The Pennsylvania State University
The Graduate School
College of Agricultural Sciences

PHEROMONE-RELATED OLFACTORY NEURONAL PATHWAYS OF MALE HELIOTHINE MOTHS

A Thesis in
Entomology
by
Seong-Gyu Lee

© 2006 Seong-Gyu Lee

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

May 2006
The thesis of Seong-Gyu Lee has been reviewed and approved* by the following:

Thomas C. Baker  
Professor of Entomology  
Thesis Adviser  
Chair of Committee

James H. Tumlinson  
Professor of Entomology

James L. Frazier  
Professor of Entomology

James H. Marden  
Professor of Biology

Gary Felton  
Professor of Entomology  
Head of the Department of Entomology

*Signatures are on file in the Graduate School.
ABSTRACT

Two sympatric species of heliothine moths, *Helicoverpa zea* and *Heliothis subflexa* were used to study aspects of sex pheromone olfaction that had previously been overlooked in this group. The objective of my research was to increase our understanding of the functional morphology of the sex pheromone olfactory pathways of these species, focusing on the portion occurring at the level of the antennal olfactory receptor neurons (ORNs) and their axonal projections into specific glomeruli in the antennal lobe of the brain.

My research showed that each type of ORN that responds to a particular pheromone-related compound and housed in specific sensillar types projects with nearly 100% fidelity to a particular glomerulus in the macrogglomerular complex (MGC) of the antennal lobe in both species. My findings show that in these noctuid species, the pheromone-component-specific olfactory pathways are linear. That is, the pheromone-component-specific information travels along the axons of ORNs tuned to a particular sex pheromone component, into a glomerulus dedicated to receiving inputs from only that type of ORN, and then directly out from that same glomerulus to higher centers in the protocerebrum via the axons of projection interneurons. My results add to the evidence from other heliothine moth species that there are only linear pathways through each pheromone-component-specific glomerulus and on to higher centers.

The ORNs of both species that are tuned to the major pheromone component, (Z)-11-hexadecenal (Z11-16:Ald) project their axons to a specific glomerulus, the cumulus, with no exceptions for either *H. zea* or *H. subflexa*. Each of the two co-
compartmentalized ORNs in another sensillum type on *H. zea* antennae always project to the two smaller glomeruli in the MGC, the dorso-medial anterioir (DMA) and dorso-medial posterior (DMP) glomeruli. One of these two ORNs is tuned to this species’ minor sex pheromone component (Z)-9-hexadecenal (Z9-16:Ald), and this is the ORN type that projects to, and arborizes in, the DMP. The other ORN type is tuned to two compounds that are antagonistic to attraction, (Z)-11-hexadecen-ol (Z11-16:OH) and (Z)-11-hexadecenyl acetate (Z11-16:Ac). This is the ORN type that projects to the DMA.

The projection destinations of ORNs from the two successful stainings I was able to obtain from a third type of sensillum having a single responsive ORN tuned to (Z)-9-tetradecenal, another antagonist to attraction, were the same as for the type of sensillum described above, housing an ORN tuned to Z9-16:Ald and a second ORN tuned to both Z11-16:Ac and Z11-16:OH. This result shows for the first time that this third class of sensillum is actually the same type as the second class described above, but with the ORNs being less responsive or entirely unresponsive to these pheromone-related compounds.

The ORN stainings in the second and third types of sensilla on *H. subflexa* antennae clarified that for this species, too, the pathways for pheromone-component-specific information are linear through the glomeruli in the MGC. As in *H. zea*, the cumulus is the primary olfactory processing center for the major pheromone component, Z11-16:Ald. Information about the minor pheromone component, Z9-16:Ald, is conveyed through a glomerulus in this species called the dorso-medial (DM) glomerulus, and information about the other minor *H. subflexa* pheromone component, Z11-16:OH, travels through the antero-medial (AM) glomerulus. Finally, the Z11-16:Ac-responsive
ORN that is co-compartmentalized with the Z11-16:OH-responsive ORN sends its excitation into a ventro-medial (VM) glomerulus. Thus my findings show that specific glomeruli of the MGC of both species are dedicated to receiving particular pheromone-compound-specific information that is critical for discriminating conspecific pheromone blends from pheromone blends emitted by females of other species.

During the cobalt staining studies of Z11-16:Ald-tuned ORNs in both *H. zea* and *H. subflexa*, I found that there were often secondary, “unresponsive” neurons that also became stained, and these consistently arborized in a particular glomerulus situated posterior to the MGC. I recognized that this glomerulus resided in a previously overlooked cluster of glomeruli, which I named the Posterior Complex, or “PCx”. The specific glomerulus in which these ORNs arborized I named “PCx1”. In *H. subflexa* I found that there was also a second type of unresponsive ORN that was co-compartmentalized with the ORN tuned to Z9-16:Ald and which consistently projected to another specific glomerulus in the *H. subflexa* PCx, which I named the PCx4.

For both species, in the process of using large arrays of different classes of odorant molecules to determine whether these secondary ORNs were truly unresponsive, I found that these very small-spiking ORNs exhibited significantly increased spike frequencies whenever the large-spiking ORN responded to Z11-16:Ald. A hydrocarbon analog of Z11-16:Ald, 1,Z12-hexadecadiene, also was effective in evoking tonic firing from these secondary ORNs in *H. zea* but was not consistently effective for the secondary ORNs of *H. subflexa*. It is possible that the excitation of the secondary ORN coincides with that of the large-spiking ORN because the ligand for the secondary ORN is a Z11-16:Ald degradation product produced in the sensillum lymph when Z11-16:Ald is
presented. In both species, the lack of significant activity of this secondary ORN in response to the large array of other, general odorant compounds that was tested is also supportive of the idea that the secondary ORNs are responding to a Z11-16:Ald metabolite. I did not find any effective ligand for the secondary ORNs in *H. subflexa* sensilla co-compartmentalized with the ORN responsive to Z9-16:Ald. It is still not possible to conclude that this ORN is truly unresponsive to any odorant.
# TABLE OF CONTENTS

LIST OF FIGURES .............................................................................................................. xi

LIST OF TABLES .................................................................................................................... xiv

ACKNOWLEDGEMENTS ................................................................................................. xv

CHAPTER 1: INTRODUCTION ................................................................................................. 1
Insect sex pheromone olfaction ......................................................................................... 1
Insect olfactory pathway .................................................................................................... 2
   General .......................................................................................................................... 2
   Upwind flight behavior depends on pheromone strands in the plume ......................... 8
Functional morphology of Elements of the olfactory pathway ...................................... 11
   Sensilla trichodium ...................................................................................................... 11
   Sensillar cuticle .......................................................................................................... 18
   Sensillar lymph .......................................................................................................... 18
Pheromone binding protein ............................................................................................ 19
Pheromone-degrading enzymes ..................................................................................... 20
Heliothine moth pheromone ODEs ............................................................................... 22
Olfactory receptor neurons ............................................................................................ 22
Antennal lobe .................................................................................................................. 24
Sex pheromone olfaction in heliothine moths ................................................................. 28
   Behavioral responses ................................................................................................. 29
   Olfactory receptor neuron tuning related to behavior .................................................. 31
The dissertation chapters ................................................................................................. 33
References ........................................................................................................................ 36
CHAPTER 2: ANTENAL LOBE PROJECTION DESTINATION OF _HELICOVERPA ZEA_ MALE OLFATORY RECEPTOR NEURONS RESPONSIVE TO HELIOTHEINE SEX PHEROMONE COMPONENTS ........45

Abstract ............................................................................................................................................46

Introduction .........................................................................................................................................46

Material and Methods .....................................................................................................................49

Insects .................................................................................................................................................49

Chemical stimuli ................................................................................................................................49

Single-sensillum recordings ............................................................................................................50

Staining of ORNs with cobalt lysine ............................................................................................51

Calcium imaging ............................................................................................................................53

Results .................................................................................................................................................55

Single sensillum recording and staining .......................................................................................58

Type-A sensilla ................................................................................................................................58

Type-B sensilla ..................................................................................................................................59

Type-C sensilla ..................................................................................................................................60

Calcium imaging ............................................................................................................................61

Discussion ............................................................................................................................................70

References ..........................................................................................................................................76

CHAPTER 3: ANTENAL LOBE PROJECTION DESTINATION OF _HELITHIS SUBFLEXA_ MALE OLFATORY RECEPTOR NEURONS RESPONSIVE TO HELIOTHEINE SEX PHEROMONE COMPONENTS ........80

Abstract ............................................................................................................................................80

Introduction .........................................................................................................................................81

Materials and Methods ....................................................................................................................84

Insect ..................................................................................................................................................84
Chemical stimuli

Single-sensillum recording

Staining of ORNs with cobalt-lysine

Immunocytochemistry

Image processing and figure preparation

Results

Type-A sensilla

Type-B sensilla

Type-C sensilla

Discussion

References

CHAPTER 4: ACTIVITY OF HELICOVERPA ZEA AND HELIOTHIS SUBFLEXA SECONDARY OLFATORY NEURONS CO-COMPARTMENTALIZED WITH NEURONS RESPONSIVE TO SEX PHEROMONE COMPONENTS

Abstract

Introduction

Materials and Methods

Insect

Chemicals

Single-sensillum recording

Data analysis

Results

Type-A sensilla of H. zea

Type-A sensilla of H. subflexa

Type-B sensilla of H. subflexa
LIST OF FIGURES

Fig. 1-1 Brain structures involved in lepidopteran insect olfaction in a top view of a *H. zea* male moth’s head .................................................................4

Fig. 1-2 Schematic diagram of an insect olfactory neuronal pathway....................5

Fig. 1-3 Internal morphology of lepidopteran olfactory sensilla ..............................6

Fig. 1-4 external morphology of heliothine moth antenna and sensilla if *H. zea* ......13

Fig. 1-5 Sexual dimorphism in heliothine moth antennae ......................................14

Fig. 1-6 Topographical distribution of sensillar types of male *H. subflexa* antennae ........................................................................................................15

Fig. 1-7 Topographical distribution of sensillar types of male *H. virescens* antennae .........................................................................................................16

Fig. 1-8 Topographical distribution of sensillar types of male *H. zea* antennae ......17

Fig. 2-1 Dorsal view the antennal lobe of *H. zea* brain .......................................56

Fig. 2-2 Schematic frontal view diagram of the glomerular topographies of the MGC and the PCx in the male *H. zea* AL .........................................................57

Fig. 2-3 Axonal projection pattern of *H. zea* type-A sensillum ORNs ..................63

Fig. 2-4 Axonal projection pattern of *H. zea* type-B sensillum ORNs ..................65

Fig. 2-5 Axonal projection pattern of *H. zea* type-C sensillum ORNs ..................66

Fig. 2-6 Calcium imaging activity during stimulation of antennal ORNs with pheromone-related compounds .................................................................67

Fig. 2-7 Schematic diagram showing the arborization destinations of ORNs from the three classes of long trichoid sensilla on *H. zea* male antenna ..................60

Fig. 3-1 Dorsal view the antennal lobe of *H. subflexa* brain .................................91

Fig. 3-2 Schematic frontal view diagram of the glomerular topographies of the MGC and the PCx in the male *H. subflexa* AL .......................................................93

Fig. 3-3 Spike trains and axonal projection pattern of *H. subflexa* type-A sensillum ORNs .................................................................94
Fig. 3-4 Dorsal views of stained neuronal arborization from a type-A sensillum

Fig. 3-5 Spike trains and axonal projection pattern of *H. subflexa* type-B sensillum ORNs

Fig. 3-6 Axonal projection pattern of *H. subflexa* type-B sensillum ORNs having additional ORNs targeting ordinary glomerulus

Fig. 3-7 Spike trains and axonal projection pattern of *H. subflexa* type-C sensillum ORNs

Fig. 3-8 Axonal projection pattern of *H. subflexa* type-C sensillum ORNs having additional ORNs targeting ordinary glomeruli

Fig. 3-9 Schematic diagrams of the sensillar compartmentalization arrangements and projection destinations of olfactory receptor neurons in type-A, 1-B, and -C trichoid sensilla of four heliothine moth species

Fig. 4-1 Electrophysiological activities of ORNs in *H. zea* type-A sensillum in response to Z11-16:Ald

Fig. 4-2 Electrophysiological activities of ORNs in *H. zea* type-A sensillum in response to 1,(Z)12-17:Hy

Fig. 4-3 Spike activities of ORNS in a *H. zea* type-A sensillum in responses to different dosages of Z11-16:Ald and the 1, Z12-17:Hy

Fig. 4-4 Activity comparisons between large-spiking ORN and secondary ORN of *H. zea* type-A sensilla in responses to Z11-16:Ald and 1, Z12-17:Hy

Fig. 4-5 Activities of secondary ORNs in *H. zea* type-A sensilla in responses to minor constituents of pheromone glands and to minor pheromone components of three heliothine moths

Fig. 4-6 Spike trains of *H. zea* type-A sensilla in responses to minor constituents of pheromone glands and to minor pheromone components of three heliothine moths

Fig. 4-7 Responses of a secondary (small-spiking) ORN (in red above) in a *H. zea* type-A sensillum to stimulation with long-chain carboxylic acids and 6 general odorant chemical sets

Fig. 4-8 ORNs Activities of *H. zea* type-A sensilla in responses to carboxylic acids (100 µg) and general odorants sets (10 µg/compound)

Fig. 4-9 Electrophysiological activities of ORNs in *H. subflexa* type-A sensillum in response to Z11-16:Ald
Fig. 4-10 Electrophysiological activities of ORNs in *H. subflexa* type-A sensillum in response to 1,(Z)12-17:Hy ................................................................. 145

Fig. 4-11 Spike activities of ORNs in a *H. subflexa* type-A sensillum in responses to different dosages of Z11-16:Ald and the 1, Z12-17:Hy ......................... 146

Fig. 4-12 Activity comparisons between large-spiking ORN and secondary ORN of *H. subflexa* type-A sensilla in responses to Z11-16:Ald and 1, Z12-17:Hy .... 147

Fig. 4-13 Activities of secondary ORNs in *H. subflexa* type-A sensilla in responses to minor constituents of pheromone glands and to minor pheromone components of three heliothine moths .................................................. 148

Fig. 4-14 Activities of secondary ORNs in *H. subflexa* type-A sensilla in responses to minor constituents of pheromone glands and to minor pheromone components of three heliothine moths .................................................. 149

Fig. 4-15 Spike trains of *H. subflexa* type-A sensilla in responses to minor constituents of pheromone glands and to minor pheromone components of three heliothine moths ........................................................................ 150

Fig. 4-16 ORNs Activities of *H. subflexa* type-A sensilla in responses to carboxylic acids (100 µg) and general odorants sets (10, and 100 µg/compound) .................................................................................................. 151

Fig. 4-17 Spike trains of *H. subflexa* type-B sensilla in responses to minor constituents of pheromone glands .................................................................. 152

Fig. 4-18 Spike trains of *H. subflexa* type-B sensilla in responses to minor pheromone components of three heliothine moths ........................................................................ 153

Fig. 4-19 Activities of the secondary ORN in *H. subflexa* type-B sensilla responding to carboxylic acids and the six general odorant sets ............ 154

Fig. 4-20 Responses of the large-spiking and secondary ORN in a *H. subflexa* type-B sensillum ........................................................................................................ 155

Fig. 5-1 Confocal images of male *H. zea* antennal lobe .............................................. 177

Fig. 5-2 Confocal images of female *H. zea* antennal lobe ........................................... 178

Fig. 5-3 Confocal images of male *H. subflexa* antennal lobe ....................................... 179

Fig. 5-4 Confocal images of female *H. subflexa* antennal lobe ................................... 180

Fig. 5-5 3-D reconstruction images of male *H. zea* MGC and PCx .......................... 181
LIST OF TABLES

Table. 2-1 Summary of the glomerular arborization destinations of olfactory receptor neurons from three different types of *H. zea* male sensilla ....................62

Table. 2-2 Summary of the focal points of glomerular activities of *H. zea* MGC.......68

Table. 3-1 Summary of the glomerular arborization destinations of olfactory receptor neurons from three different types of *H. subflexa* male sensilla ............105

Table. 4-1 Chemical sets utilized in cartridges for a high-throughput survey of the responsiveness of secondary ORNs in the type-A sensilla of *H. zea* and *H. subflexa*, and in the *H. subflexa* type-B sensilla to possible ligands. ....................138
ACKNOWLEDGMENTS

I am indebted to my advisor, Dr. Thomas C. Baker, for his guidance, limitless support, and encouragement throughout my entire graduate student life. Especially I am deeply grateful for his patience and sacrifice during the writing process. He is more than research advisor to me.

I would like to express my gratitude to Dr. James H. Tumlinson, Dr. James L. Frazier, and Dr. James H. Marden for their vision, support, and their advice to me while serving on my committee.

I also would like to thank fellow lab members: Kye-Chung Park and Bryan Banks, who provided timely materials required for my research, Mike Domingue for helping me with statistical analyses, and Jon Lelito for encouraging me over my tough times. They are not just collaborators but great friends.

I would like to acknowledge to Dr. Peter Teal, Dr. Tappey Jones, Dr. Jim Tumlinson, and Dr. Katalin Boroczki, who kindly provided me with important chemicals for my research. I also would like to acknowledge the staff in the Center for Quantitative Cell Analysis and the Electron Microscopy Facility in the Huck Institutes of the Life Science for their kind assistance in helping me get nicer images.

Words are not enough to express how much I thank to my parents. They have sacrificed all the way in the name of love.
CHAPTER 1

INTRODUCTION

Insect Sex Pheromone Olfaction

Sex pheromones have long intrigued a diverse array of biologists, including chemical ecologists, neurophysiologists, ethologists, evolutionary biologists and economic entomologists. The powerful ability of sex pheromones to elicit attraction from long distances with just nanogram amounts, their high species-specificity, and their demonstrated efficacy for applied uses in pest management systems have prompted a large amount of research and development effort.

The high sensitivity of male moths to sex pheromones is due to their exquisitely evolved olfactory system, and the first elements in this system are the olfactory receptor neurons (ORNs), which transduce the chemical signal into electrical impulses in these sensory neurons. The ORNs of the male silk moth, *Bombyx mori*, have been calculated to generate action potentials in response to even one pheromone molecule finding its way to an ORN’s receptor-laden surface on its dendrite (Kaissling and Priesner 1970; cited in Kaissling 1996). These same studies calculated that a mere one impulse per second by 1% of pheromone receptor cells can elicit a behavioral reaction. The high species-specificity of sex pheromone receptors allows the ORNs to be strongly differentially
tuned so that their differential patterns of reporting with their action potentials to higher centers in the brain allows these centers to discriminate very slight differences in blend quality, such as occurs between two closely related species (c.f., Todd and Baker 1999). However, these are just the beginning features of the insect olfactory system that contribute to sensitivity and specificity. A brief overview of this entire system will help give perspective to the aspects of the heliothine moth olfactory system that I investigated in this study and to the importance of these findings.

**Insect Olfactory Pathways**

**General**

The main elements in the insect sex pheromone olfactory pathway that result in upwind flight behavior are the sensillum trichodeum, the olfactory receptor neuron (ORN), the glomeruli in the macroglomerular complex (MGC) of the antennal lobe, and the mushroom body (see below) (Fig. 1-1, 1-2). The particular section of the insect olfactory pathway that I was interested in exploring in my study was from the sensillum trichodeum to the glomeruli via the ORNs. My goal was to chart the locations of glomeruli in the MGC to which physiologically pre-characterized ORNs projected their axons. Thus I would be using a functional anatomy approach to understanding sex pheromone olfaction for this part of the pathway.

