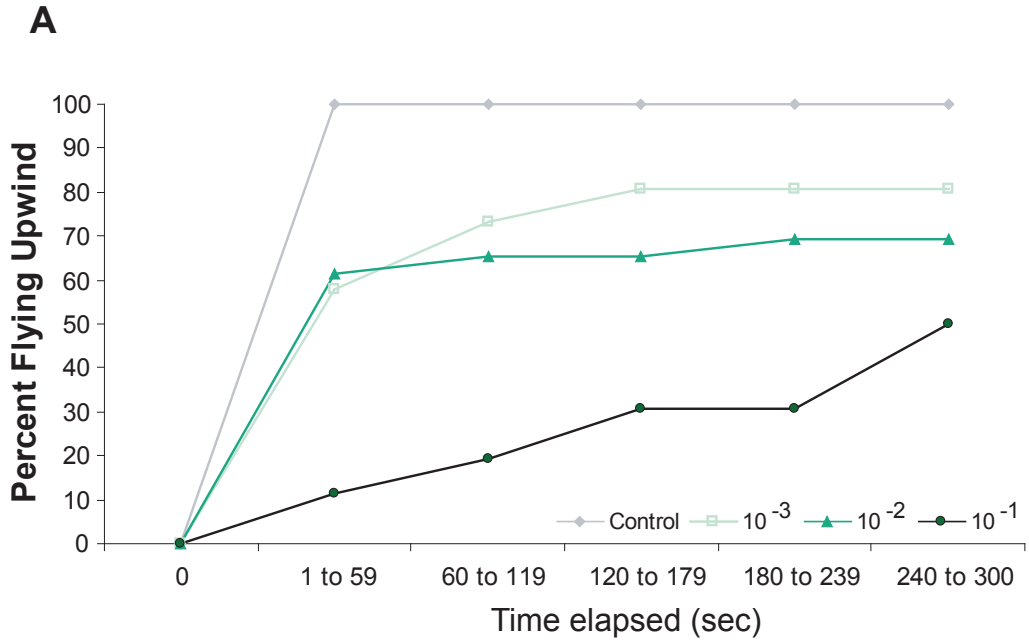


Figure 3.27

CHAPTER IV:
THE ROLE OF CO₂ AVOIDANCE BEHAVIOR IN
DROSOPHILA MELANOGASTER

Introduction:

In many insect species olfaction plays a critical role in behaviors such as locating mates, oviposition, and finding food (Faucher et al., 2006). The olfactory cue carbon dioxide (CO₂) acts as a diverse cue for insects including moth oviposition behavior as well as mosquito host-seeking behavior, among others (Guerenstein and Hildebrand, 2008). In the fruit fly *Drosophila melanogaster*, CO₂ is released from its main food sources, rotting fruits and yeast, suggesting that CO₂ could act as an attractive cue for the insect. However, it was found that when fruit flies are stressed, they emit a „stress odor“ a large component of which is CO₂, that evokes an innate avoidance behavior in naive fruit flies (Suh et al., 2004). Interestingly, sensing a concentration difference in CO₂ above ambient levels causes an avoidance response in the fly, giving rise to the paradox of how could these insects approach their food source while in the presence of this strong avoidance cue. Faucher et al. (2006) have proposed that these behavioral responses can be correlated with CO₂ released from fruits as unripe bananas emit more CO₂ than ripe ones, suggesting that a decrease in CO₂ concentration may indicate the ripeness of fruit. It was also shown that behavior to CO₂ depends on context, where inclusion of apple cider vinegar with CO₂ sensitizes female flies to the presence of CO₂ (Faucher et al., 2006).

The olfactory system of *Drosophila* includes 62 odor receptor proteins which are expressed in non-overlapping subsets in the olfactory organs, the antenna and the maxillary palp (Clyne et al., 2000; Robertson et al., 2003; Vosshall et al., 1999). Another class of chemosensory receptors expressed in antennal coeloconic sensilla, called ionotropic receptors, have also been shown to convey olfactory responses (Benton et al., 2009). Contrary to the well-established properties of *Drosophila* olfaction, it has been shown that CO₂ is detected through two receptors from the gustatory gene family, *Gr21a* and *Gr63a* (Jones et al., 2007; Kwon et al., 2007). The gustatory receptors *Gr21a* and *Gr63a* are expressed in the ab1C neuron in the fly antenna and are thought to be solely responsible for detection of CO₂. The CO₂ neurons extend their axons to converge on the V glomerulus (Jones et al., 2007; Kwon et al., 2007), which forms a dedicated uni-glomerular circuit for CO₂ detection. It has been shown that the CO₂ receptors, as well as activation of the ab1C neuron, are necessary and sufficient to convey avoidance behavior toward CO₂ (Jones et al., 2007; Suh et al., 2007). However, when using *Shibire* to block synaptic transmission in ab1C neurons, flies still avoid *Drosophila* stress-odor, suggesting there may be other compounds in stress-odor that cause avoidance which are not mediated through ab1C neurons (Suh et al., 2004).

It is thought that because the CO₂ detection machinery is so finely tuned to one odor, that this circuit could have evolved to evoke innate behaviors reminiscent of other uni-glomerular circuits, such as those activated by pheromone cis-vaccenyl acetate (Kurtovic 2007). Unlike cis-vaccenyl acetate circuitry, whose absence can cause male flies to inappropriately court other males and cause female flies to be less receptive to males (Kurtovic, 2007), activation of the CO₂ detection machinery and avoidance behavior raises a paradigm that conflicts with the fly's survival. Because CO₂ increases in concentration around the fly's food source of rotting fruits, it would seem that the fly should avoid its natural food sources. In a recent study, we have shown that odors present in fruits can directly inhibit the CO₂ receptor, which causes a profound decrease in CO₂ avoidance behavior (Turner and Ray, 2009). Most surprisingly, one of these odors, 2,3-butanedione, causes the complete abolishment of the avoidance behavior to *Drosophila* stress odor. Because stress-odor is thought to be a blend of odors that together contribute to avoidance behavior, we sought to investigate how 2,3-butanedione, a CO₂ receptor inhibitor, could be completely blocking *Drosophila* stress odor mediated avoidance behavior. In addition, we investigate the role of activation of the CO₂ neuronal circuitry by odorants that activate the CO₂ receptor, and the conservation of ab1C mediated avoidance behavior.

Results and Discussion:

Using a T-maze choice assay as described earlier (Suh et al., 2004), we first sought to compare the effect of *Drosophila* stress odor and CO₂ on two varieties of wild type *Drosophila melanogaster* strains. Both *wCS* and *OregonR* flies avoid *Drosophila* stress odor and CO₂, as expected (Figure 4.1A). We next used *Or83b*² mutant flies, which are nearly anosmic, but have fully functioning CO₂ receptors (Larsson et al., 2004). As reported previously, *Or83b*² flies avoid CO₂ to the same extent as *wCS* (Turner and Ray, 2009) (Figure 4.1A). In addition, *Or83b*² flies avoid *Drosophila* stress odor at nearly the same preference index as CO₂, suggesting that *Drosophila* stress odor avoidance is mediated largely through the CO₂ receptor. As we have found previously, when the CO₂ inhibitory odor 2,3-butanedione is included with CO₂ in the T-maze, CO₂ avoidance behavior is completely abolished in *wCS* flies (Turner and Ray, 2009) (Figure 4.1B).

To further investigate the role of the CO₂ receptor in conveying avoidance behavior to *Drosophila* stress odor, we used the *Gr63a*¹ mutant. Previously, a single null mutant allele of the *Gr63a* gene was created, which causes a complete lack of response to CO₂ from ab1C neurons (Jones et al., 2007). As shown previously, *Gr63a*¹ mutant flies show no avoidance behavior to CO₂ (Jones et al., 2007) (Figure 4.1B). Interestingly, when these flies are exposed to *Drosophila* stress odor, they show no behavioral response to the odor (Figure

4.1B). Previous work with *Drosophila* stress odor using *Gr21a-Gal4, UAS-Shi^{ts}* flies had shown that avoidance behavior toward *Drosophila* stress odor had not decreased significantly, which was interpreted to indicate that other odors acting through receptors other than the CO₂ receptor could be leading to avoidance behavior (Suh et al., 2004). Because our experiment utilizes a complete knock out of the *Gr63a* gene rather than a partial knock down of synaptic transmission in *Gr21a* expressing neurons, it stands to reason that any behavior elicited from *Gr21a-Gal4, UAS-Shi^{ts}* flies could be due to incomplete penetrance of the phenotype and incomplete inactivation of all ab1C neurons. All olfactory pathways are functional in the *Gr63a¹* mutant fly, except for CO₂ detection (Jones et al., 2007). In addition, because *wCS* flies had such a strong decrease of avoidance behavior in the presence of the CO₂ inhibitor 2,3-butanedione, it would seem unlikely that any receptor other than the CO₂ receptor /ab1c neuron is involved in avoidance behavior to dSO. Our results suggest that CO₂ is both the necessary and sufficient component of *Drosophila* stress odor which drives avoidance behavior. Perhaps activation of the CO₂ uni-glomerular circuit is sufficient to evoke the "fear" or avoidance response to *Drosophila* stress odor.

Previously, others have found that activation of the CO₂ circuit by artificial means can induce an avoidance response (Suh et al., 2007). In our previous study, we have also shown that 2-butanone, an odor structurally related to CO₂ response inhibitors, can in fact activate the CO₂ neuron in a *Gr63a* dependent manner and cause avoidance behavior (Turner and Ray, 2009). While performing an electrophysiological screen for other activators of the CO₂ neuron, we identified an additional odorant pyridine which also strongly activates the ab1C neuron (Figure 4.2A). Although pyridine has not been identified as a semiochemical, there are many pyridine derivatives which act as attractants and pheromones in a variety of insect species (Table 4.1). Interestingly, pyridine at 10⁻¹ dilution can activate the ab1C neuron nearly three times greater than that of 0.33% CO₂, whereas pyridine at 10⁻² dilution activates the neuron at nearly the same rate as CO₂. (Figure 4.2B). The activation of the ab1C neuron by pyridine is dependent on *Gr63a* (Figure 4.2C, D). Also of note, pyridine strongly activates the neighboring neuron ab1A in a dose dependent manner (Figure 4.2C). In agreement with the electrophysiological data, both *wild-type* and *Or83b²* flies avoid pyridine, while *Or83b²,Gr63a¹* flies show no preference to the odor, indicating that avoidance to pyridine is conferred through activation of the CO₂ receptor (Figure 4.3).

CO₂ is widely detected by other insects, such as in mosquitoes which are highly attracted to CO₂, as this cue signifies their prospective host (Cardé and Willis, 2008; Dekker et al., 2005; Guerenstein and Hildebrand, 2008; Thom et al., 2004). Indeed, the CO₂ receptor proteins are highly conserved across dipteran species, signifying the importance of this environmental cue across this order (Robertson and Kent, 2009). Although a few selected fruit odors like 1-hexanol and 2,3-butanedione can inhibit the CO₂ neuron of *D. melanogaster*, and provides an elegant solution for the flies inability to approach CO₂ while indicating ripeness of fruit (Turner and Ray, 2009), it remains unclear as to why this specific cue would have evolved in the animal as opposed to a more direct mechanism of host detection. How recently has avoidance behavior mediated by activation of the CO₂ neuron evolved? To further address this question, we turned to a closely related species *Drosophila simulans*. Characterization of the peripheral olfactory system has been done recently on *D. simulans* as well as 11 other *Drosophila* species, and as in *D. melanogaster*, ab1 sensilla house four neurons of which one neuron responds to CO₂ (de Bruyne et al., 2010). We find that the ab1 neurons which are activated by CO₂, are also activated by pyridine (Figure 4.4), demonstrating that the neuronal response to both CO₂ and pyridine is conserved in *D. simulans*. We then used a T-maze assay to test for avoidance behavior toward CO₂, and found that *D. simulans* avoid CO₂ to a small degree in comparison to *D. melanogaster* (Figure 4.5). Similarly, *D. simulans* show relatively weak avoidance toward *Drosophila melanogaster* stress odor and

pyridine (Figure 4.5). It remains to be seen if *D. simulans* derived stress odor would cause an avoidance response in the animal. However, *D. simulans* only shows half the degree of avoidance toward CO₂ (Figure 4.5) as of that in *D. melanogaster*. Because *D. melanogaster* dSO is mostly comprised of CO₂ and is arguably the sole avoidance cue in stress odor, it is unlikely that there is a species specific component of stress odor that *D. simulans* would respond to.

