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INSECT HOST SEEKING: INVESTIGATIONS INTO THE MOLECULAR MECHANISMS OF CHEMOSENSATION

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy

By

Walton D. Jones

June 2007

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INSECT HOST SEEKING: INVESTIGATIONS INTO THE MOLECULAR MECHANISMS OF CHEMOSENSATION

Walton D. Jones

The Rockefeller University 2007

Among other functions, chemosensory systems play a crucial role in the host-seeking behaviors of insects that allow them to find their preferred food sources. Quite often, however, these host-seeking behaviors have a negative impact on either human health or livelihood. The following details investigations into the evolution and molecular mechanisms of two distinct pathways involved in insect chemosensation.

The atypical odorant receptor gene, *Or83b*, is co-expressed with other ORs in most olfactory sensory neurons (OSNs) of the *Drosophila* antenna. OR83b acts as a generic heterodimeric partner for other ORs coupling them to the ciliary trafficking machinery, which is responsible for delivering the OR complexes to their site of action, the OSN dendrites. Flies lacking *Or83b* have marked electrophysiological and behavioral olfactory defects presumably because the OR cargo of OR83b is degraded when it cannot traffic properly. The amino acid sequence of OR83b has been remarkably well conserved over the course of evolution. Homologues have been identified in insects as diverse as beetles, moths, honeybees, and locusts. Several of these are true orthologues that can rescue the *Or83b* mutant phenotype indicating that the function of OR83b has also been conserved.

At least one population of neurons in the fly antenna is *Or83b*-independent. These neurons, which respond to changes in CO_2 concentration, co-express a pair of chemosensory receptors belonging to the gustatory receptor family, *Gr21a* and *Gr63a*. Transgenic misexpression of these two receptors can confer CO_2 sensitivity on a neuron that is normally CO_2-insensitive. *Gr63a*^{1} mutant flies lack all electrophysiological and behavioral responses to CO_2. Clear homologues of these two genes are co-expressed in the mosquito organ that responds to CO_2 implying that they act as the mosquito CO_2 receptors as well.

It seems that many insects, including the malaria mosquito, use these two pathways—one *Or83b*-dependent and one *Or83b*-independent—to track host odors, which synergize with CO_2 plumes to modulate host-seeking behavior. Thus, not only is the evolutionary history of these insect chemosensory pathways incredibly interesting, both pathways make attractive targets for the rational design of novel insect control measures designed to interrupt host seeking.

나의 아내, 영선씨에게…

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Abbreviations

AL – antennal lobe

BLAST – basic local alignment search tool

cAMP – 3'-5'cyclic adenosine monophosphate

CA – carbonic anhydrase

CB – carotid body

EAG – electroantennogram

EM – electron micrograph

GPCR – G protein-coupled receptor

GR – gustatory receptor

GPROR – OR in *Anopheles gambiae*

GPRGR – GR in *Anopheles gambiae*

GCS – globin coupled sensor

GSN – gustatory sensory neuron

HemATs – heme-containing aerotaxis transducers

HIF-1 – hypoxia inducible factor – 1

HNOB – heme-NO-binding

LN – lateral inhibitory interneuron

LPO – labial palp pit organ

LPOG – labial palp pit organ glomerulus

MCP – methyl accepting chemotaxis protein

OSN – olfactory sensory neuron

OR – odorant receptor

PAS – Period, aryl hydrocarbon receptor nuclear translocator, and Single-minded

PCR – polymerase chain reaction

PN – projection neuron

sAC – soluble adenylyl cyclase

sGC – soluble guanylyl cyclase

SOG – suboesophageal ganglion

TSA – tyramide signal amplification

1 Introduction to *Drosophila* chemosensation

1.1 Insect chemosensation

1.1.1 The agricultural, economic, and medical impact of insects

Insects account for almost 75% of all animal species and make up a larger portion of the Earth's biomass than any other Class. Insects are everywhere. Over the course of evolutionary history, insects have achieved their incredible success because of the variety of adaptive strategies with which they have solved some of nature's most difficult challenges. Yet, while insects are fascinating in their own right, we also study them for more egocentric reasons.

The relationship between humans and insects is complicated; both positive and negative depending on the particular species in question. Insects pollinate many of our crops. We farm them for honey, silk, and other products. We have also learned to use certain species in the biological control of others. Unfortunately, however, these beneficial aspects are not the whole story. To date, roughly 3,000 species of insects are known to have a negative impact on humans costing over a million lives and billions of dollars per year (Hill and Biology, 1997). Several types of beetles destroy our crops, termites destroy our homes, and biting insects directly attack us and our livestock (Daly et al., 1998). Many insects can act as human and animal disease vectors. Insects are responsible for transmitting to humans the microbial agents that cause malaria, yellow fever, dengue fever, several varieties of encephalitis, filariasis, epidemic typhus, bubonic plague, sleeping sickness, and Chagas' disease, among others.

Malaria, which is transmitted by anopheline mosquitoes, is the most devastating of these diseases, afflicting between 300 and 500 million people a year (Korenromp, 2004), leading to over 1 million deaths annually. Interestingly, much of the blame for the agricultural, economic, and human health impact of insects can be placed squarely on their highly sensitive chemosensory systems.

1.1.2 The importance of chemosensation to insects

Unlike humans, who rely primarily on vision for impressions of our environment, most other animals take a more balanced approach to the reception of environmental stimuli. In fact, although many insect species have excellent visual systems, their olfactory and gustatory systems are critical in determining the location and quality of foodstuffs, in identifying potential mates, in communicating with conspecifics, and in avoiding danger. It is through these sensory systems that agricultural pests like the medfly (*Ceratitis capitata*) can locate and ruin our orange groves. By smelling our volatile emissions (i.e. our breath and body odor), black flies, sand flies, and most importantly, mosquitoes can locate people on which to feed and potentially inoculate with disease-causing microbes. What are these sensory systems and how do they work?

1.2 What is chemosensation?

Chemosensation is the reception of chemical signals from the environment and their transformation into trains of neuronal action potentials that can be ‘read’ by the brain as a perception of odor or taste. Unlike vision or

audition, which transform continuous spectral stimuli into sensory perception, chemosensory systems have no recognizable continuous spectrum with which to work. Chemicals come in many shapes and sizes, with different functional groups, carbon chain lengths, and concentrations, and they may be present alone or in blends. Chemical stimuli have different volatilities that help us classify them as either odorants (volatile) or tastants (non-volatile). Since, however, these stimuli, especially in olfaction, are so complex, the way the brain decodes them into a particular perception is still largely a mystery. Only recently have we begun to understand olfaction and gustation. A model genetic organism, the fruit fly *Drosophila melanogaster*, has been an absolute boon to scientists seeking to understand how these systems work.

1.3 Olfaction in *Drosophila*

1.3.1 Peripheral olfactory anatomy

The main insect olfactory organ is the antenna. In many insects, another head appendage, the maxillary palp, also participates in olfactory perception (**Fig. 1.1A**). These organs are covered with hollow, porous cuticular projections, termed sensilla, which house the olfactory sensory neurons (OSNs). The branched dendrites of one to four OSNs project into the lumen of each sensillum. There they are bathed in a fluid called sensory lymph that is produced by support cells at the base of the sensillum (**Fig. 1.1B**). Olfactory sensilla in *Drosophila* come in three types, named according to their morphology: sensilla basiconica (thick and club-shaped), sensilla trichodea (long and slender), and sensilla

coeloconica (short and conical) (Stocker, 1994). These classes have been further sub-divided on the basis of high resolution morphological analysis (Shanbhag et al., 1999) and according to their odor-response profiles (de Bruyne et al., 1999; de Bruyne et al., 2001).

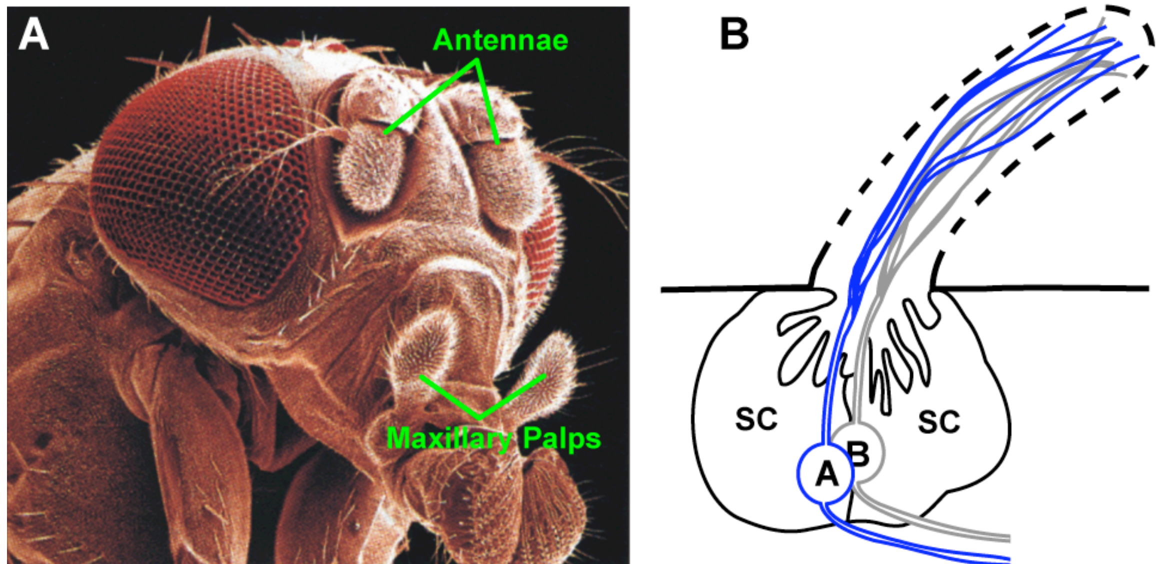

Figure 1.1: Olfactory organs of *Drosophila* and a model sensillum

A) A scanning electron micrograph of the head of *Drosophila melanogaster*. The olfactory organs are indicated with green lines. These organs are covered with small cuticular projections known as sensilla. (Photo credit: Jürgen Berger, Max Planck Institute, Tübingen, Germany). **B)** A schematic of a basiconic olfactory sensillum, which houses two OSNs (A and B) with branched dendrites. Support cells are labeled SC. Adapted from de Bruyne et al. (1999).

1.3.2 Neuroanatomical organization

Groups of OSNs that respond to the same odorant ligands bundle their axons into the antennal nerve, which projects back into the brain to a structure called the antennal lobe (AL). The *Drosophila* AL is made up of at least 45 distinguishable structural and functional units called glomeruli. It is in these glomeruli that OSN axons form synapses with the second-order olfactory

neurons, the projection neurons (PNs). PNs relay the odor-induced message to two higher brain centers. The first is the mushroom body, which is involved in odor memory. The second is the lateral horn of the protocerebrum, which is involved in odor detection and processing. Both are essential for the ultimate behavioral output (**Fig. 1.2**). Another population of cells, a network of lateral inhibitory interneurons (LNs) diffusely innervating the AL may be important in processing or refining the glomerular activation pattern that is 'read' by the PN dendrites (Ng et al., 2002).

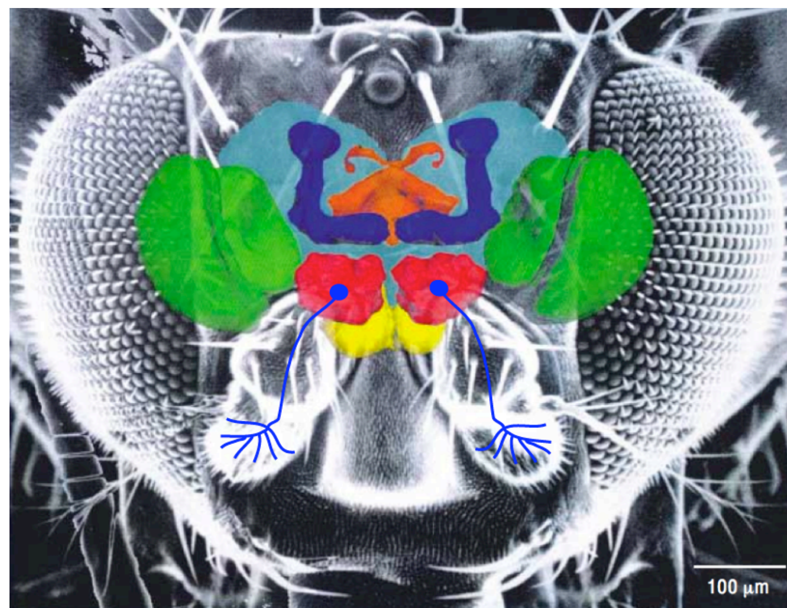

Figure 1.2: Schematic innervation of a single AL glomerulus by axons of OSNs with equivalent response profiles

A model fly brain is superimposed on a scanning EM of a fly head. Blue OSN axons coming from the 3rd segment of the antenna bundle into the antennal nerve, project back to the AL (red), and innervate a single olfactory glomerulus. Optic lobes (green), mushroom bodies (dark blue), the suboesophageal ganglion (yellow), and the central complex (orange) also appear. Adapted from (Heisenberg, 2003).

However, before any of this central brain processing occurs, the information about odor identity and intensity has already undergone a dramatic transformation in the OSNs themselves. For any perception of odor in any insect, odor molecules must pass through a sensillum's cuticular pores, dissolve in its sensillar lymph, and then diffuse to the OSN dendrites. But what happens when the odorant molecules reach the OSN dendrites? Something has to transform a complicated chemical signal into an electrical OSN activation pattern. This is the job of the odorant receptors (ORs).

1.3.3 Odorant receptors

The biggest breakthrough in our understanding of the sense of smell in all organisms came with the discovery of the rat odorant receptors by Linda Buck and Richard Axel (Buck and Axel, 1991). Their work, which was recognized with a Nobel Prize in Physiology or Medicine in 2004, shed light on the inner workings of the OSN black box. The thousands of odorants mammals are capable of smelling are recognized by their odorant receptors, which form a large sub-family of the G protein-coupled receptor (GPCR) group of seven-pass transmembrane proteins. This family, representing 1% of all mammalian genes, is the largest in mammalian genomes (Mombaerts, 1999). Its members are highly divergent, presumably indicating the vast structural diversity of the ligands they recognize. However, they retain the conserved hallmarks of the GPCR super-family, notably the DRY residue located at the intracellular face just after the third transmembrane domain (Liu et al., 2003; Sakmar, 2002). The ORs bind odorant

molecules dissolved in the mucous covering the OSN dendrites in the mammalian olfactory epithelium. Ligand-binding increases cAMP levels through a G protein-dependent mechanism and leads to neuronal activation (Ronnett and Moon, 2002).

Interestingly, that which applies in rodents and people is not necessarily true for insects. The insect ORs were discovered in 1999 in *Drosophila* (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). Despite appearing at first-glance similar to mammalian ORs in having seven transmembrane domains and discrete expression in OSNs, the insect ORs form a distinct family of receptors with inverted membrane topology (i.e. an intracellular N-terminus) that are unlikely to be GPCRs (Benton et al., 2006; Wistrand et al., 2006). *Drosophila melanogaster* has 62 ORs, most of which are expressed in distinct and stereotyped sub-populations of OSNs in both the antennae and maxillary palps. Generally, each subtype of OSN expresses a single OR, although exceptions to this rule have been described (Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldman et al., 2005). OSNs expressing the same ORs, and thus having the same odor response profiles, together project toward and innervate single stereotyped AL glomeruli (**Fig 1.2**). The ensemble of these glomeruli, all innervated by OSNs expressing different receptors, forms an odortopic map that faithfully represents a given chemical stimulus to higher brain centers through the PNs.

1.4 Gustation in *Drosophila*

As the ‘other’ chemosensory system in flies, gustation has some parallels with the olfactory system, but many striking differences. Soluble tastant molecules are detected by gustatory sensory neurons (GSNs) found in several types of sensilla on the fly labial palps, legs, wing-margins, female genitalia, and inside the pharynx (Stocker, 1994). The relatively wide distribution of taste sensilla in flies necessarily means that gustatory neuroanatomy is less straightforward than in the olfactory system. Whereas OSNs project to single glomeruli in the antennal lobe before their message reaches the central brain, GSNs project to one of a few different relay stations (i.e. the tritocerebrum, and the suboesophageal (SOG) and thoracic-abdominal ganglia) depending on their location in the body (Stocker, 1994; Wang et al., 2004). The SOG, which acts as the relay center for GSNs in the labial palps, does not have discrete neuropil subdivisions like the AL glomeruli. There are data to suggest, however, that GSNs responsible for sensing different taste modalities (i.e. sweet versus bitter) project to non-overlapping portions of the SOG that are not otherwise distinguishable (Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004).

The distinct response properties of these cells, as is the case in olfaction, are likely to result from their expression of different gustatory receptor genes. The 68 *Drosophila* gustatory receptor (GR) genes, which are scattered all over the genome, form a diverse family whose members show as little as 8-12% amino acid-level sequence identity (Amrein and Thorne, 2005; Robertson et al.,

2003; Scott et al., 2001). The degree of diversity in this family points to its ancient origin, and in support of this idea, a few genes in the nematode *C. elegans* genome appear to be distantly related to *Drosophila* GRs (Robertson, 2001). The expression pattern of GRs has been best studied in the labial palps, and there, unlike in the olfactory system, many GRs are often co-expressed in the same GSN population (Thorne et al., 2004; Wang et al., 2004).