The primary olfactory organs in insects are the olfactory sensilla, which are cuticular sensory structures arranged in various arrays across the antennae (reviewed by...
Keil 1999). A sensillum has housed within it from one to several olfactory receptor neurons (Fig.1-3). The dendrites of the ORNs reside in sensillum lymph comprised mainly of odorant binding protein (OBP) and odorant degrading enzyme (ODE). Imbedded in the membrane of the dendrite are odorant receptors (Ors) that have particular affinities for particular odorant molecules or classes of odorant molecules. When the right odorant molecules arrive at the receptor on the dendrite after passing through the pore tubules and through the binding protein, the dendrite’s membrane porosity increases following an internal G-coupled protein signal cascade, and ions into and out of the membrane, depolarizing it. If the depolarization is great enough, action potentials are generated at the base of the cell body, and these travel without synapses to the antennal lobe of the brain (Fig. 1-2). The antennal lobe is the first synaptic area in the olfactory system, and it lies at the base of each antenna. It is a highly organized structure comprised of knots of neuropil called glomeruli (Fig. 1-1). The axons of particular ORNs from the antenna that are tuned to one particular small set of odorant molecules all project to the same glomerulus, and thus that glomerulus receives action potentials only due to the excitation of the ORNs tuned to one type of odorant. The axons of another set of ORNs tuned to a different small set of odorant molecules all project to a different glomerulus, and so on (Fig. 1-2). After various kinds of signal processing by networks of local interneurons that synaptically interconnect the glomeruli and feed back in various ways to the incoming axons as well as to other interneurons, the odorant information emerges from the glomeruli in the form of action potentials generated by output interneurons called projection interneurons (Fig. 1-2). Only a very few projection interneurons come out of each glomerulus, and they send their axons up to still higher
Brain structures involved in lepidopteran insect olfaction in a top view of a *Helicoverpa zea* male moth’s head, (horizontal section). The back of the moth’s head is at the top of the page. The part below the dotted line (anterior) is the deutocerebrum (antennal lobes or olfactory lobes), and upper (posterior) part is the protocerebrum. The antennal lobes are comprised of glomeruli and neuronal cell bodies. A male-specific glomerular sub-cluster within the antennal lobe, called the macroglomerular complex (MGC), is engaged in sex pheromone olfaction. Ordinary glomeruli (Ord) are the most numerous in the antennal lobe, and are known to be involved in general odorant olfaction. A newly described glomerular sub-cluster called the Posterior Complex (PCx) lies posterior to the MGC (see Chapters 2, 3). Mushroom bodies (MB) and lateral protocerebra (LP) are the axonal destinations of olfactory projection neurons carrying odor information out of the antennal lobe (c.f. Anton and Homberg 1999, Hansson 1995). The mushroom bodies are known to be associated with odor memory. The figure was produced with small modification from Fig. 2-1C. MB mushroom body, LP lateral protocerebrum, Ord ordinary glomeruli, P posterior, L lateral. Scale bar 100 µm.
Figure 1-2
Schematic diagram of an insect olfactory neuronal pathway from one antenna and into and out of one antennal lobe. A specific class of olfactory receptor neurons (ORNs) on the antenna project their axons to the specific target glomerulus directly without intermittent synaptic junctions. Projection neuron dendrites have synaptic connections with corresponding ORN and local interneuron axons. Local interneurons modulate inter-neuronal communication between glomeruli in the antennal lobe through inhibitory synaptic junctions. The array of projection interneurons exiting the antennal lobe sends olfactory information via an across-interneuron pattern of action potentials to higher-order neurons that ‘read’ the pattern in the mushroom body and lateral protocerebrum of the protocerebrum.
Figure 1-3
Internal morphology of trichoid sensilla, with basiconic sensilla for comparison. A Cross section of a *H. zea* male antenna. Arrow indicates a section in which a long trichoid sensillum is visible in the equatorial region next to where the scale region begins. B Schematic diagram of trichoid sensillum housing two ORNs. Cell bodies of the ORNs are surrounded by the accessory cells, named tormogen (To), trichogen (Tr), and thecogen (Th). The sensillum cuticle has pores (black arrowhead) with pore tubules (yellow arrowhead). Dendrites (D) are not branched in trichoid sensilla (Diagram courtesy of Prof. Dr. Ali Steinbrecht, Max Planck Institute, Seewiesen, Germany) C-F Transmission electron micrographs of sensilla of three heliothine moth species. C Cross section image of a basiconic sensillum of *Heliothis subflexa*. Pores (black arrowheads), pore tubules (yellow arrowheads), and numerous dendrites are visualized. D Trichoid sensillum of *H. subflexa* having one dendrite in the sensillum lymph (SL). E *H. zea* trichoid sensillum having two dendrites (D) bathed in sensillum lymph (dark material). One dendrite is big and the other is small. F Basiconic sensillum of *Heliothis virescens*. Basiconic sensilla are morphologically characterized by highly branched dendrites (“D” is place over one of the branches), and more pores in a thinner cuticle wall than for trichoid sensilla. Black arrow heads indicate pores. Sc scales, AB axonal bundle, T trachea, H hemolymph hollow (hemocoel), D dendrite, SL sensillum lymph, To tormogen, Tr trichogen, Th thecogen.
integrative structures in the brain, the mushroom bodies and the lateral protocerebrum (Fig. 1-2).

**Upwind Flight Behavior Depends on Pheromone Strands in the Plume.**

Because sex pheromone systems have fairly small and simple sets of pheromone component odorants and very narrowly tuned ORNs, one can explore aspects of olfaction to establish proof of concept for principles that can be applied to olfaction related to more general odorants. Sex pheromone communication involves, by definition, upwind flight attraction behavior that proves that pheromone communication has occurred, and thus a brief description of our understanding of how olfaction affects upwind flight behavior is warranted here.

A rapid response to the temporal intermittency of strands of odor that comprise the plume is important for initiating and maintaining the orientation behavior of moths flying upwind to the source (Kennedy et al. 1984; Kaisling 1990; Marsh et al. 1978; Baker et al. 1985; Baker and Haynes 1987; Baker and Vickers 1994). The male moth surges upwind in response to contact with individual pheromone strands, and begins to cast across wind upon encounters with individual pockets of clean air between the strands. The result to an observer is a zigzagging upwind progress within the time-averaged plume, but the moth is alternating rapidly between surging and casting in response to the plume’s microstructured pheromone strands and clean air.

When the wind changes direction and causes the time-averaged plume to swing many tens of degrees, the moth might fly out into a large pocket of clean air behind the
swing and thus lose the plume for many seconds. In such cases the cross-wind casting behavior will be expressed completely with left-right 90° across wind reversals continuing for many seconds until the plume is re-contacted; zigzagging upwind flight will occur once more in response to the reiterative responses to pheromone strands and clean air (Baker and Kuenen 1982; Kennedy 1983; Baker 1985). The surging and casting latencies of oriental fruit moths to strand contact and loss can occur as fast as 0.15 s (Baker and Haynes 1987), but for moths such as heliothine species, the latencies usually are ca. 0.3s (Vickers and Baker 1996, 1997; Quero et al. 2001).

Why do the moths respond rapidly to intermittent odor-clean-air stimulation, with these two behaviors? The wind transports odor away from an odor source in straight lines for many tens of meters. Even if the wind shifts the direction in which odor leaves the source, the strands that have already left the source traveling in a certain direction continue to travel in that same direction for long distances (David et al. 1982, 1983).

Thus, the parcel of odor-bearing air serves as a ‘sign-post’ for the upwind location of the source and in shifting wind-fields a quick upwind surge reaction to individual strands plus wind direction at the moment of contact with each strand will most accurately move the moth toward the source. The quick response to a pocket of clean air is needed in order to prevent the moth from plunging too deeply into large pockets of clean air that arise from large shifts in wind direction that change the direction of the plume. If the moth does not react quickly enough, he will be moving upwind in clean air in an off-line direction from the source, and will be separated ever more greatly in lateral distance from where the plume has been shifted (Baker 1990; Kaissling 1990). The long-lasting casting response serves to keep the moth in place while optimizing its chances of
re-contacting the deviant plume off to one side, to which side the moth might not be able 
to know, hence the left-right reversals.

The rapid alteration between surging and casting, as well as the long-lasting 
casting response, are reflected very nicely in the temporal response characteristics of 
pheromone-sensitive ORNs (Baker 1990; Baker and Vickers 1994). The ORN response 
of one category of temporal response has, for decades, been described as ‘phasic-tonic’ 
(c.f. Kaisssling 1974). For the rapid response to the ONs and OFFs of odor filaments, a 
phasic response is most appropriate for keeping the olfactory system ready to react to 
each change in stimulation. This is why ORNs have been called ‘flux detectors’ rather 
than concentration monitors (Kaisssling 1998). The system must be readied to detect the 
arrival of a new strand, as well as the arrival of a new pocket of clean air. A prompt 
elimination of odor compounds in the sensillum lymph (see below) would facilitate the 
ORNs reporting of either of these events.

Some ORNs behave in a more tonic manner (Borroni and O’Connell 1992), and 
in addition, long-lasting excitation of antennal lobe projection interneurons and 
protocerebral interneurons has been reported in response to pheromone blends 
(Christensen and Hildebrand 1987; Kanzaki and Shibuya 1986, 1992). Tonic firing of 
neurons in pheromone pathways would explain how long-lasting response to pheromone 
occurs in the form of casting behavior. The behaviors of both the neurons and the moth 
helps get the moth through a long period of clean air when there is a significant wind 
Functional Morphology of Elements of the Olfactory Pathway

*Sensilla trichodea.*

The antennae and glomeruli of the antennal lobe of Lepidoptera and many other insect species are sexually dimorphic (reviewed by Keil 1999). Male moths of many species such as in the Saturniidae and Lymantriidae have highly pectinated antennae (feathered, or branched) whereas their female counterparts have either short or no branches. The antennae of heliothine moths are not branched in either sex; however, the sensillar structures are sexually dimorphic. Male heliothines have long trichoid sensilla in the region close to the scales (equatorial region) and have short trichoids in the medial region between the two long-trichoid regions (Figs. 1-4, 1-5A). Females, on the other hand, do not have long trichoid sensilla, only short trichoids over the entire sensillar region (Fig. 1-5B).

The length of trichoids of male *Helicoverpa zea* moths is ca. 50 µm on average. However, there is a wide range of lengths from 33.2 to 83.0 µm (Callahan 1969). The long trichoids of male *H. zea, Heliothis virescens* and *Helicoverpa assulta* antenna are lined up in three or four very regular rows (Callahan 1969; Jefferson et al. 1970; Grant et al. 1989; Almass et al. 1991; Koh et al. 1995), and the male *Heliothis subflexa* long trichoid sensilla also shows this similarity (Baker et al. 2004). The lengths of these sensilla diminishing gradually in each row from the equatorial regions to those located closest to the medial region (Figs. 1-6, 1-7, 1-8). Except for their lengths, there are no
obvious differences of exterior morphology among sensilla in a row that are discernable via either light- or scanning-electron microscopy.

These male specific long trichoid sensilla are known as pheromone-related types-A, -B, and -C sensilla trichodea according to the three different types of tuning profiles per species of the ORNs that reside in them (Grant et al. 1989; Almaas and Mustaparta 1990; Hansson et al. 1995; Cossé et al. 1998; Baker et al. 2004). Baker et al. (2004) reported that the three sensillar types on *H. virescens* and *H. subflexa* antennae are not evenly distributed across different loci in a row but rather have some predictable topographical differences in distribution. More type-B or -C sensilla were found toward the medial region, i.e. where more of the shorter trichoids are located (Figs. 1-6, 1-7). I found that *H. zea* also showed a similar distribution pattern of the three types (data not published) (Fig. 1-8). Such studies on topography of sensillar types in moths have not been performed very often, but in another noctuid species, *Agrotis exclamationis*, the sensilla housing the major-pheromone-component-tuned ORNs were found in the shorter, more medial sensilla. Most of the larger and equatorial-located sensilla housed ORNs responsive to the minor pheromone component, unlike my results (Hansson et al. 1986; Baker et al. 2004).

The correlation between sensillar topography and electrophysiology of pheromone-sensitive ORNs also has been studied in male *Trichoplusia ni* (Noctuidae) (O’Connell et al 1983) revealing that the density of the pores on the sensillar cuticle was an indicator of the physiological characteristics of the ORNs inside. Using the same species, Grant et al. (1998) found that the sensilla housing ORNs tuned to the major
Figure 1-4
Scanning electron micrographs showing the external morphology of heliothine moth antenna and sensilla. **A** *H. zea* antenna. Pheromone-related long trichoid sensilla are located toward the lateral (equatorial) areas on each side of the antenna, close to the scale area on the dorsal side of the antenna that cannot be seen here. The short trichoid sensilla are arranged in the medial region between the two lateral regions. The beginning of the scale region that would have been somewhat visible at the bottom of the figure does not show up because the scales were displaced during sample preparation. **B** Sensilla on an *H. virescens* female antenna. Female heliothine moths do not have long *sensilla trichodea*, only short ones (arrows). Arrowheads point to basiconic sensilla, and the asterisks indicate the locations of *sensilla coeloconica*, encircled by finger-like cuticular structures.
Figure 1-5
Sexual dimorphism in heliothine moth antennae. **A** SEM of the antenna of a male *H. zea*. The sexually dimorphic long trichoids are visible in the left and right lateral regions of the antenna. In *H. zea*, the long trichoids are arranged in four rows on each flagellomere. **B** The long trichoids are not found on female *H. zea* antennae.
Figure 1-6
Topographical distribution of sensillar types on 20 flagellomere of male *H. subflexa* antennae. (N=206 sensilla). Sensillum 1 (far left) is the longest, most equatorial of the sensilla trichodea and sensilla 2 – 7 become progressively smaller toward the most central (medial) region of the chemoreceptive antennal surface. Minor-pheromone-component-responsive types B and C sensilla are more frequently encountered in the more medial region of each flagellomere. For the number of sensilla numbered 1 – 7 that were sampled and physiologically categorized, N = 37, 33, 33, 34, 32, 21, and 16, respectively. Reported in Baker et al. 2004.
Figure 1-7
Topographical distribution of sensillar types on 20 flagellomere of male *H. virescens* antennae (N=172 sensilla). Sensillum 1 (far left) is the longest, most equatorial of the sensilla trichodea and sensilla 2 – 7 become progressively smaller toward the most ventral, central region of the chemoreceptive antennal surface. The B- and C-type sensilla are found slightly more commonly in the shorter sensilla trichodea that are located toward the medial region of the segment. For the number of sensilla numbers 1 – 7 that were sampled, N=28, 28, 26, 26, 25, 24, and 15, respectively. Reported in Baker et al. 2004.
Figure 1-8
Topographical distribution of sensillar types on male *H. zea* antennae. (N=387 sensilla). Sensillum 1 (far left) is the longest, most equatorial of the sensilla trichodea and sensilla 2 – 8 become progressively smaller toward the most ventral, central region of the chemoreceptive antennal surface. Distribution patterns of each sensillar type show slight similarity to other two species. The C-type sensilla are found slightly more commonly in the shorter sensilla trichodea that are located toward the medial region of the segment. For the number of sensilla numbers 1 – 8 that were sampled, N=68, 63, 63, 54, 47, 39, 23, and 30, respectively. Previously unpublished data.
pheromone component were more frequently located on the basal flagella of the antenna than on the distal flagella.

_*The sensillum cuticle._

The definitive morphological character for olfactory sensilla compared to other types (mechanoreceptors, hygroreceptors, etc.) is the presence of numerous pores on the cuticular walls on olfactory receptors. Gustatory sensilla have no pores along their shaft, and have only a single pore at the tip through which dissolved chemicals can enter. Olfactory sensilla have been classified into several types according to their external structures. The most abundant sensilla found on the antennae of male moths are *sensilla trichodea*, long hair-like sensilla with pointed tips (Fig. 1-4). Olfactory *s. trichodea* do not have flexible socket (joint membrane) but rather they arise directly form the cuticular wall of the antennal surface. In contrast, most mechano-sensilla or gustatory trichoid sensilla have flexible sockets at their base. There are numerous pore tubules at the base of each pore-kettle (Fig. 1-3) that invade the aqueous interior (lumen) of each sensillum (Steinbrecht 1997). Pore tubules are considered to be the structures that facilitate odorant transport by creating a transition from the hydrophobic cuticular surfaces to the aqueous sensillum lymph. The chemical nature of the pore tubules is not known despite various attempts to characterize them (Steinbrecht 1997).

_*Sensillum lymph._
Once the pheromone molecules hit the surface of a sensillum, they have been shown to diffuse within 10 msec through the pores and pore tubules into the sensillum lymph (Kanaujia and Kaissling 1985; Kaissling 2001). In the sensillum lymph there are two types of water-soluble proteins, the odorant-binding proteins (OBPs) and odorant-degrading enzymes (ODEs), which are known to be involved in the perireceptor events that regulate the flux of pheromone molecules onto the dendritic-membrane-bound odorant receptors. The common characteristics found in the OBPs that have been used as key characters for their identification are their specific expression in the lumen of olfactory sensilla, their ability to bind odors, and a sequence similarity that includes six conserved Cys residues forming disulfide bonds. OBPs having binding ability to pheromone-related molecules (or else) and found only in the male antennae, are specifically called pheromone binding proteins (PBPs).

**Pheromone binding proteins.**

The first identified OBP was the PBP of from the antennae of the male silk moth *Antheraea polyphemus* (Vogt and Riddiford 1981). Later, the binding affinity of this PBP specifically for the *A. polyphemus* pheromone component was proven (Vogt et al. 1985; Du and Prestwich 1995). Since that first identification of a PBP, additional OBPs have been identified from more than 20 species (reviewed by Vogt 2003). The OBPs from this group that have not been shown to be PBPs and for which odorant-ligands have not been identified are called general odorant binding proteins (GOBPs) (Vogt 2003).
Since the first identification of PBP, various functions of OBPs have been postulated. The most widely accepted hypothesis, first formulated by Vogt and Riddiford (1981, Vogt et al. 1985) and still currently accepted is that OBPs solubilize hydrophobic air-borne odors and transport them through the aqueous sensillum lymph to the olfactory receptors (Ors) on the dendrites of the ORNs (Vogt and Riddiford 1981; Vogt et al. 1985; Vogt 1987; Kaissling 1986; Van den Berg and Ziegelberger 1991; Pophof 2002). The responses of ORNs have been shown to depend on both the pheromone components and the PBPs, which demonstrates the role of PBP as a solubilizer and carrier of the pheromone components (Van den Berg and Ziegelberger 1991; Pophof 2002).

Earlier evidence indicated that PBPs can have two different conformations of PBP having an identical amino acid sequences (Ziegelberger 1995), and that these different shapes have to do with different binding affinities for the pheromone molecule (Kaissling 2001). It was thought that the pheromone could be both carried to the receptor with the PBP in one state, released when the PBP took the second shape, with the pheromone being reacquired and the PBP taking up the initial conformation to help reduce the number of molecules interacting with receptors, dampening the signal. It was later found that the pocket in which the PBP carries the pheromone molecule is open to receive the pheromone when the PBP is in a higher pH environment near the pore tubules, and then the pocket everts to eject the pheromone when it encounters a lower pH environment near the dendrite (Wojtasek and Leal 1999; Leal et al. 2005).

**Pheromone-degrading enzymes.**
The process of odorant molecule elimination is important for clearing the sensillum of signal from an encounter with an odor strand such that the system is ready to respond to the next strand (Todd and Baker 1999). Such activity was not envisioned early-on with regard to the clearing of individual odor strands, but rather for longer-term loss of contact by the moth straying out of the plume (Baker and Vogt 1988). The extinguishing of the pheromone signal by the sensillum lymph at first was thought to involve only odorant degrading enzymes. Then Vogt and Riddiford (1985) observed that the rate of pheromone degradation by a sensillar esterase ODE decreased with the addition of PBP. They introduced the idea of a kinetic equilibrium between the pheromone ligand, the PBP, and the ODE that allowed sufficient pheromone to get through to the receptor if the flux of pheromone molecules was high enough. A few ODEs have been identified and characterized, including sensillar esterases (SEs; Vogt and Riddiford 1981; Vogt et al. 1985), and aldehyde oxidases (AOs) and aldehyde dehydrogenases (ALDHs) (Rybczynski et al. 1989, 1990; Tasayco J and Prestwich 1990a,b,c). Aldehyde-oxidizing AOs can co-exist in sensilla with alcohol-oxidizing enzyme (Rybczynski et al. 1990). The sensilla of Manduca. sexa contain AOs degrading two aldehydic pheromone components, (E,Z)-10,12-hexadecadienal (bombykal) and (E,E,Z)-10,12,14-hexadecatrienal to carboxylic acids (Rybczynski et al. 1989). The half-life of bombykal in sensilla was estimated to be about 0.6 msec. This fast degradation makes it possible that pheromone receptor neurons can detect clean air pocket with less latency.