How has CO₂, a ubiquitous odor cue, evolved to be perceived differently in two closely related generalist species of *Drosophila* leading to a decrease in avoidance behavior? Indeed there could be a wide variety of environmental cues that indicate sub-optimal conditions for the fruit fly. In the instance of CO₂, many insects need to be able to exchange CO₂ for O₂ during respiration, especially during times of high activity (Hetz and Bradley, 2005). Perhaps “stress odor”, the largest component being CO₂, is merely a form of heightened respiration that occurs in the fly during an unnatural vortexing or electric shock procedure (see CHAPTER VI: METHODS). For example, *Drosophila melanogaster* releases CO₂ in discontinuous patterns, the rate of which increases as a fly desiccates (Williams et al., 1997). If this respiration rate was at a level detectable by the fly, perhaps CO₂ in this context could be an indicator of a low O₂ environment for *D. melanogaster*.

Although both *D. melanogaster* and *D. simulans* are attracted to and oviposit on similar substrates, both species could have minor differences in host preference simply as a means for coexistence in the same environment (Soliman, 1971; Soliman and Knight, 1984). In the case of *D. melanogaster*, the fly will avoid green fruits as this food source will emit more carbon dioxide than ripe fruits (Faucher et al., 2006; Turner and Ray, 2009). Fruits also develop volatile odors as they ripen which could not only act as attractants for the fly, but also contain unique classes of odors which inhibit CO₂ receptors (Turner and Ray, 2009). Although there is similar activation of the CO₂ neuron in *D. melanogaster* and *D. simulans*, there is a decrease in the range of avoidance between these two species. Despite this difference, it would seem plausible that CO₂-responsive uni-glomerular circuitry leads to an avoidance behavior in order to create an ecological niche that would direct the fly toward the most suitable food source.

Conclusion:

Overall, we have found that CO₂ is the necessary and sufficient component of *Drosophila* stress odor that drives avoidance behavior. In addition, we have found that odors activate the CO₂ neuron in two *Drosophila* species. Future studies will be needed to determine if this observation is conserved across other *Drosophila* species. It will also be important to see if CO₂ avoidance behavior is conserved across various species of *Drosophila*. These types of studies will help reveal the role of CO₂, as well as the role of CO₂ neuronal activators in the ecology of the fly.

Figure Legends:

Figure 4.1: Avoidance behavior to CO₂ and *Drosophila* stress odor is abolished by CO₂ inhibitory odorant.

T-maze behavior assay: **A**, Mean preference index of *OregonR*, *wCS*, and *Or83b*² flies given a choice between room air in a 15ml tube and either 0.1ml of pure CO₂ (CO₂), odor collected from 70 untreated flies (mock), *Drosophila* stress odor (dSO) collected from 70 vortexed “emitter” flies, or a binary mixture of 0.1ml pure CO₂ and 2,3-butanedione at 10⁻² dilution (CO₂+d4on). **B**, Mean preference index of *Gr63a*^{-/-} and *wCS* flies given a choice between room air in a 15ml tube and either 0.1ml of pure CO₂ (CO₂), odor collected from 70 untreated flies (mock), *Drosophila* stress odor (dSO) collected from 70 vortexed “emitter” flies, a binary mixture of 0.1ml pure CO₂ and 2,3-butanedione at 10⁻² dilution (CO₂+d4on), or a binary mixture of dSO and 2,3-butanedione at 10⁻² (dSO + d4on). n=6-9 trials (~40 flies each), error bars=s.e.m.

Figure 4.2: Pyridine activates the ab1C neuron in *D. melanogaster*.

A, Representative traces of the ab1c neuron to a 0.5-sec stimulus of pyridine 10^{-1} dilution. **B**, Mean responses of the ab1C neuron in *Or83b²* flies to 0.5-sec stimuli of paraffin oil (PO), 0.33% CO₂, or pyridine (pyr) at indicated concentrations. **C**, Mean responses of *Gr63a^{-/-}* flies to 0.5-sec stimuli of paraffin oil (PO), 0.33% CO₂, ethyl acetate (2Ac), pentyl acetate (5Ac), or pyridine (pyr) at indicated concentrations. **D**, Mean responses of *Or83b²Gr63a¹* flies to paraffin oil (PO), 0.33% CO₂, or pyridine (pyr) at indicated concentrations. n=5, error bars=s.e.m.

Figure 4.3: Avoidance to pyridine is dependent on *Gr63a*.

T-maze behavior assay: Mean preference index of wCS **A**, *Or83b²* **B**, or *Or83b²Gr63a¹* **C** flies given a choice between paraffin oil or pyridine (pyr) at indicated concentrations. n=6, error bars=s.e.m.

Figure 4.4: The CO₂ sensitive neuron of *D. simulans* responds to pyridine.

A, Representative traces of the CO₂ sensitive neuron to a 0.5-sec stimulus of 0.33% CO₂ or pyridine (pyr) 10^{-1} dilution. **B**, Mean response from the CO₂ sensitive neuron to 0.5-sec 0.33% CO₂, or pyridine at the indicated concentrations. n=5, error bars=sem.

Figure 4.5: Avoidance behavior in *D. simulans*.

T-maze behavior assay: Mean preference index of *D. simulans* flies given a choice between paraffin oil and 0.1ml pure CO₂ in 15ml air, *Drosophila* stress odor (dSO) collected from 70 vortexed “emitter” *wCS* flies, or pyridine (pyr) at indicated concentrations. n=7, error bars=s.e.m.

Table 4.1: Pyridine derivatives as semiochemicals

Chemical name	common name	CAS #	Class	Genus	Ref
3-Pyridinecarboxylic acid	Ethyl nicotinate	614-18-6	A	Orocrambus	1
3-Pyridinecarboxylic acid	Ethyl nicotinate	614-18-7	K	Taeniothrips	2
3-Pyridinecarboxylic acid	Ethyl nicotinate	614-18-8	K	Neohydatothrips	2
3-Pyridinecarboxylic acid	Ethyl nicotinate	614-18-9	K	Limothrips	3
Methyl 3-pyridinecarboxylate	Methyl nicotinate	93-60-7	P	Aenictus sp	4
2-(1-Methylpyrrolidin-2-yl)-pyridine	nicotine	23950-04-1	Al	Sclerobunus	5
3-(2-Piperidinyl)-pyridine	anabasine	40774-73-0	P	Aphaenogaster	6,7
3-(2-Piperidinyl)-pyridine	anabasine	40774-73-1	P	Messor	8
2-(Pyridin-3-yl)-pyridine	2,3-bipyridyl	581-50-0	P	Aphaenogaster	9
2-(Pyridin-3-yl)-pyridine	2,3-bipyridyl	581-50-1	P	Messor	8
Butan-2-one	4-2Kt	78-93-3	A	Coquillettidia	10
Butan-2-one	4-2Kt	78-93-4	A	Psorophora	10
Butan-2-one	4-2Kt	78-93-5	A	Aedes	10
Butan-2-one	4-2Kt	78-93-6	A	Culex	10
Butan-2-one	4-2Kt	78-93-7	P	Cimex	11
Butan-2-one	4-2Kt	78-93-8	P	Myrmica	12

A=Attractant, K=Kairomone, Al=Allomone, P=Pheromone

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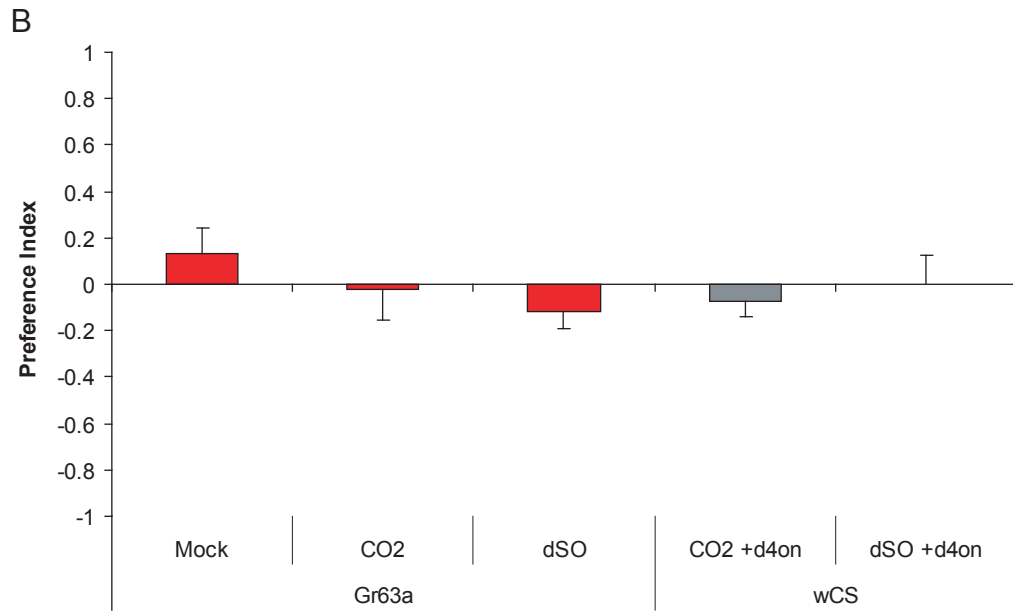
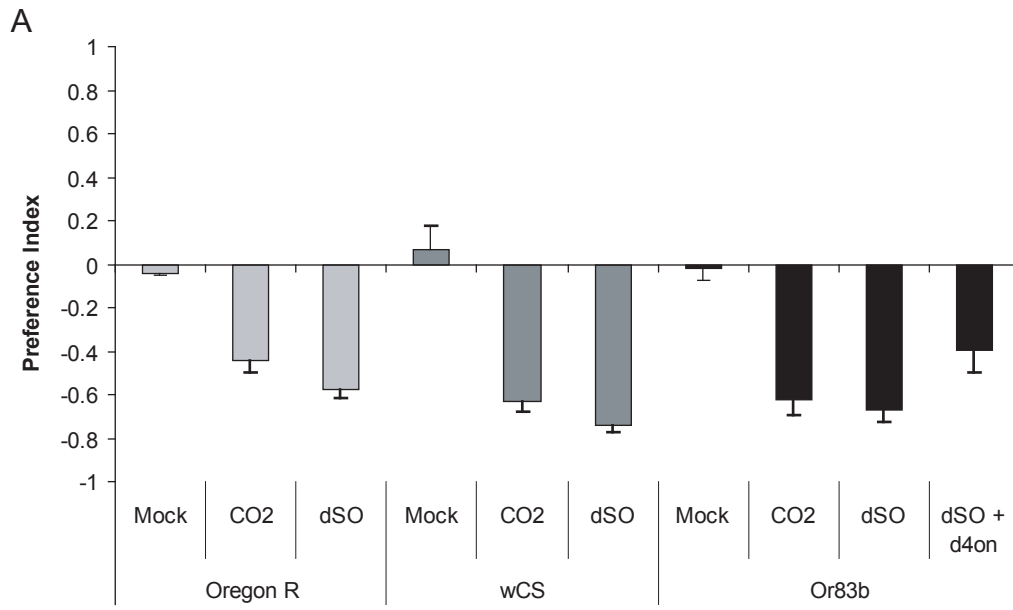
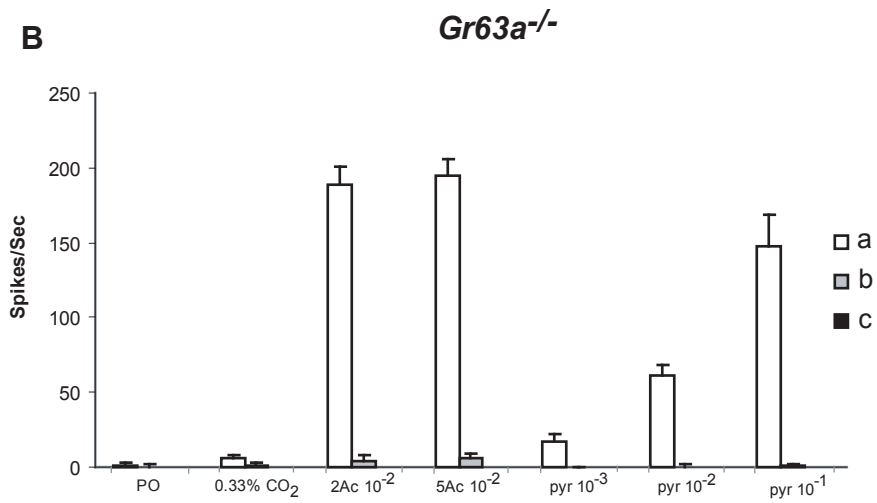
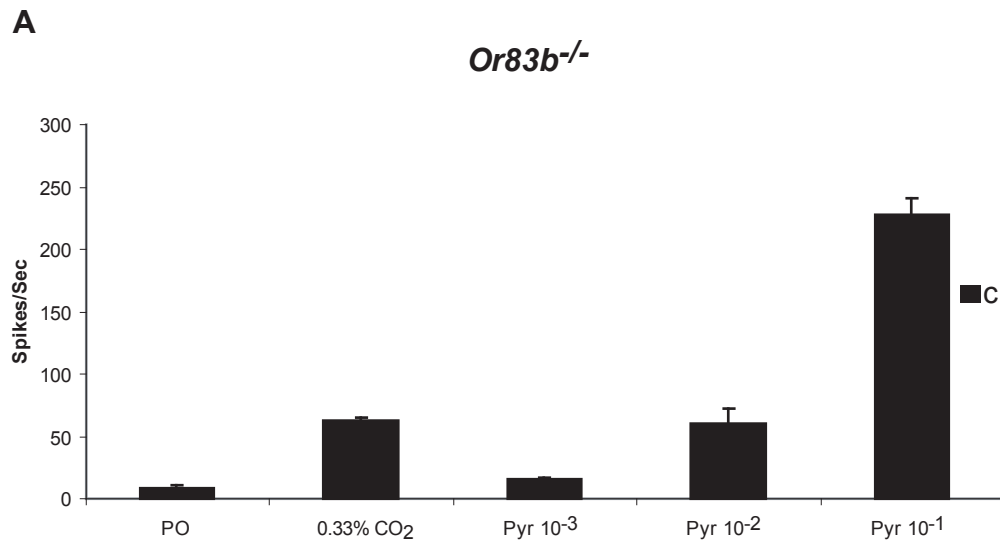