1.5 Evolution of the OR/GR superfamily and its role in host-seeking

Both *Drosophila* ORs and GRs share a limited degree of similarity, leading to the hypothesis that these two gene families may, in fact, represent subdivisions of a larger superfamily of insect chemosensory receptor genes (Robertson et al., 2003). Understanding the origin of this superfamily and when its functional split into olfactory and gustatory halves occurred will require tracing its development through the sequencing of many more insect genomes, especially those of primitive insects at the base of the hexapod lineage. Since insects were some of the earliest animals to inhabit the land over 400 million years ago (Grimaldi and Engel, 2005), it is possible that the subdivision of their chemosensory systems may have occurred with the transition from aquatic life to terrestrial life and the corresponding switch from sensing soluble ligands to airborne volatiles (Robertson et al., 2003).

It is also tempting to speculate that the further evolution of insect chemosensory systems can actually have a causative role in altering both mate

and host selection preferences, which in turn could spur the evolution of new species. The hedonic value an insect assigns to a given chemical stimulus likely has little to do with the repertoire of chemosensory genes from this superfamily possessed by the insect. This repertoire would, however, determine the spectrum of chemicals that an insect can detect, and to which this hedonic value can be assigned through processing in higher brain centers. Thus, variable repertoires of chemosensory genes (mainly ORs for long range chemical cues) provide the evolutionary raw materials for changes in host selectivity, which in turn may drive speciation itself.

Evidence in support of this hypothesis can be found in a comparison of the OR repertoires of *Drosophila melanogaster*, a strict fruit feeder, and *Anopheles gambiae*, which feeds on either nectar or blood depending on sex and life stage. Although some direct homologues of *Drosophila* ORs exist in *Anopheles*, there are also several species-specific groups. This has led to the compelling hypothesis that these non-homologous groups of ORs may be responsible for detecting volatiles associated with species-specific host preferences (Hill et al., 2002). Electrophysiological studies on mosquito olfactory organs reveal them to be remarkably sensitive to many human-derived odors (Meijerink et al., 2001; Meijerink and van Loon, 1999; Qiu et al., 2006; van den Broek and den Otter, 1999). One particular OR specific to *Anopheles gambiae*, *GPRor1*, is expressed in blood-feeding adult female mosquitoes and not in nectar-feeding adult males (Fox et al., 2001). In addition, when *GPRor1* is expressed in an “empty” *Drospophila* OSN lacking its endogenous ORs it confers sensitivity to 4-

methylphenol, an odorant found in human sweat that may be important in mosquito host-seeking (Hallem et al., 2004b).

Additional evidence supporting the role of chemosensory receptors and host preference in evolution can be found in the recent (<150 years ago) switch in host preference of a population of apple-maggot flies (*Rhagoletis pomonella)* from hawthorn fruit to apples. It is clear that their choice between food sources is made on the basis of fruit olfactory cues (Linn et al., 2003), and that F1 hybrids between the two parental populations have reduced electrophysiological and behavioral responses to these host volatiles (Linn et al., 2004; Olsson et al., 2006). This likely represents a significant barrier to productive mating in that offspring of the two groups are less able to find appropriate food resources. Thus it seems that by observing these simple changes in the olfactory system, which have affected host preference, this population of flies may have been caught in the act of sympatric speciation (i.e. speciation without geographic isolation). It is difficult to determine whether the olfactory changes themselves are the cause of or a result of speciation, but it is clear that the two are closely linked.

The remainder of this dissertation will focus on two distinct investigations into members of this chemosensory receptor gene family that seem to play separate but complementary roles in insect host-seeking behavior. First, I will discuss the results of studies on the both the function and evolution of *Or83b,* an atypical member of the OR subfamily that has a pivotal role in olfactory perception and host-seeking behaviors in flies, and most likely in all insects. This will be followed by a discussion of carbon dioxide chemosensation in insects, the

various roles it plays in species-specific host preferences, and the molecular mechanisms of its detection.

2 The function and evolution of *Or83b*

2.1 Introduction

2.1.1 Initial discovery

In the early days of experiments on the molecular nature of insect olfaction, *Drosophila* was used as a model organism to gain insight into the far more complicated mammalian olfactory system with its hundreds of receptors and thousands of glomeruli. Even after the initial discovery of the insect ORs, because of their apparently similar seven-pass transmembrane structure and the fact that they seemed to follow the reasonably well-accepted one-neuron one-receptor rule found in the mammalian system (Mombaerts, 2004; Serizawa et al., 2003), this model organism relationship was seldom questioned. One curious finding, however, in the initial descriptions of the insect OR gene family cast some doubt on the system's similarity to the mammalian olfactory system. One member of the family, initially known as *A45* (Vosshall et al., 1999) or *AN5* (Gao and Chess, 1999), but now known as *Or83b,* was found to have an expression pattern unlike any other. While most ORs are expressed in small (~20 cells) non-overlapping groups of OSNs, *Or83b* is expressed broadly in all OSNs of the maxillary palps and almost all OSNs of the antennae (**Fig. 2.1)**.

Figure 2.1: *Or83b* is expressed in most OSNs of the fly antenna

Alkaline phosphatase visualization of RNA *in situ* hybridization on antennal sections reveals expression of *Or83b* in many OSNs.

2.1.2 *Or83b* mutant reveals function

The unique function of this highly conserved atypical OR became clear only when a mutant fly lacking *Or83b* was generated. *Or83b* mutants have severe deficits in both electrophysiological and behavioral assays of olfactory function. Electroantennograms (EAGs), which give a gross readout of local field potentials in the antenna, reveal little if any electrical activity in *Or83b* mutant flies. *Or83b* mutant larvae are unable to chemotax toward an odor source, and adults show impaired odor-guided attraction (Larsson et al., 2004).

The broad antennal expression pattern of *Or83b* led to the hypothesis that it may act as some sort of universal co-factor for the typical ORs with which it is co-expressed. Consistent with this hypothesis, the dendritic trafficking of both OR22a (**Fig 2.2**) and OR43a is completely abolished in flies lacking *Or83b* (Larsson et al., 2004).

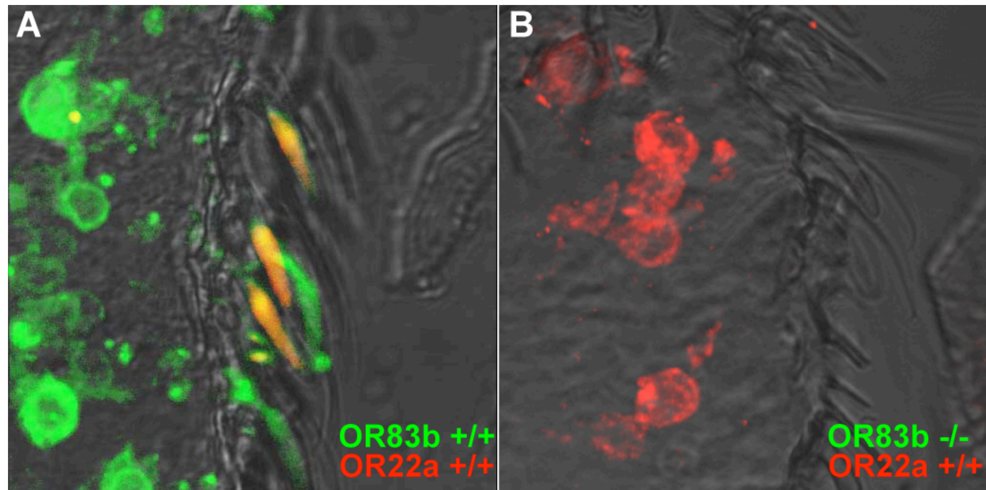

Figure 2.2: OR83b is required for dendritic trafficking of OR22a

Immunostaining of antennal sections using antibodies against OR83b (green) and OR22a (red). **A)** Wild type antennae have normal dendritic trafficking of the OR22a antigen, with very little staining in the OSN cell bodies. Only a small number of OR83b-positive cells also stain for OR22a. **B)** Staining of *Or83b* mutant antennae reveals a complete lack of OR83b antigen, and a mislocalization of OR22a antigen in the OSN cell bodies instead of the dendrites.

Instead of being trafficked to the OSN dendrites where they can encounter odorant molecules, in the absence of OR83b the OR proteins remain stuck in the endomembrane system of the OSN cell body and are degraded. It is now clear that OR83b physically associates with other ORs (Benton et al., 2006; Neuhaus et al., 2005) and couples them to the ciliary trafficking machinery of the OSNs so that they can be transported to their site of action (Benton et al., 2006). OR83b does not seem to recognize ligands by itself. OSNs lacking a given odorant receptor but retaining OR83b fail to show odor responses (Dobritsa et al., 2003; Elmore et al., 2003). However, OR83b appears to perform a generic function necessary for all odorant receptors. If conventional ORs are transgenically expressed in mutant sensilla lacking their native complement of ORs, but containing *Or83b*, these genetic reconstitutions faithfully recapitulate odor

responses of the sensilla where the transferred ORs are normally expressed (Hallem and Carlson, 2006; Hallem et al., 2004a). This means OR83b performs a critical, but generic function in each of the sensilla in which it is expressed.

2.1.3 Evolutionary implications

Reinforcing this unique position of *Or83b* in the chemosensory superfamily, when the primary protein structure of the *Drosophila* ORs and GRs are aligned in order to generate a phylogenetic tree, OR83b is a clear outlier between the two subfamilies as the OR closest in sequence to the GRs (Robertson et al., 2003). Thus, in the insect lineage, *Or83b* may be the link between the OR subfamily and the more ancient GRs. In addition, although most other OR and GR family members are highly divergent in both intra- and inter-species comparisons, OR83b is well-conserved in all insect orders tested so far (Jones et al., 2005; Krieger et al., 2004; Krieger et al., 2003; Melo et al., 2004; Pitts et al., 2004).

Despite its conservation in insects, there are no obvious homologues of Or83b in any mammalian genome. This reinforces the idea that the organizational similarities shared by the insect and mammalian olfactory systems may stem from their relationship as analogous structures formed through convergent evolution and not because they are directly related. Regardless of the implications for *Drosophila* as a model system for studying the mammalian olfactory system, the dramatic evolutionary conservation of *Or83b* in insects reinforces the role of *Drosophila* as an important model organism for olfactory research in disease vector insects. Since the olfactory systems of these insects,

including the malaria mosquito *Anopheles gambiae*, are of more immediate relevance to global human health than even our own olfactory systems, the function and evolution of *Or83b* becomes an important object of research.

The investigation of the functional importance of *Or83b* in the olfactory systems of insects that negatively affect both human health and agriculture is difficult without the sophisticated molecular genetic tools that have been developed in *Drosophila melanogaster*. Therefore, in order to investigate the function of *Or83b* in other species we used *Drosophila* as an expression system to determine whether or not distantly related *Or83b* homologues from a few pest insect species could functionally rescue the fly *Or83b* mutant phenotype.

2.2 Materials and methods

2.2.1 *Drosophila* stocks

Fly stocks were maintained on conventional cornmeal-agar-molasses medium at 25ºC. Constructs for the transgenic stocks were injected into either *yw* or *w1118* embryos. Single transformants were then isolated and balanced according to standard fly genetic methods. The following flies were used in this study: wild type Berlin (M. Heisenberg); *Or83b-GAL4*; *Or22a-GAL4*; $Or83b^2/Or83b^3$; *UAS-Or83b*; *UAS-CcOr83b*; *UAS-GPRor7*; *UAS-HzOr83b*.

2.2.2 Cloning of *Or83b* Orthologues

The following full-length cDNA sequences have been deposited in Genbank: *Anopheles gambiae*, AY843205; *Ceratitis capitata*, AY843206; *Helicoverpa zea*, AY843204.

2.2.2.1 Cloning of *Anopheles gambiae GPRor7* cDNA

The *Drosophila melanogaster Or83b* cDNA sequence was used with the BLAST to search a collection of random *Anopheles gambiae* genomic DNA sequences, which were compiled by Genoscope and the Institut Pasteur, France. Two sequence fragments with significant similarity to the query sequence were identified. These *A. gambiae* sequences were used to design oligonucleotide primers to amplify a portion of the *A. gambiae AgOr83b* gene (since renamed GPRor7) via PCR using mosquito genomic template DNA. The resulting 3kb PCR product was used to screen an *A. gambiae* genomic DNA library.

Several genomic clones were isolated and sequenced. The intron/exon structure of *GPRor7* was predicted using GENSCAN. Oligonucleotide primers designed to amplify the predicted *GPRor7* open reading frame were synthesized and used in RT-PCR of *A. gambiae* adult head mRNA. PCR products were purified, cloned into pGEM-T Easy and sequenced. One clone was chosen and subcloned into pUAST (Brand and Perrimon, 1993) for fly injection.

2.2.2.2 Cloning of *Ceratitis capitata CcOr83b* cDNA

The *D. melanogaster* Or83b cDNA was used to screen a *Ceratitis capitata* genomic DNA library at low stringency as described above in section 2.2.2.1. Several clones were isolated and sequenced. The intron/exon structure of the *C. capitata CcOr83b* gene was predicted using GENSCAN. Oligonucleotide primers flanking the predicted *C. capitata* Or83b ORF were synthesized and used to amplify *CcOr83b* cDNA by RT-PCR from *C. capitata* adult antennal mRNA. PCR

products were purified, cloned into pGEM-T Easy and sequenced. One clone was chosen and subcloned into pUAST (Brand and Perrimon, 1993) for fly injection.

2.2.2.3 Cloning of *Helicoverpa zea HzOr83b* cDNA

An *H. zea* antennal cDNA library constructed in lambdaZAP was screened with *D. melanogaster Or83b* cDNA at low stringency by prehybridizing at 42ºC in 5X SSCP (10X Denhardt's, 25% formamide, 0.1% SDS, 0.25 mg/ml salmon sperm DNA). Filters were hybridized with a ^{32}P-labelled probe in the same buffer overnight at 42ºC. Filters were washed at low stringency (2x30 minutes in 2X SSC/0.1% SDS at 25ºC; 2x30 minutes in 0.5X SSC/0.1% SDS at 42ºC). Plasmids containing positively hybridizing inserts were obtained by *in vivo* excision and sequenced. One clone was chosen and subcloned into pUAST (Brand and Perrimon, 1993) for fly injection.

2.2.3 Multi-protein alignment and phylogenetic tree construction

Protein sequences for *Or83b* orthologues were predicted from cDNA sequences and aligned using the default settings of the ClustalW algorithm supplied with the sequence analysis package MacVector. A best-fit phylogenetic tree was constructed using the neighbor-joining method also supplied in MacVector.

2.2.4 RNA *in situ* hybridization

Antennae of each insect were embedded in OCT freezing medium and sectioned on a cryostat. RNA *in situ* hybridization using DIG-labeled antisense riboprobes was carried out as described except that detergents were omitted

(Vosshall et al., 1999). In addition, all sample manipulations after sectioning were carried out horizontally without cover slips to avoid losing the tissue.

2.2.5 Antennal immunostaining

Visualization of OR22a dendritic trafficking was carried out with several modifications to a previously described protocol using a rabbit anti-OR22a antibody (J. Carlson) (Dobritsa et al., 2003). Briefly, 10 μm fresh frozen sections of transgenic *Drosophila* antennae were collected on SuperFrost slides (Fisher) and fixed in 4% paraformaldehyde in PBS for 7 minutes. Slides were washed 2x5 minutes in PBS, and permeabilized in P/T (PBS, 0.1% TritonX-100) for 30 minutes. Slides were then blocked horizontally for 30 minutes with 500 μl P/T/S (P/T + 5% heat inactivated normal goat serum). The primary antibody was diluted 1:100 in P/T/S, and 500 μl was added to the slides, which were then incubated overnight at 4ºC. The next day, slides were washed 3x10 minutes in P/T, blocked again for 30 minutes, and incubated with a goat anti-rabbit-Cy3 conjugated secondary antibody (1:800 in P/T/S) for 2 hours in the dark. After three more 5 minute washes the slides were mounted with Vectashield (Vector Labs) and visualized on a Zeiss LSM510 confocal microscope.

2.2.6 Electroantennograms

EAGs were performed with several modifications to a previously described protocol (Ayer and Carlson, 1992). Using a fly aspirator two to four day old flies (equal numbers of males and females) were inserted into the end of a plastic pipet tip cut at an angle of roughly 25 degrees from horizontal. The fly was

pushed forward from behind with cotton such that part of the thorax and the anterior head were protruding, while the mouthparts remain inside the pipet tip. The other end of the pipet tip was secured using modeling clay and the fly was visualized in a dissection microscope.

Glass needle electrodes were pulled from borosilicate glass capillary tubes (1.5mm OD, 0.75mm ID from F.H.C.) using a PB-7 micropipette puller (Narishige) and filled with 0.1 M KCl and 0.5% polyvinyl propylene. These were placed in electrode holders and attached to a high impedance guarded input AC/DC probe (Syntech) attached to a manual micromanipulator. The reference electrode penetrated the upper thorax and entered the hemolymph. The recording electrode was pushed against the dorsomedial portion of one antenna and adjusted to obtain satisfactory electrical contact. Odor-evoked voltage changes were recorded using an IDAC-USB attached to a PC loaded with the EAGPro software (Syntech) and analyzed offline.

Odorants of the highest grade available (Sigma-Aldrich) were diluted 1:100 in paraffin oil (Fluka) and 30µl was added to strips of filter paper. These were then loaded into 1ml glass tuberculin syringes (Becton Dickinson) fitted with 1/16” tubing. One-second odor pulses, under the control of the CS-05 stimulus controller (Syntech), were added to a constant air stream directed at the antenna.

To verify consistent contact with the antenna, all *Or83b* mutant antennae were tested with carbon dioxide before and after application of other odorants. CO_2-responsive OSNs are *Or83b*-independent (Larsson et al., 2004) and produce robust potentials.