Odorant binding proteins such as PBPs are still thought to possibly protect the odorants from degradation by odorant-degrading enzymes in the sensillum lymph during
transport to the ORNs (Vogt and Riddiford 1981, 1986; Ziegelberger 1995; Kaissling 1998, 2001), but the role of ODEs in extinction of the pheromone signal has been heightened recently (Maibèche-Coisne et al. 2004; reviewed by Vogt 2003).

**Heliothine moth pheromone ODEs**

Aldehyde-oxidation activities of conversion Z11-16:Ald to its corresponding carboxylic acid in the antenna by AOs and aldehyde dehydrogenase (ALDH) have been described for heliothine moths, *H. zea*, *H. subflexa*, and *H. virescens* (Tasayco J and Prestwich 1990a,b,c). AOs found in the antennal extracts of *H. virescens* were able to oxidize benzaldehyde and two pheromone components, (Z)-11-hexadecenal (Z11-16:Ald) and (Z)-9-tetradecenal (Z9-14:Ald) to (Z)-11-hexadecenoic acid (Z11-16:COOH) and (Z)-9-tetradecenoic acid (Z9-14:COOH) respectively. Unlike AOs, ALDHs presumably require the catalytic cofactor NADH. Tasayco and Prestwich (1990a,b,c) postulated that AOs reside in the lumen of each sensillum trichodeum, whereas the ALDHs reside in the cells underlying the cuticle.

**Olfactory receptor neurons**

Transduction of odorant molecules into action potentials by ORNs is initiated with the binding of odorant molecules to the corresponding receptor proteins on the ORN dendrites. Since the discovery of the Or gene family in vertebrates (Buck and Axel 1991),
our understanding of olfactory transduction mechanisms has been expedited. The Ors are members of the G-protein coupled receptor (GPCR) super family with a conserved hydrophobic region of seven transmembrane domains (Buck and Axel 1991).

Although the mechanism of ORN activation by OBP-odorant complex is not completely understood, Ors have been proven to be the key molecules being able to discriminate specific chemical ligands and therefore to selectively activate particular ORNs. Intensive examination using ectopic expression of 31 Or genes in a mutant ab3A neuron lacking its original receptor genes Or22a/b (the “empty neuron”, Dobritsa et al. 2003), demonstrated the functional odor response spectra of 13 heterologous Ors resembles the response spectra of 13 identified ORNs. These odor response spectra of heterologously expressed Or genes strongly suggested that the Ors are the primary determinants of any particular ORN’s differential tuning profiles in response to arrays of odorants (Hallem et al. 2004). Further work showed that all of the characteristics of an ORNs response to odorants, including their relative spike amplitude, their tendency to have a tonic or phasic temporal response pattern, in addition to their tuning profiles in response to arrays of odorant-ligands, were dictated entirely by the Or that was ectopically expressed on the empty neuron.

The Carlson group has found two ORNs, in Drosophila maxillary palp and antenna, that two different Or genes can be simultaneously expressed on the same ORN (Dobritsa et al. 2003; Goldman et al. 2005). The antennal ab3A ORN expresses two genes, Or22a/Or22b (Dobritsa et al. 2003), and the maxillary palp pb2A ORN expresses both Or85e and Or33c (Goldman et al. 2005). Olfactory receptor genes Or22a and Or22b are thought to be the products of recent gene duplication events because of several lines
of evidence. They are tightly clustered, lying within 650 bp of each other in the genome, and they show a 78% amino acid identity; the average identity among DOr genes in a cluster is ~45% (Dobritsa et al. 2003). In contrast, Or85e and Or33c are not linked, being located on two different chromosomes. Also, they show only 16% amino acid identity (Goldman et al. 2005). Thus the ‘one ORN-one Or’ concept that has thus far held true for vertebrates has now been shown to not be valid in insects. The dendrite of a single ORN of an insect can express at least two different Ors, and it has been suggested that the resulting tuning profile of the ORN could be a result of the combined profiles of each of the individual Ors seen in isolation (Goldman et al. 2005).

Antennal lobe

The action potential activities of single classes of ORNs are not sufficient for the pheromone blend discrimination that is necessary for pheromone olfaction and successful upwind flight behavior to a female of the correct species. To do this, the ORN action potential frequencies from several differentially tuned types of ORNs must be integrated and ‘read’ by interneurons for recognition of the correct pattern of activity across the array.

The insect antennal lobe (AL) (sometimes called the olfactory lobe (OL) or deutocerebrum) corresponds to the olfactory bulb (OB) of vertebrates (Hildebrand and Shepard 1997), and is the first integrative area in the olfactory pathway for blend discrimination through pattern recognition in insect, as in vertebrates. The ALs of insects and OBs of vertebrates are comprised of large numbers of spheroidal neuropil masses
called glomeruli, in which the axonal terminals of ORNs establish synaptic connections with local interneurons and with projection neurons transmission of processed signals to still higher levels of the brain for further integration. Each glomerulus is separated from other glomeruli by layers of glial structures. In insects, the number and the arrangement of glomeruli vary according to species: The fruit fly, *Drosophila melanogaster* has 43 glomeruli (Laissue et al. 1999); *Aedes aegypti* has 35 glomeruli (Bausenwein and Nick 1998, cited in Laissue et al. 1999); male *M. sexta* (Sphingidae) have 66 glomeruli (Rospars and Hildebrand, 1992); there are 65 glomeruli and 3 male-specific glomeruli present in the AL of male *H. assulta* (Noctuidae); 62 glomeruli are present in female *H. virescens* (Noctuidae) and male *H. virescens* have 4 additional glomeruli (Berg et al. 2002).

Sexual dimorphism in the antennal lobe has been reported not only in moths, but also in other insect groups such as cockroaches and honeybees (Anton and Homberg 1999). The most conspicuous difference between male moths ALs from those of females is the presence of an enlarged sub-cluster of glomeruli called the macrogglomerular complex (MGC) (Matsumoto and Hildebrand 1981; Christensen and Hildebrand 1987). The MGC is most dorsally situated at the entrance of antennal nerve into the AL, and consists of several individual glomeruli. The largest glomerulus and the one closest to the antennal nerve is called ‘cumulus’ with the smaller MGC glomeruli arranged around the cumulus being named for their spatial positions (dorso-ventral, lateral-medial) (Matsumoto and Hildebrand 1981, Christensen et al. 1991). After thorough investigations using with back-filling of identified olfactory neurons and activity-dependent optical imaging in many moths, the MGC has been proven unequivocally to be a primary
integration center for conspecific and interspecific pheromone component information. This work has been conducted on many species, including Agrotis segetum (Hansson et al. 1992), B. mori (Koontz and Schneider 1987; Kanzaki et al. 2003), A. polyphemus (Koontz and Schneider 1987), Antheraea perni (Boeckh and Boeckh 1979), Lymantria dispar (Koontz and Schneider 1987), M. sexta (Christensen and Hildebrand 1987, Hansson et al. 1991), Spodoptera littoralis (Ochieng et al. 1995), Trichoplusia ni (Todd et al. 1995), H. virescens (Hansson et al. 1995; Christensen et al. 1995; Berg et al. 1998; Galizia et al. 2000), H. subflexa (Vickers and Christensen 2003), H. zea (Christensen et al. 1991, Vickers et al. 1998), H. assulta (Berg et al. 2005), and H. zea (Lee et al. 2006).

Each glomerulus in the MGC is devoted to receiving inputs from ORNs tuned to specific pheromone components. The high specificity of each MGC glomerulus for receiving pheromone-odorant-specific information is not different from what is known for olfactory processing in the AL of Drosophila (Gao et al. 2000; Vosshall et al. 2000; Scott et al. 2001) or the OB of vertebrates (Ressler et al. 1994; Vassar et al. 1994; Wang et al. 1998) in which a single class of ORNs tuned to a specific repertoire of odorants converges onto one specific glomerulus to create a pure pool of odorant information at that one location. The differential activities across glomeruli can then be integrated to create an across-glomerular pattern for any blend of odors that can be recognized by higher centers wired to receive such patterns from the AL.

Much supporting evidence has been established for this one-ORN-one-glomerulus arrangement. However, the question arises as to how axons of ORN find their corresponding target glomerulus with such precision? One of the most decisive factors of axon targeting in the vertebrate OB is believed to be the olfactory receptor proteins
themselves. Consequently, ORNs expressing the same Ors converge onto the same
distinct glomerulus. Deletion of Or genes in vertebrate ORNs causes a disarray of
corresponding axons in the OB rather than convergence onto specific glomeruli.
Furthermore, Or gene replacement with other Or results in convergence onto a new
glomerulus, the glomerulus of the donor ORN corresponding to the donor Or (Mombaerts
et al. 1996; Wang et al. 1998; Gogos et al. 2000; Belluscio et al. 2002; Bozza et al. 2002;
Feinstein et al. 2004; Feinstein and Mombaerts 2004).

However, the situation is entirely different in insects. The establishment of precise
axonal targeting does not require Ors in insects. First, it has been observed that the axon-
targeting to the glomerulus precedes the expression of receptor proteins, and so other
axon-guiding factors have been proposed to be involved in axonal path-finding (Clyne et
al. 1999; Elmore and Smith 2001). Second, Dobritsa et al. (2003) demonstrated that the
“empty neurons” (see above) were able to target the same, normal glomerulus with
100% fidelity regardless of whether or not a variety of other Ors were ectopically
expressed on that ORN or not. Hence, they concluded that the axonal targeting of a
particular glomerulus by an ORN does not depend on Or gene expressed on that ORN.

Then, what other factors are essential for axonal guidance? Recently, several
well-know axon guidance proteins in vertebrates and in Drosophila have been proven to
play a critical role in the axonal wiring of ORNs into the target glomeruli. In Drosophila,
the Dreadlock (Dock) and serine/threonine kinase (Pak) are necessary for precise
guidance to glomeruli, rather than an outgrowth toward the antennal nerve (Ang et al.
2003). Some ORN classes of Drosophila require Dscam (Down Syndrom Cell Adhesion
Molecule) to find a specific target glomerulus, but are not involved in general axon
guidance upstream (Hummel et al. 2003). ORN targeting in the *Drosophila* AL is reported to be determined also by the signaling through Robos (Roundabouts) receptors through chemorepulsive reaction with Slit in the AL (Jhaveri et al. 2004).

The glomerulus is a mass of neuropil forming synaptic connections between axons of ORNs and dendrites of second-order neuron such as local interneurons (LNs) and/or projection interneurons (PNs). Hence, precise axonal finding for a glomerulus implicates that there be precise synaptic connections with second-order neurons. In other words, not only the ORNs but also second-order interneurons need to be coordinated so that wiring occurs only with the proper glomerulus at the proper location.

**Sex pheromone olfaction in heliothine moths**

For the research reported in this dissertation, two heliothine moths, *H. zea*, and *H. subflexa*, were used. The pheromone communication systems of these two species, plus a third species *H. virescens*, have been thoroughly investigated previously, first of all in order to produce better pest management tools for these agricultural pests. *H. zea* is a serious crop pest of corn, soybeans, cotton, vegetables, and more. The larvae of *H. subflexa* feed on plants in the genus *Physalis* and are considered to be a pest of tomatillo in Mexico. *H. subflexa* got attention as a potential biological control method for *H. virescens* by means of producing sterile hybrid males that are perpetuated through the following generations (Laster 1972).
Behavioral responses

These three sympatric North American heliothine species share (Z)-11-hexadecenal (Z11-16:Ald) as the major pheromone component in their pheromone blends (Roelofs et al. 1974; Tumlinson et al. 1975; Klun et al. 1979; Teal et al. 1981). However, males are not attracted to females of the other species due to differences in their pheromone blends with regard to their minor pheromone components. For each species, these are essential for the attraction of conspecific males and are antagonistic to the other sympatric species.

The minor pheromone component of *H. zea* (Z9-16:Ald) is an essential secondary component for male attraction in a proportion of about 2% relative to the Z11-16:Ald. Two other compounds, (Z)-7-hexadecenal (Z7-16:Ald) and hexadecanal (16:Ald) are found in the pheromone gland but have no effect on upwind flight or courtship behavior of males when combined with the binary pheromone blend of Z11-16:Ald and Z9-16:Ald in both field and wind tunnel studies (Klun et al. 1980; Vetter and Baker 1984).

The addition of (Z)-9-tetradecenal (Z9-14:Ald), an essential secondary pheromone component of *H. virescens* (Vetter and Baker 1983; Vickers et al. 1991), to the *H. zea* blend significantly decreases *H. zea* male attraction (Shaver et al. 1982). Wind tunnel studies showed that Z9-14:Ald induced arrestment of upwind flight in *H. zea* when it was added to the *H. zea* blend, but interestingly this compound was found to be capable of substituting for Z9-16:Ald when it was blended at 1% or less in binary blends with Z11-16:Ald (Vickers et al. 1991). Two pheromone components of *H. subflexa*, (Z)-11-hexadecenyl acetate (Z11-16:Ac) and (Z)-11-hexadecen-1-ol (Z11-16:OH), also are
behavioral antagonists to male *H. zea* attraction by reducing, on a strand-by-strand basis, the length and speed of each strand-induced upwind surge (Shaver et al. 1982; Fadamiro and Baker 1997; Quero and Baker 1999; Quero et al. 2001). These compounds will thus reduce male upwind flight in response to the pheromone blend of female *H. subflexa*.

*H. subflexa* also use Z11-16:Ald and Z9-16:Ald as pheromone components, and these would otherwise attract *H. zea* if it were not for Z11-16:OH being emitted by *H. subflexa* females as an essential third pheromone component (Heath et al. 1990, Vickers 2002). The Z11-16:Ac that is emitted by *H. subflexa* females will also antagonize the attraction of *H. virescens* males to *H. subflexa* females that emit the two *H. virescens* pheromone components that can evoke *H. virescens* attraction: Z11-16:Ald and Z9-14:Ald. The Z11-16:Ac added to this two-component *H. virescens* pheromone blend causes this upwind flight antagonism when present in the blend at percentages as low as 0.1 to 1.0% of Z11-16:Ald (Vickers and Baker 1997).

The function of Z11-16:OH with regard to *H. virescens* attraction is somewhat uncertain. When it is added to the two-component *H. virescens* sex pheromone blend at comparatively high proportions (>3.0%), it appears to be antagonistic to attraction (Vetter and Baker 1983). On the other hand, in *H. virescens* field trapping as well as wind-tunnel tests, Z11-16:OH occasionally appeared capable of acting to increase male attraction (Ramaswamy et al. 1985; Sparks et al. 1979; Hartstack et al. 1980).

Male *H. subflexa* will not be attracted to the *H. virescens* blend because too little Z9-16:Ald is emitted by *H. virescens* females (Pope et al. 1982), and also because Z11-16:OH is not emitted by *H. virescens* females (Teal et al. 1986; Pope et al. 1982) even though it is found in the pheromone gland (Klun et al. 1980; Pope et al. 1982; Teal et al.
1986). As mentioned above, Z9-14:Ald is requisite pheromone component of *H. virescens*, and therefore male *H. virescens* are not attracted to the pheromone blend of *H. zea*.

**Olfactory receptor neuron tuning related to behavior**

These behavioral effects (above) caused by these pheromone-related compounds have been correlated with neurophysiological tuning profiles of ORNs housed within three different physiological types of sexually dimorphic long trichoid sensilla on the male antennae of all three species (Cossé et al. 1998, Baker et al. 2004). The majority of the sensilla that were encountered were labelled type-A and house an ORN that responds exclusively to the major pheromone component (Z11-16:Ald). Approximately 71% of the *H. zea* long trichoid sensilla (Cossé et al. 1998), ca. 72% of *H. subflexa*’s and ca. 81% of male *H. virescens*’s are type-A (Baker et al. 2004). In addition to the Z11-16:Ald-responsive ORN, the occasional presence of spontaneous, very small-spiking secondary neuron activity had been reported in type-A sensilla of heliothines (Hansson et al. 1995; Berg et al. 1998; Baker et al. 2004). In these instances several candidate ligands had been applied, but specific reliable stimulants were not identified. Thus, due to the unresponsiveness of these small-spiking neurons, they were provisionally labeled “silent” or “unresponsive” neurons until a specific ligand might be found.

Approximately 10% of the sampled sensilla of male *H. zea* have an ORN that responds to Z9-14:Ald only (type-B sensilla). Type-C sensilla (19% of sampled sensilla) contain one ORN exhibiting large spikes that responds equally to both Z9-16:Ald and Z9-
14:Ald, as well as a second ORN that has smaller spikes that responds to the behavioral antagonists Z11-16:Ac and Z11-16:OH. The *H. virescens* secondary pheromone component Z9-14:Ald also stimulates this ORN, but only at higher concentrations (Cossé et al. 1998). This tuning profile means that Z9-14:Ald can activate the agonistic ORN in type-C sensilla without causing the antagonistic ORN to fire, only at low concentrations. These low levels should allow Z9-14:Ald to substitute for Z9-16:Ald in the *H. zea* blend, and it does (Vickers et al. 1991). The higher-concentration presence of Z9-14:Ald as a substitute for Z9-16:Ald in the pheromone blend will activate both the agonistic and the antagonistic ORNs, and should result in antagonism of the attraction that would otherwise be caused by the increased activity of the agonistic large-spiking ORN acting alone, and it does (Vickers et al. 1991).

The second population (type-B sensilla) of long trichoid sensilla on male *H. subflexa* antennae (ca. 18%) house receptor neurons that are tuned to the minor sex pheromone component, Z9-16:Ald, and also respond to Z9-14:Ald with less sensitivity. Type-C sensilla (ca. 8%) of *H. subflexa* exhibit spiking activities from two different ORNs. A small-spike-amplitude ORN responds to another essential secondary pheromone component Z11-16:OH, and an ORN with a large spike amplitude responds to both Z11-16:Ac and Z9-14:Ald (Baker et al. 2004).

The type-B sensilla (ca. 4%) of *H. virescens* are tuned specifically to the minor sex pheromone component, Z9-14:Ald. Approximately 16% of trichoids sampled on *H. virescens* are type-C, having two ORNs in which the small-spiking ORN is responsive to Z11-16:OH. The co-compartmentalized large-spiking ORN is tuned to the behavioral antagonist Z11-16:Ac (Baker et al. 2004).
The dissertation chapters

A large amount of information about insect olfaction, and specifically insect sex pheromone olfaction, has been generated over the past 45 years or so since the first chemical characterization of a pheromone. As can be seen above, knowledge about heliothine sex pheromone communication systems is particularly rich with information about behavior, sensory physiology including neurophysiology, biochemistry, and molecular biology of receptors, and central nervous system integration including functional morphology of the antennal lobe. This prior knowledge provides a particularly rich landscape upon which to place new information that addresses gaps in our knowledge. I have tried to address these knowledge gaps with my research in order to further enrich this field of research into heliothine moth sex pheromone olfaction. Perhaps these findings will enlighten researchers in these areas to new avenues of inquiry that otherwise would have been overlooked.

In Chapter 2 of this dissertation I try to track the axons of ORNs from types-A, -B, and -C sensilla trichodea on H. zea male antennae to their glomerular destinations in the MGC. This research was needed to fill in a gap as to what is happening in this part of the H. zea sex pheromone olfactory pathway, by physically (optically) tracing the paths of individual specific-odorant-tuned ORNs to their reporting stations in the brain. Chapter 2 has already been published in the Journal Comparative Physiology A (Lee et al. 2006).

This chapter is entirely my own work except for the one portion dealing with calcium imaging of the antennal lobe. The calcium imaging was performed in collaboration with researchers Bill Hansson and Mickael Carlsson of Swedish
Agricultural University at Alnarp. Apart from unequivocally delineating the axonal pathways that had been inferred from antennal lobe interneuron recordings but never before experimentally determined, my research produced new findings. I found that there were secondary ORNs usually co-compartmentalized in sensilla with the ORN tuned to the major sex pheromone component, and I discovered that these secondary ORNs always projected to the same location in the antennal lobe. I characterized for the first time a previously unrecognized sub-cluster of glomeruli residing immediately posterior to the MGC, which I named the Posterior Complex, or “PCx”. The glomerulus in which the secondary ORN terminates with its arborizations lies within the Posterior Complex, and I named this glomerulus the PCx1.

The research in Chapter 3 was, similar to that reported in Chapter 2, was performed to increase our understanding of the olfactory pathways in the (until recently) lesser-researched species, *H. subflexa*. In addition, I wanted to do this work to take a comparative approach to understanding heliothine sex pheromone olfaction and by doing so gain an extra appreciation for the possible evolutionary convergence and divergence that may have occurred in this group.