Figure 4.1



C

Or83b², Gr63a¹

	PO	0.33% CO ₂	Pyr 10 ⁻³	Pyr 10 ⁻²	Pyr 10 ⁻¹
average (spikes/sec)	0	0	0	0	0

Figure 4.2

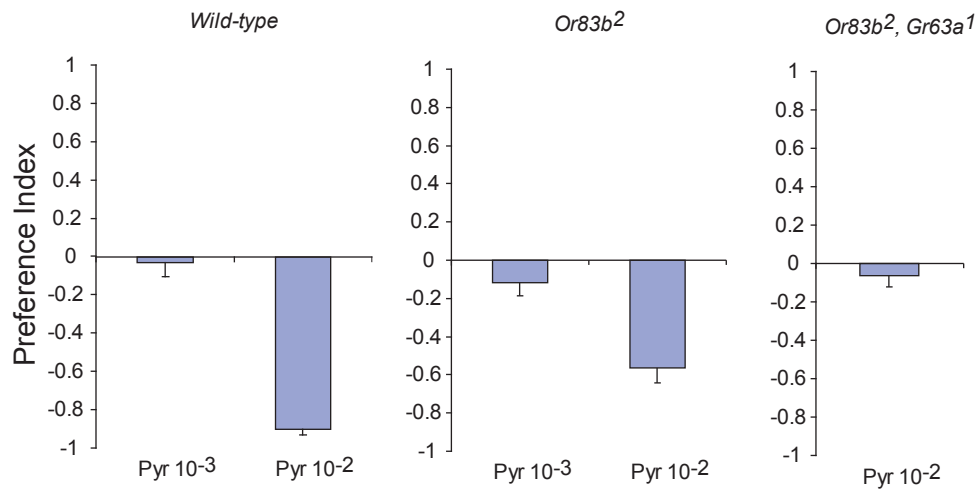
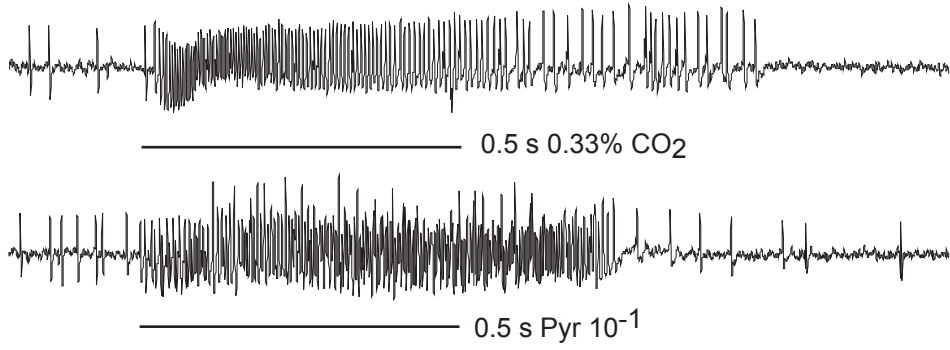


Figure 4.3

A



B

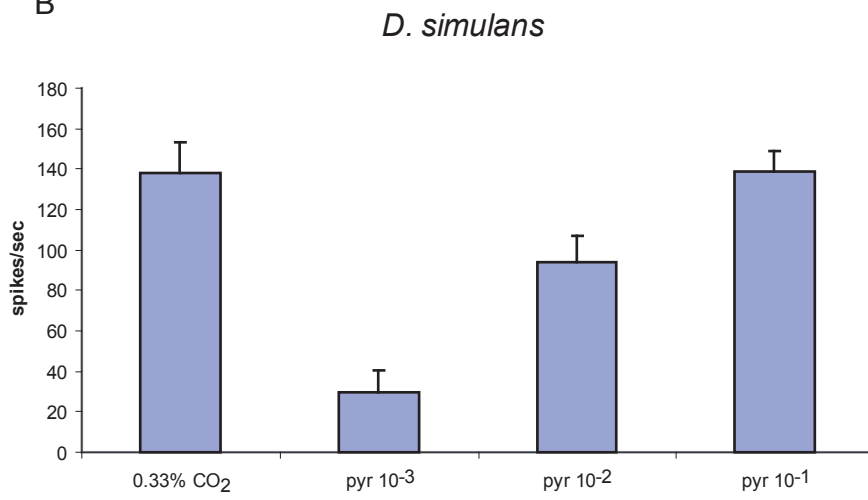


Figure 4.4

D. simulans

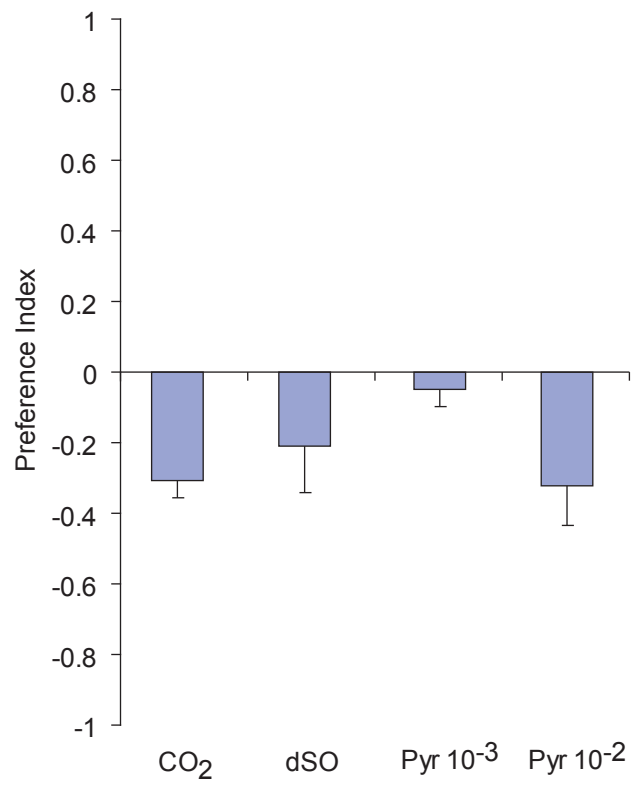


Figure 4.5

CHAPTER V:
ACTIVITY-DEPENDENT EXPRESSION MODULATION OF CHEMOSENSORY
RECEPTORS IN *DROSOPHILA MELANOGASTER*

Introduction:

The olfactory map in *Drosophila* has been highly elucidated and once formed, has been shown to be highly stable (Berdnik et al., 2006; Wong et al., 2002). Odor receptor neurons (ORNs) from the antenna or the maxillary palp, the olfactory organs of the fly, extend their axons to the antennal lobe of the fly's brain. ORNs which express the same odor receptor will all target the same region of the antennal lobe and form a spherical region known as a glomerulus. This organization within the antennal lobe creates what is known as a stereotypical map where expression of odor receptors can be traced to specific glomeruli. Activation of ORNs by odors will lead to the activation of sub-sets of glomeruli, which is thought to produce a neuronal code for output of specific odor-derived behaviors.

Although the olfactory map in *Drosophila* is highly stable, olfactory circuits within the confines of a given glomerulus appear to have the ability to change upon contact with olfactory stimuli (Sachse et al., 2007). It has been recently demonstrated that prolonged odor exposure can cause morphological changes in a 1-day old adult antennal lobe, which can lead to changes in fly behavior (Devaud et al., 2001; Sachse et al., 2007). Flies used in these experiments are

adults. However, in many insect species the imago olfactory system is plastic (Berdnik et al., 2006; Sigg et al., 1997) and subject to experience-dependent structural and functional modifications (Jean-Marc Devaud et al., 2003). Recent studies suggest that it is only during this “critical period” that experience-dependent plasticity can take place, and may be necessary to tune the olfactory system to its surrounding environment (Devaud et al., 2001; Jean-Marc Devaud et al., 2003; Sachse et al., 2007).