2.3 Results

2.3.1 Conservation of sequence and gene expression pattern

Since *Or83b* has the distinction among members of the OR family of having clear orthologues in other species (i.e. several moths, a beetle, the honeybee, several flies, and two mosquitoes), we asked whether this remarkable sequence conservation also reflects an essential function that has been conserved through insect evolution. To this end, we cloned *Or83b* orthologues from three major insect pests: the medfly, a citrus pest; the corn earworm moth, which damages corn and tobacco; and the malaria mosquito. The medfly and the mosquito, along with the fruit fly, all belong to the order Diptera. The corn earworm moth, from the order Lepidoptera, is the most evolutionarily divergent from *Drosophila*. Orthologous cDNAs were obtained either by library screening or RT-PCR. RNA *in situ* hybridization using antisense riboprobes specific to each orthologous gene reveals widespread OSN-specific expression in antennal sections from each respective insect (**Fig. 2.3**).

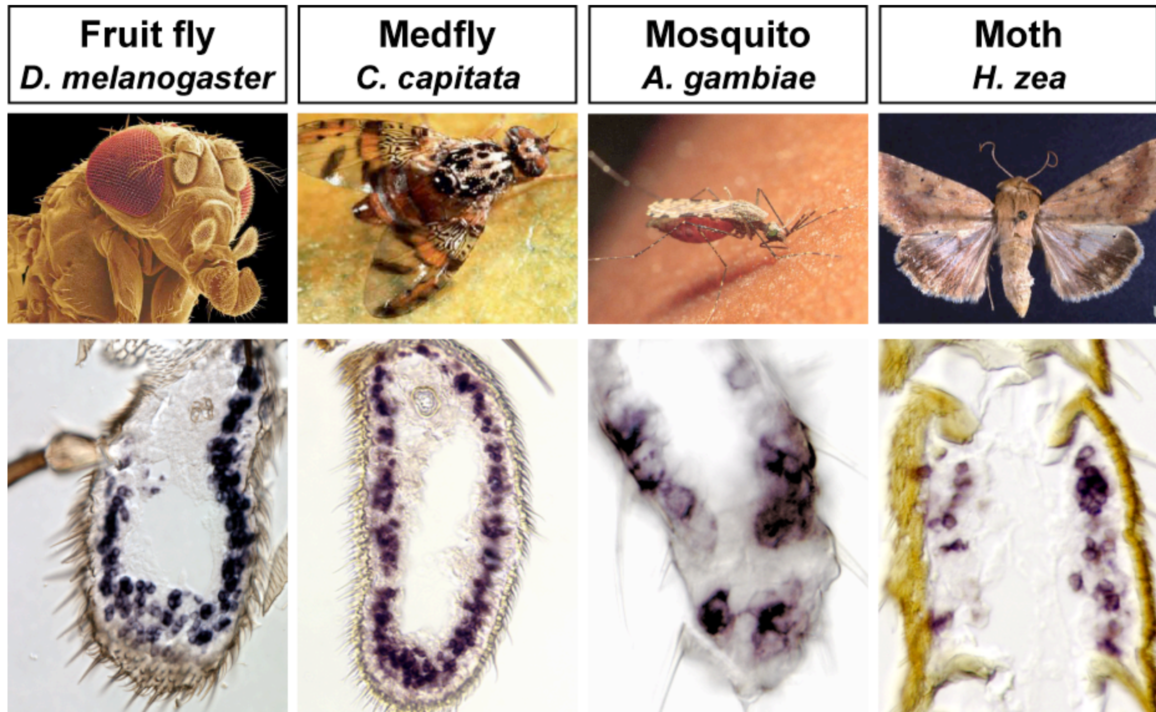

Figure 2.3: *Or83b* orthologues have a conserved gene expression pattern

Broad expression of *Or83b* orthologues in large populations of antennal OSNs from four divergent species as revealed by RNA *in situ* hybridization. Insect photo credits: fruit fly (Jürgen Berger, Max Planck Institute, Tübingen, Germany); medfly (USDA); mosquito (Ekisei Sonoda); moth (John L. Capinera, University of Florida).

A multi-protein alignment constructed with the predicted protein coding sequence from each gene reveals 65-87% amino acid identity in whole-sequence pair-wise comparisons to *Drosophila* OR83b. Certain portions, near the C-terminus in particular, are noticeably more conserved than others (**Fig. 2.4**).

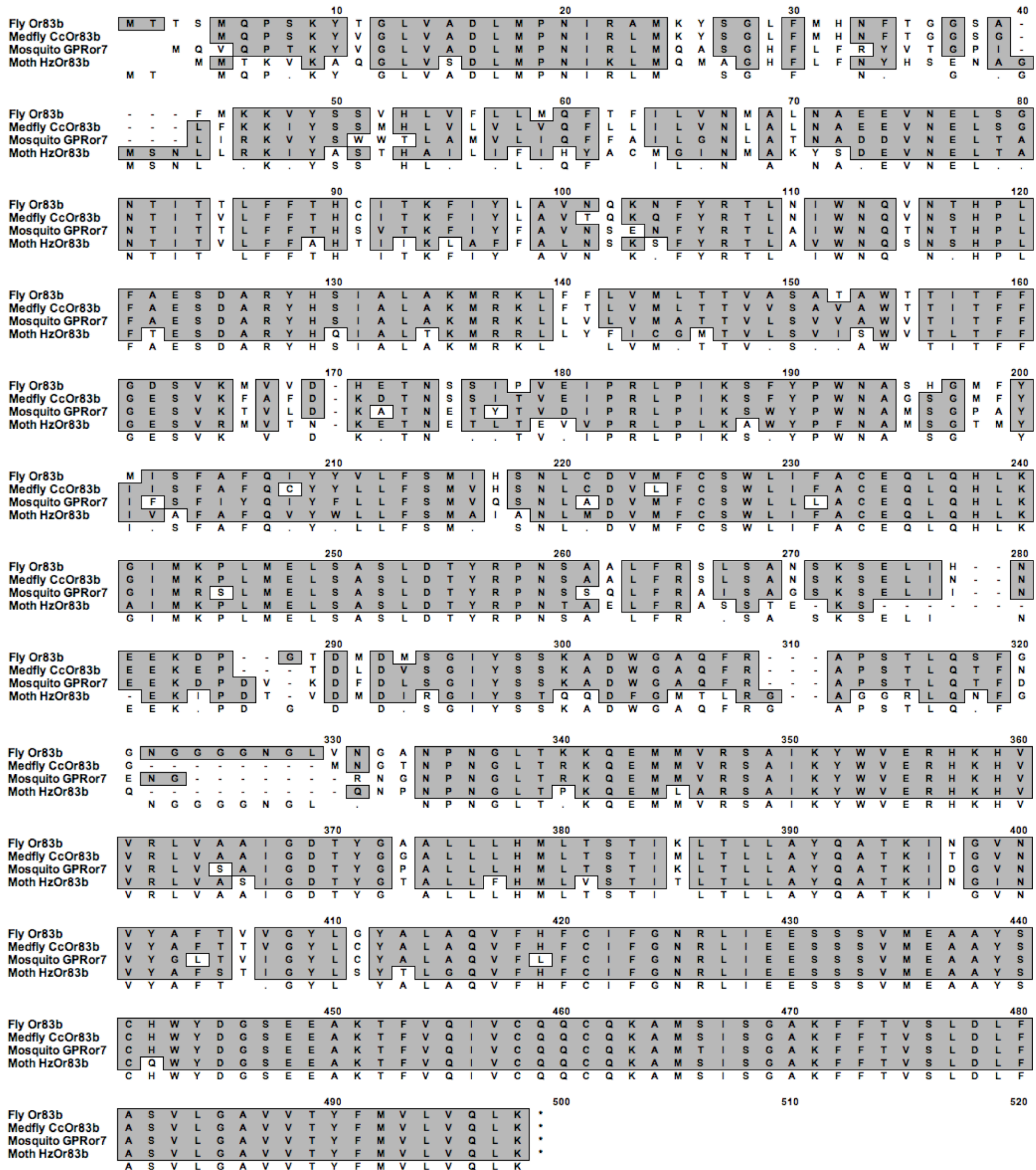

Figure 2.4: Multi-protein alignment of *Or83b* orthologues

Proteins predicted from cDNA sequence were aligned using the ClustalW algorithm (MacVector). Identical amino acids are indicated with gray shading.

A phylogenetic tree constructed using this protein alignment is consistent with the inferred phylogeny of these insects based on morphological characteristics and fossil evidence (**Fig. 2.5**).

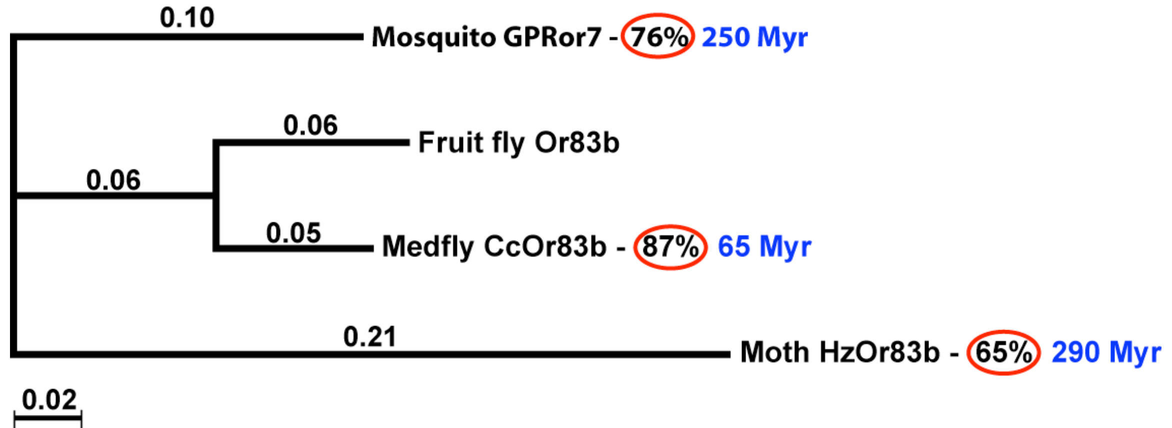

Figure 2.5: *Or83b* phylogenetic tree

A best-fit *Or83b* phylogenetic tree. Values above the branches are uncorrected ('p') distance, with the scale indicated at the lower left. Percent amino acid identities of *Or83b* orthologues as compared to *Drosophila Or83b* are circled in red. The phylogenetic distance of each species to *Drosophila* (in millions of years, Myr) is indicated in blue.

Since both *Drosophila* and *Ceratitis* are brachyceric Dipterans they are the most closely related, and probably diverged around 65 million years ago (Grimaldi and Engel, 2005). The mosquito is also a member of Diptera, but is much more distantly related having diverged from the common ancestor it shares with *Drosophila* roughly 250 million years ago (Kulathinal et al., 2004; Yandell et al., 2006). Dating the last common ancestor of *Drosophila* and *Helicoverpa* (Lepidoptera) is considerably more difficult. The oldest Lepidopteran fossil is 190 million years old, but the divergence of the group Panorpida (to which both Lepidoptera and Diptera belong) probably occurred around 290 million years ago (Grimaldi and Engel, 2005).

2.3.2 Conservation of function

We next examined whether these *Or83b* orthologues can functionally complement the physiological defects found in *Or83b*$^{-/-}$ flies, namely the mislocalization of typical ORs and the defect in odor-evoked activity in the antenna (Larsson et al., 2004). Using the binary GAL4/UAS gene expression system (Brand and Perrimon, 1993), *Drosophila Or83b* and its orthologous cDNAs were expressed in *Or83b*$^{-/-}$ OSNs under the control of the *Or83b* promoter. This transgenic expression rescues both the block in OR22a/b trafficking to the sensory dendrites (**Fig. 2.6**) and the odor-evoked electrophysiological defects (**Fig. 2.7**) of the *Or83b* mutant.

Multiple fly stocks representing several independent insertions of each transgenic rescue construct were tested giving variable degrees of rescue. In the immunostaining experiments, both the medfly and the mosquito still had OR22a/b antigen in the OSN cell bodies, but the fruit fly and moth rescues were indistinguishable from wild type. In the EAG experiments, the mosquito *UAS-GPRor7* rescue construct gave consistently lower amplitude responses than the other rescuing transgenes and lower responses than wild type to all four odorants tested. Transgenic position effects, sequence divergence, or both may cause this variability in the degree of rescue. We favor the position effect hypothesis, because the *Or83b*$^{-/-}$ rescue experiment expressing the most divergent *Or83b* orthologue, moth *HzOr83b*, is indistinguishable from wild type.

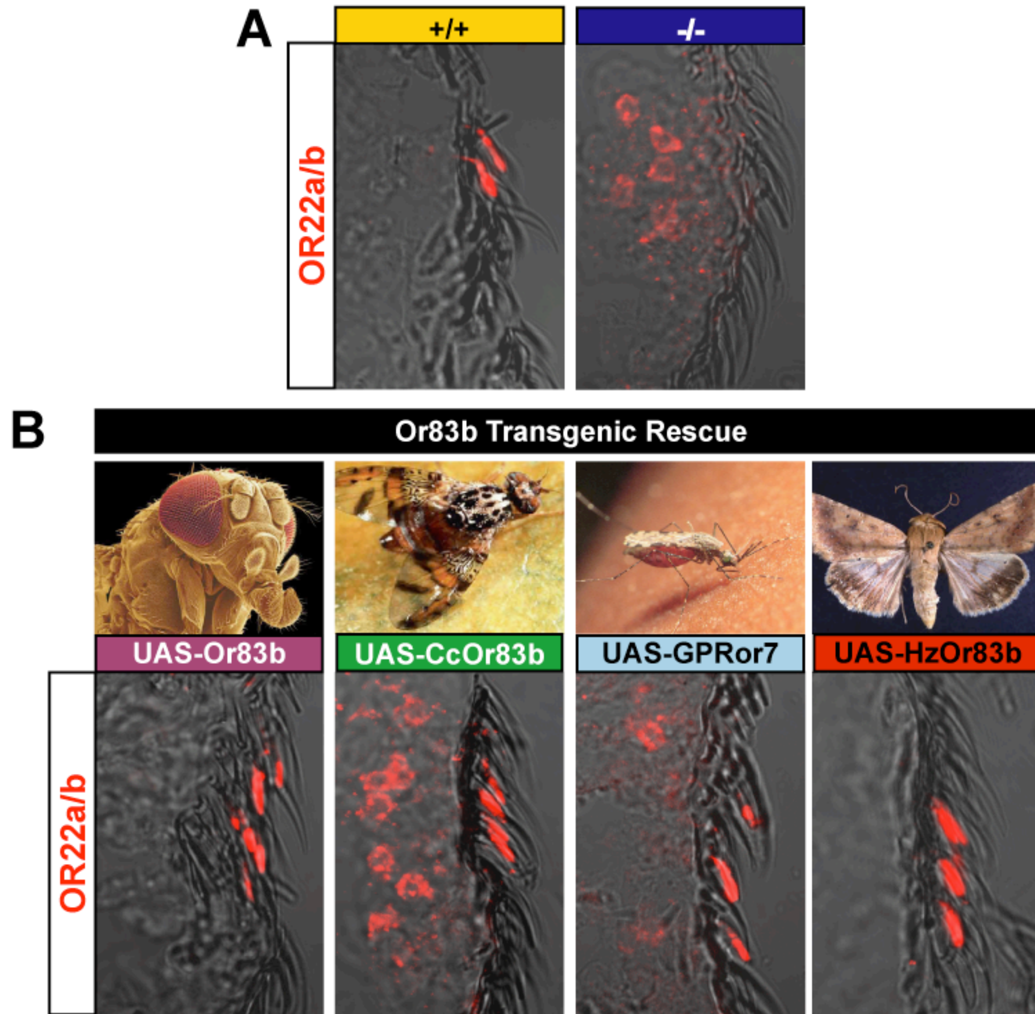

Figure 2.6: Transgenic rescue using *Or83b* orthologues restores OR22a dendritic localization in *Or83b*$^{-/-}$ flies

A) Normal localization of OR22a/b to the OSN dendrites in +/+ (wild type Berlin) flies is blocked in *Or83b*$^{-/-}$ (*Or83b-GAL4; Or83b*^{2}*/Or83b*^{3}) flies. The only OR22a/b antigen is not degraded is located in the OSN cell bodies. **B)** Transgenic rescue with *Drosophila Or83b* and with the medfly (*UAS-CcOr83b*), mosquito (*UAS-GPRor7*), and moth (*UAS-HzOr83b*) *Or83b* orthologues restores OR22a/b localization in the OSN dendrites. Genotypes: *Or83b-GAL4/UAS-species; Or83b*^{2}*/Or83b*^{3}).