In this work on *H. subflexa* I discovered that there is again a previously unrecognized sub-cluster of glomeruli, a Posterior Complex, for *H. subflexa* that is quite similar to that of *H. zea*, and to what I am quite sure is also present in the other heliothines *H. virescens* and *H. assulta*. As in *H. zea*, I characterized for the first time that the secondary ORNs co-compartmentalize in the type-A sensilla with the ORN tuned to Z11-16:Ald project to and arborizes in the PCx1 glomerulus. This glomerulus is located in exactly the same location in *H. subflexa* as it is in the *H. zea* PCx. In Chapter 3 I also
am able to begin forming a broader generalization that the glomeruli in the PCx cluster receive inputs from specific co-compartmentalized pairs of secondary ORNs paired with pheromone-component-tuned ORNs. I found that the secondary ORNs paired in the type-B sensillum of *H. subflexa* with the ORN tuned to the Z9-16:Ald minor component projects with 100% fidelity to a different glomerulus in the PCx, which I named the PCx4.

Chapter 3 has been submitted for publication in the journal, Chemical Senses.

Chapter 4 deals with the question now needing to be answered following the results presented in Chapters 2 and 3. Are these ORNs that project to the PCx truly unresponsive, given their fidelity of arborizing in a given glomerulus, or are there odorant ligands that they do respond to that I hadn’t challenged them with yet? Another question pertains to the reason for the existence of a sub-cluster of glomeruli in the antennal lobe, such as the PCx. Is it non-functional with respect to contributing to odor discrimination because the ORNs themselves are non-functional? Is it a vestigial organ because the ORNs have lost some previous odorant-reporting capability? Or is it a still-active place contributing to odor blend discrimination, and if so, what kinds of information are these glomeruli processing? One idea would be that the information is pheromone-related because the secondary (unresponsive) ORNs reside in the long trichoid sensilla that are nearly always associated only with pheromone olfaction in moths.

In examining the behavior of the unresponsive (secondary) ORNs of *H. zea* and *H. subflexa*, I found that they are not completely ‘silent’ as previously stated. The spike amplitudes in both species’ type-A sensilla are so small that most of the time they disappear into the background noise. However, when they can be discriminated from noise, they show activity related to the activity of Z11-16:Ald or also for *H. zea*, the
hydrocarbon analog of Z11-16:Ald, lending support to the idea that the secondary ORNs are reporting pheromone-related information. Why such information would not go to the MGC, but rather to the PCx is an interesting discussion-point. I found that the behavior of the secondary ORN co-compartmentalized with the Z9-16:Ald-tuned ORN in the H. subflexa type-B sensilla do not behave like the secondary ORNs in the type-A sensilla.

Chapter 4 will be broken into two parts to produce two manuscripts reporting the secondary ORN physiologies of H. zea separate from those concerning H. subflexa. I anticipate submitting the papers for publication in the journal, Chemical Senses very shortly.

In Chapter 5, I attempt to summarize what I have learned from these studies, and put it in the context of heliothine moth sex pheromone olfaction in general. In addition, I present high-resolution confocal images that I have recently made of both male and female antennal lobes using new antibody staining techniques plus 3-D reconstruction. I argue that these images lend support to the idea that at least some of the glomeruli in the PCx are the receiving points for information from sex-pheromone-related ORNs. A paper concerning the confocal image results will be submitted for publication in the near future, probably in the journal, Chemical Senses.

**References**


Berg BG, Almaas TJ, Bjaalie JG, Mustaparta H (2005) Projections of male-specific receptor neurons in the antennal lobe of the oriental tobacco


Callahan PS (1969) The exoskeleton of the corn earworm moth, *Heliothis zea* (Lepidoptera: Noctuidae) with special reference to the sensilla as polytubular dielectric arrays. Research Bulletin 54: University of Georgia, College of Agriculture Experiment Station.


Fadamiro HY, Baker TC (1997) *Helicoverpa zea* males (Lepidoptera: Noctuidae) respond to the intermittent fine structure of their sex pheromone plume and an antagonist in a flight tunnel. Physiol Entomol 22:316-324


Hansson BS, Christensen TA, Hildebrand JG (1991) Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth *Manduca sexta*. J Comp Neurol 312:264-278


Heath RR, Mitchell ER, Cibrian-Tovar J (1990) Effect of release rate and ratio of
(Z)-11-hexadecen-1-ol from synthetic pheromone blends on trap capture
of Heliothis subflexa (Lepidoptera: Noctuidae). J Chem Ecol 16:1259-
1268
Hildebrand JG, Shepherd GM (1997) Mechanisms of olfactory discrimination:
converging evidence for common principles across phyla. Ann Rev
Neurosci 20:595–631
Hummel T, Vasconcelos ML, Clemens JC, Fishilevich Y, Vosshall LB, Zipursky
SL (2003) Axonal targeting of olfactory receptor neurons in Drosophila is
controlled by Dscam. Neuron 37: 221-23
Jefferson RN, Rubin RE, McFarland SU, Shorey HH (1970) Sex pheromone of
Noctuid moths. XXII. The external morphology of the antennae of
Trichoplusia ni, Heliothis zea, Prodenia ornitogalli, and Spodoptera
exigua. Ann Entomol Soc Am 63:1227-1238
the olfactory lobe of Drosophila by Robo signaling. Development
131:1903-1912
Jaenicke L (ed.) Biochemistry of Sensory Functions. Springer-Verlag,
Berlin pp. 243–273
Annu Rev Neurosci 9:121-145
Kaissling K-E (1990) Antennae and noses: their sensitivities as molecule
detectors. In:Torre V, Cervetto L, Borsellino A (eds), Sensory
Transduction. Plenum, New York, pp 81-97
Chem Senses 21:257-68
Kaissling K-E (1998) Flux detectors versus concentration detectors: Two types of
chemoreceptors. Chem Senses 23:99-111
Kaissling K-E (2001) Olfactory perireceptor and receptor events in moths: A
kinetic model. Chem Senses 26:125-150
Naturwiss 57:23-28 (in German)
Kanaujia L, Kaissling K-E (1985) Interactions of pheromone with moth antennae:
absorption, desorption and transport. J Insect Physiol 31:71-81
Kanzaki R, Shibuya T (1986) Descending protocerebral neurons related to the
mating dance of the male silkworm moth. Brain Res 377:378-382
neurons in the pheromone-processing pathways of the male moth Bombyx
from subdivisions of the antennal lobe macroglomerular complex of the
male silkmoth. Chem Senses 28:113-130
Koontz MA, Schneider D (1987) Sexual dimorphism in neuronal projections from the antennae of silk moths (Bombyx mori, Antheraea polyphemus) and the gypsy moth (Lymantria dispar). Cell Tissue Res 249:39-50
Laster ML (1972) Interspecific hybridization of Heliothis virescens and H. subflexa. Environ Entomol 1:682-687


Ramaswamy SB, Randle SA, Ma WK (1985) Field evaluation of the sex pheromone components of *Heliotthis virescens* (Lepidoptera: Noctuidae) in cone traps. Environ Entomol 14:293–296


Tasayco J ML, Prestwich GD (1990a) Aldehyde oxidases and dehydrogenases in antennae of five moth species. Insect Biochem 20:691-700
represented in distinct combinations of uniquely identifiable glomeruli. J Comp Neurol 400:35-56


CHAPTER 2

Antennal lobe projection destinations of Helicoverpa zea male olfactory receptor neurons responsive to heliothine sex pheromone components

Seong-Gyu Lee, Mikael A. Carlsson, Bill S. Hansson, Julie L. Todd, and Thomas C. Baker

ABSTRACT

We used single sensillum recordings to define male Helicoverpa zea olfactory receptor neuron physiology followed by cobalt staining to trace the axons to destination glomeruli of the antennal lobe. Receptor neurons in type-A sensilla that respond to the major pheromone component, (Z)-11-hexadecenal, projected axons to the cumulus of the macrogglomerular complex. In approximately 40% of these sensilla a second receptor neuron was stained that projected consistently to a specific glomerulus residing in a previously unrecognized glomerular complex with six other glomeruli stationed immediately posterior to the macrogglomerular complex. Cobalt staining corroborated by calcium imaging showed that receptor neurons in type-C sensilla sensitive to (Z)-9-hexadecenal projected to the dorsomedial posterior glomerulus of the macrogglomerular
complex, whereas the co-compartmentalized antagonist-sensitive neurons projected to the dorsomedial anterior glomerulus. We also discovered that the olfactory receptor neurons in type-B sensilla exhibit the same axonal projections as do those in type-C sensilla. Thus, it seems that type-B sensilla are anatomically type-C with regard to the projection destinations of the two receptor neurons, but physiologically one of the receptor neurons is now unresponsive to everything except \((Z)-9\)-tetradeccenal, and the other responds to none of the pheromone-related odorants tested.

**INTRODUCTION**

The larvae of *Helicoverpa zea* are serious crop pests having a wide host range including corn, soybean, cotton, sorghum, peanuts, and lettuce in North America (Neunzig 1963; Martin et al. 1976). Adult moths use \((Z)-11\)-hexadecenal \((Z11-16:Ald)\) as a major sex pheromone component (Roelofs et al. 1974; Klun et al. 1979). The female also produces \((Z)-9\)-hexadecenal \((Z9-16:Ald)\) as an essential secondary component for male attraction in a proportion of \(~2\)% relative to the Z11-16:Ald. Two other compounds, \((Z)-7\)-hexadecenal \((Z7-16:Ald)\) and hexadecanal \((16:Ald)\), are found in the pheromone gland but have no effect on upwind flight or courtship behavior of male *H. zea* when combined with the binary pheromone blend of Z11-16:Ald and Z9-16:Ald in both field and wind tunnel studies (Klun et al. 1980; Vetter and Baker 1984). Two other North American heliothine moths, *Heliothis virescens* and *Heliothis subflexa*, also use Z11-16:Ald as a major pheromone component. However, *H. zea* males are not attracted to
females of other sympatric heliothine species due to the behaviorally antagonistic effects of several pheromone components released by females of these species. Addition of \((Z)-9\text{-tetradecenal (Z9-14:Ald)}\), an essential secondary pheromone component of \textit{H. virescens} \cite{Vetter1983, Vickers1991}, to the \textit{H. zea} blend significantly decreases \textit{H. zea} male attraction \cite{Shaver1982}. Interestingly, this compound, blended with \textit{Z11-16:Ald}, was capable of substituting for \textit{Z9-16:Ald}, the essential secondary component \cite{Vickers1991}. Two pheromone components of \textit{H. subflexa}, \((Z)-11\text{-hexadecenyl acetate (Z11-16:Ac)}\) and \((Z)-11\text{-hexadecen-1-ol (Z11-16:OH)}\), also are behavioral antagonists to male \textit{H. zea} attraction \cite{Shaver1982, Fadamiro1997, Quero1999, Quero2001}.

The behavioral effects of these inter- and intraspecific pheromonal compounds have been linked with neurophysiological activities of olfactory receptor neurons (ORNs) housed within three different physiological types of trichoid sensilla on the antennae of male \textit{H. zea} \cite{Cossé1998}. The majority (71%) of examined sensilla have an ORN that responds exclusively to \textit{Z11-16:Ald} (type-A sensilla). Approximately 10% of the sensilla have an ORN that responds to \textit{Z9-14:Ald} only (type-B sensilla). Type-C sensilla (19%) contain one ORN exhibiting larger spikes that responds equally well to both \textit{Z9-16:Ald} and \textit{Z9-14:Ald} as well as a second ORN that has small spikes and responds to the behavioral antagonists \textit{Z11-16:Ac} and \textit{Z11-16:OH}. The \textit{H. virescens} secondary pheromone component \textit{Z9-14:Ald} also stimulates this ORN, but at a higher concentration. The presence of antagonistic ORNs responding to the interspecific pheromone components helps explain how male \textit{H. zea} discriminate conspecific pheromone blends from others \cite{Baker1998, Cossé1998}. The dose-dependent agonistic and
antagonistic effects of Z9-14:Ald on *H. zea* male behavior (Vickers et al. 1991) correlate with the physiological responses to Z9-14:Ald of the two different co-compartmentalized ORNs in the type-C sensillum. However, whether the Z9-14:Ald-selective ORNs in the type-B sensilla contribute to agonistic or antagonistic behavioral effects has remained unclear.

Histophysiological studies of projection neurons (PNs) in the *H. zea* macroglomerular complex (MGC) of the antennal lobe (AL) have helped us begin to understand how information about the presence of pheromone-related compounds in blends is relayed to higher centers (Christensen et al. 1991; Vickers et al. 1998). Because the glomeruli are known to be structural and functional units of olfactory information processing in the antennal lobe, staining and tracking physiologically identified PNs has elucidated the output characteristics of such neurons exiting each target glomerulus. However, the glomeruli-specific location and output activities of noctuid moth PNs have occasionally been found not to correspond to the activities and glomerular arborization locations of ORNs (Anton and Hansson 1999), despite there being a good correspondence in the majority of cases (Berg et al. 1998; Galizia et al. 2000). In the present article, we have examined the arborization destinations of *H. zea* ORNs in the type-A, -B, and -C sensilla through both cobalt staining (Hansson et al. 1992) and calcium imaging (Joerges et al. 1997; Galizia et al. 2000; Berg et al. 2002; Carlsson et al. 2002). Our goal was to relate these ORN input destinations with PN outputs found in previous studies (Vickers et al. 1998).
MATERIALS AND METHODS

Insects

The *H. zea* colony was maintained on a 16:8 (L:D)-h photoperiod at 25°C, 40-50% RH. Larvae were reared on a modified pinto bean diet (Shorey and Hale 1965). Males and females were separated in the pupal stage and housed in separate growth chambers in different rooms. Male pupae were sent to Sweden for the calcium imaging experiments through express mail.

Chemical stimuli

The synthetic pheromone-related compounds Z11-16:Ald, Z9-16:Ald, Z9-14:Ald, Z11-16:Ac, and Z11-16:OH (Bedoukian Research Inc., Danbury, CT; purities >98%, verified by gas chromatography), were obtained from our laboratory stock. Serial dilutions of these compounds were made in HPLC-grade hexane and stored at –20°C. To prepare stimulus cartridges for each compound, 10 µl of each diluted solution was loaded onto a 0.7 × 2.5-cm filter-paper strip, the solvent was allowed to evaporate, and then the paper strip was placed in a glass Pasteur pipette. For the mixture of Z11-Ald and (±)-linalool, 10 µg (±)-linalool in 10 µl hexane was added to a filter paper onto which 10 µg Z11-16:Ald had already been loaded, and the solvent was allowed to evaporate. New
cartridges were prepared every 5 days and stored at –20°C when not in use after being sealed with aluminium foil.

For attempts to stimulate co-compartmentalized “silent” ORNs in sensilla, we used 28 different volatile organic compounds at a loading of 100 µg on filter paper in Pasteur pipettes. These compounds were purchased from Aldrich Chemical Co. (Milwaukee, WI), Fluka Chemika (Buchs, Switzerland), and Bedoukian Research Inc., and were >97% free of other volatile impurities. The compounds were (Z)-3-hexenol, (Z)-3-hexenyl acetate, (E)-2-hexenal, 1-hexanol, phenylacetaldehyde, ocimene, α-humulene, cis-nerolidol, α-pinene, 2-phenylethanol, benzaldehyde, geraniol, (±)-linalool, indole, terpinyl acetate, 3-carene, α-copaene, (–)-(E)-caryophyllene, ethyl caproate, citronellal, allyl isothiocyanate, methyl jasmonate, methyl salicylate, dimethyltrisulfide, isovaleric acid, 1-octen-3-ol, (E)-β-farnesene, and 6-methyl-5-hepten-2-one.

**Single sensillum recordings**

The cut-sensillum technique (Kaissling 1974; Van der Pers and Den Otter 1978) was used to record the activities of ORNs housed within individual antennal sensilla. A male *H. zea* was restrained inside a disposable pipette tip; the narrow end of the pipette tip was cut to allow the male’s head to pass through. The head and antennae were immobilized in the proper orientation using dental wax. A Ag/AgCl wire was inserted into the abdomen to serve as a reference electrode and was then secured with an alligator clip attached in turn to the end of one of the micromanipulators. By maneuvering this
micromanipulator, the tip of one of the many sexually dimorphic long trichoid sensilla was placed on the edge of a vertically positioned metal knife fashioned by sharpening an insect pin. The sensillum tip was cut off using a horizontally positioned glass knife held in place by a second micromanipulator. The cut end of the sensillum was then contacted by a saline-filled glass micropipette, and an electrical connection was established. The AC signals from the ORN were amplified using a high-impedance amplifier (DAM50, World Precision Instruments, Sarasota, FL), and the action potentials were digitally recorded on VHS tapes or directly onto a PC computer by means of data processing software (Syntech Autospike 3.2, Syntech, Hilversum, The Netherlands).

Olfactory stimuli (50-ms pulses at 40 ml/s flow rate) were delivered into a continuous charcoal-filtered humidified airstream (10 ml/s) by means of a stimulus flow-controller device (SFC-2, Syntech). The odor-laden air pulse passed through a 14-cm-long glass tube (8 mm ID) whose outlet was positioned 2 cm from the antenna. Linear flow through this continuous airstream was ~0.3 m/s. At least 30 seconds was allowed to elapse between consecutive stimulations in order to minimize adaptation.

**Staining of ORNs with cobalt lysine**

To try to find the axonal target arborization destination in particular glomeruli of the antennal lobe for each electrophysiologically recorded ORN, we used a cobalt lysine staining technique, with a minor modification, following the protocol of Hansson et al. (1995), Ochieng et al. (1995), and Todd et al. (1995). The recording electrode was replaced by a new electrode filled with 0.5 M cobalt lysine solution after the ORNs
residing in the sensillum had been identified with regard to their response specificity. The cobalt lysine electrode then was used to contact the sensillum for 10 min, during which time one of the pheromone-related compounds to which one of the ORNs was tuned was puffed at a frequency of 0.7 Hz with a 50-ms pulse duration at 40 ml/s flow rate. Afterwards, the moth was kept at 4°C for 2 days. The brain was then dissected in saline (150 mM NaCl, 3 mM KCl, 3 mM CaCl₂, 10 mM TES, and pH 7.0) and then was treated with ammonium sulfide solution for the cobalt precipitation. Brains were washed four times during 5 min each with saline with 2% sucrose. After fixation, the stained neurons were intensified with silver (Bacon and Altman 1977). Brains were dehydrated through a graded series of ethanol, cleared with methyl salicylate, and viewed as whole mounts under a light microscopy. Brains were preliminarily counterstained for differentially enhancing cell bodies and glomeruli using modified Lee’s Methylene Blue-Basic Fuchsin solution (Bennett et al. 1976). Our modified solution used 0.5% methylene blue + 0.5% azure II in 1% borate: 0.5% basic fuchsin in 95% ethanol: 100% ethanol = 1:2:1, or 0.15% methylene blue, 0.15% azure II, and 0.3% basic fuchsin in 70% alcohol for 10 min. The stained whole brains were washed with 70% alcohol and dehydrated sequentially with 95%, pure ethanol (twice), and twice with propylene oxide. The infiltration procedure was performed using a mixture of propylene oxide and Durcupan resin (Sigma-Aldrich, St. Louis, MO). The propylene oxide was allowed to evaporate at room temperature overnight, and then the brains were embedded in pure Durcupan resin. Most brains were oriented for frontal sections, but a few were prepared for horizontal sections. After curing at 60°C for 2 days, brains were sectioned at 10-µm thickness and digitally
captured. The levels of digitally captured images were modified using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA) for better contrast.

**Calcium Imaging**

Preparation of animals and optical recordings were performed as described previously (Carlsson et al. 2002; Carlsson and Hansson 2003). Briefly, the animals were restrained in plastic tubes and firmly fixed with dental wax. A window was cut in the head between the compound eyes, and then glands and muscles and tracheae removed to allow AL observations. A calcium-sensitive dye (CaGR-2-AM, Molecular Probes, Eugene, OR) was bath-applied to the uncovered brain. The dye was dissolved in 20% Pluronic F-127 in dimethyl sulfoxide (Molecular Probes) and diluted in saline (Christensen and Hildebrand 1987) to a final concentration of ~30 µM. After incubation (~60 min in 10-12°C) and rinsing in saline, recordings were performed in vivo.