Early olfactory experience may cause plasticity in the antennal lobe, leading to behavioral modifications. In a recent experiment (Jean-Marc Devaud et al., 2003), flies exposed to a high concentration of benzaldehyde for 5 days caused a volume reduction in two glomeruli which were chosen based on traceability: DM2 as well as the CO₂-specific V glomerulus. During this study, it was not yet known what odor receptor neurons (ORNs) innervated these glomeruli, and what receptors they expressed. Neither Or22a/Or22b (expressed in ORNs of DM2) nor Gr63a/Gr21a (expressed in ORNs of V) respond to or are inhibited by benzaldehyde (Couto et al., 2005; Hallem and Carlson, 2006). How can an odor effect neuronal plasticity of a glomerulus that normally does not respond to that specific odor? The glomeruli of the antennal lobe are innervated primarily by two other forms of neurons, local interneurons (IN) and projection neurons (PN) and could explain how glomeruli which are not innervated by ORNs that respond to a specific odor still experience odor induced plasticity (For more information, please see CHAPTER I: INTRODUCTION). For

example, the glomeruli which are activated by benzaldehyde, DL5 and DM6 (Couto et al., 2005; Hallem and Carlson, 2006) could transmit this information through interneurons to other glomeruli, causing a more global activation of glomeruli. The resulting information from many glomeruli would then be transmitted through projection neurons to the higher brain centers. However, in the case of the Devaud study more experiments would have to be performed in order to demonstrate the possibility of a “global” effect in neuronal plasticity by odors.

Recently, the CO₂ detecting uni-glomerular circuit was used in order to demonstrate the ability of odors to cause specific odor induced activity-dependent neuronal plasticity (Sachse et al., 2007). CO₂ detection is exclusive to the V glomerulus and when activated causes an innate avoidance behavior (Suh et al., 2004). This circuit allows for examination of how a specific odor might effect neuronal modulation in a specific glomerulus without interaction from other activated odor receptors. It was shown that constant exposure to 5% CO₂ over a five day period (166 times above ambient CO₂ levels) caused a specific volume increase of 38% in the V glomerulus, and not to other glomeruli (Sachse et al., 2007). Flies exposed to air only, with an ambient CO₂ level of 0.03%, do not see any volume change in the V glomerulus. Also, activation of the CO₂ neuronal circuit was sufficient to induce neuro-anatomical modulation in the V glomerulus. After long term exposure to CO₂, flies show a decreased behavioral response to CO₂ in terms of distance walked in comparison to air exposed flies.

Although, this behavioral paradigm does not reveal any information about CO₂ induced avoidance behavior, this experiment does suggest that volume changes in the V glomerulus do contribute to an overall motility reduction (Sachse et al., 2007). These experiments demonstrate olfactory neuron circuit plasticity or neuro-anatomical modulation which can only be induced during a "critical period" of development. Devaud et al. demonstrated that activation of an olfactory neuron circuit may cause a broad change in glomerular modulation in the antennal lobe, whereas Sachse et al. showed unequivocally that one odor can cause a very specific modulation to a single glomerulus.

A few mechanisms that dictate odor receptor gene expression during development patterning have been identified. For example, POU-domain transcription factors Acj6 and Pdm3, have been implicated in mediating both expression of odor receptors (*Or*) as well as synaptic targeting in *Drosophila* (Certel et al., 2000; Tichy et al., 2008). In addition, regulatory elements also have been identified that act combinatorially to promote or repress the expression of specific *Or* genes in olfactory sensilla of the maxillary palp (Ray et al., 2008)(For more information, please see CHAPTER I: INTRODUCTION). However, it remains to be determined if these transcriptional regulators and cis-regulatory sites that direct developmental expression of *Or* genes are also used in controlling *Or* gene expression in response to the environment.

The CO₂ receptor is comprised of two gustatory receptor (Gr) proteins, Gr21a and Gr63a and are both required for CO₂ detection and CO₂ mediated avoidance behavior (Jones et al., 2007; Kwon et al., 2007). The CO₂ receptor is required not only for the detection of CO₂, but is necessary for neuro-modulation of the V-glomerulus after extended exposure to CO₂ (Sachse et al., 2007). In a recent study, we demonstrated that fruit odor 2,3-butanedione can inhibit a CO₂ response by interacting directly with the CO₂ receptor Gr21a/Gr63a (Turner and Ray, 2009). Because 2,3-butanedione has such a dramatic effect on CO₂ response, we sought to determine what effect this odor would have on the CO₂ uni-glomerular circuit during the flies “critical period” or the first days of the flies adult life.

Results and Discussion:

To assess the effect of CO₂ response inhibitor 2,3-butanedione on the antennal lobe, flies were exposed to the odorant during the first days immediately following eclosion. *Gr63a-Gal4; UAS-mcd8:GFP* and *Gr21a-Gal4; UAS-mcd8:GFP* flies were exposed to 2,3-butanedione at a 10⁻² dilution from two to five days in an air-tight container, and then imaged for expression of GFP in the CO₂ receptor expressing ab1C neurons of the antenna, as well as the V glomerulus, which receives axonal input from ab1C neurons (Suh et al., 2004). Surprisingly, *Gr63a* promoter driven GFP signal in the V glomerulus was completely abolished after 5 days of exposure to the odor for all brains collected (Figure 5.1A). GFP signal in antennal ab1C neurons was also lost after 5 days (Figure 5.1B). Similar results are seen for *Gr21a*-driven expression (Figure 5.2A), where expression of GFP was lost in both the V-glomerulus and ab1C neurons (Figure 5.2B). This phenomenon is dependent on duration of odor exposure, including the exact timing of exposure: flies need to be exposed to odor while they are still virgins (within the first few hours after eclosion). GFP expression was not lost gradually over time as might be expected, but rather at a very specific time point, suggesting that there is a duration threshold of odor exposure during the first few days of a fly's life.

To test the specificity of CO₂ receptor expression loss, we exposed flies to another CO₂ response inhibitor, 1-butanol. Again flies were exposed to the odorant for 5 days at a 10⁻² dilution, and as a result GFP expression was reduced in both *Gr21a-Gal4; UAS-mcd8:GFP* and *Gr63a-Gal4; UAS-mcd8:GFP* strains, albeit with less of an expression loss in comparison to treatment with 2,3-butanedione (Figure 5.3, 5.4). Although loss of GFP expression in the V glomerulus was observed, a fraction of flies treated with the odorant were not affected. Correspondingly, there was a slight loss of GFP expression in the antennal ab1C neurons marked using *Gr21a-Gal4; UAS-mcd8:GFP* flies or *Gr63a-Gal4; UAS-mcd8:GFP* flies (Figure 5.3B and 5.4B). 1-Butanol is a strong CO₂ response inhibitor, while 2,3-butanedione strongly inhibits the CO₂ neuron for an extended period of time (Turner and Ray, 2009), which may explain why a fraction of flies still expressed GFP while flies treated with 2,3-butanedione did not express GFP at all. Perhaps temporal properties of receptor inhibition by 2,3-butanedione may be an important prerequisite for GFP expression modulation.

Loss of gustatory receptor expression in this system could best be explained by two of the following scenarios: Odor exposure causes change in gene expression through either direct or indirect transcriptional regulation, or prolonged odor exposure causes neuronal cell death. To test for the possibility of neuronal cell death, we performed the following experiment. After exposing *Gr63a-Gal4; UAS-mcd8:GFP* and *Gr21a-Gal4; UAS-mcd8:GFP* flies to 2,3-butanedione for 6 days, we then allowed the flies to recover in clean air for 5 days and looked at GFP expression. Importantly, during both of these stages in treatment, flies remain healthy and mobile and show no signs of poor health. After 6 days of odor exposure, GFP expression is lost as expected. However, after 5 days of recovery in clean air GFP expression is regained in the V-glomerulus as well as in antennal ab1C neurons, indicating that both *Gr21a* and *Gr63a* expression has been restored (Figure 5.5). Because neurogenesis does not occur in the adult olfactory system in the fruit fly (Berdnik et al., 2006; Sigg et al., 1997), we can conclude that this gain in expression is most likely due to a change in gene regulation and not due to neuronal cell death.

In addition, to test for neuronal cell death we used electrophysiology to track neuronal activity of the CO₂ detecting ab1C neurons both after odor exposure and after recovery in clean air. Here we use the *Or83b*² mutant as a means of measuring response to CO₂. These flies are thought to be anosmic as Or83b is required for proper function of conventional odor receptors (Larsson et al., 2004). Because the CO₂ receptor Gr63a/Gr21a does not require Or83b for function, we can sort the activity of the CO₂ neuron easily due to the lack of activity of the remaining three neurons of the ab1 sensilla (Kwon et al., 2007; Larsson et al., 2004). After treatment to 2,3-butanedione for 6 days, 75% of sensilla on the antenna display spontaneous activity, and 22% responded to 0.33% CO₂ at an average of 29.5 spikes per second (Table 5.1). These results indicate that most of the ab1C neurons were unable to respond to CO₂ after such an odor treatment with 2,3-butanedione, as untreated flies of the same age responded at an average of 134 spikes per second (data not shown). Of the flies which were allowed to recover for 5 days after the odor treatment, 71% of sensilla tested displayed spontaneous activity, and 60% of sensilla responded to 0.33% CO₂ at an average of 86 spikes per second (Table 5.1). In agreement with our confocal data, the recovery of neuronal activity demonstrates that cell death is not taking place after treatment with the odor, and that CO₂ response recovers to near normal levels after the recovery period.

We next asked if the gene expression decrease induced by 2,3-butanedione was specific to *Gr21a/Gr63a*. Was 2,3-butanedione, a CO₂ response inhibitor, only causing decreased expression in CO₂ receptor expressing cells? First, antennal expressed odor receptors were tested for this possibility. *Or88a-Gal4; UAS-mcd8:GFP* and *Or22a-Gal4; UAS-mcd8:GFP* flies were exposed to 2,3-butanedione for 6 days, and were found to lose GFP expression in both VA1d and DM2 glomeruli as well as antennal at4C and ab3A neurons, respectively (Figure 5.6). These results indicate that the observed expression loss phenomenon is not specific to the CO₂ receptor *Gr21a/Gr63a*, but observed in other antennal *Ors* as well.

To test if a reduction in gene expression is limited to antennal *Ors/Gr*s, we also exposed maxillary palp specific *Or46a-Gal4; UAS-mcd8:GFP* flies to 2,3-butanedione. After six days of exposure, GFP signal was lost in the VA7I glomerulus as well as palp pb2B neurons, indicating down regulation of *Or46a* (Figure 5.7). These experiments demonstrate that 2,3-butanedione dramatically effects chemosensory receptor expression in both the antenna and the maxillary palp, indicating that down regulation of gene expression may not be due to a specific regulatory element or transcription factor limited to *Gr21a/Gr63a*.

Could 2,3-butanedione have a global effect on odor and gustatory receptor expression? To test this possibility we used *Gr5a-Gal4; UAS-mcd8:GFP* flies. *Gr5a* confers response to the sugar trehalose and is expressed in sugar neurons located in gustatory sensilla of the fly labellum, whose axons project into the subesophageal ganglion (SOG) (Dahanukar et al., 2001). Flies were exposed to the odorant for 6 days, and interestingly, did not show a change in expression levels in either labellar sugar neurons, or in the SOG (Figure 5.8). Because the morphology of the gustatory sensilla which house taste neurons are quite different from antennal sensilla, and requires contact for stimulation, it is possible that the odorant is not able to act on the gustatory receptor neurons (GRN) of the labellum as it would with ORNs and GRNs in the antenna or maxillary palp. It would be necessary to repeat this experiment with *Gr10a-Gal4; UAS-mcd8:GFP* flies, another antennal expressed gustatory receptor, to determine if 2,3-butanedione can cause gene expression changes of other *Gr* genes.

To answer definitively whether gene expression is down regulated at the transcriptional level by 2,3-butanedione we used quantitative-RT-PCR. Wild-type flies that were exposed to 2,3-butanedione for 6 days showed dramatic down regulation in all receptor genes tested (Figure 5.9). Antennal odor receptor *Or88a* showed less than 50% of normal expression, as did *Gr63a*. However, after a 5-day recovery period *Gr63a* gene expression recovered beyond normal levels, whereas *Or88a* gene expression did not recover at all. Of all the genes

tested antennal receptor *Or47a* is the only *Or* gene which is down regulated upon treatment with the odorant, and is still able to recover to normal expression levels. However, unlike the observations with confocal imaging data, *Gr21a* expression levels appear just below normal expression levels after treatment with the odorant. This result could be a reflection of inadequate Q-RT-PCR optimization for *Gr21a*, as the highest amplification level seen in control samples is 27-28 cycles, whereas *Gr63a* control samples have amplification at 26 cycles.