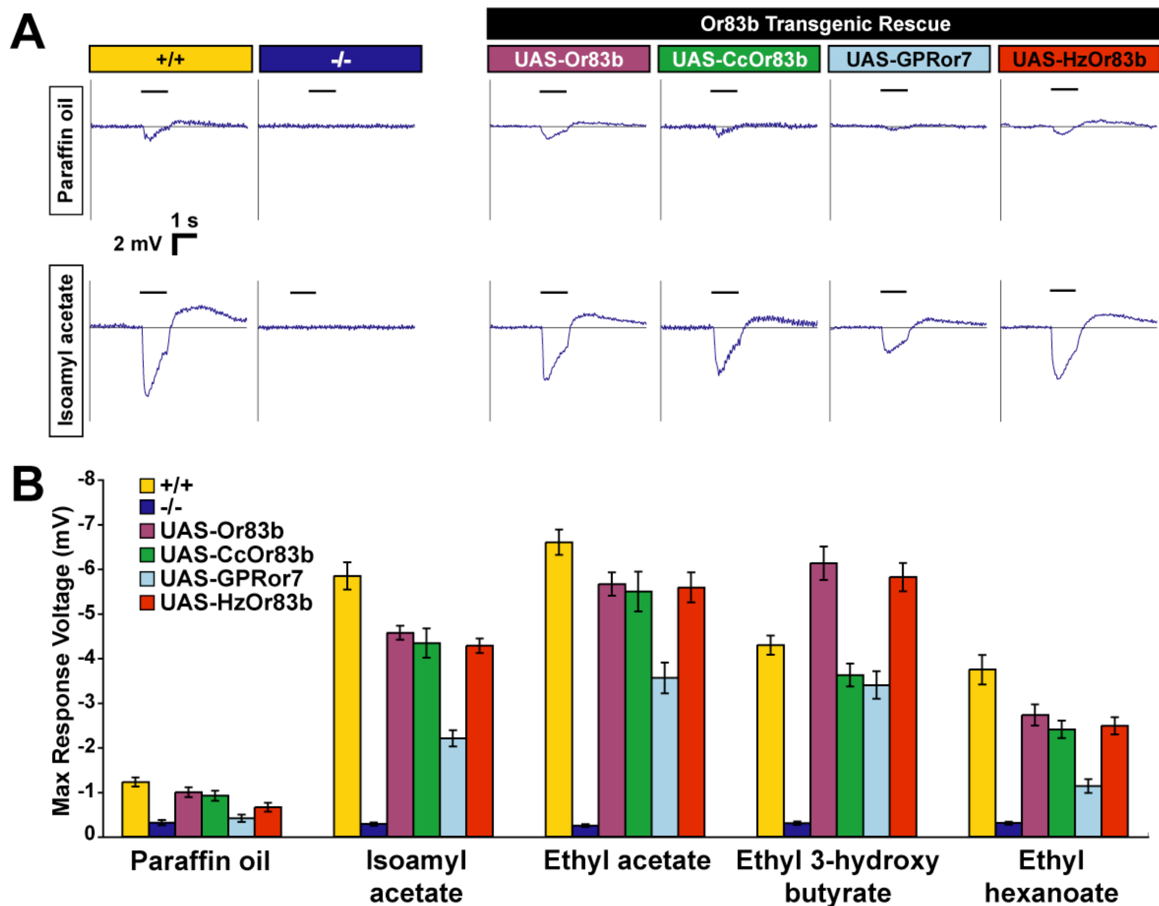

Figure 2.7: Transgenic rescue using *Or83b* orthologues restores odor-evoked antennal potentials in *Or83b*$^{-/-}$ flies

A) Electroantennograms (EAGs) measure robust odor-evoked activity in wild type antennae that is abolished in *Or83b* mutants (Left two panels). *Or83b* and its orthologues from medfly (*CcOr83b*), mosquito (*GPRor7*), and moth (*HzOr83b*) rescue this odor-evoked activity (right). Representative plots for isoamyl acetate and the solvent control (paraffin oil) are shown. Genotypes as in Fig 2.6. **B)** Summary of EAG data plotted as mean ± SEM of the peak response voltage, n = 9 or 10 antennae per genotype per odorant. Responses of each transgenic rescue to the four odorants tested were significantly different from *Or83b*$^{-/-}$ mutant responses to the same odorants in pair-wise comparisons ($p<0.001$; two-tailed t test).

2.4 Conclusion

These data reveal that an atypical OR gene has been functionally conserved across almost 300 million years of evolution since the evolutionary divergence of the Lepidopteran and Dipteran lineages. The strong selective pressure required to maintain this level of conservation must reflect the critical importance of this gene in insect olfaction. As we now know, OR83b is required for proper localization of insect ORs to their site of activity, the ciliated OSN dendrites (Benton et al., 2006). OR83b likely does not directly participate in receptor-ligand interactions in the dendrites, but its heterodimerization with other insect ORs is required for coupling to the ciliary trafficking machinery. The conserved nature of its function across insect olfaction and its absence in the mammalian system suggests that OR83b may represent the insects' "Achilles heel." It may soon be possible to express insect ORs reliably in an *in vitro* expression system. This would permit high-throughput screening of chemical compound libraries for small molecule inhibitors of insect OR protein complexes. Novel insect control strategies that specifically target OR83b could very well succeed in interrupting the diverse olfactory host-seeking behaviors that allow insects to act so efficiently as disease vectors.

3 Carbon dioxide chemosensation

3.1 Introduction to gas sensation

3.1.1 The role of hemoproteins in sensing diatomic gases

In addition to the pervasive requirement for chemical odorant detection in the animal kingdom, organisms also need to be able to detect and behaviorally regulate internal concentrations of several metabolic gases on both short- and long-term time scales. Oxygen, carbon monoxide, and nitric oxide are all diatomic gases that readily bind the heme moieties of hemoproteins. Hemoproteins make up a large, structurally and functionally diverse group of proteins responsible for storage, transport, and catalysis of various ligands. Almost every protein known to directly detect gases is a hemoprotein (Rodgers, 1999). Heme-based sensor proteins must be able to detect their ligand at appropriate concentrations and translate its presence or absence into an appropriate response. In general, when the heme moiety of a heme-based sensor binds its ligand an allosteric change activates another domain of the same protein or even a separate protein. It is critical that a sensor protein be able to distinguish between true and false positive signals, because their gaseous ligands are small and similar in structure (e.g. they are all diatomic gases) (Gilles-Gonzalez and Gonzalez, 2005). To this end, recent advances have elucidated some basic principles in the pairing of protein structure (i.e. the amino acid residues coordinating the heme-moiety) with ligand specificity (Chan, 2001).

There are at least four types of heme-based sensors categorized on the basis of their heme-binding domains or their mechanisms of signaling: those that

bind DNA, proteins with a PAS domain, the GCS family, and the HNOB family (Gilles-Gonzalez and Gonzalez, 2005). Heme-coupled DNA binding proteins include both the e75 nuclear receptor in *Drosophila* and CooA. The e75 nuclear receptor is thought to respond to either nitric oxide or carbon monoxide and play a role in fly development (Reinking et al., 2005). CooA is a transcription factor initially identified in the photosynthetic bacterium *Rhodospirillum rubrum*. Upon binding carbon monixide, CooA homodimerizes and binds the *coo* operon, which is a genetic element that controls the expression of genes necessary for utilizing carbon monoxide as an energy source (Roberts et al., 2004; Shelver et al., 1997).

The heme-binding PAS domain is found in several different kinds of proteins responsible for detecting various signals in addition to the presence of diatomic gases. These include light, redox potential, voltage, and xenobiotics (Gilles-Gonzalez and Gonzalez, 2004). The most well studied example of a PAS-domain containing protein is FixL (Gilles-Gonzalez and Gonzalez, 2005). FixL is an membrane-bound oxygen sensor found in the symbiotic *Rhizobia* bacteria responsible for nitrogen fixation in the root nodules of many plants. In the absence of oxygen, the PAS domain of FixL regulates its own histidine kinase domain through auto-phosphorylation. This leads to the phosphorylation of a transcription factor, FixJ, which initiate the expression of genes involved in nitrogen fixation (Gilles-Gonzalez et al., 1991; Lois et al., 1993).

Globin-coupled sensors, or GCS proteins, combine the globin fold found in myoglobins and hemoglobins with a signaling domain that was initially found in

the *E. coli* serine chemoreceptor *Tsr* called the methyl-accepting chemotaxis protein (MCP) domain (Gilles-Gonzalez and Gonzalez, 2005). GCSs were originally identified in both the archaeon *Halobacterium salinarum* (the HemAT-Hs) and the bacterium *Bacillus subtilis* (the HemAT-Bs) as oxygen sensors that mediate aerotaxis (Hou et al., 2000). GCSs are now known to form a family of at least 30 sensors across Archaea and Prokaryota (Freitas et al., 2003).

Another group of hemoproteins, the heme-NO-binding (HNOB) family, have higher affinity for nitric oxide than for the other diatomic gases. The mammalian soluble guanylate cyclase (sGC) is a well-characterized member of this family. sGC is a cytosolic "receptor" that mediates several physiological responses including neurotransmission, smooth muscle relaxation, and photoreception depending on the tissue subtype in which it is expressed. The alpha and beta subunits of sGC heterodimerize and produces cGMP from GTP when activated by NO (Bredt and Snyder, 1992; Hobbs, 1997). Nitric oxide sensation is not specific to vertebrates, however, as homologues of sGC exist in other organisms like *Drosophila*, where they also seem to play a role in NO-mediated neurophysiology (Liu et al., 1995; Shah and Hyde, 1995). Atypical members of the sGC family have been characterized in *C. elegans* and *Drosophila* that may be more specific to oxygen than nitric oxide (Gray et al., 2004; Vermehren et al., 2006; Wingrove and O'Farrell, 1999). In *C. elegans*, GCY-35, an atypical sGC, is expressed in chemosensory neurons and is responsible for mediating aerotaxis (Gray et al., 2004).

3.1.2 Mammalian oxygen sensing

Despite all that is known about heme-based gas sensors, the molecular mechanism underlying one of the most well-studied examples of physiologic oxygen sensation has yet to be identified. Mammals detect hypoxia nearly instantaneously with the carotid body (CB), a vascular bulb located at the bifurcation of the carotid arteries. The CB feeds into the sympathetic nervous system, which affects the cardiovascular and respiratory systems with the net effect of increasing blood oxygenation (Lahiri et al., 2006). Several molecular mechanisms, including a hemoprotein (i.e. hemoxygenase-2), have been proposed as candidate CB oxygen sensors, but none of these candidates have been proven (Baysal, 2006; Wenger, 2000).

Although the short-term hypoxia sensor is unknown, the mechanism mammals use to respond to falling oxygen levels on a longer time scale, such as those encountered by moving to a higher altitude, is clearer. It is accomplished through indirect activation of a transcription factor, hypoxia inducible factor (HIF)-1 (Ratcliffe et al., 1998). This transcription factor is activated by non-hemoprotein oxidases whose activities are modulated by oxygen. HIF-1 initiates many downstream genetic adaptations to chronic hypoxia including erythropoietin production and the expression of angiogenic factors (Stockmann and Fandrey, 2006).

3.1.3 Introduction to CO_2 chemosensation

One interesting footnote to the investigation of the function of *Or83b* was the discovery of at least one group of cells in the antenna of *Drosophila* that is completely independent of *Or83b*. OSNs designated ab1C (see section 3.1.4) respond specifically to carbon dioxide (CO_2) (de Bruyne et al., 2001) and remain fully functional in *Or83b* mutant flies (Larsson et al., 2004). In addition, there is a large behavioral and electrophysiological literature on CO_2 as an important environmental stimulus for a wide variety of insects (Nicolas and Sillans, 1989; Stange, 1996; Stange and Stowe, 1999). Thus, the *Or83b*-dependent olfactory pathway responsible for sensing host odorants and the *Or83b*-independent CO_2 chemosensory pathway may play synergistic roles in the host-seeking behaviors that allow some insects to act as disease vectors.

3.1.3.1 Environmental CO_2 gradients

Carbon dioxide is a pervasive chemical stimulus that is important in the ecology of many insect species (Nicolas and Sillans, 1989). Current, post-industrial atmospheric CO_2 levels hover around 0.035% or 350 ppm, but insects, depending on the species, detect behaviorally relevant CO_2 concentrations from atmospheric levels up to around 10-15% in some microenvironments (Anderson and Ultsch, 1987; Ziesmann, 1996). Although meaningful to insects, these environmental gradients, often originating from multiple CO_2 sources, are invisible to humans because we are only capable of sensing CO_2 concentrations above 20% via trigeminal nerve nociception (Thurauf et al., 2002). An

environmental CO_2 concentration gradient does not, however, have the same ethological meaning to every insect. The separation of insects into two major categories, blood-feeders and non-blood-feeders (i.e. hematophagic categorization), is very useful in understanding carbon dioxide's role in insect behavior.

3.1.3.2 CO_2-evoked behavior

3.1.3.2.1 Non-hematophagous insects

Plant and nectar feeders use environmental CO_2 information in two main ways. By sensing CO_2 gradients, insects can locate sources and sinks of CO_2, which can help guide them to food sources or oviposition sites. In addition, by monitoring changing ambient CO_2 concentrations, social insects can respond accurately to help control their living conditions.

Tephritid fruit flies like the medfly (see chapter 2) and the Queensland fruit fly (*Bactrocera tryoni*) are major agricultural pests. They use their piercing ovipositors to lay eggs in oranges and apples. As the fly larvae grow they eat the fruit from the inside, destroying it. Even though these flies are capable of penetrating the tough skins of their host fruits, they prefer to use an existing lesion, which leaks CO_2, for oviposition. In fact, even small increases in CO_2 concentration, anywhere from 0.04% to 3%, are highly attractive and stimulate oviposition behavior in *Bactrocera* (Stange, 1999).

Most Lepidopteran larvae are phytophagous, and most plants generate CO_2 gradients by alternately acting as sources or sinks for atmospheric CO_2

depending on the time of day and season. Although the nature of CO_2 sensation is unknown in the larval stage, these CO_2 gradients should be useful cues containing specific information about food quality (i.e. those plant tissues undergoing active photosynthesis). Consistent with this idea, at least some moth larvae seem to orient toward their preferred food sources using CO_2 gradients (Rasch and Rembold, 1994). Furthermore, adult hawkmoths (*Manduca sexta*) use the information contained in CO_2 gradients while foraging for higher quality sources of their preferred food, *Datura wrightii* flowers; newly opened flowers emit more CO_2 and contain more nectar (Thom et al., 2004).

Not all insects, however, use CO_2 information to locate and judge the quality of food sources. Social insects, living in large groups, face different environmental challenges than solitary insects. With so many individuals living together, respiratory CO_2 production often accumulates faster than it can diffuse away. Thus, many social insects have evolved sensitive CO_2 detection systems that alert them to the danger overcrowding. These systems and their corresponding behavioral output allow the social insects to maintain strict climate control in their hives, nests or mounds. In both honeybees and bumblebees, workers respond to increases in hive CO_2 by moving to the entrances and performing a wing fanning response that ventilates the hive, maintaining homeostatic CO_2 levels (Seeley, 1974; Southwick and Moritz, 1987; Weidenmuller et al., 2002).

Another social Hymenopteran, leaf-cutter ants (*Atta vollenweideri*) also have specialized CO_2 detection systems (Kleineidam et al., 2000). However,

instead of fanning like bees, ants maintain optimal climate conditions by manipulating a wind-driven nest ventilation system; using soil and plant matter to seal and unseal nest openings (Kleineidam et al., 2001; Kleineidam and Roces, 2000). Similarly, when mound CO_2 concentrations rise, some termites modify mound walls to be more porous and CO_2-permeant (Ziesmann, 1996). Outside the nests, some subterranean termites also seem to use CO_2 gradients to locate better food sources (Bernklau et al., 2005).

3.1.3.2.2 Hematophagous insects

In contrast to a relatively flat landscape of atmospheric CO_2 at 0.035%, a filamentous plume of 4-5% CO_2 emitted periodically with each breath of a respiring host animal seems to be a ubiquitous activator of upwind search behavior in all hematophagous insects studied thus far (Stange, 1996). This discussion, however, is far from simple, especially in the most highly studied vector insect, the female mosquito. Therefore, I will focus mainly on the behavioral responses of mosquitoes toward CO_2, followed by a brief outline of work in other hematophages.

Mosquito host-seeking in general is a complex multi-sensory behavior involving mainly olfaction at long to mid-range, olfactory and visual cues for the final approach, and mechanical, olfactory, and gustatory cues immediately prior to biting. Blood feeding behaviors by mosquitoes can be divided into several sub-behaviors that are each differentially modulated by a variety of environmental stimuli. The stages include rest, flight and searching, settling on the host, probing

and biting, and finally leaving the host (Kalmus and Hocking, 1960). Host-seeking behavior is a subset of blood-feeding behavior that really only includes activation from rest and upwind guided flight toward the host.

Olfactory stimuli are the most important cues involved in host-seeking (Takken and Knols, 1999), but the precise ethological relevance of each of the various olfactory stimuli modulating the steps of host-seeking behavior is still not clear despite hundreds of papers being published on the subject. In fact, one researcher working in the field said, “Rarely has so much work yielded so little consensus of opinion; results which are apparently contradictory abound, even in the same paper and more so among different workers,” (Hocking, 1971). Much of the inconsistency in the literature stems from non-uniform and/or poorly controlled experimental methods in both the laboratory and the field. In addition, if the entire sequence of host-seeking behavior is thought of as a complex chain of events, it is understandable that experiments designed to test single steps along the chain, to the neglect of the rest, may come to different conclusions than experiments beginning with the first step (Hocking, 1971).

CO_2 is probably the most controversial of all olfactory stimuli that have been linked to mosquito host-seeking, in that numerous reports exist claiming CO_2 as being alternatively attractive or repulsive (Reeves, 1990; Willis and Roth, 1952). Both stimulus-specific and insect-specific factors are likely to blame for the controversy that exists in this field. First, CO_2 stimuli used in different studies are never standardized; multiple sources and delivery methods have been used. CO_2 from a block of dry ice, which produces pure CO_2 along with a massive

temperature gradient, is not the same as purified CO_2 diluted to 5% in a pre-mixed compressed air tank at room temperature. CO_2 delivered at a constant flow rate is less attractive than CO_2 that is pulsed from a point source producing filamentous plumes (Geier et al., 1999). Very few studies in the laboratory, and no field studies control all of the variables necessary to mimic the CO_2 output of a respiring human unless an actual human is used as bait.