We used an air-cooled imaging system (TILL Photonics, Gräfelfing, Germany) with a 12 bit slow-scan charge-coupled device camera. Filter settings were dichroic, 500 nm, and emission LP, 515 nm, and the preparation was excited at 475 nm. Sequences of 40 frames and a sampling rate of 4 Hz (200-ms exposure time) were recorded through an upright microscope (Olympus, Tokyo, Japan) with a 20× (numerical aperture 0.50, Olympus) air objective. Stimulation started at frame 12 and lasted either 100 ms or 1 s. On-chip binning (2 × 2) was performed, which resulted in a final image size of 320 × 240
pixels. Execution of protocols and initial analyses of data were made using the software Till-vision (TILL Photonics).

A moistened and charcoal-filtered continuous airstream (30 ml/s) was used to flush away odors from the antenna ipsilateral to the recorded AL through a glass tube (7 mm ID). The glass tube ended ~10 mm from the antenna. An empty Pasteur pipette was inserted through a small hole in the glass tube, blowing an airstream of ~5 ml/s. Air was blown (~5 ml/s) through the odor-loaded pipette by a manually triggered puffer device (Syntech) into the continuous airstream. During stimulation, the airstream was switched from the empty pipette to the odor-loaded pipette, thereby minimizing mechanical influences. Odorants (conspecific pheromones or behavioral antagonists) were diluted in hexane and applied on filter papers (5 × 15 mm). Filter papers were inserted in Pasteur pipettes that were then sealed with Parafilm and stored in freezer (–20°C) until experiment started. A syringe with a filter paper loaded with solvent served as a control. Every fifth stimulation was done with a control.

Background fluorescence (\(F\)) was defined as an average of frames 2-11, i.e., before onset of stimulation. \(F\) was subtracted from all frames to yield \(dF\), and signals were expressed as \(dF/F\), i.e., a relative change in fluorescence over background fluorescence. For image presentation, an average of frames 14-18 (peak of activity) of a bleaching-corrected sequence was calculated, and an average of frames 2-11 (prestimulation) was subtracted. The resulting image was subsequently filtered with a spatial average low-pass filter (13 × 13 pixels) and false-color coded to its entire intensity range.
RESULTS

Our histological examinations of the *H. zea* antennal lobe (Fig. 2-1) were consistent with other reports (Vickers et al. 1998) showing an MGC comprised of three glomeruli called the cumulus, the dorsomedial posterior (DMP), and the dorsomedial anterior (DMA) (Fig. 2-1A), along with numerous ordinary glomeruli clearly set apart from these three MGC glomeruli (Fig. 2-1B, 2-1C). However, we also identified a previously undescribed complex of glomeruli residing just posterior to the MGC in every male that we examined, and we have named this the posterior complex, or PCx (Figs. 2-1C, 2-2). The PCx is comprised of seven glomeruli in which the MGC is nestled, with many of the glomeruli in the complex representing the most posterior area of this most dorsal portion of the antennal lobe (Fig. 2-1). The PCx also is characterized by a confluent bundle of fibers whose main trunk is oriented toward the center of the antennal lobe and that also eventually merges with the fiber bundle from the MGC (Figs. 2-1B, 2-2-2C). The numbers we assigned to the PCx glomeruli were determined first according to the order of their dorsal-to-ventral positions, after examining serial sections from six males (8 antennal lobes), followed by anterior-posterior and medial-lateral number assignments.
Figure 2-1
Dorsal view the antennal lobe of *H. zea* brain (horizontal sections) showing position of the PCx relative to MGC and ordinary glomeruli (Ord). A section at ~70 µm depth from the most dorsal surface. Three MGC compartments (Cu, DMP, and DMA) and three glomeruli of the PCx (PCx1, 2, 3) are visible. B Depth of 130 µm showing the cumulus (Cu) of the MGC and four more glomeruli of the PCx (PCx4, 5, 6, 7). A confluent bundle of the fibers from interneurons from the MGC and the PCx is indicated (arrowhead). C Low-magnification overview of the brain, showing the positions of MGC, Ord, and PCx relative to the protocerebrum. Mushroom bodies (MB) of the protocerebrum are indicated at the posterior part of the brain, and the central body (CB) and lateral protocerebrum (LP) also are shown. Cu, cumulus; DMA, dorsomedial anterior; DMP, dorsomedial posterior; Ord, ordinary glomeruli; MB, mushroom body; CB, central body; LP, lateral protocerebrum; A, anterior; M, medial; L, lateral. Scale bar, 100 µm.
Figure. 2-2
Schematic frontal view diagram of the glomerular topographies of the MGC and the PCx in the male *H. zea* AL. **A** MGC is comprised of three glomeruli: cumulus, dorsomedial anterior (DMA), and dorsomedial posterior (DMP). **B** The PCx is comprised of seven glomeruli: PCx1-7. The numbers after PCx are designated by the order of the glomerular arrangement in the AL; dorsal to ventral, anterior to posterior, and medial to lateral. The PCx1 is the most dorsally positioned glomerulus in this complex and is the arborization destination of the second (“silent”) ORN from the type-A sensilla. This schematic diagram represents an average male antennal lobe reconstructed after examination of serial 10-µm sections from eight individual *H. zea* males. **C** Combined view, showing that the PCx is situated posterior to the MGC. Outgoing neurons from both the MGC and the PCx seem to form a common tract. D, dorsal; L, lateral; A, anterior.
Single sensillum recording and staining

Most of the examined sensilla were selected from between the 10th and 20th flagellomeres, counted from the base of the antenna. The average number of flagella comprising a male antenna was 75 (n = 6) with a range of 72 to 79. This result is slightly different from the reports of Callahan (1969) and Grant et al. (1989) who counted an average of 82 flagellomeres on *H. zea* antennae.

Type-A sensilla

We physiologically identified 134 type-A sensilla. In these, action potentials from a large-spiking ORN were evoked after stimulation by Z11-16:Ald only, and not by any of the other heliothine pheromone components (Fig. 2-3A). A second ORN having a spike amplitude barely discernible from background noise could occasionally be seen in recordings from these sensilla (Fig. 2-3A, top trace). None of the 28 volatile organic compounds that we tested for 15 type-A sensilla caused firing from either this small spiking ORN or the large-spiking ORN.

Attempts to stain ORNs in these 134 sensilla resulted in 40 successful stains. Of these, 23 (~57.5%) had single stains, with one ORN projecting to the cumulus glomerulus of the MGC (Vickers et al. 1998) (Table 2-1). In the other 17 preparations (42.5%), two ORNs were stained. In these preparations, one ORN projected to the cumulus (Fig. 2-3B)
and the other projected to a glomerulus, glomerulus 1 (PCx1) of the posterior complex, located on average more than 100 µm posterior to the cumulus (Figs. 2-3C, D).

During the application of cobalt stain into the cut sensillum, the antenna was exposed to repetitive stimulation (0.7 Hz) with either 10µg of Z11-16:Ald, 10µg of (±)-linalool, or a mixture of 10µg Z11-16:Ald and 10 µg (±)-linalool. (±)-Linalool was used as a stimulus because of the enhanced firing that occurs in pheromone-sensitive ORNs when the antenna is stimulated with a mixture of (±)-linalool and Z11-16:Ald (Ochieng et al. 2002). We thought there might possibly be activity-dependent cobalt uptake in one of the ORNs with (±)-linalool-Z11-16:Ald mixtures. However, the uptake of cobalt stain was not influenced by which stimulus was used, with approximately one-half of all preparations resulting in one stained ORN and the other half showing two stained ORNs. Single stains always involved one ORN projecting only to the cumulus glomerulus, and those showing two stained ORNs always included one projecting to the cumulus and the other projecting to the PCx1.

Type-B sensilla

In trying to find type-B sensilla, 20 sensilla were sampled between the basal and the middle region of the antenna (from the base to approx. the 25th flagellomere), 133 were sampled from the middle region (between the 25th and the 50th flagellomere), and 24 were even more distally located. These sensilla, housing ORNs exhibiting action potentials only in response to Z9-14:Ald (Fig. 2-4A), were rarely encountered. However, of the three such type-B sensilla we found (at flagellomeres 34 and 35), two produced
ORNs that were successfully stained. These both exhibited two stained axons, one projecting into the DMA glomerulus and the other into the DMP glomerulus, respectively (Fig. 2-4B–D; Table 2-1), which are the same arborization locations as the ORNs from type-C sensilla (see below). The stimulus used during cobalt introduction for both sensilla was Z9-14:Ald.

**Type-C sensilla**

The physiological identification of type-C sensilla was done by means of their response profiles, which were consistent with the findings of Cossé et al. (1998). Larger amplitude action potentials were evoked after stimulation with Z9-16:Ald and also Z9-14:Ald, whereas smaller action potentials were elicited after stimulation with Z11-16:Ac Z11-16:OH, and Z9-14:Ald (Fig. 2-5A). Previous studies using differential adaptation had already shown that it is one large-spiking ORN that responds to both Z9-16:Ald and Z9-14:Ald (Cossé et al. 1998). Similarly, there is only one smaller-spiking ORN that responds to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald (Cossé et al. 1998).

Attempts to stain ORNs in these 58 sensilla resulted in 26 successful stains. Out of these preparations, 24 (~92%) showed two separate ORNs each projecting to the two smaller glomeruli of the MGC, the DMA and the DMP glomeruli (Vickers et al. 1998) (Fig. 2-5B, C; Table 2-1). Although nearly all of preparations with multiple stains (23/24) showed two stained neurons that targeted two glomeruli, one preparation showed three stained neurons. Two of these neurons projected axons to the DMP glomerulus and one to the DMA glomerulus. The other two preparations (out of 26) had a single ORN
projecting to the DMA glomerulus. No apparent differences in resulting stains were found when either Z11-16:Ac, Z9-16:Ald, or a mixture of the two were used to stimulate the ORNs during cobalt uptake; two stained axons projecting to the same two glomeruli were obtained, except in two cases when only one stained ORN was obtained (Figs. 2-5B, C; Table 2-1).

**Calcium imaging**

With the use of the calcium imaging technique, increases in intracellular calcium concentrations in glomeruli in response to antennal stimulation with pheromone-related compounds were observed in eight male moths (Table 2-2). Two different doses (1 and 10 µg) of pheromone components were applied. Stimulation with the major component, Z11-16:Ald, increased calcium concentration prominently in the cumulus (Fig 2-6A). Exposure of the antenna to the behavioral antagonist Z11-16:Ac showed maximum activity in the DMA glomerulus (Fig 2-6B), whereas stimulation with the minor pheromone component Z9-16:Ald showed maximum activity in the DMP glomerulus in all attempts (Fig. 2-6C). These three volatiles elicited consistent results at any dosage (Table 2-2). In cases in which Z9-14:Ald was used for stimulation, however, the location of calcium activity in the AL was dependent on the dosage. For the two animals in which 1 µg of Z9-14:Ald was used, this dose activated the DMP glomerulus. In contrast, animals exposed to 10 µg of Z9-14:Ald displayed predominant activity in the DMA glomerulus, with some activity also occurring in the DMP glomerulus (Fig 2-6D; Table 2-2).
Table 2-1. Summary of the glomerular arborization destinations of olfactory receptor neurons from three different types of *H. zea* male sensilla, as determined by cobalt back-filling of physiologically characterized neurons.

<table>
<thead>
<tr>
<th>Sensilla type</th>
<th>Stained sensilla (attempts)</th>
<th>Target glomeruli</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Single target</td>
<td>Double targets</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cumulus</td>
<td>DMA</td>
</tr>
<tr>
<td>A-type</td>
<td>40 (134)</td>
<td>23</td>
<td>0</td>
</tr>
<tr>
<td>B-type</td>
<td>2 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C-type</td>
<td>26 (58)</td>
<td>0</td>
<td>2(^a)</td>
</tr>
</tbody>
</table>

DMA, dorsomedial anterior; DMP, dorsomedial posterior; PCx1, posterior complex glomerulus 1.

\(^a\) One of these occasions stained two axons but one axon faded out in its pathway with no target glomerulus

\(^b\) One sensillum possessed three stained axons with two projecting into DMP
A

Z11-16:Ald 10 µg

Z9-16:Ald 10 µg

Z11-16:Ac 10 µg

Z11-16:OH 10 µg

Z9-14:Ald 10 µg

10 mV

200 ms
**Figure. 2-3**

**A** Spike train of an ORN from an *H. zea* type-A sensillum. Action potentials were triggered only by stimulation with Z11-16:Ald. **B–D** Frontal view of a male *H. zea* antennal lobe, showing the arborization destinations of two ORNs that were stained within one type-A sensillum. **B** One stained axon (arrowhead) arborized in the cumulus (Cu). **C, D** A second ORN arborized in the dorsal-most glomerulus (PCx1) of the posterior complex. The section in B is ~80 µm posterior from the frontal surface. The section in C is 100 µm posterior to B. In D, the section is ~10 µm posterior to C and the axonal path of the stained second ORN (arrowhead) is shown traveling (in C) around glomerulus PCx5 of the posterior complex to arborize in glomerulus PCx1 (arrowhead in D). Cu, cumulus; DMA, dorsomedial anterior; DMP, dorsomedial posterior; Ord, ordinary glomeruli; D, dorsal; M, medial. Scale bar, 50 µm.
Figure. 2-4
A Responses of ORNs from a type-B sensillum. Only Z9-14:Ald evokes a spike train. B–D Frontal view of antennal lobe showing glomerular arborization destinations of two ORNs housed within one type-B sensillum. B One axon (arrowhead) arborizes in the DMA glomerulus. C The next section, 10 µm posterior to B shows a second axon starting to arborize (arrowheads) in the DMP glomerulus. D About 20 µm posterior to C, showing more arborization of this ORN (arrowhead) in the DMP glomerulus. The DMA glomerulus is no longer visible in this section. Cu, cumulus; DMA, dorsomedial anterior; DMP, dorsomedial posterior; D, dorsal; M, medial. Scale bar , 50 µm.
A

Z11-16:Ald 10 µg

Z9-16:Ald 10 µg

Z11-16:Ac 10 µg

Z11-16:OH 10 µg

Z9-14:Ald 10 µg

200 ms

10 mV

Figure. 2-5

A In type-C sensilla, tracings of ORN activity show a larger amplitude spiking ORN that responds to both Z9-16:Ald and Z9-14:Ald and a smaller amplitude spiking ORN that responds to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald. B-C Neuronal projection patterns of the two ORNs housed within a type-C sensillum (frontal view). B One axon arborized in the DMA glomerulus (arrowheads). C The second axon arborized in the DMP glomerulus (arrowhead). The DMA glomerulus is no longer visible in this section, which is ~50 µm posterior to B. Cu, cumulus; DMA, dorsomedial anterior; DMP, dorsomedial posterior; D, dorsal; M, medial. Scale bar, 50 µm.
Figure. 2-6
Frontal view of the calcium imaging activity from the left antennal lobe during stimulation of antennal ORNs with pheromone-related compounds. The images were left-right reversed from the originals in order to better conform to the orientations of Figs. 2-2 – 2-5 of this paper. The activity maps were cropped to 50% of maximal activity and superimposed on gray-scale images from the same recordings. A Stimulation from a cartridge loaded with 1 µg of Z11-16:Ald. B Stimulation from a cartridge loaded with 1 µg of Z11-16:Ac. C Stimulation from a cartridge loaded with 1 µg of Z9-16:Ald. D Stimulation from a cartridge loaded with 10 µg of Z9-14:Ald. The focal activity of glomeruli is distributed between both the DMA and the DMP glomeruli at the higher emission rate of Z9-14:Ald used with this animal, consistent with the physiological responses of both the large- and small-spiking ORN in type-C sensilla to Z9-14:Ald (Cossé et al. 1998; this study, Fig. 2-5A). There are also some weaker signals from a few other glomeruli, which is probably due to the aldehyde group that could activate to a lesser degree some other types of general-odorant-tuned ORNs projecting to ordinary glomeruli. DMA, dorsomedial anterior; D, dorsal; M, medial. Scale bar, 100 µm.
Table 2-2. Summary of the focal points of maximum calcium release in the MGC glomeruli of *H. zea* males, as indicated by highest levels of calcium-sensitive dye activity after antennal stimulation with the indicated pheromone-related compounds. Numbers indicate the number of individual moths in which successful recordings were made.

<table>
<thead>
<tr>
<th>Stimulants</th>
<th>Dose (µg)</th>
<th>No. moths</th>
<th>Active glomerulus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cumulus</td>
</tr>
<tr>
<td>Z11-16:Ald</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Z9-16:Ald</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Z11-16:Ac</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Z9-14:Ald</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

DMA, dorsomedial anterior; DMP, dorsomedial posterior.
Figure 2-7.
Schematic diagram showing the arborization destinations of ORNs from the three classes of long trichoid sensilla on *H. zea* male antenna, constructed according to the combined results from single ORN physiological recordings (Cossé et al. 1998), cobalt stainings, and calcium imaging (this study). Axonal projections from the ORNs responsive to Z11-16:Ald in type-A sensilla arborize in the cumulus glomerulus (Cu), and the “silent” ORNs from type-A sensilla arborize in the PCx1 glomerulus. Type-B sensilla house two ORNs; those responsive to Z9-14:Ald arborize in the DMA glomerulus and are associated with behavioural antagonism just as are the small-spiking ORNs from type-C sensilla. The ‘silent’ ORNs from type-B sensilla arborize in the DMP glomerulus just as do the Z9-16:Ald/Z9-14:Ald-responsive ORNs from type-C sensilla (see below). The large-spiking ORNs from type-C sensilla responsive to both Z9-16:Ald and Z9-14:Ald project to the DMP glomerulus and are associated with attraction as part of the *H. zea* pheromone blend. The small-spiking ORNs in type-C sensilla that are responsive to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald project to the DMA glomerulus and their activities are associated with cessation of upwind flight. Cu, cumulus; DMA, dorsomedial anterior; DMP, dorsomedial posterior; PCx1, posterior complex glomerulus 1.
DISCUSSION

The results presented here, combined with those from previous studies (Christensen et al. 1991; Cossé et al. 1998; Vickers et al. 1998) show that in *H. zea*, as in other heliothine moths (Hansson et al. 1995; Berg et al. 1998; Vickers and Christensen 2003), there is a correspondence between pheromone odorant-specific input to, and output from, the different MGC compartments. These relationships for *H. zea* are depicted in Fig. 7.

Without exception, cobalt stains showed that at least one axon of the examined type-A sensilla (40/40) projected into the cumulus (Table 2-1), which is also the arborization location of Z11-16:Ald-responsive PNs (Christensen et al. 1991, Vickers et al. 1998). Our calcium imaging experiments show that excitation from the entire population of ORNs across the antenna that are responsive to Z11-16:Ald converges onto the cumulus (Table 2-1; Fig. 2-3A). Therefore, in *H. zea*, as shown in two other North American heliothine moths (Hansson et al. 1995; Berg et al. 1998; Vickers et al. 1998; Vickers and Christensen 2003), the cumulus receives input from ORNs about the presence of the major pheromone component, Z11-16:Ald.

The unresponsive, silent ORNs co-residing with the Z11-16:Ald-sensitive ORNs in the type-A sensilla project with 100% fidelity (17 of 17 cobalt stains) to the most dorsal glomerulus, PCx1, of the newly described posterior complex (Figs. 2-1, 2-2, 2-3D, 2-7; Table 2-1). We did not find any ORNs from any sensilla other than from the type-A
that projected to the PCx1. This leads us to conclude that the secondary ORNs in the

type-A sensilla are not arbitrarily co-compartmentalized with the Z11-16:Ald-responsive

neurons and are biochemically similar due to their axonal projection addresses into this

specific PCx1 glomerulus. In *H. subflexa*, we have found a similar arrangement involving

silent ORNs that are co-compartmentalized within type-A sensilla with Z11-16:Ald-
sensitive ORNs. The axons of these *H. subflexa* silent ORNs always project to a dorsal-
most glomerulus similar in position to the PCx1 in *H. zea*, which also is located in a

data).