In this gene expression screen we also tested *Or83b*, a ubiquitously expressed non-canonical receptor which forms heteromeric pairs with conventional odor receptors to confer proper function (Larsson et al., 2004). After prolonged exposure to the odor, *Or83b* expression was down regulated by 50%. This expression does recover slightly after a 5-day recovery period, but still remains below normal levels, again indicating that 2,3-butanedione generally down regulates many chemosensory genes expressed in both the antenna and the maxillary palp.

In summary, the CO₂ response inhibitor 2,3-butanedione caused a decrease in gene expression on the V-glomerulus and antennal ab1C neurons, suggesting a novel phenomenon where an inhibitory odor can directly effect CO₂ gustatory receptor expression through some yet to be determined mechanism. After further investigation we have found that 2,3-butanedione reduces expression of antennal as well as palp odor receptors. Perhaps more striking, down regulation of the ubiquitously expressed *Or83b* suggests that odor exposure could be acting on some cellular pathway that leads to down regulation of several receptor genes. Although *Or83b* has been shown to be down regulated as both male and female flies age (Zhou et al., 2009), the decrease in expression of *Or83b* in our studies was not due to a general aging effect, but was caused by exposure to the odorant. It is also important to note that gene expression levels of house-keeping gene ribosomal protein 49 (*RP49*) remain nearly identical when comparing odor treated versus air treated flies, suggesting that the mechanism of action of 2,3-butanedione downstream effectors act specifically on odor and gustatory receptors and not on other expressed genes.

It has been shown that in the *Drosophila* olfactory system, the formation of neuronal circuitry is independent of *Ors* or neuronal activity (Dobritsa et al., 2003), and it has been demonstrated that this circuitry is most likely fully formed during the pupal stage where the developing insect has no contact with odors (Technau, 2008). However, as in mammals, olfactory neuron maintenance requires neural activity (Woo et al., 2006). It was previously shown that silencing of olfactory neurons which innervate a single glomerulus led to degeneration of those neurons (Chiang et al., 2009). Although previous reports demonstrate that neuron activity is necessary for neuron maintenance, and neurons die as a result of inactivity, the prolonged treatment of CO₂ response inhibiting odor used in this study caused *Gr* gene expression to go down, while not causing neuronal cell death. Importantly, in our study, an exposure time of 6 days had not led to induction of neuronal degeneration. Data from previous studies have demonstrated that in their model of neuro-degeneration, by 6 days complete clearance of degenerating neurons may have occurred (Chiang et al., 2009; MacDonald et al., 2006).

Conclusion:

Overall, this project demonstrates a novel phenomenon where an odor can induce down regulation of chemosensory receptors. Further research will be needed to understand the mechanism that causes a decrease of such gene expression. 2,3-butanedione is an inhibitor of the CO₂ neuron, but also causes a decrease in odor receptors. It will be interesting to see if inhibitors of specific odor receptors can also cause a decrease in gene expression after prolonged odor exposure. It will also be important to see why down regulation of only some genes is reversible, and if extended odor exposure periods could contribute to this phenomenon.

Figure Legends:

Figure 5.1: CO₂ inhibitory odor 2,3-butanedione causes down regulation of CO₂ receptor gene *Gr63a*.

A, Representative confocal micrographs of whole mount brain (top) and antennal (bottom) staining of *Gr63a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 2,3-butanedione 10⁻² dilution (Treated) or air for 2 to 5 days (see CHAPTER VI: METHODS). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections. **B**, Mean of ab1C neurons in the antenna expressing GFP after indicated days of odor exposure to 2,3-butanedione. d4on=2,3-butanedione. n=6, error bars=s.e.m.

Figure 5.2: CO₂ inhibitory odor 2,3-butanedione causes down regulation of CO₂ receptor gene *Gr21a*.

A, Representative confocal micrographs of whole mount brain (top) and antennal (bottom) staining of *Gr21a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 2,3-butanedione 10⁻² dilution (Treated) or air for 2 to 5 days (see CHAPTER VI: METHODS). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections. **B**, Mean of ab1C neurons in the antenna expressing GFP after indicated days of odor exposure to 2,3-butanedione. d4on=2,3-butanedione. n=6, error bars=s.e.m.

Figure 5.3: CO₂ inhibitory odor 1-butanol causes down regulation of CO₂ receptor gene *Gr21a*.

A, Representative confocal micrographs of whole mount brain (top) and antennal (bottom) staining of *Gr21a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 1-butanol 10⁻² dilution or air for 5 days (see methods). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections. **B**, Mean of ab1C neurons in the antenna expressing GFP after 5 days. 4al= 1-butanol, n=6, error bars=s.e.m.

Figure 5.4: CO₂ inhibitory odor 1-butanol causes down regulation of CO₂ receptor gene *Gr63a*.

A, Representative confocal micrographs of whole mount brain (top) and antennal (bottom) staining of *Gr63a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 1-butanol 10⁻² dilution or air for 5 days (see methods). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections. **B**, Mean of ab1C neurons in the antenna expressing GFP after 5 days. 4al= 1-butanol, n=6, error bars=s.e.m.

Figure 5.5: Down regulation of the CO₂ receptor is reversible.

Representative confocal micrographs of whole mount brain (top) and antennal (bottom) staining of *Gr63a-Gal4; UAS-mcd8:GFP* and *Gr21a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 2,3-butanedione 10⁻² for 6 days (treated 6 days), and allowed to recover in clean air for 5 days (treated 6 days/recovered 5 days), or exposed to air for 11 days (control day 11). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections.

Figure 5.6: 2,3-butanedione causes decreased expression of antennal odor receptors Or22a and Or88a.

Representative confocal micrographs of whole mount brain (top) and antennal (bottom) staining of *Or22a-Gal4; UAS-mcd8:GFP* and *Or88a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 2,3-butanedione 10⁻² for 6 days (treated), or exposed to air for 6 days (air). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections.

Figure 5.7: 2,3-butanedione causes decreased expression of palp odor receptor Or46a.

Representative confocal micrographs of whole mount brain (top) and palp (bottom) staining of *Or46a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 2,3-butanedione 10^{-2} for 6 days (treated), or exposed to air for 6 days (air). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections.

Figure 5.8: 2,3-butanedione causes decreased expression of labellar gustatory receptor Gr5a.

Representative confocal micrographs of whole mount brain (top) and labellar (bottom) staining of *Gr5a-Gal4; UAS-mcd8:GFP* flies. Flies were exposed to 2,3-butanedione 10^{-2} for 6 days (treated), or exposed to air for 6 days (air). Neuropil marker nc82 (red) and anti-GFP (green). Micrographs are compiled as Z-stack projections.

Figure 5.9: Odor and gustatory gene expression is down regulated by exposure to 2,3-butanedione.

Flies were exposed to 2,3-butanedione 10^{-2} for 6 days (Day 6 treated), or exposed to 2,3-butanedione 10^{-2} for 6 days and allowed to recovery in air for 5 days (Day 6 treated, 5 day recovery). Gene expression by Q-RTPCR is compared to flies raised in air for the equivalent amount of days. Expression data is based on fold expression normalized to RP49. Fold expression=1 indicates no change in expression. n=2 for *Gr21a* and *Gr63a*, n=1 for *Or83b*, *Or88a* and *Or47a*.

Table 5.1: Electrophysiology on odor treated flies

Or83b ² flies treated 6 days with 2,3-butanedione			
From 6 flies and 40 sensilla			
% with no Spontaneous activity	% with spontaneous activity	% with response over 20 s/s	% with Response over 50 s/s
25	75	22.5	0
10/40	30/40	9/40	0/40
		ave: 29.5 s/s	
		sem: 2.92 s/s	

Or83b ² flies treated 6 days with 2,3-butanedione, 5 day recovery			
From 7 flies and 35 sensilla			
% with no Spontaneous activity	% with spontaneous activity	% with response over 20 s/s	% with response over 50 s/s
28.57142857	71.42857143	65.71428571	60
10/35	25/35	23/35	21/35
		ave: 82.78	ave: 86.9
		sem: 5.37	sem: 4.95

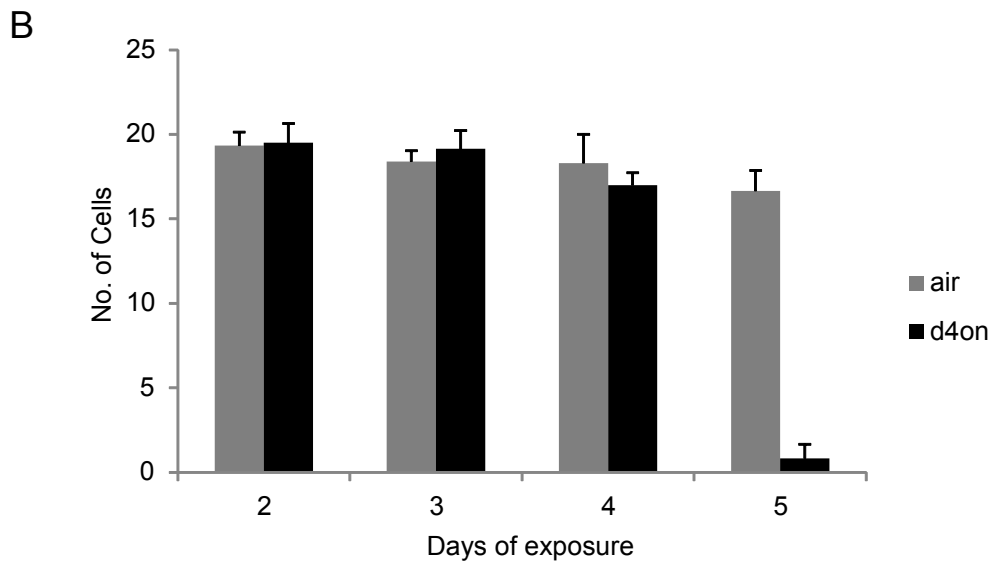
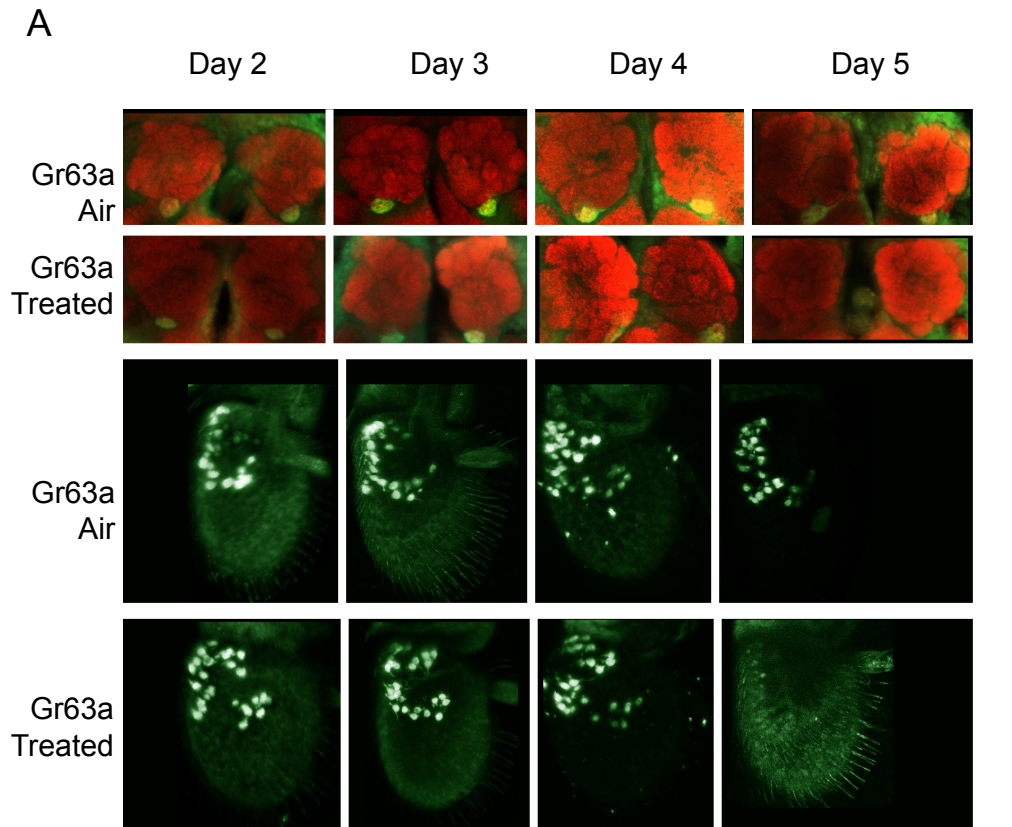