In natural settings such as those using a human as bait, CO_2 is not encountered alone, but always in combination with numerous other host odorants. Certain mosquitoes, especially the *Anopheles gambiae* species complex, are more anthropophilic than others, and this seems to have an olfactory basis (Dekker et al., 2001; Gibson, 1996). CO_2, however, is a non-specific signal that does not distinguish human from animal, much less account for the dramatic differences in attractiveness seen among individual humans (Kelly, 2001; Mukabana et al., 2002). Almost 350 different volatile chemicals are emitted from human skin, but aside from a few that have been confirmed in behavioral studies, the relevance of each compound to mosquito behavior is unknown (Bernier et al., 2000). Two specific volatiles, L-lactic acid and 1-octen-3-ol, have been confirmed to act synergistically with CO_2 to enhance attraction in several species (Bosch et al., 2000; Kemme et al., 1993; Steib et al., 2001; Takken and Kline, 1989; Van Essen et al., 1994). Artificial combinations of bait chemicals still fail, however, to even come close to the attractive power of real human baits or extracted human skin odors supplemented with CO_2 (Costantini et al., 1996; Dekker et al., 2005).

Apart from all of these stimulus-specific variables, the host-seeking behaviors of female mosquitoes also vary widely with respect to many other factors. Some of the most important variables include the particular mosquito species or sub-species the experimental location (atmospheric conditions and mosquito density), the time of year, the time of day, the age of the mosquitoes and their stage of maturity, their nutritional state and mating status, and the time since their last blood meal (Bowen, 1991; Bowen, 1996; Gibson, 1996; Klowden, 1996). In summary, identical stimuli that elicit host seeking in one mosquito at one time, may not elicit the same behavior in the same mosquito a few hours later.

All this being said, and despite the controversy surrounding the various stimuli used by mosquitoes for human host-seeking, it is clear that CO_2 is the most important single compound acting as an olfactory kairomone capable of both activating mosquitoes and drawing them upwind toward a human host (Gillies, 1980). This is best evidenced in the large number of studies published on mosquito trap designs, whose effectiveness is always dramatically enhanced by adding CO_2 (Knols et al., 1994; Mboera et al., 2000; Njiru et al., 2006).

In addition, mosquitoes are not the only hematophagous insects that use CO_2 as a cue for host seeking. Many hematophagic flies that act as disease vectors (e.g. sleeping sickness, filariasis, leishmaniasis etc.) are also known to be attracted to CO_2 (Gibson and Torr, 1999). These include tsetse flies (Glossinidae) (Voskamp et al., 1999), horse flies (Tabanidae) (McElligott and McIver, 1987), black flies (Simulidae) (Fallis and Raybould, 1975), the stable fly

(*Stomoxys calcitrans*) (Alzogaray and Carlson, 2000), biting midges (Ceratopogonidae) (Ritchie et al., 1994), and sandflies (Phlebotominae) (Pinto et al., 2001).

Ticks and fleas, responsible for spreading Lyme disease and the bubonic plague respectively, are both attracted to host CO_2 emissions (Benton and Lee, 1965; Steullet and Guerin, 1992). *Triatoma infestans*, the reduviid bug, is the disease vector for the trypanosome *T. cruzi*, which causes Chagas' disease. This insect, a member of the order Hemiptera, orients upwind in response to pulsatile release of CO_2 (Barrozo and Lazzari, 2006). Similar to that found in mosquitoes and other hematophagous insects, *Triatoma* also use CO_2 as a synergistic cue with other host volatiles to locate their next blood meal (Barrozo and Lazzari, 2004a; Barrozo and Lazzari, 2004b).

3.1.3.3 CO_2 receptor neurons

Carbon dioxide is distinct among odorants because of its small size, relative inertness, extremely high volatility and diffusibility in air, and its solubility in both water and lipids. CO_2 is also environmentally ubiquitous at a relatively high concentration (350 ppm) in comparison to more typical odorants. As such, behaviorally relevant information about environmental CO_2 includes both its ambient concentration and any rapid changes in concentration encountered in CO_2 gradients.

The electrophysiological profiles of all the insect CO_2 receptors studied thus far support this idea, in that they all display a phasic-tonic response profile.

Basically, in response to a change in CO_2 concentration, a phasic burst of action potentials responds quickly, clearly resolving the onset of the stimulus. Then, if the change in CO_2 concentration is prolonged (>5 seconds), this phasic response plateaus into a sustained firing frequency that conveys information about the absolute concentration currently being detected. In most insects this tonic plateau is non-adapting in contrast with the responses to more typical odorants, which do adapt (Kaissling et al., 1987). One experiment in an ant species stimulated the CO_2 receptor neurons for over an hour without any evidence of fatigue or adaptation (Kleineidam et al., 2000). The only known exception to this rule of non-adaptation is in the moth *Cactoblastis cactorum* (Stange et al., 1995). Discounting this exception, the non-adapting, phasic-tonic dual functionality of insect CO_2 receptor neurons allows both the tracking of a filamentous plume of 5% CO_2 by mosquitoes and the precise following of ambient CO_2 levels by honeybees that is necessary for climate control in the hive.

Despite these apparent functional similarities, however, CO_2 receptor neurons display many distinct variations among and even within the major insect orders. These include species-specific response modalities (i.e. excitatory or inhibitory), adaptation properties, receptive ranges, sensitivities, and variations in anatomic location and morphology (Stange, 1996; Stange and Stowe, 1999) (summarized in **Table 1**).

Order – sensory organ Common name – *Genus species*	Type of CO_2 response	Response to other odors	Adaptation	Working range
Isoptera – antenna Termite – *S. lamanianus*	–	+	–	0-100%
Chilopoda – temporal organ Centipede – *T. hilgendorfi*	–	–	–	0.01-5%
Acarina – Haller's organ Tick – *Amblyomma variegatum*	Cell type 1: – Cell type 2: +	– –	– –	0.04-1% 0.1-5%
Hymenoptera – antenna Honeybee – *Apis mellifera* Ant – *Atta sexdens*	 + +	 – ?	 – –	 0-100% ?
Lepidoptera – labial pit organ Moth – *Rhodogastria spp.* Moth – *Cactoblastis cactorum*	 + +	 + ?	 – + at high [CO_2]	 0-5% 0-5%
Diptera –maxillary palp/antenna Biting midge – *Culicoides furens* Mosquito – *Aedes/Anopheles* Fruit fly – *D. melanogaster* Tephritid fruit fly – *B. tryoni* Tsetse – *Glossina morsitans*	 + + + + +	 – – – + ?	 – – – ? ?	 0-0.5% 0-5% 0-10% 0-5% 0-5%

Table 1: Summary of CO_2 response properties in several arthropods

Type of response: "–" indicates inhibitory; "+" indicates excitatory. "?" indicates conflicting or inadequate information. Adapted from (Ziesmann, 1996) with additional information from (Bogner et al., 1986; Grant and Kline, 2003; Grant et al., 1995; Hull and Cribb, 2001; Kellogg, 1970; Kleineidam et al., 2000; Lacher, 1964; Stange et al., 1995; Steullet and Guerin, 1992; Suh et al., 2004; Yamana et al., 1986)

Most CO_2 receptor neurons are excited by increases in CO_2 concentration, but neurons that are inhibited by CO_2 have been reported in one species of termite, the Japanese house centipede (an carnivorous arthropod, but not an insect), and in the tropical bont tick (also an arthropod, but not an insect) (Steullet and Guerin, 1992; Yamana et al., 1986; Ziesmann, 1996). These CO_2 receptor neurons still respond in a non-adapting phasic tonic way, but in the opposite direction. The termite is particularly interesting, because, unlike in most insects, the CO_2 receptor neurons of *Schedorhinotermes* are inhibited by CO_2 and

excited by other odors. The responses do not saturate, even at 100% CO_2, perhaps because the termite can be exposed to extremely high CO_2 levels inside its enclosed mound. At the elevated background concentrations of CO_2 inside a termite mound these neurons do not respond to odors, but outside the mound in atmospheric CO_2 their full response profile becomes available (Ziesmann, 1996). Presumably, this resulting location-dependent modulation of olfactory perception results in odor-evoked behaviors that are matched to the environment in which the termite finds itself.

The tropical bont tick, *Ambylomma variegatum*, is not an insect and has no antennae. It does, however, have two different types of CO_2 receptor neurons located within a specialized encapsulated sensory structure on the dorsal side of the first leg (tarsus) called Haller's organ. One is inhibited by CO_2 and very sensitive to small changes in concentration around ambient levels. The other type is excited by CO_2 and tuned to concentrations from 0.1% to 5% (Steullet and Guerin, 1992). This arrangement, using two receptors with complementary receptive ranges, seems odd considering the ability of other insect CO_2 receptor neurons to cover ranges from 0-100%.

Moths may have nature's most sensitive CO_2 receptor neurons located in a specialized organ at the tip of their labial palp appendages called the labial palp pit organ (LPO). The LPO can contain anywhere from one to almost 2,000 CO_2 receptor neurons depending on the species (Stange et al., 1995). By recording from LPO neurons, individual species from many Lepidopteran groups including Arctidii, Noctuidae, Pieridae, Nymphalidae, Pyralidae, Danainae, Sphingidae,

and Saturniidae have all been shown to respond specifically and sensitively to minute changes in CO_2 concentration (Bogner, 1990). A set of experiments on the sensitivity of the CO_2 neurons in one particular moth, *Heliothis armigera*, found significant resolution of changes in CO_2 concentration as small as 9 ppm around background (350 ppm), but by using sinusoidal pressure modulation to quickly and reliably simulate CO_2 concentration changes, these neurons were found to be sensitive enough to detect changes as small as 0.5 ppm (Stange, 1992).

Poorly controlled amputation experiments initially placed the mosquito CO_2 receptor neurons in the antenna (Willis and Roth, 1952), but later palpectomy experiments corrected the mistake (Omer and Gillies, 1971). Mosquito CO_2 receptor neurons are located on the maxillary palps and innervate thin-walled capitate peg sensilla (Sutcliffe, 1994). These neurons have been best characterized electrophysiologically in *Aedes aegypti*, and display specific non-adapting phasic-tonic excitation to changes in CO_2 concentration (Grant et al., 1995; Kellogg, 1970). In *Anopheles gambiae* there is a significant sexual dimorphism in the number of capitate peg sensilla, and therefore CO_2 neurons; females have almost 70 on palp segments 2-4, and males have less than 20 only on segment 4. At first this seems consistent with the fact that only females display human host-seeking behaviors, but because other mosquitoes, such as *Aedes aegypti*, that also feed on blood do not have this sexual dimorphism, its role in behavior is unclear (McIver, 1982; McIver and Siemicki, 1975).

Despite the vast amount of work that has gone into describing the diversity of form and function among insect CO_2-evoked behaviors and CO_2 receptor neurons, the molecular mechanism of CO_2 chemosensation is still unclear. Insight into this mechanism has been difficult to obtain because of a lack of genetic resources in all of these insects. One particular Dipteran, however, the model organism *Drosophila*, has a wealth of molecular genetic tools with which to study CO_2 chemosensation.

3.1.3.4 CO_2 chemosensation in *Drosophila*

Adult *Drosophila* antennae have a small population of about 25 CO_2-responsive OSNs that are designated ab1C (de Bruyne et al., 2001). This designation indicates that they innervate the first class of antennal basiconic sensilla, which houses four OSNs. The CO_2 neuron gives the third largest spike amplitude out of these four neurons, hence its designation as the “C” neuron. These neurons respond in much the same way the mosquito CO_2 neurons do, they are excited in a non-adapting phasic-tonic way to changes in CO_2 concentration. The ab1C neurons seem to be very specific to CO_2, in that they do not respond to any of the odorants with which they have been tested (de Bruyne et al., 2001; Stensmyr et al., 2003a; Stensmyr et al., 2003b).

In the *Drosophila* antennal lobe, a single bilateral ventrally-situated glomerulus (V) responds selectively in a dose-dependent manner to changes in CO_2 concentration from 0%-10% (Suh et al., 2004). The V glomerulus is located in between the AL and the SOG. This is reminiscent of the central projection

pattern of CO_2 receptor neurons in the only other insects in which it has been studied, moths and mosquitoes. In moths, information about changing CO_2 concentrations is relayed to the central brain via the labial palp nerve to the LPO glomerulus (LPOG), which lies ventrally between the AL and the SOG (Bogner et al., 1986; Guerenstein et al., 2004; Kent et al., 1986). The CO_2 neurons in mosquitoes project via the maxillary nerve to a single, bilateral, but slightly more dorso-medial glomerulus in the AL, which is much larger than neighboring glomeruli (Anton et al., 2003; Distler and Boeckh, 1997). The *Drosophila* V glomerulus is innervated by a population of neurons expressing the gustatory receptor *Gr21a* (Scott et al., 2001).

The behavioral relevance of CO_2 in *Drosophila* is unclear, but unlike the attraction seen in mosquitoes, increasing concentrations of CO_2 repel both larvae and adults. In larvae, which have a single bilateral *Gr21a*-expressing neuron innervating a gustatory structure called the terminal organ, CO_2 concentration could indicate the age and suitability of rotting fruit, the fly's preferred food source (Faucher et al., 2006). In adults, however, it seems to be a component of a stress odorant released by flies when physically stressed (i.e. by shaking or electrical shock) (Suh et al., 2004). Regardless, both of these CO_2-avoidance behaviors are eliminated upon genetic silencing of *Gr21a*-expressing neurons, confirming that these neurons are the only CO_2-sensitive neurons in *Drosophila* (Faucher et al., 2006; Suh et al., 2004). Using a combination of electrophysiological, behavioral, and molecular genetic techniques, we asked whether *Gr21a* is

directly involved in CO_2 chemosensation or whether it is merely a marker for these neurons.

3.2 Materials and methods

3.2.1 *Drosophila* stocks

Transgenic fly stocks were produced and maintained as described in section 2.2.1. The following flies were used in this study: wild type-Berlin (M. Heisenberg); *w1118; 70FLP,70I-SceI/Cyo* and *w1118; 70FLP* (K. Golic); *UAS-CD8-GFP* (L. Luo); *Gr21a-GAL4* (K. Scott); *Or22a-GAL4; UAS-GFP-Gr21a* (R. Benton). *Gr63a-sytRFP* flies were constructed by fusing the coding region of n-synaptotagmin to RFP and placing this under the control of the *Gr63a* promoter (P. Cayirlioglu, I. Grunwald Kadow, and S.L. Zipursky).

3.2.2 RNA *in situ* hybridization

Adult flies (specific genotypes listed with the description of each experiment) or adult mosquitoes (*Anopheles gambiae* G3; MRA-112) were placed in custom-machined stainless steel fly collars and covered in O.C.T. prior to freezing. After cutting 14 µm antennal sections with a cryostat, the slides processed using Cy5- and FITC-TSA kits (Perkin Elmer) to amplify RNA signals as described without protocol modifications (Fishilevich and Vosshall, 2005). Mosquitoes were provided by P. Howell and M.Q. Benedict of MR4 at the CDC, Atlanta, GA.

3.2.3 Whole-mount brain and antennal section immunostaining

Whole mount brain immunostaining of *Gr63a-syt-RFP*; *Gr21a-GAL4, UAS-CD8-GFP* flies was performed as previously described (Laissue et al., 1999) using 1:1000 anti-GFP (Molecular Probes) and 1:10 nc82 (a gift of Reinhard Stocker) with 1:100 anti-rabbit-Alexa488 (Molecular Probes) and 1:100 anti-mouse-Cy3 (Jackson ImmunoResearch) secondary antibodies. Ten micron antennal sections were fixed and stained as previously described using mouse anti-GFP 1:1000 (Molecular Probes) and rabbit anti-OR83b (EC2) 1:5000 (Larsson et al., 2004).

3.2.4 GR transgene generation

Gr10a was amplified from Oregon-R antennal cDNA using primers 5'- ATGACATCGCCGGATGAGCGT-3' and 5'-CTAGGACTTCTTGCGCAAATA-3'. *Gr63a* was amplified from *yw* genomic DNA using primers 5'- ATGCGTCCGTCTGGCGAAAAA -3' and 5'-CTAGCCTTTCCGGCCCTTTAG -3'. PCR products produced using the Expand High Fidelity PCR kit (Roche) were subcloned into pGEM-T Easy (Promega). Fly GRs were subcloned into pUAST (Brand and Perrimon, 1993) or a modified pUAST containing GFP (Benton et al., 2006) and transgenic animals were produced (Genetic Services Inc., Cambridge, MA, USA) and balanced using standard methods. *GPRgr22* was amplified from *Anopheles gambiae* G3 antennal cDNA using primers 5'-ATGATTCACACACAGATGGAA-3' and 5'-TTAGGTGTTCACTTTGTCTGC-3'. The first exon of *GPRgr23* was amplified

from *Anopheles gambiae* G3 antennal genomic DNA using primers 5'-ATGCGCTGGAACGGTTGT-3' and 5'- CGATGTGAGCAGTTCCCG-3'. *GPRgr24* was amplified from *Anopheles gambiae* G3 antennal cDNAs using primers 5'-ATGGTGTTTGAAAGCTCCAAA-3' and 5'-CTAAGAATGAGACGAATTACT-3'. These PCR products were cloned into pGEMT-Easy and used to generate DIG or FITC labeled riboprobes for RNA *in situ* hybridization.