Spontaneous activity is often encountered from a secondary ORN co-

compartmentalized with the Z11-16:Ald-responsive ORN on the male antennae of *H.

virescens* and *Helicoverpa assulta* as well, but as in *H. zea* and *H. subflexa*, none of the
tested compounds have been found to elicit activity from this ORN (Hansson et al. 1995;
Berg et al. 1998; Baker et al. 2004; Berg et al. 2005). In *H. virescens*, the target

glomerulus of this secondary ORN seems to correspond to the position of PCx1 in *H. zea,

with respect to the dorso-lateral position, relative to the cumulus, of what was described
as an “ordinary” glomerulus in the figures of Berg et al. (1998). Similarly, the staining of

secondary ORNs in *H. assulta* that are co-compartmentalized with Z11-16:Ald-sensitive

ORNs resulted in arborizations that appear to occur in a PCx glomerulus (Berg et al.
2005).

For *H. zea*, we cannot say with certainty that there is no ligand that this silent

ORN in the type-A sensillum is tuned to, but it responded only occasionally and

inconsistently to some of the compounds in the array of 28 compounds that we
challenged it with in this study, plus nearly 60 others in a separate exhaustive study (S.-G. Lee, N.J. Vickers, and T. C. Baker, unpublished data). In the latter study we also tried stimulation with the C16 acids corresponding to the enzymatic degradation products of heliothine aldehyde pheromone components (Prestwich et al. 1989; Tasayco and Prestwich 1990) because in other noctuid species such as Trichoplusia ni and Agrotis segetum, the ORNs that are co-compartmentalized with major component-sensitive ORNs (Hansson et al. 1990; Todd et al. 1992, 1995) are tuned to the corresponding degradation products (alcohols) resulting from esterases acting on the acetate major components (Prestwich et al. 1989). In heliothines, the enzymatic degradation product of Z11-16:Ald is (Z)11-hexadecanoic acid (Z11-16:COOH), which results from the activity of aldehyde oxidase and dehydrogenase operating in the sensillar lymph (Prestwich et al. 1989). However, attempts thus far to stimulate this secondary ORN with C16 acids have not produced consistent results (S.-G. Lee et al. unpublished).

In T. ni and A. segetum, stimulation of co-compartmentalized ORNs tuned to the corresponding alcohols of the acetate pheromone components results in antagonism of upwind flight to pheromone. These ORNs project to an MGC glomerulus and not into a separate complex such as the PCx in H. zea. If the silent ORNs in H. zea were indeed found to be sensitive to Z11-16:COOH, we would predict that this acid should be behaviorally antagonistic to H. zea upwind flight. However, why these ORNs should project to a glomerulus in a separate kind of complex close to the MGC and not to the MGC itself as in other noctuids is intriguing.

This question relates not just to H. zea but to other heliothines as well. In recordings of ORNs residing in the type-A sensilla on the male antennae of the three
North American heliothines that have been extensively researched (H. zea, H. virescens, and H. subflexa), spontaneous activity from an ORN co-compartmentalized with the Z11-16:Ald-responsive ORN has often been reported, but none of the pheromone-related test compounds have been found to elicit activity from these ORNs (Grant et al. 1989; Hansson et al. 1995; Berg et al. 1998, 2005; Baker et al. 2004).

Although not described before now, structures similar to the posterior complex can be seen in the antennal lobes of other male heliothine moths, including Helicoverpa assulta (Berg et al. 2002) and H. subflexa (S.-G. Lee, N.J. Vickers, and T.C. Baker, unpublished data). For example, in the confocal micrograph of male H. assulta AL (Berg et al. 2002), a cluster of seven glomeruli (G61, 50, 46, 47, 48, 49, and 60) is located posterior to the MGC that seems to have a structural conformity with the PCx of H. zea. In particular, the glomeruli G61 of H. assulta (Berg et al. 2002) and G54 of H. virescens (Berg et al. 2002) seem to coincide with the PCx1 that receives input from the silent ORNs from type-A sensilla on H. zea (this study) and H. subflexa antennae (S.-G. Lee N.J. Vickers, and T.C. Baker, unpublished data).

The combination of cobalt staining and calcium imaging has now also clarified the relationship of the arborization destinations of the two ORNs housed in the type-C sensilla compared with the arborization locations of the PNs related to attraction and behavioral antagonism (Cossé et al. 1998; Vickers et al. 1991, 1998). The minor pheromone component Z9-16:Ald and the behavioral antagonist Z11-16:Ac, respectively, stimulate two different ORNs that are co-compartmentalized within type-C sensilla (Cossé et al. 1998). Similarly, two different types of H. zea PNs are responsive to either Z9-16:Ald or to Z11-16:Ac, and their arborization in the DMP and DMA glomeruli,
respectively had strongly suggested that similarly tuned ORNs also should project to the DMP and DMA glomeruli (Vickers et al. 1998). In our cobalt staining studies with type-C sensilla, two stained axons from these sensilla consistently arborized within the DMP and DMA glomeruli, and nowhere else (Table 2-1), thereby confirming the linkage between the ORNs in type-C sensilla and these two target glomeruli. Subsequently, our calcium-imaging experiments were definitive, showing that Z9-16:Ald stimulation produced an increase in calcium concentration only in the DMP glomerulus (Fig. 2-6; Table 2-2). Conversely, antennal stimulation with the behavioral antagonist Z11-16:Ac consistently increased calcium concentrations only in the DMA glomerulus (Fig. 2-6; Table 2-2). The combined results from cobalt staining and calcium imaging are consistent with the conclusion that the large-spiking ORNs in type-C sensilla that are related to attraction and are sensitive to both Z9-16:Ald and Z9-14:Ald (Cossé et al. 1998) transmit their action potentials to the DMP glomerulus. PNs responsive to Z9-16:Ald also arborize in the DMP glomerulus (Vickers et al. 1998); therefore, there is a linear arrangement of Z9-16:Ald-driven input from ORNs through this glomerulus followed by Z9-16:Ald PN output.

Our cobalt stains plus the calcium imaging experiments together clarify that the small-spiking ORN in type-C sensilla related to behavioral antagonism and responsive only to Z11-16:Ac, Z11-16:OH, and Z9-14:Ald (Cossé et al. 1998) projects to the DMA glomerulus. PNs responsive to these compounds also arborize here (Vickers et al. 1998), and now we can say that they relay information about these compounds along this line to higher order central nervous system interneurons.
Almaas et al. (1991) concluded that an ORN that they found responding both to Z9-14:Ald and Z9-16:Ald in *H. zea* was far more responsive to Z9-14:Ald and therefore its activity was related to behavioral antagonism. They did not take into account, however, the great difference in volatility of these two compounds and they did not measure the actual amounts emitted from, not loaded into, the odor cartridges as did Cossé et al. (1998). Moreover, Almaas et al. (1991) showed no awareness that they were recording the spike activities from two ORNs (the large- and the small-spiking ORNs of Cossé et al., 1998), not just one, in the four sensilla they recorded from when stimulating with Z9-14:Ald. Because Almaas et al. (1991) lumped the separate activities of these two ORNs that are both equally responsive to Z9-14:Ald as having come from one ORN, they will have overestimated the apparent sensitivity of this ‘neuron’ by 2-fold in response to Z9-14:Ald compared to what occurs in only the large-spiking ORN in response to Z9-16:Ald (Cossé et al. 1998). The difference in emitted versus loaded amounts of Z9-14:Ald will have added an additional 10-fold overestimation of sensitivity to Z9-14:Ald.

The results of our successful cobalt stainings of type-B sensilla suggest that type-B sensilla are anatomically type-C sensilla, but the large-spiking ORN is now silent, being unresponsive to Z9-14:Ald and Z9-16:Ald, and the small-spiking ORN that responds to Z9-14:Ald is no longer responsive to Z11-16:Ac and Z11-16:OH (Fig. 2-7). The dendrites of a projection neuron responding exclusively to Z9-14:Ald, as do those of an ORN in the type-B sensillum, are restricted to arborizing in the DMA glomerulus (Vickers et al. 1998). Recent studies of *Drosophila melanogaster* olfactory receptor genes have shown that receptors can be expressed or repressed without the ORN changing its glomerular arborization destination (Dobritsa et al. 2003). That the fidelity
of targeting a particular glomerulus does not depend on which receptors are expressed on an ORN would explain how the type-B sensillar ORNs can continue to target the same glomeruli as type-C ORNs, even though their responsiveness to pheromone components has apparently been repressed. Maintaining a constant glomerular destination by an ORN that has shifted its tuning spectrum due to pheromone receptor gene enhancement or repression of expression would potentially provide rich opportunities for evolutionary shifts in pheromone blends (T.C. Baker et al., submitted). There are obviously great differences in the sensitivities of what seem to be homologous ORNs in response to different subsets of pheromone components across these heliothine species. There are also striking similarities in the occurrence of co-compartmentalized silent ORNs, such as in type-A sensilla across these species. A lot may be learned from studying these heliothine ORNs with regard to the expression and repression of pheromone component receptor genes.

REFERENCES

Baker TC, Cossé AA, Todd JL (1998) Behavioral antagonism in the moth Helicoverpa zea in response to pheromone blends of three sympatric heliothine moth species is explained by one type of antennal neuron. Ann NY Acad Sci 855:511-513


Callahan PS (1969) The exoskeleton of the corn earworm moth, *Heliothis zea* (Lepidoptera: Noctuidae) with special reference to the sensilla as polytubular dielectric arrays. Research Bulletin 54: University of Georgia, College of Agriculture Experiment Station.


Fadamiro HY, Baker TC (1997) Helicoverpa zea males (Lepidoptera: Noctuidae) respond to the intermittent fine structure of their sex pheromone plume and an antagonist in a flight tunnel. Physiol Entomol 22:316-324


Martin PB, Lingren PD, Greene GL (1976) Relative abundance and host preferences of cabbage looper, soybean looper, tobacco budworm, and corn earworm on crops grown in northern Florida. Environ Entomol 5:878-882


CHAPTER 3

Antennal lobe projection destinations of Heliothis subflexa male olfactory receptor neurons responsive to heliothine sex pheromone components

Seong-Gyu Lee, Neil J. Vickers and Thomas C. Baker

ABSTRACT

I used single-sensillum recordings to define male Heliothis subflexa antennal olfactory receptor neuron physiology in response to compounds related to their sex pheromone. These were then followed by cobalt staining in order to trace the neurons’ axons to their arborization destinations in the antennal lobe glomeruli. Receptor neurons responding to the major pheromone component, \((Z)-11\)-hexadecenal, in the first type of sensillum, type-A, projected axons to the cumulus of the macroglomerular complex. In approximately forty percent of the type-A sensilla, a secondary receptor neuron was stained that projected consistently to the PCx1, a specific glomerulus in an 8-glomerulus complex that I call the Posterior Complex. I found that receptor neurons residing in type-B sensilla and responding to a secondary pheromone component, \((Z)-9\)-hexadecenal, send their axons to the dorsal medial glomerulus of the macroglomerular complex. As in the
type-A sensilla, I found a co-compartmentalized secondary neuron within type-B sensilla that sends its axon to a different glomerulus of the Posterior Complex, PCx4. One neuron in type-C sensilla tuned to a third pheromone component, (Z)-11-hexadecenol, and a secondary neuron responding to (Z)-11-hexadecenyl acetate projected their axons to the antero-medial and ventro-medial glomeruli of the macroglomerular complex, respectively.

INTRODUCTION

*Heliothis subflexa* (Lepidoptera, Noctuidae) larvae are known to feed on plants in the genus *Physalis*, such as ground cherry. In Mexico, *H. subflexa* larval feeding on tomatillos can be so extensive that it is now considered to be a pest of this agricultural crop (Mitter et al. 1993). Even though they are not yet serious agricultural pests in the U.S., the successful hybridization of *H. subflexa* with the well-known pest species, *Heliothis virescens*, has interested researchers as a potential control technique for *H. virescens* because hybrid male offspring are sterile, as are backcross males (Laster 1972; Teal and Oostendorp 1995; Sheck and Gould 1995, 1996).

Morphological and molecular phylogenies of heliotheine moths are in agreement that these two species are closely related (Mitter et al. 1993; Cho et al. 1995; Fang et al. 1997). They are sympatric in North America and share the same female-emitted major pheromone component, (Z)-11-hexadecenal (Z11-16:Ald) (Roelofs et al. 1974; Tumlinson et al. 1975; Klun et al. 1979, 1980, 1982). However, interspecific attraction of males and cross-mating do not occur in nature due to the addition of different minor pheromone components to the blends of the two species. *H. subflexa* females release (Z)-
9-hexadecenal (Z9-16:Ald) and (Z)-11-hexadecenol (Z11-16:OH) (Teal et al. 1981; Heath et al. 1991) as minor components that significantly increase attraction of *H. subflexa* males and are therefore part of this species’ sex pheromone blend (Heath et al. 1990; Vickers 2002). Hexadecenal (16:Ald), (Z)-11-hexadecenyl acetate (Z11-16:Ac), (Z)-9-hexadecenyl acetate (Z9-16:Ald), (Z)-7-hexadecenyl acetate (Z7-16:Ac), and (Z)-9-hexadecenol (Z9-16:OH) have been identified as other constituents in the female pheromone gland (Teal et al. 1981; Heath et al. 1991; Klun et al. 1982), but as yet these compounds have not been shown to play a pheromonal role.

Another sympatric heliothine pest, *Helicoverpa zea*, like *H. subflexa*, emits Z11-16:Ald and Z9-16:Ald as essential pheromone components for male attraction (Pope et al. 1984; Vetter and Baker 1984). However, the *H. subflexa* blend will not attract *H. zea* males because the Z11-16:OH present in this blend has been shown to be antagonistic to *H. zea* attraction when added to its pheromone blend (Shaver et al. 1982; Quero and Baker 1999).

Much work has now been performed on the sex pheromone olfactory systems of these three North American heliothine moth species. These studies include examination of the electrophysiological response profiles of the olfactory receptor neurons (ORNs) (Cossé et al. 1998; Berg et al. 1995; Almaas et al. 1991; Baker et al. 2004; Lee et al. 2006) as well as neuroanatomical studies of the central nervous system (CNS) pathways that integrate and relay the inputs from the ORNs (Vickers and Christensen 2003; Vickers et al. 1998; Christensen et al. 1991, 1995). Neuroanatomical studies of the ORN pathways into the antennal lobe, the first integrative center of the moth olfactory system, also now have begun to delineate exactly how ORN pheromone-component-specific
information converges onto specific glomeruli (knots of neuropil within which synaptic contacts are made between ORNs and central neurons) as well as how pheromone blend quality information is conveyed to higher centers by central olfactory projection neurons.

Such studies have been illuminating and have for instance shown how the pheromone-component-tuned ORNs of *H. virescens* (Berg et al. 1998), *Helicoverpa assulta* (Berg et al. 2005), and *H. zea* (Lee et al. 2006) project their axons to component-specific homologous glomeruli in these species’ macroglomerular complexes (MGC). However, similar information about the ORNs of *H. subflexa* has been lacking. I therefore sought to clarify the primary ORN pathways to the MGC for *H. subflexa* by using the ORN cobalt staining technique that has proven to be so informative for other noctuid moth species (Hansson et al. 1992; Hansson 1995; Ochieng et al. 1995; Todd et al. 1995; Berg et al. 1998, 2005; Lee et al. 2006).

I report here that the glomeruli in which male *H. subflexa* component-specific ORNs arborize correspond well to the glomerular arborization locations of similarly tuned projection interneurons exiting the *H. subflexa* antennal lobe to higher centers (Vickers and Christensen 2003). In addition, I found that the secondary ORNs (Lee et al. 2006) that are co-compartmentalized with ORNs tuned to Z11-16:Ald or to Z9-16:Ald project their axons to a cluster of glomeruli located just posterior to the MGC, which I call the Posterior Complex (PCx) (Lee et al. 2006). The secondary ORNs co-compartmentalized with Z11-16:Ald-sensitive ORNs always project to the same glomerulus, the PCx1, in the Posterior Complex, and the secondary ORNs co-compartmentalized with Z9-16:Ald-sensitive ORNs always project to a different glomerulus in this complex, the PCx4.
MATERIALS AND METHODS

Insects

A colony of *H. subflexa* was maintained at the University of Utah as described by Vickers (2002). Pupae were separated according to sex and shipped overnight to the Baker laboratory. Emerging adults were segregated into separate cages daily to obtain individuals of known age. Adults were maintained on a 16:8 L:D photoperiod at 25°C, 40-50% RH.

Chemical stimuli

Five heliothine moth sex pheromone compounds were utilized to identify sensillar types according to Baker et al. (2004). The synthetic compounds, Z11-16:Ald, Z9-16:Ald, Z9-14:Ald, Z11-16:Ac and Z11-16:OH were purchased from Bedoukian Research Inc. (Danbury, Connecticut, USA; purities >98% verified by gas chromatography). Serial dilutions were made in HPLC-grade hexane to 0.1 µg/µl and 1 µg/µl, and stored at -20°C. To prepare stimulus cartridges for each compound, 10 µl of each diluted solution was loaded onto a 0.7 X 2.5 cm filter-paper strip, the solvent allowed to evaporate, and then the paper strip was placed in a glass Pasteur pipette. New cartridges were prepared every 5 days and stored at -20°C when not in use after being sealed with aluminum foil.
Single-sensillum recording

The cut-sensillum technique (Kaisling 1974; Van der Pers and Den Otter 1978; Cossé et al. 1998) was used to record the activities of ORNs housed within individual antennal sensilla. A male *H. subflexa* was restrained inside a disposable pipette tip; the narrow end of the tip was cut to enlarge it to allow the male’s head to pass through. The head and antennae were immobilized in the proper orientation using dental wax. A Ag/AgCl wire was inserted into the abdomen to serve as a ground, and was then secured with an alligator clip attached in turn to the end of one of the micromanipulators. By maneuvering this micromanipulator, the tip of one of the many sexually dimorphic long trichoid sensilla was placed on the edge of a vertically positioned metal knife fashioned by sharpening an insect pin. The sensillum tip was cut off using a horizontally positioned glass knife held in place by a second micromanipulator. The cut end of the sensillum was then contacted first by a saline-filled glass micropipette and a connection established in order to determine the type of sensillum that was contacted, according to the differential responses of the ORNs to the array of pheromone odorants. The AC signals from the ORNs were amplified using a high impedance amplifier (DAM50, World Precision Instruments, Sarasota, FL, USA) and the action potentials were digitally recorded directly onto a PC computer by means of data processing software (Syntech Autospike 3.2; Hilversum, The Netherlands).

Volatile stimuli (50-ms pulses at 40 ml/s flow rate) were delivered into a continuous charcoal-filtered humidified airstream (~15 ml/s) by means of a stimulus flow-controller device (SFC-2; Syntech). The odor-laden air pulse passed through a 14-
cm-long glass tube (8 mm ID) whose outlet was positioned 2 cm from the antenna. Linear flow speed through this continuous airstream was \(~0.3\) m/s.

**Staining of ORNs with cobalt-lysine**

I used a cobalt-lysine staining technique, with a minor modification, following the protocol of Hansson et al. (1995), Ochieng et al. (1995), and Todd et al. (1995) in order to try to find the glomerular arborization destinations of the axons of each electrophysiologically identified ORN. A glass electrode filled with 0.5 M cobalt-lysine solution replaced the saline recording electrode after the ORNs residing in the sensillum were characterized using the saline electrode to discern their response specificity. The cobalt-lysine electrode then was used to contact the sensillum for 10 min, during which time one of the pheromone-related compounds to which one of the ORNs was tuned was puffed at a frequency of 0.7 Hz. For these repetitive stimulations to type-A sensilla, 10 µg Z11-16:Ald or a mixture of 10 µg Z11-16:Ald plus 10 µg (±) linalool was used. For type-B sensilla, only 10 µg Z9-16:Ald was used, and for type-C sensilla, 10 µg Z11-16:Ald, 10 µg Z11-16:OH, or blank cartridges were used.

The male was then removed from the electrophysiology rig and refrigerated for 2 days at 4°C to allow sufficient time for the cobalt to migrate along the axons to the antennal lobe. The brain was then dissected from the head in saline (150 mM NaCl, 3 mM KCl, 3 mM CaCl₂, 10 mM TES, and pH 7.0) and treated with ammonium sulfide solution for the cobalt precipitation step. Brains were washed four times for 5 min each with saline that included 2% sucrose. After fixation, any cobalt-stained ORNs’ axons
were intensified with silver (Bacon and Altman 1977). Brains were dehydrated through a graded series of ethanol, cleared with methyl salicylate, and viewed as whole mounts under a light microscopy. All brains were stored at 4°C in 70% alcohol until being used for the next procedure.