Figure 5.1

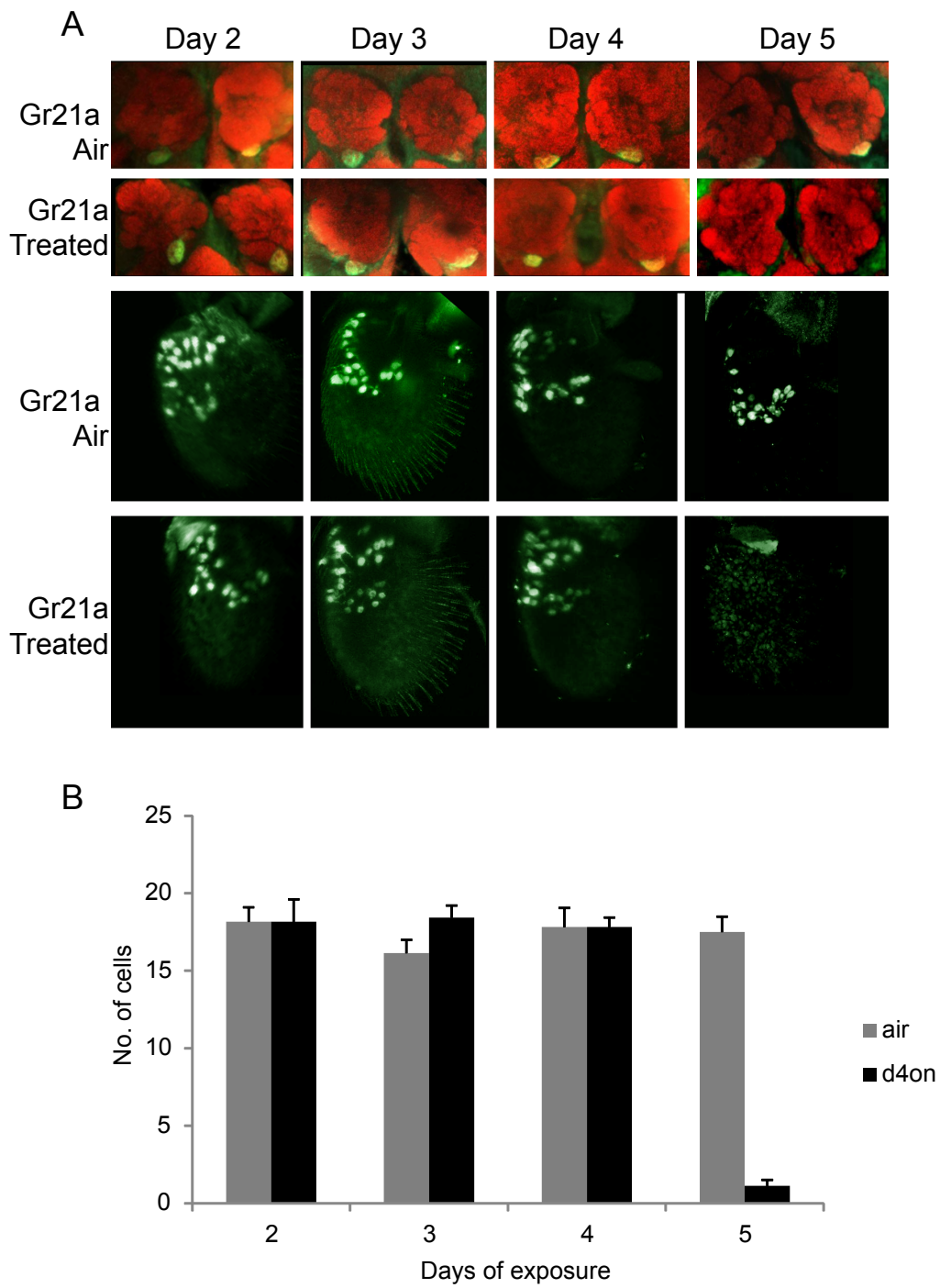


Figure 5.2

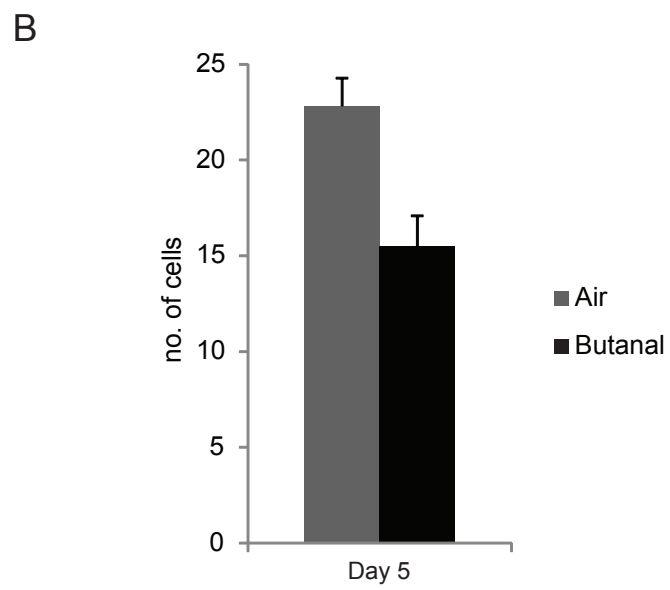
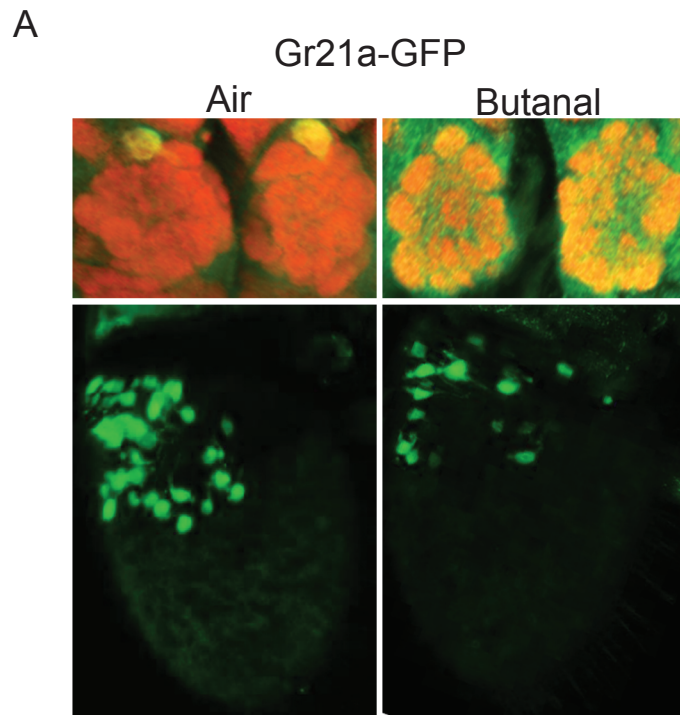


Figure 5.3

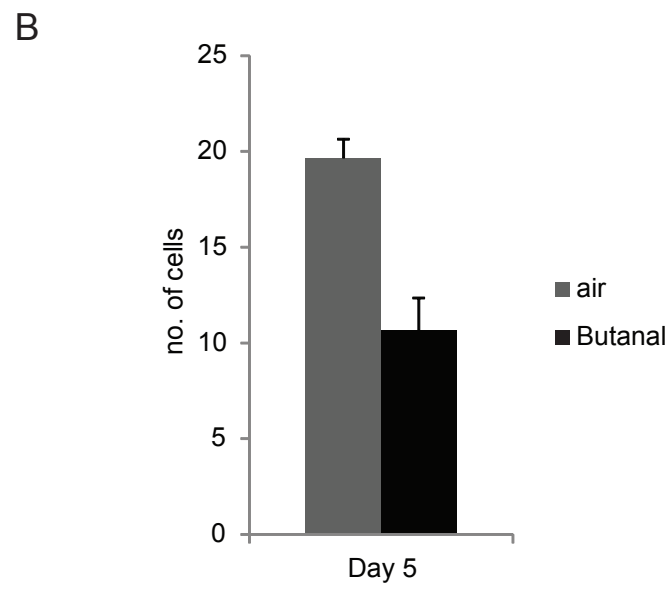
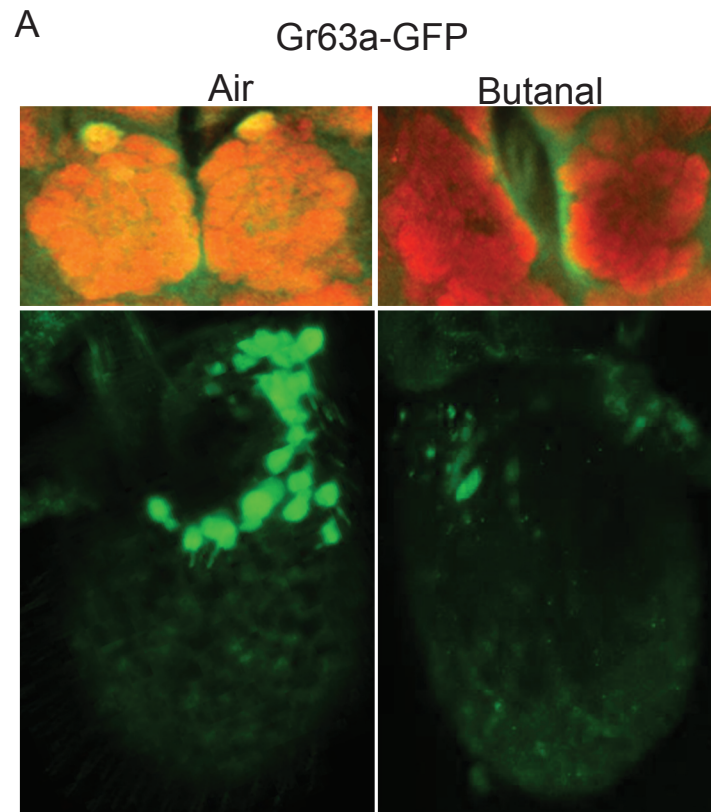