3.2.5 Single sensillum electrophysiology

Extracellular recordings of ab1 and ab3 sensilla from individual flies (2-10 days old) were made as described (de Bruyne et al., 2001; Larsson et al., 2004) using a 10X AC probe connected to the Syntech IDAC-4 acquisition controller and analyzed offline using the software Autospike (Syntech, Hilversum, The Netherlands). Thirty µl of odorant, diluted 10^{-4} in paraffin oil, were added to filter paper strips and placed inside 1ml plastic tuberculin syringes. One-second odor stimuli were added to a constant air stream under the control of the Syntech CS-55 Stimulus controller. Prior to CO_2 recordings each sensillum was identified by its published characteristic odorant response profile: ab3A, ethyl hexanoate; ab3B, 2-heptanone; ab1A, ethyl acetate; ab1B, acetoin; ab1D, methyl salicylate (de Bruyne et al., 2001; Stensmyr et al., 2003b). All odorants were obtained from Sigma-Aldrich and were of the highest purity available. CO_2 stimuli were applied by filling 20 ml syringes from pre-mixed CO_2 in air tanks from Matheson Tri-Gas (Parsippany, NJ). Maximum stimulus concentrations (odors ~$3x10^{-5}$; CO_2 ~2-3%) at the exit point of the stimulus device were calculated by measuring system

airflows. Responses were quantified by counting all spikes within a 500 ms window from the onset of the response. Air responses were then subtracted from CO_2 responses from the same sensillum and the resulting number of spikes was doubled to obtain a corrected CO_2 response in spikes/second. After checking the response distributions for normality, we proceeded with parametric means comparisons using the Tukey HSD test.

3.2.6 *Gr63a* targeting construct and mutant screen

Genomic DNA both 5' and 3' of the *Gr63a* coding sequence was amplified from *yw* flies using Expand High Fidelity PCR kit (Roche) and TA cloned into pGEM-T Easy (Promega, Madison, WI, USA). Inserts were end-sequenced and internally sequenced to verify coding regions embedded in the arms and subcloned into CMC105 (Larsson et al., 2004). 5' homologous arm: primers corresponding to nucleotides 3880204-3880228 and 3883041-3883062 of *Drosophila melanogaster* chromosome 3L (Genbank entry NT_037436) amplified a 2.859 kb fragment.

Four independent insertions of the targeting construct were screened as described (Larsson et al., 2004). The progeny of approximately 16,500 virgin mosaic or white-eyed females (~330,000 flies) were screened for re-insertion on the 3rd chromosome, and we recovered a single mutant line, *Gr63a*^{1}. PCR confirmation of *Gr63a*^{1} was performed on genomic DNA preparations of the mutant line and its corresponding wild type parental targeting construct insertion with primers within *Gr63a* itself (nucleotides 3879912-3879930 and 3880180-

3880204 of Genbank entry NT_037436) and within the neighboring gene *CG1079* (nucleotides 3877256-3877274 and 3877702-3877720 of Genbank entry NT_037436). A similar screen for a *Gr21a* mutant produced no mutants among ~350,000 progeny derived from five independent targeting construct insertions.

3.2.7 CO_2 avoidance behavior

CO_2 T-maze avoidance experiments were performed essentially as described (Suh et al., 2004). Avoidance Index is calculated as # flies on CO_2 side - # flies on air side/ total # flies. Flies that failed to choose one of the two stimulus tubes were excluded from the Index. Experiments were carried out in the dark at 25ºC and 70% relative humidity with a 15W red-light positioned behind and perpendicular to the T-maze. Pure CO_2 (0.28 ml) was added to 14 ml tubes for a final concentration of ~2%. The stimulus was added to alternating sides to preclude any side bias. Each individual experiment included between 15 and 50 flies (mean=30).

3.2.8 Phylogenetic tree

Multiple protein alignments were made using ClustalX version 1.83 with default parameters (Chenna et al., 2003). A neighbour-joining tree was generated with PHYLIP v. 3.6 using default settings [Felsenstein, J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle] and viewed

using the web-based Phylodendron tree viewer

(http://iubio.bio.indiana.edu/treeapp/treeprint-form.html).

3.3 Results

3.3.1 CO_2-responsive OSNs co-express two GR family members

Knowing that the CO_2-responsive neurons express *Gr21a*, and guided by previous reports of co-expression of multiple GRs in GSNs of the fly proboscis, we began by looking for the expression of other GRs in ab1C neurons. Two other GRs are known to be expressed in the *Drosophila* antenna, *Gr63a* and *Gr10a* (Scott et al., 2001). Fluorescent double *in situ* hybridization reveals that *Gr63a* is co-expressed with *Gr21a* (**Fig. 3.1A**), but that *Gr10a* is expressed in the adjacent ab1D neuron (**Fig. 3.1B**) (Fishilevich and Vosshall, 2005). Thus, the full complement of receptors expressed in the ab1 sensillum has been discovered (**Fig. 3.1C**) (Couto et al., 2005).

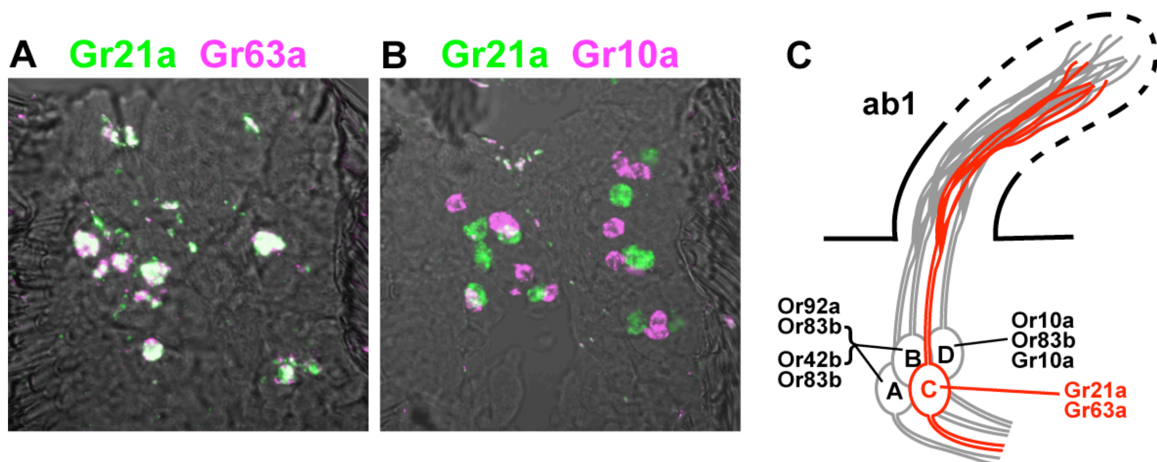

Figure 3.1: OSNs expressing *Gr21a* also express *Gr63a*, but not *Gr10a*.

A) Fluorescent double *in situ* hybridization on the third antennal segment of *Drosophila* (wild type Berlin) reveals co-expression of *Gr21a* (green) and *Gr63a*

(magenta). **B**) *Gr21a* (green) is not co-expressed with the only other GR expressed in the antenna, *Gr10a* (magenta). **C**) Schematic receptor expression profile for ab1 sensilla. ab1C (red) expresses both *Gr21a* and *Gr63a*. ab1D expresses one GR and two ORs. The receptor pairs expressed in the remaining OSNs are known, but have not been definitively assigned to either ab1A or ab1B.

In confirmation of the co-expression of *Gr21a* and *Gr63a* in the OSNs responsible for sensing CO_2, genetic markers constructed with the promoters of either gene label the neurons that project to the CO_2-responsive V glomerulus (**Fig. 3.2**).

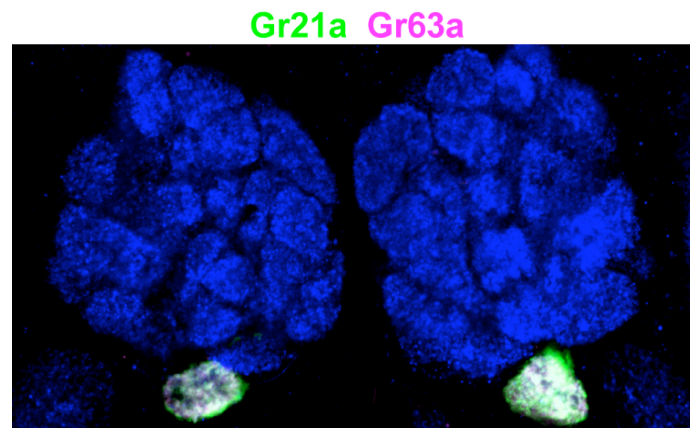

Figure 3.2: Both *Gr21a* and *Gr63a* promoter regions drive expression in OSNs that project to the V glomerulus.

Gr21a-GAL4 drives expression of UAS-CD8-GFP (green) in the same OSNs that express a marker constructed by fusing the *Gr63a* promoter with synaptotagmin-RFP (magenta). These neurons project to the bilateral ventral-most antennal lobe glomerulus, the V. This whole mount brain immunofluorescence preparation is counter-stained with the neuropil marker nc82 (Laissue et al., 1999) (blue) to reveal AL glomerular structure.

3.3.2 Receptor misexpression confers CO_2-sensitivity

To investigate the role of these GRs as putative CO_2 receptors, we ectopically expressed the antennal GRs both alone or in pairs in neurons normally unresponsive to CO_2 using the Gal4/UAS system (**Fig. 3.3**).

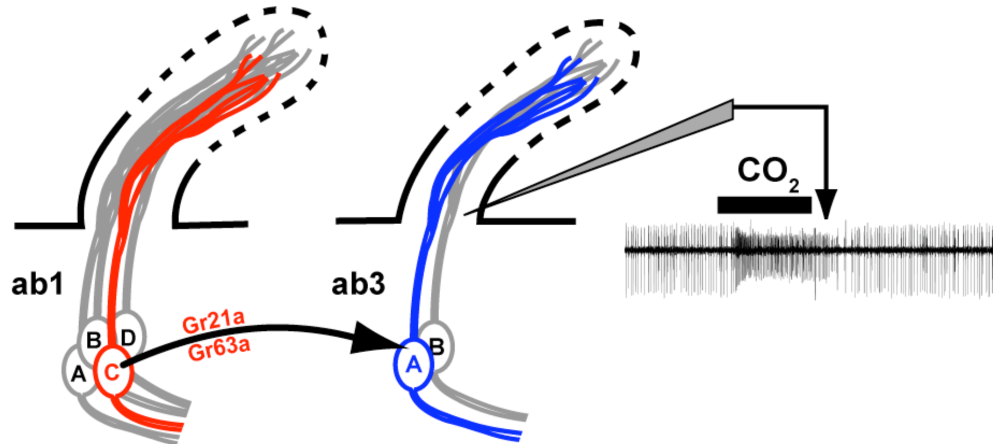

Figure 3.3: Schematic outlining the receptor mis-expression experiments

The *Or22a-GAL4* line is used to drive the expression of candidate CO_2 receptors normally expressed in ab1C neuron (red) in the ab3A neuron (blue). Single sensillum recordings can then reveal the conferred CO_2-sensitivity of a neuron that is normally unresponsive to CO_2 (see figure 3.4).

Or22a-GAL4 drives expression in ~75% of the electrophysiologically accessible ab3A neurons that express *Or22a/b* (Dobritsa et al., 2003). No single antennal GR was capable of conferring CO_2 responsiveness on the ab3A neurons (**Fig. 3.4**). Since, however, it has been previously demonstrated that *Drosophila* ORs are obligate OR/OR83b heterodimers (Benton et al., 2006), we asked whether a combination of two GRs could function as a CO_2 receptor. Neither *Gr21a* nor *Gr63a* confer responses to CO_2 when combined with *Gr10a*, but the combination of *Gr21a* and *Gr63a* produces a significant response to a stimulus of ~3% CO_2 (**Fig. 3.4**). It is therefore the specific combination of these two GRs that is sufficient to induce CO_2 sensitivity rather than a generic requirement for the co-expression of any two antennal GRs.

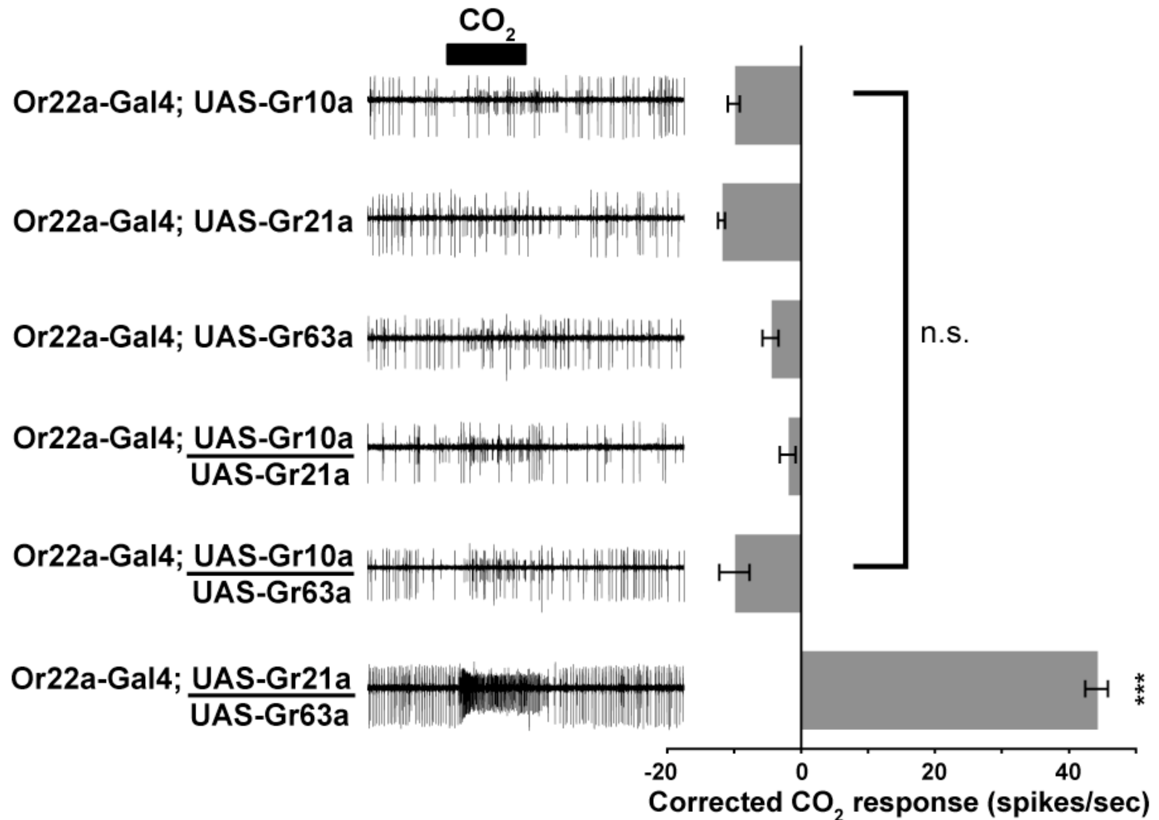

Figure 3.4: CO_2 responses of ectopically expressed GR combinations

The combinations of antennal GRs indicated on the left were ectopically expressed in ab3A (large spikes) neurons using the *Or22a-GAL4* driver. Single sensillum electrophysiological recordings on ab3 sensilla, recognized by their characteristic response to ethyl hexanoate (ab3A) and 2-heptanone (ab3B) (Dobritsa et al., 2003), were made for both room air (~0.035% CO_2) and ~3% CO_2. The number of spikes in a 500 ms window following air stimulation were tallied and subtracted from a similar window following CO_2 stimulation. The resulting number was multiplied by two to get a corrected response in spikes per second. Representative traces (stimulus bar = 1 second) and mean responses (± SEM; n = 15-18 sensilla per genotype with roughly equal numbers of males and females) are shown. Significant responses to CO_2 are only found with the combination of *Gr21a* and *Gr63a* (Tukey HSD test; $p<10^{-6}$). In all other cases, ab3A seems to be slightly inhibited by CO_2. The slight activation of ab3B during the stimulus is due to either mechanical stimulation or slight odor contamination.

Gr21a and *Gr63a* together also increase the level of spontaneous activity in the ab3A neuron. We considered the possibility that this reflects activity in response to ambient CO_2 levels, but found that the activity of these neurons is

not reduced in response to a CO_2-free airstream (data not shown). Prior results indicate that ORs have some substantial odor-independent activity (Hallem and Carlson, 2006), and this suggests that GRs share this property.

Further analysis of ectopically expressed *Gr21a/Gr63a* reveals a dose-dependent increase in response to stimuli of increasing CO_2 concentration, but this response is less sensitive than that of the native ab1C neuron (**Fig. 3.5**).