Brains were preliminarily counterstained for differentially enhancing cell bodies and glomeruli using modified Lee’s Methylene Blue-Basic Fuchsin solution (Bennett et al. 1976). My modified solution used 0.5% methylene blue + 0.5% azure II in 1% borate: 0.5% basic fuchsin in 95% ethanol: 100% ethanol = 1:2:1, or 0.15% methylene blue, 0.15% azure II, and 0.3% basic fuchsin in 70% alcohol for 10 min. The stained whole brains were washed with 70% alcohol and dehydrated sequentially with 95%, pure ethanol (twice), and twice with propylene oxide. The infiltration procedure was then performed using a mixture of propylene oxide and Durcupan resin (Sigma-Aldrich, St. Louis, MO). The propylene oxide was allowed to evaporate at room temperature overnight, and then the brains were embedded in pure Durcupan resin. Most brains were oriented for frontal sections, but a few were prepared specifically for horizontal sections. After curing at 60°C for 2 days, brains were sectioned at a thickness of 10-µm.

**Immunocytochemistry**

I used a monoclonal anti-synaptotagmin of *Drosophila* from mouse as a primary antibody, and goat anti-mouse IgG antibody conjugated with Alexa Fluo555 (Molecular Probe, Eugene, OR, USA) as secondary antibody. The anti-synaptotagmin antibody
developed by Dr. Kai Zinn was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Brains were dissected in 4% paraformaldehyde in phosphate buffered saline (PBS) with 0.2% Triton X-100 (PBST) and washed with PBST four times for 30 min at room temperature (RT). After washing, brains were pre-incubated in 5% normal goat serum (NGS) in 0.5% PBST overnight at 4°C, and then incubated in primary antibody (1:100 in 2% NGS PBST) for 3 days at 4°C. Brains were washed for 6 hrs at RT, and then washed with PBST overnight at 4°C and then incubated in secondary antibody (1:100 in 2% NGS PBST) for 3 days at 4°C, always in darkness from this procedure. After washing with PBST for 6 hrs at RT and then washing overnight at 4°C, and with PBS 3 times for 1hr each at RT, brains were dehydrated through a graded series of ethanol and cleared with methyl salicylate. They were then observed using a 1 or 2 µm thickness of optical sections with the laser scanning confocal microscope (Olympus Fluoview 300) housed in the Huck Institutes for the Life Sciences at Penn State University. Alexa Fluo555 was excited with a green HeNe laser (543 nm) and images collected through a 565 longpass filter.

**Image processing and figure preparation**

Histological sections were captured with Spot RT II digital camera (Diagnostic Instrument, Sterling Heights, MI, USA) attached to a compound microscope (Olympus BX50). The color, brightness, and contrast levels of digitally captured images were adjusted using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA) for better
contrast. Schematic diagrams of Figures 3-2A, 3-B, and 3-C were digitally drawn using Canvas 9 (ACD Systems, Miami, FL, USA). Captured images of confocal microscopy were reconstructed using software developed by J. C. Fiala and K. M. Harris (downloaded from www.synapses.bu.edu).

RESULTS

My histological examinations of the *H. subflexa* antennal lobe (Fig. 3-1) revealed an MGC the same as described by Vickers and Christensen (2003), comprised of four glomeruli called the cumulus (Cu), the dorsomedial (DM), the anteromedial (AM), and the ventromedial (VM) (Figs. 3-1, 3-2). There were also numerous ordinary glomeruli clearly set apart from the four MGC glomeruli. In addition, I also found a distinct cluster of 8 glomeruli just posterior to the MGC in every male that I examined, and I named this the Posterior Complex (PCx) (Figs. 3-1, 3-2). Many of the PCx glomeruli lie in the most posterior area of this most dorsal portion of the antennal lobe (Fig. 3-1). The PCx is also clearly separated from the large cluster of ordinary glomeruli (Figs. 3-1, 3-2). A large bundle of fibers projects out of the PCx, the main trunk of which is a confluence of the bundles from the various levels all oriented toward the center of the antennal lobe (Figs. 3-1B, 3-2C). The fiber bundle from the PCx eventually merges with the bundle from the MGC (Fig. 3-2C). The *H. subflexa* PCx appears similar to the PCx described for the first time in *H. zea* (Lee et al. 2006), except that the *H. subflexa* PCx has one more glomerulus than does *H. zea*. The numbers I assigned to the PCx glomeruli were determined as for *H.
zea, according to the order of their dorsal-to-ventral, anterior-posterior, and medial lateral positions, respectively, after examining serial sections from twelve males (16 antennal lobes).

**Type-A sensilla**

I attempted to stain type-A sensilla, as determined by their electrophysiological profiles in which ORN action potentials occurred only in response to the major pheromone component, Z11-16:Ald (Fig. 3-3A). Among the 72 such sensilla that were located, 28 produced successfully stained preparations showing arborizations in the antennal lobe. Out of these 28 preparations, 17 (~60.7 %) exhibited a single axonal stain projecting exclusively to the largest of the MGC glomeruli, the cumulus (Fig. 3-1; Table 3-1).

The other 11 preparations (~39.3 %) exhibited two or more stained ORNs (Table 3-1). Without exception, one of these axons projected to the cumulus and a second axon projected to the most dorsal of the PCx glomeruli, which I have labeled “PCx1” (Figs. 3-3, 3-4).

An additional two sensilla out of those exhibiting projections to the cumulus and PCx1 showed two or three extra axons projecting into ordinary glomeruli (Figs. 3-4B, C). Also, an additional preparation exhibiting ORNs projecting to the cumulus and PCx1 had its third axon projecting to the PCx4 of the Posterior Complex (Table 3-1).
Figure. 3-1
Dorsal view the antennal lobe of *H. subflexa* brain (horizontal sections) showing position of the Posterior Complex (PCx) relative to MGC and ordinary glomeruli (Ord). **A.** A section at a depth of 60 µm from the most dorsal surface. Three MGC compartments (Cu, DM, and AM) and two glomeruli of the PCx (PCx1, and 2) are visible. A stained axon’s arborizations are visible in the Cu. **B.** Depth of 80 µm showing the cumulus (Cu; again with stained arborizations) and VM of the MGC, and three more glomeruli of the PCx (PCx3, 4, 5). **C.** A depth of 110 µm (a more ventral section) showing four glomeruli of the PCx (PCx4, 6, 7, 8). Cu, cumulus; DM, dorsomedial glomerulus; AM, anteromedial glomerulus; VM, ventromedial glomerulus; Ord, ordinary glomeruli; P, posterior; M, medial. Scale bar = 50 µm.
Figure. 3-2
Schematic frontal view diagram of the glomerular topographies of the MGC and the Posterior Complex (PCx) in the male *H. subflexa* AL. A. The MGC is comprised of four glomeruli; cumulus (Cu), dorsomedial (DM), anteromedial (AM), and ventromedial (VM) glomeruli. B. The PCx is comprised of eight glomeruli, PCx1-8. The numbers after “PCx” are designated by the order of the glomerular arrangement in the AL; dorsal to ventral, anterior to posterior, and medial to lateral. The PCx1 glomerulus is positioned most dorsally in this complex, and is the arborization destination of the second secondary ORN from the type-A sensilla. The PCx4 glomerulus is positioned anteroventrolaterally, and is the destination of the secondary ORN of type-B sensilla. C. A combined view, showing that the PCx is situated posterior to the MGC. Outgoing neurons from both the MGC and the PCx appear to form a common tract. D. Three dimensional reconstruction image from the series of optical sections with laser scanning confocal microscopy after fluorescence immunostaining for synapse-specific proteins. D dorsal; L lateral; A anterior.
A

Z11-16:Ald 1 µg

B

C

PCx2

DM

Cu

PCx4

PCx3

PCx4

PCx1

PCx8
Figure. 3-3
A. Spike train of an ORNs from a type-A sensillum in response to Z11-16:Ald puffed for 50 msec. Larger amplitude action potentials were elicited only by stimulation with Z11-16:Ald among test compounds used for the identification. A neuron with small spontaneous action potentials was not responsive consistently to any of the test compounds. Inset shows different waveforms from two different neurons; smaller action potential from an secondary neuron, and larger action potential from Z11-16:Ald-responsive ORN. B-C. Frontal view of a male *H. subflexa* antennal lobe, showing the arborization destinations of two neurons that were stained within one type-A sensillum. B. One stained axon arborized (arrowheads) in the cumulus (Cu). C. A second axon arborized (arrowhead) in the dorsal-most glomerulus (PCx1) of the posterior complex. The section in (B) is 80 µm posterior from the frontal surface. The section in (C) is 40 µm posterior to (B). Cu, cumulus; DM, dorsomedial glomerulus; D, dorsal; L, lateral. Scale bar = 50 µm.
Figure. 3-4
Dorsal views of stained neuronal arborization from a type-A sensillum. A. A section at ca. 30 μm depth from the most dorsal surface. Axonal arborizations can be seen in PCx1 (black arrowhead) and the cumulus (light arrowheads). B. The next section, 10 μm more ventral than (A), shows an extensive axonal arborization in the cumulus (light arrowhead), and a second stained axon projecting to an ordinary glomerulus (black arrowhead) situated near the DM glomerulus of the MGC. C. A section at a depth 40 μm more ventral than (B) showing another stained axon (arrowhead) terminating in a second ordinary glomerulus. Cu, cumulus; DM, dorsomedial glomerulus; AM, anteromedial glomerulus; Ord, ordinary glomerulus; P, posterior; M, Medial, Scale bar = 50 μm.
Stimulation with repetitive puffs of either 10µg Z11-16:Ald or a blend of 10µg Z11-16:Ald and 10µg linalool during contact with the cobalt-filled electrode did not significantly influence the selective uptake of cobalt stain by the ORNs in these type-A sensilla. Approximately 60% of the stained preparations, regardless of stimulus, ended up with a single-stained ORN projecting to the cumulus.

Type-B sensilla

Type-B sensilla were identified by their characteristic ORN-responsiveness to Z9-16:Ald, an important minor pheromone component of this species, plus a low degree of cross-responsiveness to Z9-14:Ald (Fig. 3-5A) (Baker et al. 2004). Spontaneous activities in clean air showed spikes having two distinct waveforms. The larger-spiking of these ORNs showed a more symmetrically bipolar waveform than the smaller-spiking ORN (Fig. 3-5A). Responses during stimulation showed that it is this larger-spiking ORN that is sensitive to Z9-16:Ald and Z9-14:Ald stimulation (Fig. 3-5A) (Baker et al. 2004). The second ORN, the one having the more asymmetric, more unipolar action potential, showed no consistent activity in response to any of my test compounds (Fig. 3-5A).

I obtained 28 successful stains from ORNs in type-B sensilla in 58 preparations. The majority of these, 23 out of 28 (~82.1%), resulted in two or more stained ORNs. In all cases the two axons arborized in two specific glomeruli, the DM and the PCx4 (Table 3-1; Figs. 3-2, 3-5B). Eighteen of these 23 exhibited only two stained axons projecting to these two locations. The remaining five of these 23 exhibited an additional third or even a
fourth stained ORN projecting either to an ordinary glomerulus (Fig. 3-6A) or to the cumulus (Table 3-1).

The ORNs from the remaining five out of 28 stained type-B sensillar preparations (~18%) showed only a single-stained ORN (Table 3-1). One such ORN had its single axon projecting only to the PCx4, and the ORNs from the other 4 sensilla stained only the DM. One of the DM-targeting sensilla had one or two extra axon(s) that were stained in the antennal nerve, but the stains faded out before reaching a target glomerulus (data not shown).

**Type-C sensilla**

The physiology of *H. subflexa* type-C sensilla is characterized by differential activity profiles of two co-compartmentalized, pheromone-odorant-sensitive neurons (Baker et al. 2004). Stimulation with the minor pheromone component Z11-16:OH elicits activity from an ORN exhibiting smaller action potentials, whereas larger action potentials are generated from the second ORN upon stimulation with either Z11-16:Ac or Z9-14:Ald (Fig. 3-7A) (Baker et al. 2004). I obtained 19 successfully stained type-C sensillar preparations out of 23 attempts. Seventeen of these (~89.5%) exhibited two stained axons projecting to the AM and VM glomeruli, the two smaller glomeruli of the MGC (Table 3-1; Figs. 3-2, 3-7B, C, 3-8A). The remaining two preparations resulted in a single stained axon projecting to the VM glomerulus. Three of these 17 exhibited additional stained
Figure. 3-5
A. Responses of an ORN from a type-B sensillum. This larger spiking ORN with a fairly symmetrically bipolar waveform is responsive to stimulation with Z9-16:Ald and Z9-14:Ald. Other test compounds did not evoke any neuronal responses. Asymmetric waveforms of the action potentials from the second, smaller spiking neuron (black dots) were spontaneously generated; this neuron did not respond to any of the compounds tested. Inset shows waveforms from two different neurons in extended time scale. B-C. Frontal sections showing neuronal projection patterns of the two ORNs housed within a type-B sensillum. B. A section at about 90 µm depth from the frontal surface of the antennal lobe revealed an axonal arborization in the DM glomerulus of the MGC (arrowhead). C. A section 90 µm posterior to (B) showing a second axon arborizing in the PCx4 glomerulus (arrowhead). Cu, cumulus; DM, dorsomedial glomerulus; VM, ventromedial glomerulus; Ord, ordinary glomeruli; D, dorsal; L, lateral, Scale bar = 50 µm.
Figure. 3-6
Frontal sections showing neuronal projection patterns of a type-B sensillar neuron that projects to an ordinary glomerulus in addition to neurons projecting to the DM and PCx4. A. One axon arborized in the DM glomerulus (light arrowhead) and another arborized in the water-drop-shaped ordinary glomerulus (black arrowheads). B. Another stained axon arborizing in the PCx4 (arrowhead) at a depth 80 μm more posterior to (A). Cu, cumulus; DM, dorsomedial glomerulus; VM, ventromedial glomerulus; Ord, ordinary glomeruli; D, dorsal; L, lateral, Scale bar = 50 μm.
A

Z11-16:Ac 1µg
Z9-14:Ald 1µg
Z11-16:OH 1µg

2 ms
10 mV
200 ms
10 mV
2 ms

B

AM
VM

C

Cu
AM
VM
Ord
Figure. 3-7
A. Spike trains of ORNs from a type-C sensillum. The tracings show a larger-amplitude spiking ORN that responds to both Z11-16:Ac and Z9-14:Ald, and a smaller-amplitude spiking ORN that responds to Z11-16:OH. Insets show waveforms from larger- and smaller-spiking ORNs in extended time scale. B. Frontal section showing axonal projections and arborizations of the ORNs in the AM and VM glomeruli (arrows). C. A section 10 µm more posterior to (B) showing arborizations in the AM and VM glomeruli. Cu, cumulus; AM, anteromedial glomerulus; VM, ventromedial glomerulus; Ord, ordinary glomeruli; D, dorsal; L, lateral, Scale bar = 50 µm.
Figure. 3-8
Frontal sections showing neuronal projection patterns of type-C sensillar neurons that project to ordinary glomeruli in addition to those having arborizations in the AM and VM glomeruli. A. Arborizations in the AM and VM glomeruli (arrows). Two axonal projections to VM and AM each are visible on dorsal to the cumulus (black arrowheads). A faint axonal projection to an ordinary glomerulus is also visible close to the dorsal AL surface (light arrowhead). B. A section 40 µm posterior to (A) showing remains of arborizations in the VM glomerulus, but in addition with a third axon arborizing in an ordinary glomerulus (arrowhead) situated adjacent to the DM glomerulus. A fourth axon shows its projection (asterisk) to its target glomerulus. Inset: magnified image showing arborization (arrowhead) as well as projection of fourth axon (asterisk). C. Section ca. 40 µm posterior to (B) showing this fourth axonal arborization (arrowheads) in the water-drop-shaped ordinary glomerulus. Inset: magnified image of the target glomerulus. Cu, cumulus; DM, dorsomedial glomerulus; AM, anteromedial glomerulus; VM, ventromedial glomerulus; Ord, ordinary glomeruli; D, dorsal; L, lateral. Scale bar = 50 µm.
Table 3-1.

A-type (N=28)

<table>
<thead>
<tr>
<th>Target Glomeruli</th>
<th>Stained</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single target Cu</td>
<td>17</td>
<td>60.7</td>
</tr>
<tr>
<td>Multiple</td>
<td>11</td>
<td>39.3(^a)</td>
</tr>
</tbody>
</table>

\(^a\)Two of these occasions stained ordinary glomeruli and one occasion stained PCx4 in addition to the cumulus and PCx1

B-type (N=28)

<table>
<thead>
<tr>
<th>Target Glomeruli</th>
<th>Stained</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single target DM</td>
<td>4</td>
<td>14.3</td>
</tr>
<tr>
<td>PCx4</td>
<td>1</td>
<td>3.6</td>
</tr>
<tr>
<td>Multiple targets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM+PCx4</td>
<td>18</td>
<td>64.3</td>
</tr>
<tr>
<td>DM+PCx4+Ord(s)</td>
<td>4</td>
<td>14.3</td>
</tr>
<tr>
<td>DM+PCx4+Cu</td>
<td>1</td>
<td>3.6</td>
</tr>
</tbody>
</table>

C-type (N=19)

<table>
<thead>
<tr>
<th>Target Glomeruli</th>
<th>Stained</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single target VM</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>AM</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Multiple targets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VM+AM</td>
<td>14</td>
<td>73.7</td>
</tr>
<tr>
<td>VM+AM+Ord(s)</td>
<td>3</td>
<td>15.8</td>
</tr>
</tbody>
</table>
A

Heliothis subflexa

Type-A

Z11-16:Ald

“Lig. Unkn.”

Type-B

Z9-16:Ald
Z9-14:Ald

Type-C

Z11-16:Ac

Z11-16:OH

B

Heliothis virescens

Type-A

Z11-16:Ald

“Lig. Unkn.”

Type-B

“Lig. Unkn.”

(Z9-14:Ald)

Z9-14:Ald

Type-C

Z11-16:OH

Z11-16:Ac
Figure. 3-9
Diagrams of the sensillar compartmentalization arrangements and projection destinations of olfactory receptor neurons in type-A, B, and C trichoid sensilla of four heliothine moth species. The schemes follow the results of studies using cobalt staining of physiologically characterized neurons within specific sensilla (Berg et al. 1998, 2005; Lee et al. 2006; Lee et al. this paper), staining of characterized projection interneurons (Vickers et al. 1998, Vickers and Christensen 2003) and also additional results from calcium imaging (Galizia et al. 2000; Lee et al. 2006). Glomeruli in gold are MGC glomeruli known to be involved in pheromonal attraction. Glomeruli in pink are MGC glomeruli known to be involved in behavioral antagonism. Glomeruli in blue are those known through anatomical analyses to reside in the PCx, and those in gray are speculated to reside in a PCx that has not been anatomically characterized yet. A. *H. subflexa* (this paper) showing pheromone-component-responsive ORNs (black axons) projecting to their respective MGC glomeruli, and the co-compartmentalized secondary ORNs from types A and B sensilla (blue axons) projecting to the PCx1 and PCx4 glomeruli, respectively. B. *H. virescens* (Berg et al. 1998; Galizia et al. 2000), showing pheromone-component-responsive ORNs projecting to respective MGC glomeruli. I speculate from the reports of Berg et al. (1998, 2002) that co-compartmentalized secondary neurons from type-A and type-B sensilla (gray axons) project to PCx1 and PCx4 glomeruli (in gray) that have not yet been anatomically characterized as such for *H. subflexa*. C. *H. zea* (Lee et al. 2006). The significant features of this projection pattern include the projection of the secondary ORN in type-A sensilla to the PCx1, and also the finding that the ORNs in type-B sensilla project to the same MGC glomeruli as the ORNs from type-C sensilla, but type-B ORNs do not respond to pheromone compounds except for Z9-14:Ald. D. *H. assulta* (Berg et al. 2005), in which the major pheromone component is Z9-16:Ald and the ORNs tuned to this component project to the cumulus. ORNs tuned to the minor component, Z11-16:Ald project from their type-A sensilla to the ventral (V) glomerulus in the MGC. I speculate from Berg et al. (2002, 2005) that the secondary ORN from type-A sensilla (gray axon) arborizes in a PCx glomerulus that has not yet been anatomically characterized (in gray). Cu, cumulus; DM, dorsomedial glomerulus; AM, anteromedial glomerulus; VM, ventromedial glomerulus; DMA, dorsomedial anterior glomerulus; DMP, dorsomedial posterior glomerulus; V, ventral glomerulus; Lig. Unkn., ligand unknown.
axons targeting one or two ordinary glomeruli (Table 3-1; Figs. 3-8B, C). There appeared to be no differential, activity-dependent ORN cobalt uptake resulting from stimulation with either 10µg Z11-16:Ac or 10µg Z11-16:OH.