Figure 5.4

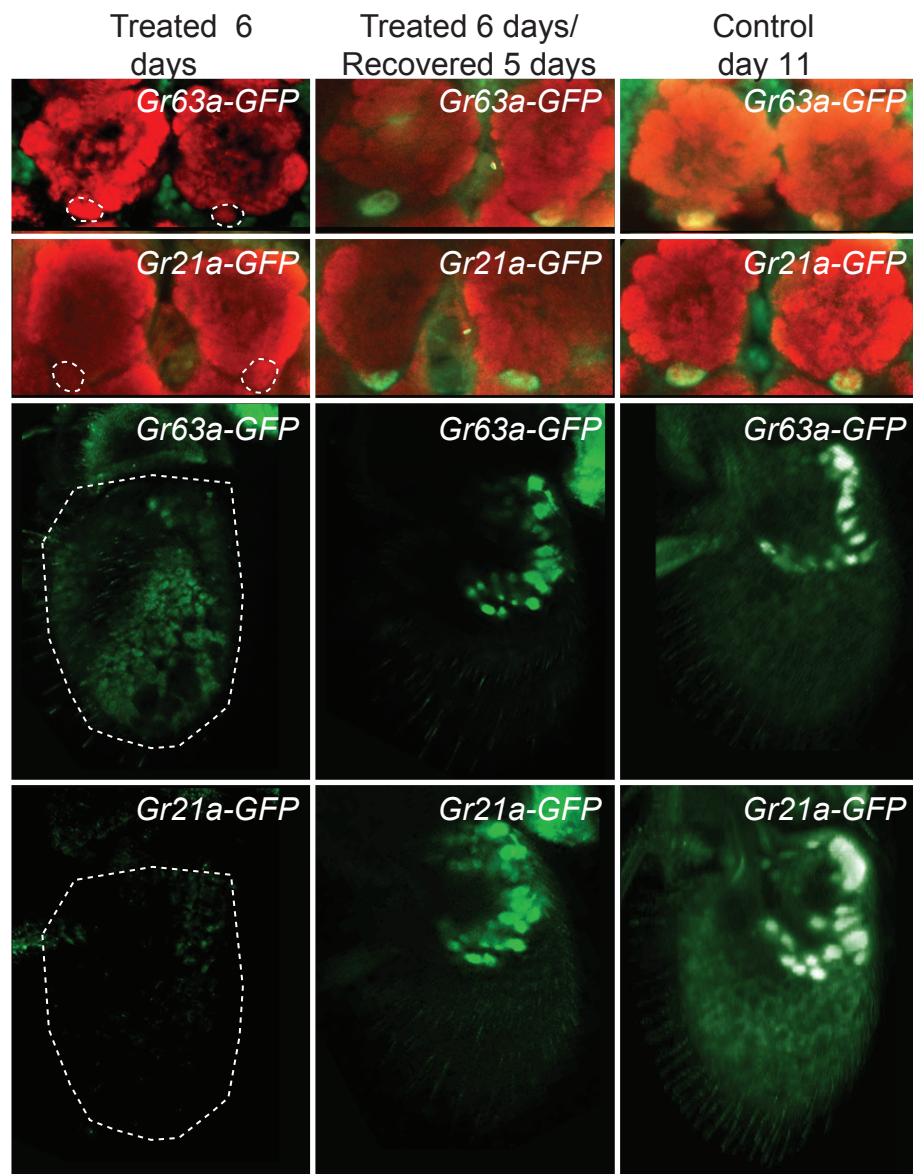


Figure 5.5

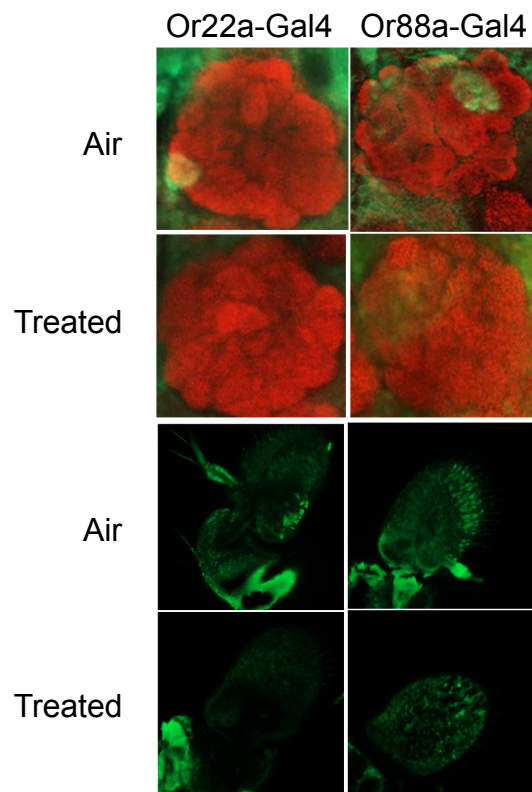


Figure 5.6

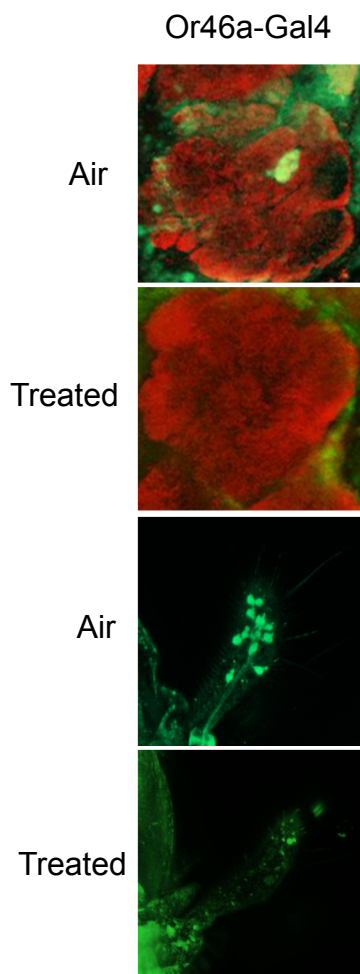


Figure 5.7

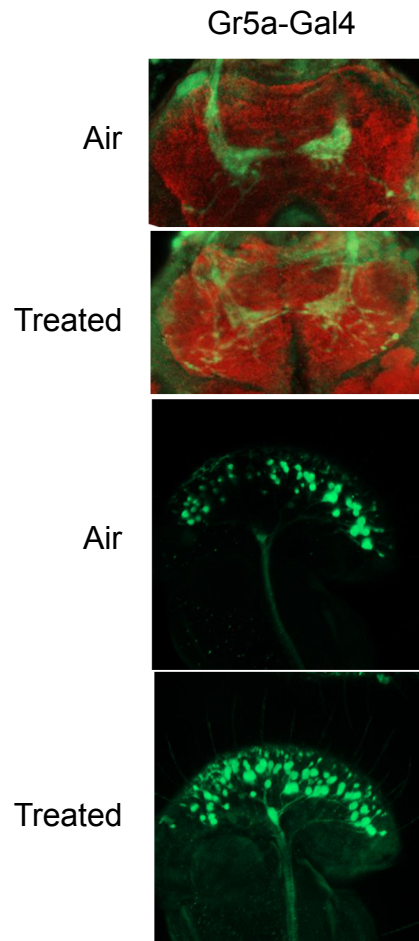


Figure 5.8

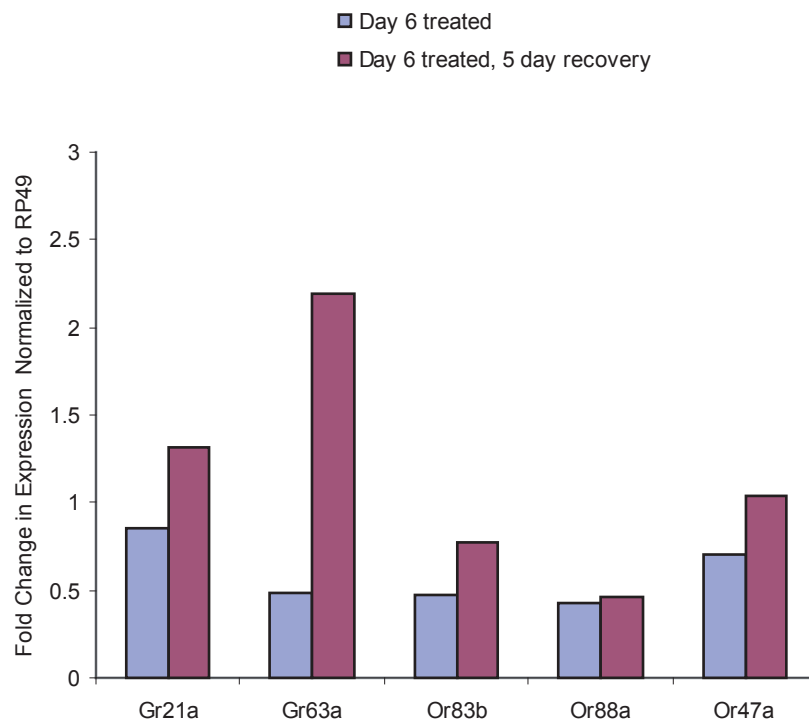


Figure 5.9

CHAPTER VI:

METHODS

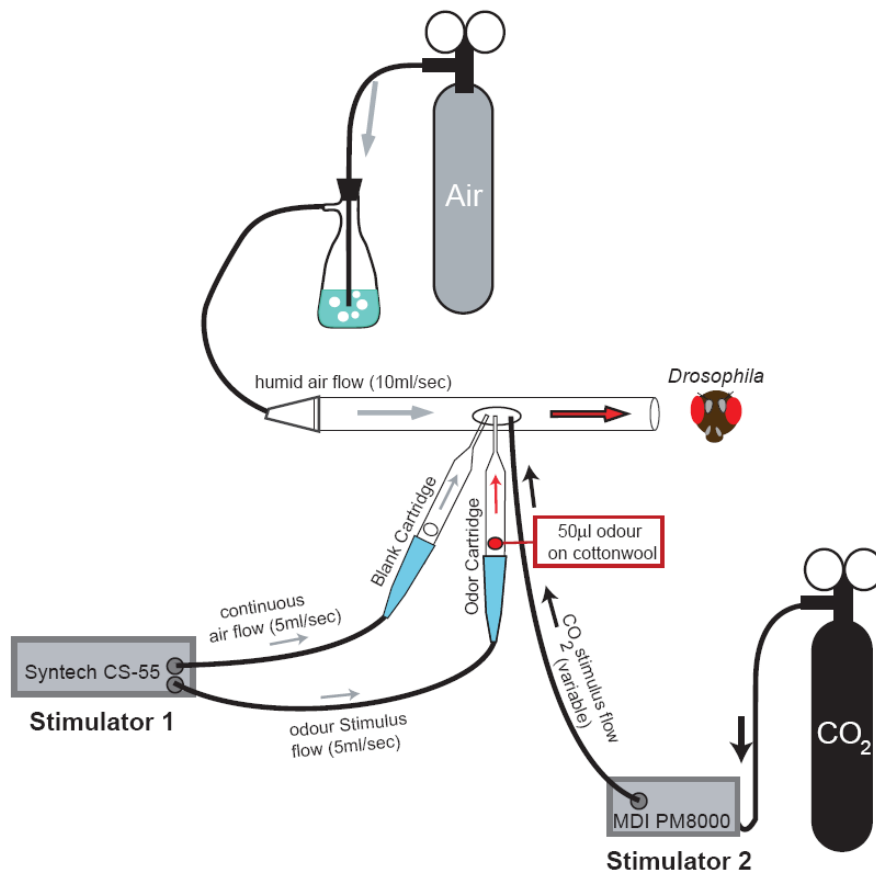
Fly Stocks

Fly stocks were maintained on standard cornmeal medium at 25°C. Wild-type stock is *w*¹¹¹⁸ backcrossed 5 generations to Canton S. The *Or83b*² mutant was obtained from the Bloomington stock center. Stocks for Δ *halo*; *Or22a-Gal4*, *UAS-Gr21a*, *UAS-Gr63a*, *Gr63a-Gal4*; *UAS-mcd8:GFP*, *Gr21a-Gal4*; *UAS-mcd8:GFP*, *Or88a-Gal4*; *UAS-mcd8:GFP*, *Or22a-Gal4*; *UAS-mcd8:GFP*, *Or46a-Gal4*; *UAS-mcd8:GFP*, and *Gr5a-Gal4*; *UAS-mcd8:GFP* were kind gifts from John Carlson, Yale University. Additional lines of *Or22a-Gal4* were generated by mobilizing the original P-element insertion line using standard genetic techniques. The Δ *halo*; *Or22a-Gal4/UAS-Gr21a*, *UAS-Gr63a* flies were raised on standard cornmeal medium at 25°C.