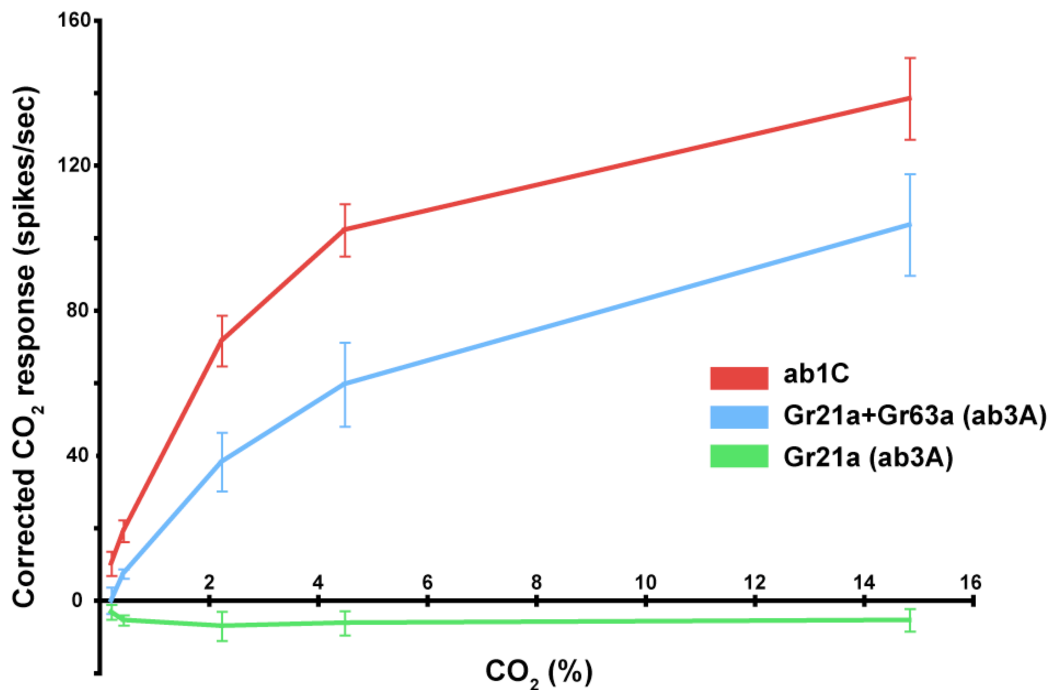

Figure 3.5: CO_2 dose response curves in native and non-native sensilla
Misexpression of *Gr21a* and *Gr63a* confers dose-dependent CO_2 sensitivity on the ab3A OSN (blue), but expression of *Gr21a* alone does not (green). The sensitivity of the reconstituted CO_2 receptor is lower than that of the native ab1C neuron (red). N = 10 antennae per genotype per CO_2 stimulus concentration.

This decreased sensitivity of the ab3A CO_2 receptor reconstruction when compared to the receptor in its native ab1C OSN is concerning, but there are several reasonable explanations. Since the *Or22a-GAL4* line is only expressed in

75% of ab3A neurons and we have no way of distinguishing an ab3 sensillum that expresses the GRs from one that does not, non-transgenic ab3 sensilla are likely dampening the size of the response when they are averaged into the total. In addition, the slight inherent inhibition of ab3A by CO_2 (seen in the green line of figure 3.5) probably also dampens the response. The other ORs that are expressed in the ab3A neuron (*Or22a/b* and *Or83b*) likely interfere with proper function of the CO_2 receptors. One group found that when a particular OR, *Or47a*, was expressed in these same ab3A neurons it retained the proper ligand specificity, but gave lower responses to its signature odors. If *Or47a* was expressed in a mutant ab3A neuron lacking its native ORs, *Or22a* and *Or22b*, both the response profile and response amplitudes matched those of the *Or47a* neuron itself (Dobritsa et al., 2003). In addition to these straightforward explanations, the existence of sensillum-specific co-factors could also play a role. Insect CO_2 receptor neurons typically have highly branched dendrites when compared to other olfactory neuron dendrites, and *Drosophila* is no exception (Shanbhag et al., 1999; Stange and Stowe, 1999). These specialized dendrites in the native neuron, coupled to higher receptor gene expression levels, may allow more receptor proteins to contact environmental CO_2 stimuli than when the receptors are expressed in the dendrites of a non-CO_2 neuron that are better suited to typical odorant detection. Regardless of the reason for the reduced sensitivity, it is clear that *Gr21a* and *Gr63a* together are sufficient to confer dose-dependent CO_2-responsivity on OSNs that are normally unresponsive to CO_2.

3.3.3 Generating a CO_2 receptor mutant

To investigate the role of these GRs in the CO_2 responses of the native ab1C neuron, we screened for *Gr21a* and *Gr63a* null mutants by homologous recombination (Gong and Golic, 2003; Larsson et al., 2004) (**Fig. 3.6**).

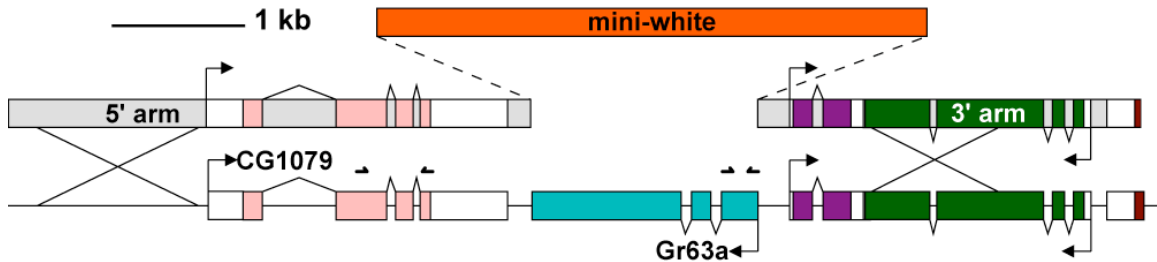

Figure 3.6: *Gr63a* homologous recombination strategy

Schematic of the *Gr63a* homologous recombination gene targeting construct and the *Gr63a* genomic locus. Upstream and downstream arms were cloned into the CMC105 vector (Larsson et al., 2004) surrounding the mini-white eye marker gene. A precise homologous recombination event would thus replace the coding region of *Gr63a* with mini-white. The location of PCR primers for the experiment in figure 3.7A are indicated with small arrows above *CG1079* and *Gr63a*.

Gr21a proved to be resistant to mutagenesis, but we obtained a single null mutant allele of *Gr63a*. PCR analysis of *Gr63a*^{1} indicates the selective loss of *Gr63a* coding sequence without affecting a neighboring gene, *CG1079* (**Fig. 3.7A**). *Gr63a*^{1} flies lack the *Gr63a* transcript when compared with parental controls, but have normal levels of *Gr21a* (**Fig. 3.7B**).

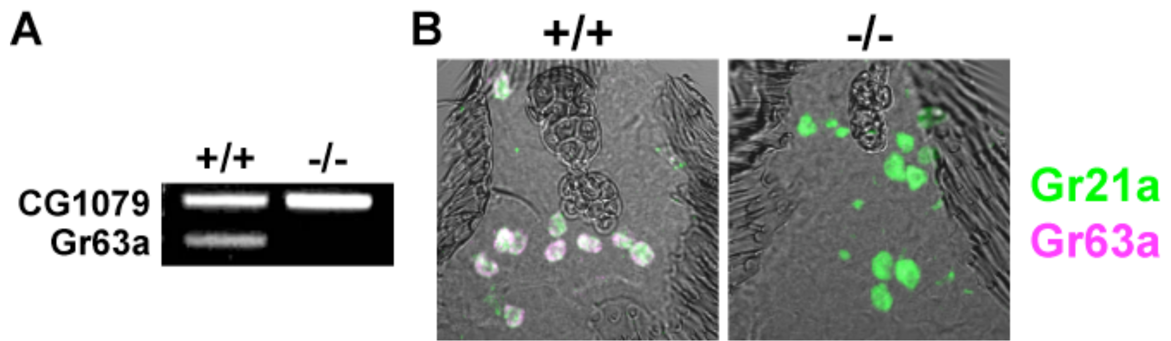

Figure 3.7: *Gr63a[1]* flies lack *Gr63a* coding sequence and mRNA transcript

A) PCR primers corresponding to the locations denoted in figure 3.6 amplify fragments of *Gr63a* and a neighboring gene in wild type flies, but reveal the selective loss of the *Gr63a* coding sequence in *Gr63a[1]* flies. **B)** Fluorescent double *in situ* hybridization of *Drosophila* antennae reveals a selective loss of *Gr63a* mRNA transcript (magenta) in *Gr63a[1]* flies when compared to the wild type parental control strain. *Gr21a* mRNA (green) levels remain unchanged.

Electrophysiological recordings of ab1 sensilla in *Gr63a[1]* flies reveal a complete indifference to stimuli of ~2.25% CO_2, in stark contrast to wild type parental control flies, whose ab1C neurons strongly respond. The *Gr63a[1]* allele is genetically recessive, because sensilla of heterozygous individuals have an essentially wild type CO_2 response. CO_2 responses in *Gr63a[1]* flies are restored by rescuing *Gr63a* expression in the ab1C neurons using the Gal4/UAS system, while control *Gr63a[1]* flies bearing either the *Gr21a*-GAL4 transgene or the UAS-*Gr63a* transgene alone fail to respond (**Fig. 3.8**).

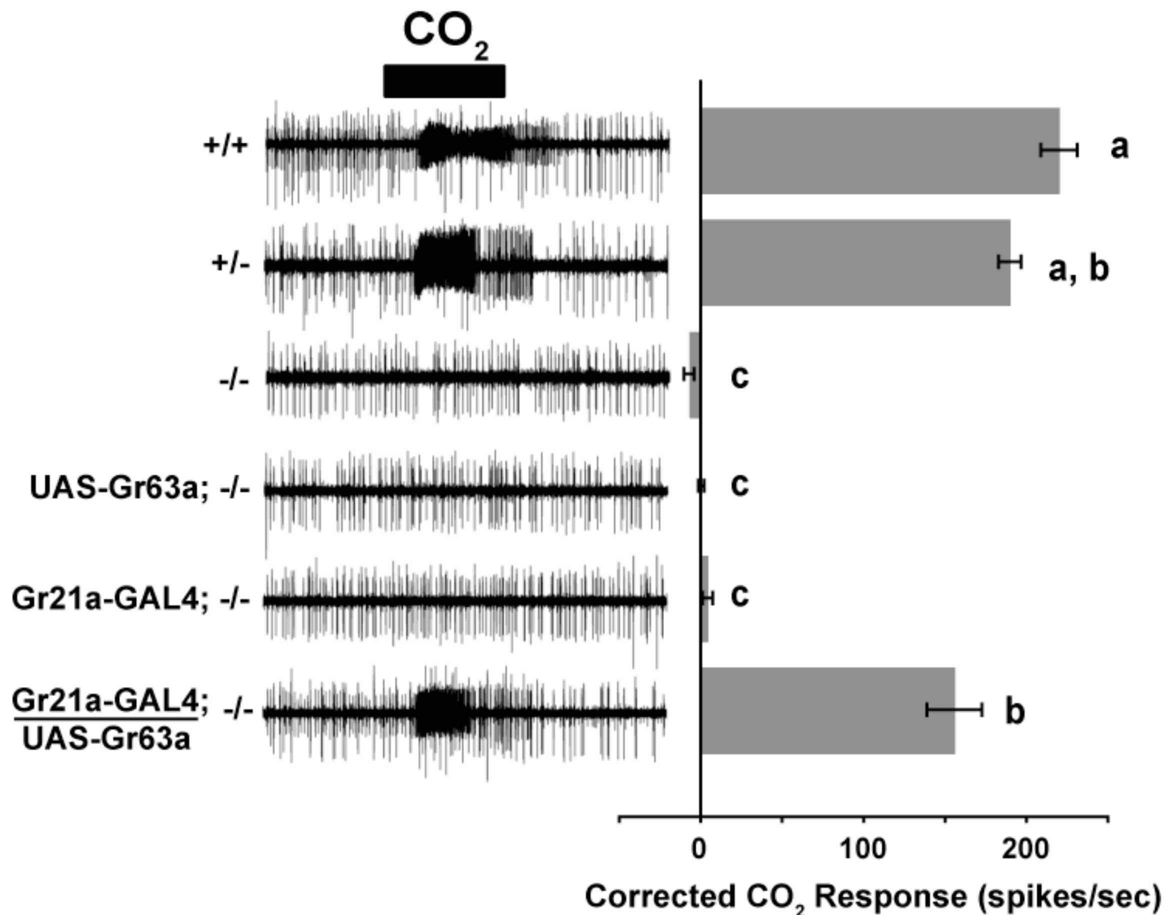

Figure 3.8: *Gr63a*[1] flies are electrophysiologically insensitive to CO_2

Gr63a[1] mutant ab1 sensilla (-/-) do not respond to ~2.25% CO_2 when compared to parental wild type (+/+) or heterozygous (+/-) flies. Responses are rescued by the combination of *Gr21a*-GAL4 and UAS-*Gr63a* in the mutant *Gr63a*[1] background, but not by either transgene alone. Representative traces of sensillar recordings whose response was closest to the mean response are on the left (stimulus bar = 1 sec). Mean corrected CO_2 responses (± SEM; n = 12 antennae for all genotypes with equal numbers of males and females) quantified as in Fig. 3.4 are on the right. After checking the data distributions for normality, statistical significance was calculated using a Tukey HSD test comparing all pairs of means ($p<0.001$). Bars labeled with different letters are significantly different.

Since genetic silencing of the ab1C neurons expressing *Gr21a* eliminates olfactory CO_2 avoidance behavior in a T-maze (Suh et al., 2004), we asked whether *Gr63a*[1] flies also have CO_2 avoidance defects. Whereas the wild type parental control flies robustly avoid CO_2 in a T-maze, *Gr63a*[1] flies fail to

distinguish room air from a ~2% CO_2 stimulus. Consistent with their electrophysiological responses, *Gr63a*[1] heterozygotes show a wild type avoidance response, while *Gr63a*[1] flies bearing either the *Gr21a*-GAL4 or UAS-*Gr63a* transgenes fail to differentiate room air from 2% CO_2. When combined, however, these two transgenes rescue olfactory CO_2 avoidance in the mutant (**Fig. 3.9**).

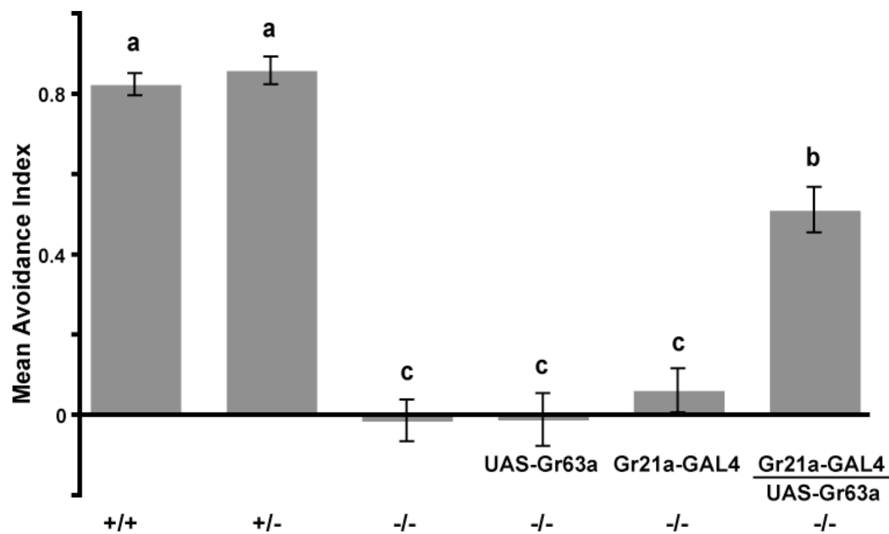

Figure 3.9: *Gr63a*[1] flies are behaviorally insensitive to CO_2

Gr63a[1] flies and the GAL4 and UAS controls are all indifferent to CO_2 in a T-maze, while wild type and heterozygous *Gr63a*[1] flies show robust avoidance. This deficit is rescued in *Gr21a*-GAL4/UAS-*Gr63a*; *Gr63a*[1] flies. Mean avoidance ± SEM is indicated (n = 15 for each genotype). Statistical significance was calculated using a Tukey HSD test comparing all pairs of means ($p<0.01$) after checking the distributions for normality. Bars labeled with different letters are significantly different.

The failure of the rescue to reach wild type levels in either the electrophysiological recordings or the behavior is likely a consequence of the lower levels of *Gr63a* expression in rescued ab1C neurons when compared to wild type ab1C neurons (data not shown). These results prove that *Gr63a* is

necessary for CO_2 chemosensation in *Drosophila* and support our hypothesis that the *Drosophila* CO_2 receptor comprises a heterodimer of both GR21a and GR63a.

3.3.4 Dendritic trafficking of GFP tagged GRs

The requirement of the co-expression of two members of the gustatory receptor family for proper function of the CO_2 receptor is reminiscent of the requirement for heterodimerization of typical ORs with OR83b. We therefore wondered if one of the two CO_2 receptors acts as a chaperoning co-factor delivering its partner to the sensory dendrite in the same way that OR83b functions with the ORs. A version of GR21a with an N-terminal GFP tag traffics to the sensory dendrites in its own ab1C neurons, which lack OR83b. In OR83b-expressing neurons GFP-GR21a cannot reach the dendrites, and instead accumulates in the cell bodies (Benton et al., 2006). This indicates that some unknown factor, present in ab1C neurons, but absent in others, permits the dendritic localization of GFP-GR21a. The most obvious hypothesis is that GR63a provides the missing functionality to traffic GFP-GR21a in non-GR21a neurons. The best way to test this hypothesis would be with custom antibodies against one or the other GR, but we were unable to create a working antibody despite multiple attempts. GFP-tagged GRs were, therefore, the only option to test this hypothesis.

GFP-GR21a never traffics properly in *Or22a/b* neurons even when it is expressed with a functional, untagged version of GR63a. A small amount of

GFP-GR63a traffics to the dendrites alone, but this is dramatically enhanced in the presence of a functional, untagged version of GR21a (**Fig. 3.10**). Thus, it seems that these two GRs are co-dependent in a similar way to that seen in OR/OR83b heterodimers.