Electrophysiology

Extracellular single-unit recordings of action potentials was performed as described previously (Dobritsa et al., 2003; Turner and Ray, 2009) by placing an electrode through the sensillum wall into contact with sensillar lymph. Signal from the recording electrode was amplified using a $>10^{12}$ -input impedance amplifier (IsoDam, WPI, Sarasota, Florida), and filtered with a 100Hz high-pass filter. Recording were obtained using Axoscope software (Molecular Devices). Responses were quantified by counting the number of action potentials during a 0.5 sec stimulus period (unless otherwise specified). Chemicals were of the highest purity available, typically $>99\%$ (Sigma-Aldrich). All odorants were diluted in paraffin oil at indicated concentration and unless indicated 50ul applied/ cartridge, each cartridge used for 3 stimuli. A controlled volume of air 5ml/sec was puffed through the odour cartridge containing vapours in equilibrium, and was delivered into a constant humidified airstream of 10ml/sec that was passed over the fly antenna. The odorant vapor present in the cartridge is thus diluted 3 fold, and the concentration of inhibitory odorants in the air stream that passes over the fly is significantly lower than that applied to the cartridge (indicated 10^{-1} stimulus = ~ 0.43 ug in cartridge/application; 10^{-2} stimulus = ~ 0.043 ug in cartridge/application). CO₂ stimulus was pulsed through a separate delivery system that delivered controlled pulses using a PSM 8000 microinjector (variable 2.5ml/sec – 6.5ml/ sec) into the same humidified airstream, from either a 1% or 5% tank of CO₂ (Airgas) . The baseline constant humidified airstream was

generated from a purified air tank (Airgas) with 0.03% CO₂. For delivery of binary mixtures of CO₂ with another odorant, we ensured a steady concentration of CO₂ to the fly preparation (Figure 6.1). Unless mentioned, responses were quantified by subtraction of spontaneous activity from activity during the stimulus. For each inhibitory odorant (some that had a long-term effect on CO₂ response), each recording was obtained from a distinct fly or mosquito.



$$\text{CO}_2 \text{ concentration} = \frac{\text{Conc. of CO}_2 \text{ in tank} \times \text{Volume of CO}_2 \text{ Stimulus flow}}{\text{Continuous air flow} + \text{odour stimulus flow} + \text{CO}_2 \text{ stimulus flow}}$$

Figure 6.1: CO₂ delivery apparatus.

Behaviour in a wind tunnel

Wind tunnel behavior experiments were performed as described previously (Dekker et al., 2005) with some modifications. Briefly, female mated non-blood fed *A. aegypti* mosquitoes between 7-14 days age that have been raised in a 14:10 hour light-dark cycle were individually collected in release cages and held for 21 hours at 25°C and 70% relative humidity. Mosquitoes in the cage were then transferred into an upended 1L glass beaker with 100uL of odor diluted in paraffin oil at the indicated concentrations. The mosquito in the release cage was removed from the odor containing beaker and transferred in room air across the room to the wind tunnel where it was carefully manipulated into the release cage. Air from outside the building was presented in the wind tunnel in a laminar flow at a controlled rate of 30 cm³/sec, of ~70% relative humidity, temperature ~23°C. A turbulent plume of 1% CO₂ was generated by mixing purified CO₂ and air from cylinders (Airgas) and delivered through a glass ring with 8 outlets pointing inwards. The cage was opened remotely to release an individual female mosquito. Two video cameras were used to record the flight path. Analyses of videos were performed offline on computers.

T-maze Behavioral Testing

All T-maze assays were performed as described (Suh et al., 2004) where flies were sorted in groups of 40 (equal parts male and female), and placed into vials stuffed with 2 kimwipes and wetted with 3ml di-water. Flies were starved for 24hrs unless otherwise specified. Flies were then put into the T-maze and allowed to choose between a test odor and solvent in the dark for 1-min. T-maze behavioral testing using *Drosophila* stress odor, CO₂, and mixtures was performed as described (Suh et al., 2004), with the following modifications: The entire headspace from 15ml capped “emitter” or “mock” fly tubes was withdrawn using fresh syringes and needles and infused into fresh capped 15ml plastic tubes immediately prior to use in the T-maze. dSO was generated by performing the following: 70 wCS flies were sorted into a vial with food and set aside until ready to do the experiment. Separately, ~40 flies (of about equal parts male and females) were sorted and placed into behavior vials (as described above) to starve for 24hrs. The 70 wCS flies set aside in a food vial were then placed into 15ml culture tube (not anesthetized), with a wad of cotton on top to keep flies from escaping. This vial was then used capped and vortexed for 3-sec bouts every 5-sec over a 1 min period. Vortexed flies are then set aside for 10-min. The plastic tubing is then taken off of the syringe, and the syringe is then pierced through a new capped 15ml tube labeled dSO. Mock fly odor was collected similarly, were instead of odor collected from vortexed flies, flies from a food vial are freely able to traverse up a funnel attachment to a clean 15ml culture tube for

10-min. Fly odor from the 15ml culture tube is taken using the same syringe method used above.

To test the response to mixtures, 10ul of odorant diluted in paraffin oil (at the concentrations indicated) was placed on a Whatman filter paper (6mm diameter) and placed carefully at the bottom of a fresh 15ml plastic tube and capped ~5-min prior to start of assay. The additional component (0.1ml pure CO₂ or 15ml dSO) was injected directly into this capped tube using a syringe, which was then used as the test arm in the T-maze. The tube in the control arm contained filter paper with 10ul of paraffin oil solvent. The avoidance response was calculated as a Preference Index (PI) = (number of flies in test arm - number of flies in control arm)/(total number of flies in assay).

Behavioral responses to CO₂ were tested using the T-maze by injecting 0.1ml of pure CO₂ into a capped 15-ml tube with a syringe and needle immediately before the choice assay. For over-ripe fruits, fruits were allowed to ripen and ferment in a sealed plastic container for ~3 weeks, at which point 5gm of fruit paste was transferred to a fresh 50ml plastic tube and sealed. After 5-min at room temperature, 15ml of headspace was removed using a syringe, and transferred to a fresh 15ml plastic tube that was used directly as the test arm of the T-maze. Yeast (1gm) was used to make a paste with 1ml of 15% sucrose solution, and incubated at room temperature for 1 hour in a 50ml sealed tube. The cap was removed to release volatiles and then replaced; 15ml of headspace was collected 5-min later and tested as described above. Similarly, 5-min

collections of headspace were taken from 5gm of green fruits, and 5ml of beer (Stone Pale Ale: Stone brewing company, San Diego, CA). Prior to being tested for responses to headspace from fruit, beer and yeast, flies were pre-exposed to the same odors in separate 15ml tubes for 2 minutes. To test the response to CO₂ or other odors after prior exposure to odorants, 10ul of odorant diluted in paraffin oil (10⁻² unless otherwise indicated in legend) was loaded on a Whatman filter disc (6mm diameter), which was placed carefully at the bottom of a fresh 15ml plastic tube approximately 5 minutes prior to start of assay. A small piece of cotton wool was inserted into the tube such that the flies were unable to make physical contact with the odorant-laden filter paper. Flies starved 24hrs were carefully put in the odor prepared tube for 1 minute and then transferred to a fresh tube containing room air for nearly 2 minutes. Just prior to the 2 min mark, the flies were transferred to the T-maze, and 0.1ml of pure CO₂ was injected into one arm. The assay was started precisely at the 2 min mark and performed as usual for 1 min in the dark. For all assays including *D. simulans*, a 10hr starvation period was used.

Odor exposure experiments

Newly eclosed male wCS flies were sorted in groups of 30 into vials with standard cornmeal medium. Vials were closed with two overlapping 3"x3" polypropylene mesh squares, and tied with cotton twine. Vials were used within 6 hours. Four vials were placed in a 1000ml Nalgene straight sided jar with a 10ml glass beaker with 10ml of 2,3-butanedione 10^{-2} in paraffin oil. Jars were closed for exposure periods ranging from 2-6 days. In recovery experiments, vials were removed from the jars and were placed in a 25°C incubator for the remainder of the recovery period.

Immunohistochemistry

Flies were anesthetized on ice, sacrificed in ethanol, and then immediately put into 1X phosphate buffered saline with 0.3% triton-x (PTX). Brains or antenna were put in 4% paraformaldehyde in PTX and incubated for 30min while rotating at 25°C. Samples were washed 5 times in PTX, and blocked in 5% natural goat serum in PTX for 1 hr while rotating at 25°C. Samples were then incubated in primary antibody with 5% goat serum in PTX, mouse-nc82 (1:20) (Development Studies Hybridoma Bank, University of Iowa) and rabbit-antiGFP (1:150) (Invitrogen) in PTX for 48 hours in 4°C while rotating. After washing 5 times in PTX samples were incubated in secondary antibody with 5% goat serum in PTX, rabbit-anti-Alexa488 (1:400) (Invitrogen) and mouse-antiAlexa568 (1:400) (Invitrogen) for 48 hours in 4°C. Samples are washed 5 times in PTX and stored in 70% glycerol in PTX. Images were taken using a Ziess 510 laser scanning confocal microscope, and image analysis was done using Image J software.

Q-RT-PCR

Drosophila heads (with antenna) were collected on liquid nitrogen and stored at -80°C until processing. 300 heads were collected for each sample, and ground on liquid nitrogen. mRNA was extracted using a PolyAtract kit (Promega), and was reverse transcribed using Superscript III (Invitrogen) according to the manufacturer's protocol. The quality of the cDNA was assessed on a 1.5% agarose gel. cDNA was added to individual reactions of SYBR green master mix (BioRad) and run on a BioRad-MyQ thermocycler. The program began with a single cycle for 3-min at 95°C, followed by 40 cycles of 15-sec at 95°C, 30-sec at 58°C and 30-sec at 72°C. Afterward, the PCR products were heated to 95°C for 1-min, and cooled to 55°C for 1-min, to measure the dissociation curves. The efficiency of each primer set was first validated by constructing a standard curve and three 10x serial dilutions of first strand cDNA. For each serial dilution, the CT value was plotted against the log (template dilution) and the slope and r^2 value of each regression line calculated. Expression of each *Or* or *Gr* gene was assessed in triplicate. Dissociation curves were used to assess the purity of the PCR reactions. *Or* and *Gr* transcript levels were normalized by using the transcript levels of *ribosomal protein 49*. Expression levels were calculated using the Pfaffl equation (Pfaffl, 2001).

In general, primers were designed by using coding sequences close to the 3' end of the gene, and where possible, primers spanned an intron.

Primers:

Gr21a F: CGATCGTCTTTCCGAATCTC

Gr21a R: GGCTCAGATCCACCCATAGA

Gr63a F: AAATGAACTCCGCCTCCTTT

Gr63a R: CGCAATTTTCAGAGGCAAAC

RP49 F: CTGCCCACCGGATTCAAG

RP49 R: GTTTCATGCGGCGAGATCG

Or47a F: ATCACAGGCCACATTGAACA

Or47a R: TCCCCGCAGTAGCAGTAGAT

Or88a F: TTAAAGTGGCCTTCCTGGTG

Or88a R: ATGCGGCAATAAAGTTCCAC

Or83b F: TTCTTGGCATTTCGCTTTTCT

Or83b R: TCCCTGGATTTGTTTGCTTC

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