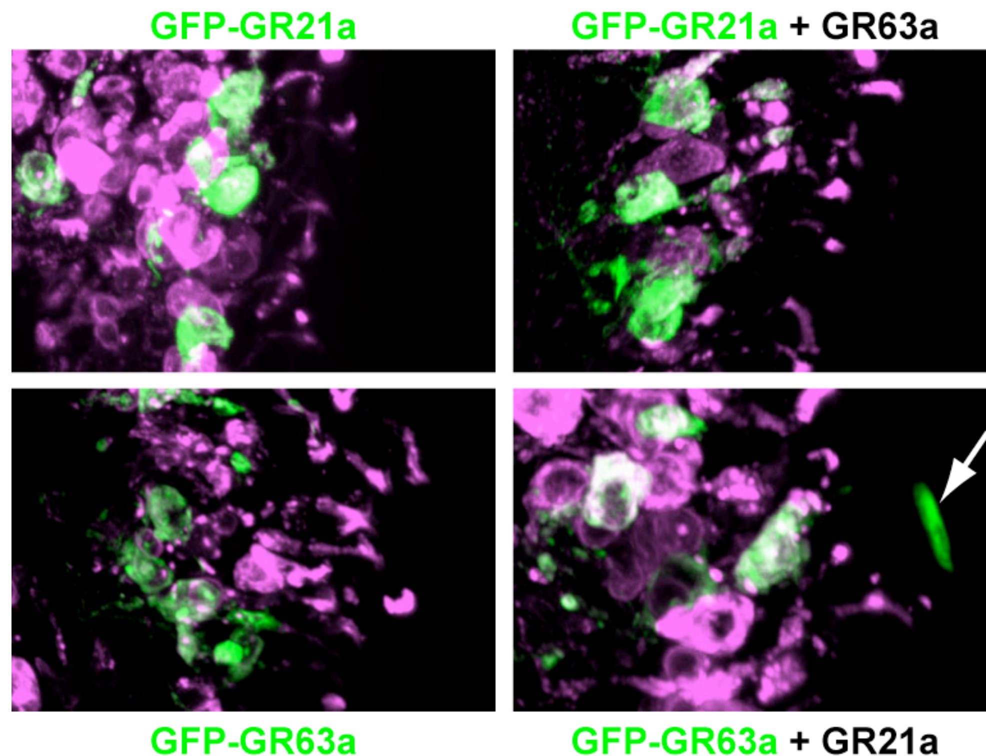

Figure 3.10: Dendritic localization of GFP-GRs in *Or22a/b* neurons

GFP-GR21a (green) expressed either alone or with an untagged GR63a using the *Or22a/b*-GAL4 reagent fails to traffic to the sensory dendrites (top). Dendritic localization of GFP-GR63a (green) is enhanced (white arrow) when it is expressed with an untagged GR21a (bottom). OR83b is counterstained in magenta.

Since ORs generally tolerate amino-terminal tags but not carboxy-terminal tags (Benton et al., 2006), these GR constructs were tagged on the N-terminus in hopes of retaining function. Unfortunately, however, single sensillum recordings

from ab3 sensilla expressing these GFP-tagged combinations fail to respond to CO_2 like the untagged versions do (see Fig. 3.4). Since the GFP tags disrupt the function of these receptors it is impossible to know whether or not the enhanced dendritic trafficking seen in the combination of GFP-GR63a and GR21a is meaningful.

3.3.5 Phylogenetic analysis of the *Drosophila* CO_2 receptors

In order to generalize our results to other insects, we searched the sequenced insect genomes for *Gr21a* and *Gr63a* homologues using the BLAST. *Gr21a* and *Gr63a* are closer in sequence to each other than to other GR genes implying a close evolutionary relationship, but clear homologues of each are easily recognizable in other species. Very similar genes exist in several sister *Drosophila* species, the malaria mosquito *Anopheles gambiae*, the yellow fever mosquito *Aedes aegypti*, and the red flour beetle *Tribolium castaneum*. The other Drosophilids have both *Gr21a* and *Gr63a* homologues, but mosquitoes have *Gr21a* and *Gr63a* homologues (*GPRgr22* and *GPRgr24* respectively in *Anopheles gambiae* (Hill et al., 2002)) and an additional related gene. This gene, *GPRgr23* is slightly closer in sequence to *GPRgr22* than *GPRgr24*. Interestingly, the red flour beetle has *a Gr21a homologue* and a *GPRgr23* homologue, but no recognizable *Gr63a* homologue (**Fig. 3.11**).

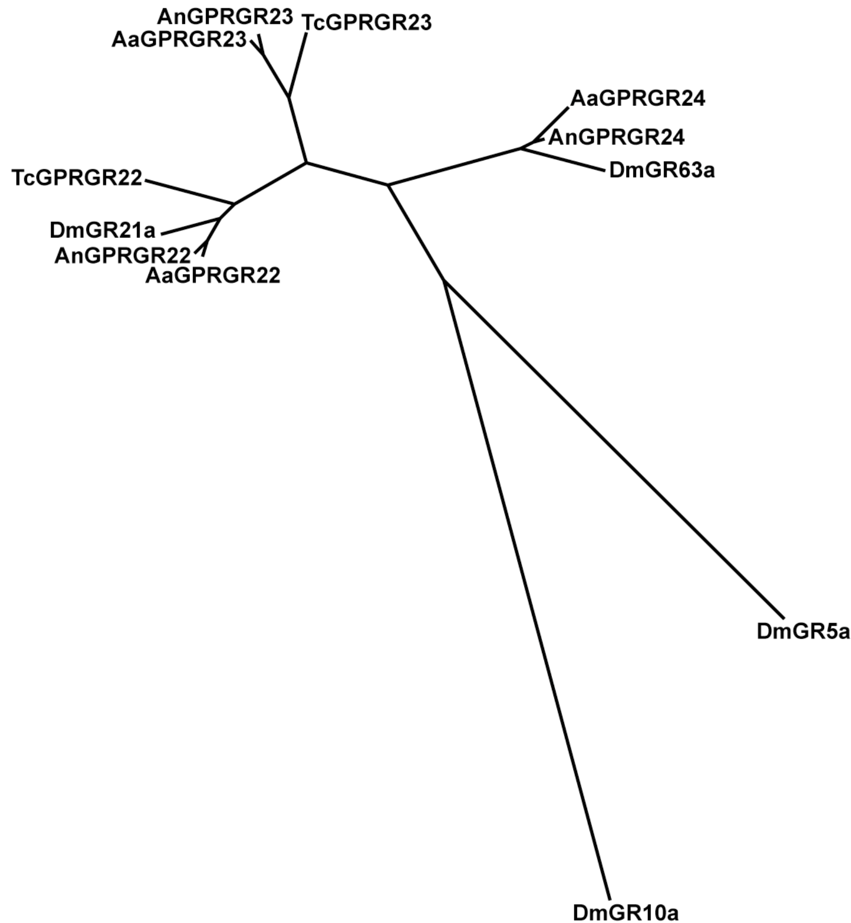

Figure 3.11: CO_2 receptor homologues

This neighbor-joining tree based on an alignment of predicted amino acid sequences reveals the phylogenetic relationships of the CO_2 receptor homologues from several species. *Drosophila* genes are preceded by Dm, *Anopheles* by An, *Aedes* by Aa, and *Tribolium* by Tc. *Drosophila* GR5a and GR10a were added as outgroups.

GPRgr22, *GPRgr23*, and *GPRgr24* are all co-expressed in a population of *Anopheles* maxillary palp neurons, the sensory organ that detects CO_2 in mosquitoes. This expression pattern is consistent with the hypothesis that these receptor homologues, as in *Drosophila*, detect CO_2 in malaria mosquitoes. Also, as in *Drosophila*, these receptors seem to be independent of the *Anopheles* *Or83b* orthologue, *GPRor7* (**Fig. 3.12**). It is unclear, however, if the two

homologues closest to *Gr21a* and *Gr63a* are the only genes required for CO_2 chemosensation or if the mosquito genes have evolved to function as heterotrimers instead of heterodimers.

Figure 3.12: Co-expression of mosquito CO_2 receptor homologues.

A) Fluorescent RNA *in situ* hybridization of *Anopheles gambiae* maxillary palps reveals co-expression of *GPRGr22* (green) and *GPRGr23* (magenta). **B**) *GPRGr22* (green) and *GPRGr24* (magenta) are also co-expressed. **C**) *GPRGr22* (green) and the *Or83b* orthologue *GPROr7* (magenta) are not co-expressed.

In honeybees the situation seems to be quite different. Although bees clearly sense elevated CO_2 levels in their hives and respond with stereotyped climate control behaviors, there are no recognizable homologues of *Gr21a* or *Gr63a* in the newly annotated honeybee (*Apis mellifera*) genome (Robertson and Wanner, 2006). Without homologues of the fly CO_2 receptors, honeybees must use another mechanism. Consistent with this idea, the honeybee CO_2 receptors, which are tuned to CO_2 concentrations ranging from 0-100%, are electrophysiologically distinct from the Dipteran CO_2 receptors, which saturate between 5-10% CO_2 (see Table 3.1). This is also consistent with recent advances in understanding of the evolutionary relationships between the four

major orders of holometabolous insects (i.e. insects with complete metamorphosis); Diptera (flies), Lepidoptera (butterflies and moths), Coleoptera (beetles), and Hymenoptera (bees, wasps, ants). Instead of two sister groups pairing Diptera with Lepidoptera and Coleoptera with Hymenoptera as was previously thought, extensive nuclear gene sequence analysis suggests that Hymenoptera may have branched off first from the base of the holometabolous radiation (Savard et al., 2006). Thus, the absence of *Gr21a* and *Gr63a* homologues in bees could mean that a CO_2 receptor precursor gene arose in a gustatory receptor subfamily expansion over 275 million years ago and then diversified such that its derivatives exist in all other extant holometabolous insects. With the sequencing of more insect genomes, especially those of Lepidopterans, the phylogeny of the CO_2 receptors may become more clear.

3.4 Conclusion

Taken together, these data reveal the role of a pair of chemosensory receptors as the proteins responsible for CO_2 detection in *Drosophila melanogaster*. *Gr21a* and *Gr63a* are co-expressed in *Drosophila* CO_2 neurons. Transgenic misexpression confers dose-dependent CO_2 sensitivity on neurons that do not normally respond to CO_2. Selective deletion of *Gr63a* disrupts CO_2 detection and CO_2-evoked behaviors. Homologues of these receptors exist in mosquitoes, which along with *GPRgr23* are likely responsible for the CO_2-evoked host seeking behaviors that allow mosquitoes to act as human disease vectors.

4 Implications of the current study and prospects for future research

This dissertation describes the results of two separate investigations, both using the model organism, *Drosophila melanogaster*, into distinct chemosensory pathways that insects use in host-seeking behaviors. The first pathway involves the atypical OR *Or83b,* which heterodimerizes with other ORs to form a functional chemosensory complex in OSN dendrites. *Or83b* has been conserved over millions of years of evolution as a generic partner that is broadly co-expressed with more typical ORs. This pathway is responsible for sensing most of the host volatiles that direct insects to their preferred food sources; fruit flies to rotting fruit and female mosquitoes to humans.

The second pathway is independent of *Or83b* and is responsible for detection of another chemical cue that is of general interest to most insects, carbon dioxide. In *Drosophila*, CO_2 is detected by a pair of chemosensory receptors belonging to the gustatory receptor family of proteins. These receptors, unlike *Or83b* seemed to have co-evolved and are specifically paired with each other in both mosquitoes and flies (although *Anopheles* CO_2 neurons also express another related receptor). Although its significance is unclear, it is interesting to note that both the *Or83b*-dependent odor perception pathway and the *Or83b*-independent CO_2 pathway rely on presumed heteromeric receptor complexes. Since it exists in both ORs and GRs, this is likely an ancient feature originating in the earliest insect lineages that merits further investigation.

The division of the chemosensory receptor superfamily in *Drosophila* into ORs and GRs was made on the basis of sequence similarity, but we now know that some proteins classified as GRs are expressed in olfactory organs and respond to volatile ligands. Thus, the division into gustatory and olfactory halves may not be as clean as previously thought. Understanding the interface between these divisions of the chemosensory receptor superfamily may lie in both the sequencing of many more insect chemosensory receptor repertoires and in further analysis of the relationship of the structure of *Or83b* to its function, as it is the closest OR in sequence to the GRs. It will be interesting to learn what structural motifs are responsible for various properties of ORs and GRs; specifically, the protein domains responsible for trafficking, coupling to other receptors or the signal transduction machinery, ligand specificity, the promoter elements that determine expression patterns in olfactory versus gustatory neurons, etc.

The CO_2 receptors themselves also merit further research. CO_2 is an important environmental cue in so many insect lineages, but the CO_2 receptor neurons in different insects have distinct electrophysiological properties. In addition, beetles are the most divergent insects that have homologues of the *Drosophila* CO_2. Thus, CO_2 receptors seem to have originated at multiple points over the course of evolutionary history. Identification of these other molecular mechanisms for CO_2 detection and understanding their evolutionary history will be very interesting.

At this point, it is also unclear exactly what CO_2 receptors are detecting. Molecular CO_2 is only slightly soluble in water, and therefore in the sensory lymph surrounding the CO_2 neuron dendrites. It is possible that the CO_2 receptors actually detect changes in pH or the concentration of bicarbonate (HCO_3^-) ions, which are immensely more water-soluble. In this case, the activity of antennal carbonic anhydrase (CA) enzymes, which catalyze the hydration of molecular CO_2 into bicarbonate, would be crucial for the quick response to changing CO_2 concentrations that have been observed in a number of insects. In rather weak support of this idea, CA activity has been found in subpopulations of OSNs in several organisms (not insects) that are known to respond to CO_2 (Brown et al., 1984; Coates et al., 1998; Kimoto et al., 2004; Wong et al., 1983). Although it may have no relevance to the Dipteran CO_2 receptors because of their lack of *Gr21a* and *Gr63a* homologues, honeybee CO_2 neurons are dramatically affected by the CA inhibitor acetazolamide (Stange, 1974).

In a mechanism coupled to bicarbonate production through CA enzymes, CO_2 detection in species lacking *Gr21a* and *Gr63a* homologues may occur through a cytoplasmic signal transduction mechanism involving soluble adenylyl cyclase (sAC). Rodent sAC is a sensitive intracellular bicarbonate sensor required for the activation of spermatozoa that makes them competent to fertilize an egg (Chen et al., 2000). Related transmembrane versions of the sAC catalytic domain function in CO_2/HCO_3^- sensing in fungal pathogens (Klengel et al., 2005). This gene, although not present in *Drosophila* (Roelofs and Van Haastert, 2002), is present in red flour beetles (Genbank accession number XP 966646.1) and

mosquitoes (Kobayashi et al., 2004). Since honeybees have the only published genome that lacks homologues of the fly CO_2 receptors, it will be especially interesting to learn if bees use sAC in olfactory CO_2 detection. In addition to the sACs that are activated by bicarbonate, a bacterial adenylyl cyclase that is activated by molecular CO_2 instead of bicarbonate has also been reported (Hammer et al., 2006). Many more experiments will be required to determine the active chemical ligand in insect CO_2 chemosensation.

The non-adapting nature of the CO_2 receptors, which sets them apart from other chemosensory receptors, could imply the existence of two distinct signal transduction pathways. Many investigators in the field of insect olfaction maintain that, like the vertebrate ORs, the insect chemosensory receptors must be GPCRs because of their seven predicted transmembrane segments. No reasonable experimental evidence supports this conclusion, and, in fact, some evidence points to the opposite. The insect chemosensory receptors seem to be a novel family of seven-pass transmembrane proteins, and although it is purely speculative, they may be odor-gated ion channels, bypassing the need for a distinct signal transduction system (Benton, 2006).

It is also possible that the insect CO_2 receptors are not receptors at all, but gas channels required for the entrance of CO_2 into the cells where it can reach the actual receptor proteins. As far fetched as this may sound, there is precident for this hypothesis. The Amt proteins of plants, bacteria, and yeast form gas channels for the passage of ammonia (Khademi et al., 2004). Some evidence exists implicating the only homologues of these proteins in higher eukaryotes, the

Rh proteins, as gas channels for molecular CO_2 (Kustu and Inwood, 2006; Soupene et al., 2002). For any hypothesis that relegates GR21a/GR63a to a accessory role in the process of CO_2 chemosensation to prove accurate, whatever the true CO_2 receptor is, it must at least be present in the acetate sensitive ab3A neurons, because GR21a and GR63a were sufficient to confer CO_2 sensitivity on these neurons.

Experimental evidence supporting any of these ideas will be hard to come by until reliable *in vitro* assays can be developed to make biochemical and biophysical analysis of insect chemosensory receptors feasible. Determining the rules of proper receptor trafficking and protein folding for *in vitro* cell expression systems, now in their infancy, is clearly the next important hurdle to overcome in order to advance our understanding of insect receptor molecular biology.

These expression systems will also be crucial in the development of novel insect control measures. Despite living in the modern era of molecular medicine, millions of people are still at risk of contracting and dying from largely treatable illnesses that are spread by disease vector insects. In almost all of the developing world the control of malaria, which is the greatest parasitic threat to human health, is grossly inadequate (Phillips, 2001). It is clear that the fight against malaria could greatly benefit from new insect control measures rationally designed to target the chemosensory systems that mosquitoes use to detect their human hosts. Understanding the molecular mechanisms of host odor and CO_2 detection by mosquitoes coupled with an *in vitro* system optimized for high-throughput screening of chemical libraries will speed the discovery of OR/GR

inhibitors that could help make humans harder to detect. Although it is always wise to temper our enthusiasm with significant pragmatism, the combination of such chemical inhibitors with existing and future infection control and disease treatment methods could make malaria the next modern medical and public health success story.

Publications

The original findings described herein are also reported in the following:

Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., and Vosshall, L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for Drosophila olfaction. Neuron *43*, 703-714.

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