**DISCUSSION**

I have now described the pathways of axons from *H. subflexa* pheromone-component-tuned ORNs residing in trichoid sensilla to their glomerular destinations in the antennal lobe. When I couple my findings with those of Vickers and Christensen (2003), who described pheromone-component-specific projection interneuron arborizations into each of the four *H. subflexa* MGC compartments, I conclude that there is a correspondence between pheromone odorant-specific input to, and output from, the different MGC compartments. These relationships for *H. subflexa* are depicted in Figure 3-9A.

Although large numbers of my single-sensillar preparations exhibited two stained ORNs each projecting to a different glomerulus, the ORNs reached the same glomeruli with high fidelity from preparation to preparation. Pairs of glomeruli exhibiting stained axons were nearly perfectly correlated with their origin from either type-A, type-B, or type-C sensilla. Preparations exhibiting only single stains were similarly highly correlated with their sensillar-type origin.

Sixty percent of the type-A sensillar preparations showed a single stained ORN projecting its axon without exception to the cumulus. Another forty percent of type-A
sensillar preparations had two stained ORNs, with all these preparations exhibiting an ORN arborization in the cumulus, plus a second stained axon targeting the PCx1 glomerulus of the Posterior Complex. No pheromone-component-sensitive ORN in moths has ever been found to arborize anywhere but in the MGC, and I conclude that it is the Z11-16:Ald-tuned ORNs from these preparations that target the cumulus.

This interpretation is consonant with what has been shown for the projections of Z11-16:Ald-tuned ORNs in *H. virescens* and *H. zea* (Berg et al. 1998; Lee et al. 2006). In addition, projection interneuron staining patterns in the *H. subflexa* antennal lobe (Vickers and Christensen 2003) show definitlely that the cumulus is where Z11-16:Ald-tuned projection interneurons arborize. Given the linear pathways of ORN-to-gemerulus-to-projection-interneuron that have been found in *H. virescens* and *H. zea* (Fig. 3-9; Berg et al. 1998; Vickers et al. 1998; Lee et al. 2006), this again indicates that the Z11-16:Ald-tuned ORNs arborize in the cumulus of the *H. subflexa* MGC.

The secondary ORNs from these preparations are therefore the ones that target the PCx1 glomerulus in the newly described Posterior Complex. The targeting of the PCx by the co-compartmentalized secondary ORN in type-A sensilla was definitively shown in *H. zea* (Lee et al. 2006). One of the most striking results from the current study is the finding that secondary ORNs in *H. subflexa* that are co-compartmentalized with pheromone-component-sensitive ORNs in either type-A or type-B sensilla project into a specific glomerulus in a cluster of glomeruli located in the PCx that has a similar morphology to that described for the first time in *H. zea* (Figs. 3-9A, C; Lee et al. 2006). Moreover, as in *H. zea*, the secondary ORNs from type-A sensilla in *H. subflexa* always project into the most dorsal and medial of the eight PCx glomeruli in *H. subflexa*, which I have similarly
labeled the PCx1 (Lee et al. 2006). Again as in *H. zea*, the PCx1-targeting neurons in *H. subflexa* were not found originating from any sensilla other than type-A; I conclude that these secondary ORNs are not arbitrarily co-compartmentalized with the larger-spiking ORNs in type-A sensilla.

Although I did not record any obvious activity from the secondary ORN in type-A sensilla in response to the odorants used in the present study, I subsequently have undertaken an intensive search for potential ligands for this ORN using a lower-noise system of excised antennae rather than the whole body. I did see activity from an extremely small-spiking ORN. The results thus far suggest that there is concomitant responsiveness of this secondary ORN whenever there is activity from the large-spiking ORN in response to Z11-16:Ald (S.-G. Lee and T. C. Baker, unpublished data).

In that same study I have tested other potential heliothine sex pheromone-related compounds including non-natural analogs and about 60 general odorants. I have also tried stimulation with the C16 acids corresponding to the enzymatic degradation products of heliothine aldehyde pheromone components (Prestwich et al. 1989; Tasayco and Prestwich 1990a, 1990b) because in other noctuid species such as *Trichoplusia ni* and *Agrotis segetum*, the ORNs that are co-compartmentalized with major component-sensitive ORNs (Hansson et al. 1992; Todd et al. 1992, 1995) are highly responsive to the corresponding degradation products (alcohols) of the acetate major component (Prestwich et al. 1989). None of this large array of odorants evoked responses from the secondary ORN. The ORN responded only when the large-spiking ORN fired in response to Z11-16:Ald or a structurally similar analog (S.-G. Lee and T. C. Baker, unpublished data).
The anatomical conformity of the target glomeruli of the secondary ORNs in type-A sensilla of these North American heliothine moths implies that if there is any odorant-specific processing performed within the PCx1, it may be similar across heliothine species. I have identified a morphologically similar PCx glomerular arrangement in the male *H. virescens* olfactory lobe (S.-G. Lee and T. C. Baker unpublished data) and have pointed out previously (Lee et al. 2006) the morphological similarities of the PCx I described in *H. zea* to glomeruli that have previously been labeled “ordinary” in *H. virescens* and *H. assulta*; all receiving input from secondary co-compartmentalized ORNs having unknown tuning properties (Berg et al. 1998, 2005). In Figure 3-9 I have summarized the current status of ORN input projections to the MGC and PCx for these four species of heliothine moth.

Although there were two ORNs consistently stained in type-B sensilla with one projecting to the DM and the other to the PCx4, I conclude that the DM glomerulus receives input from Z9-16:Ald-tuned ORNs, because in moths no pheromone-component-sensitive ORNs, and only rarely projection interneurons, have been found to arborize outside of the MGC (Hansson and Christensen 1999; Vickers and Christensen 2003). This assignment is also consonant with the arborization of Z9-16:Ald-responsive projection interneurons that always target the DM glomerulus (Vickers and Christensen 2003). In addition, calcium images I have started to accumulate for *H. subflexa* show that the focal point of calcium activity in response to Z9-16:Ald antennal stimulation is in the DM glomerulus (M. A. Carlsson, B. S. Hansson, S.-G. Lee, T. C. Baker, unpublished data). There is thus a linear arrangement through the DM glomerulus of Z9-16:Ald-related input from ORNs and Z9-16:Ald-related output from projection interneurons. The
PCx4 glomerulus of *H. subflexa* is therefore functionally and morphologically engaged in receiving information from the secondary ORN that is co-compartmentalized with the ORN responsive to Z9-16:Ald/Z9-14:Ald in type-B sensilla. This companion, secondary ORN did not respond consistently to any of the ca. 70 test compounds with which it was challenged (Lee and Baker, unpublished data). This B-type secondary ORN produces spontaneous asymmetric action potentials, and the identical waveform of this type of ORN across all the type-B sensilla I recorded from suggests that it has a distinctive signal generating system that is different from that of the Z9-16:Ald/Z9-14:Ald-responsive ORN in terms of its ion channel characteristics, especially during the outward current of hyperpolarization.

In *H. virescens*, one of two preparations of Berg et al. (1998) for the staining of B-type sensilla containing Z9-14:Ald-responding ORNs showed one axon projecting to an “ordinary glomerulus” located ventral to the MGC, in addition to an ORN projecting to the dorsomedial (DM) compartment of the MGC. The DM glomerulus was concluded to be the location receiving Z9-14:Ald-related input based, among other factors, on the DM arborization location of Z9-14:Ald-tuned projection interneurons (Vickers et al. 1998). Subsequently, a calcium imaging study confirmed the *H. virescens* DM glomerulus as the MGC glomerulus receiving ORN excitation related to Z9-14:Ald (Galizia et al. 2000). The ordinary glomerulus that is depicted in Berg et al. (1998) displays a similar, topographic, ventral proximity to the PCx4 glomerulus of *H. subflexa* (Figs. 3-9A, B). No mention was made of the posterior depth of this glomerulus in relation to the DM glomerulus (Berg et al. 1998). The secondary ORN that targets it has
an undefined tuning profile, because no compound was found that excited it (Berg et al. 1998).

The projections of the ORNs I found in type-C sensilla exhibit highly consistent patterns of labeling into the AM and VM, the two smaller glomeruli in the MGC. I were not able to directly identify which of these two ORNs targets the AM or the VM glomerulus due to the non-selectivity of cobalt-dye uptake during cobalt application. However, the arborization of a Z11-16:OH-responsive projection interneuron in the AM glomerulus (Vickers and Christensen 2003), and the increasingly well-established linearity of pheromone component olfactory pathways in heliothine moths (Berg et al. 1998; Vickers and Christensen 2003; Vickers et al. 1998) suggest that the AM glomerulus is the primary receiving center for action potentials from Z11-16:OH-tuned ORNs. By default, the VM glomerulus should be the target glomerulus for the ORN that responds to Z11-16:Ac and Z9-14:Ald. The first calcium images from *H. subflexa* now also support this assignment (M. A. Carlsson, B. S. Hansson, S.-G. Lee, T. C. Baker, unpublished data).

The frequency with which ordinary glomeruli received stains from third or fourth ORNs residing in *H. subflexa* types A, B, and C long trichoids was higher in *H. subflexa* than in other heliothines. This would imply that in this species there may be more ORNs tuned to general odorants that are co-compartmentalized in these sensilla with ORNs tuned to pheromone components than there are in either *H. zea* (Lee et al. 2006) or *H. virescens* (Berg et al. 1998). The ordinary-glomerulus-staining ORNs arose with similar likelihood from type-A, type-B and type-C sensilla, and so no one type of sensillum was associated with the presence of these ORNs. The stained arborizations were not in a wide
variety of ordinary glomeruli, but rather were restricted with high fidelity to but a few specific glomeruli. Arborizations of seven of the 16 ORNs targeting ordinary glomeruli terminated in an ordinary glomerulus most closely adjacent and medial-posterior to the DM (Figs. 3-4B, 3-8B). In addition, two ORNs from type-A, two from a type-B and three from a type-C sensillum targeted a water-drop-shaped glomerulus situated medially and several tens of micrometers posterior to the ordinary glomerulus mentioned above (Figs. 3-4C, 3-6A, 3-8C). I also found a type-A and a type-B sensillum each having an ORN projecting to the most anterior ordinary glomerulus in the antennal lobe. These results imply that these 16 ORNs terminating in ordinary glomeruli and originating variously from types A, B, and C trichoid sensilla might show three distinctive tuning spectra to odorant ligands.

It is unclear why, in addition to the MGC and ordinary glomeruli, that a third subcluster of glomeruli, the PCx, should exist in these heliothine moth species. Such a grouping implies a separate, broad function of this structure, just as the MGC cluster is related to pheromone compound inputs and the large cluster of ordinary glomeruli is related to all other odorant inputs. I have only performed staining experiments on physiologically identified ORNs in males of these species thus far. I hope to perform similar studies and find sensilla in females that have Z11-16:Ald-responsive ORNs in order to try staining any co-compartmentalized secondary ORNs to determine their target glomeruli in the female antennal lobe. My preliminary analysis of the female antennal lobes of *H. zea* and *H. subflexa* has thus far revealed a structure in both species similar to the male PCxs, but appearing slightly different than those of the males. A more thorough morphological sex-specific comparison coupled with physiological and cobalt staining
studies using females should help provide more insight regarding any functional and morphological dimorphisms of the PCx in these species.

REFERENCES


Laster ML (1972) Interspecific hybridization of Heliothis virescens and H. subflexa. Environ Entomol 1:682-687


Tasayco J ML, Prestwich GD (1990a) Aldehyde oxidases and dehydrogenases in antennae of five moth species. Insect Biochem 20:691-700


CHAPTER 4

Activity of Helicoverpa zea and Heliothis subflexa secondary olfactory neurons co-compartmentalized with neurons responsive to sex pheromone components

Seong-Gyu Lee and Thomas C. Baker

ABSTRACT

The olfactory receptor neurons co-compartmentalized in sensilla on male Helicoverpa zea and Heliothis subflexa with a neuron tuned to the major pheromone component of both species were challenged with a wide array of odorants and their activities electrophysiologically recorded to see if these were truly ‘silent’ neurons, as they had previously been labeled. These secondary ORNs in both species exhibited such small spike amplitudes that their activity, if any, could not be discerned in the majority of sensillar. Recordings for H. zea, when spike amplitudes of the secondary neurons were discernable above the noise, I found that the neurons exhibited increased action potential frequencies whenever the large-spiking neuron exhibited increased spike frequency. This correlation between the activity of the large-spiking and secondary neurons occurred in H.
zea in response to both the major component, \((Z)\)-11-hexadecenal, and a hydrocarbon analog, 1, \((Z)\)-12-heptadecadiene. In \(H.\ subflexa\) the correlation only occurred in response to \((Z)\)-11-hexadecenal; the hydrocarbon evoking a dose-related increase in response only from the large-spiking neuron. Seven long-chain carboxylic acid derivatives of aldehyde oxidation were tested, and none evoked consistent responses from the secondary neuron or the large-spiking neuron. It may be that the coactivity of the two neurons is related to a breakdown product of \((Z)\)-11-hexadecenal other than the corresponding acid. Application of 58 general odorants, 4 minor pheromone components and 3 minor constituents in the pheromone glands of three heliothine moths did not consistently activate these secondary neurons in either \(H.\ zea\) or \(H.\ subflexa\). This again bolsters the conclusion that the secondary neuron activity is related in some way to the major pheromone component’s precise structure.

**INTRODUCTION**

The sexually dimorphic long trichoid sensilla of three male heliothine moths, \(Helicoverpa\ zea, Heliothis\ virescens,\) and \(Heliothis\ subflexa\) have been categorized into three physiological sub-types with respect to the electrophysiological activities of their olfactory receptor neurons (ORNs) in response to heliothine pheromone components (Cossé et al. 1998, Baker et al. 2004). Type-A sensilla have an ORN that is most responsive to the major pheromone component common to all three species, \((Z)\)-11-hexadecenal (Z11-16:Ald). Type-B sensilla of \(H.\ zea\) have an ORN that is optimally tuned to \((Z)\)-9-tetradecenal (Z9-14:Ald), a minor pheromone component of \(H.\ virescens,\)
a behavioral antagonist to *H. zea*, and an apparently behaviorally neutral compound for *H. subflexa*. Type-C sensilla contain two different olfactory receptor neurons (ORNs), one that responds to one of the minor pheromone components of the conspecific female, and the other that responds to one of the minor pheromone components emitted by interspecific females. The latter ORNs contribute to behavioral antagonism of male attraction when added to the *H. zea* or *H. virescens* blends. These pheromone-component-responsive ORNs transmit olfactory signals to a particular glomerulus in the antennal lobe that is part of a male-specific glomerular cluster called the macrogglomerular complex (MGC). In all three species, the Z11-16:Ald-responsive ORNs project axons to the cumulus, the largest and lateral-most glomerulus among MGC glomeruli (Hansson et al. 1995, Almass et al. 1991, Berg et al. 1998, Vickers et al. 1998, Vickers and Christensen 2003, Lee et al. 2006a, 2006b).

In the course of performing cobalt-staining of heliothine ORNs and tracing them to their target glomeruli in the MGC (Lee et al. 2006a, 2006b), I found that in the type-A and type-B sensilla there was commonly a second cobalt-stained neuron that appeared to have no activity to any heliothine pheromone component. This secondary neuron had been given the temporary name “silent neuron” (Lee et al. 2006a), and because I could not seem to evoke activity from it using any compound, I wondered if it indeed could even be called an ORN.

The secondary ORNs of *H. virescens* type-A sensilla project axons to what has been called an ordinary glomerulus outside the MGC (Hansson et al. 1995; Berg et al. 1998). The axons of secondary ORNs in *H. zea* and *subflexa* type-A sensilla target a
specific glomerulus that I named the PCx1, one of the glomeruli in the array of glomeruli forming the Posterior Complex (PCx) (Lee et al. 2006a). This cluster of glomeruli is situated posterior to the MGC, butting up against it in this most dorsal region of the antennal lobe (Lee et al. 2006a, 2006b). Lee et al. (2006b) also reported stains from a co-compartmentalized secondary ORN in the *H. subflexa* type-B sensilla that is co-compartmentalized with the ORN responsive to the minor pheromone component, \((Z)-9\)-hexadecenal (Z9-16:Ald), and is also weakly responsive to Z9-14:Ald. Interestingly, the axonal projection destination of these secondary ORNs also has 100% fidelity to the PCx4, a different glomerulus in the PCx of *H. subflexa*.

A high-spontaneous-activity, small-amplitude-spiking secondary neuron that is insensitive to any of the heliothine pheromone components has been reported during electrophysiological recordings of the large-spiking ORN from type-A sensilla of *H. virescens*, *H. zea*, and *H. subflexa* (Berg et al. 1998; Baker et al. 2004; Lee et al. 2006a). It is this small-spiking, secondary ORN that projects to the PCx1 in *H. zea* and *H. subflexa*, and because it projects there with 100% fidelity, I suspected that it must have, or did have at one time in the past, a specific odorant to which it is tuned (Lee et al. 2006a, 2006b). Occasionally, the increased frequency of a small-spiking ORN could be discerned during stimulation with Z11-16:Ald along with activity from the large-spiking ORN (Lee et al. unpublished). However in these earlier recordings using a whole-body preparation, small-amplitude noise was usually produced just after pheromone stimulation, apparently due to antennal muscle movement from the scape or pedicel, and it usually obscured the extremely low-amplitude spikes of the secondary ORNs that might otherwise have been observed (unpublished observation).
In this study I therefore sought to search for possible odorants to which the secondary neurons of *H. zea* and *H. subflexa* are tuned, using an excised antennal preparation in order to minimize noise. I challenged the ORNs with a large array of volatiles comprising many chemical classes, as well as using previously untested secondary pheromone gland constituents and long-chain acids related to breakdown products of the aldehydes as a result of aldehyde oxidase known to be present in the sensillar lymph (see Chapter 1).

**MATERIALS AND METHODS**

**Insects**

Colonies of *H. zea* and *H. subflexa* were maintained in the laboratory on a pinto bean diet as previously described (c.f., Lee et al., 2006a, 2006b). Pupae of both species were segregated by sex and males were placed in a growth chamber on a 16:8-h (L:D) photoperiod at 25°C, 40-50% RH. Adult males emerged into screen cages and were given access to 5% sucrose water. Males between day of emergence and 4 days old were used for recordings.
Chemicals

Synthetic pheromone components of the two species, Z11-16:Ald, Z9-16:Ald, (Z)-11-hexadecenyl acetate (Z11-16:Ac), and (Z)-11-hexadecenol (Z11-16:OH), as well as Z9-14:Ald, a minor pheromone component of *H. virescens*, were purchased from Bedoukian Research Inc. (Danbury, Connecticut, USA; purities >98% verified by gas chromatography). A structural analog of Z11-16:Ald, the hydrocarbon 1,(Z)-12-heptadecadiene (Grant et al. 1989) (purity >99%) was synthesized in the laboratory of Dr. Tappey Jones (Virginia Military Institute, Virginia, USA) and kindly donated to us for this work. The hydrocarbon was further purified in the laboratory of Dr. Jim Tumlinson, Penn State University. Synthetic samples of three other constituents of heliothine pheromone glands, hexadecanal (16:Ald), (Z)-7-hexadecenal (Z7-16:Ald), and tetradecanal (14:Ald) with purity of >97% were kindly provided by Dr. Peter Teal (USDA/ARS, Gainesville, FL, USA). Samples of long-chain carboxylic acids related to pheromone components were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). The general odorant compounds listed in Table 1 were purchased from Aldrich Chemical Co., Fluka Chemica (Buchs, Switzerland), and Bedoukian Research Inc (Danbury, CT, USA). The purity of these compounds was >97% except for citronellal (>90%), as checked by GC in our laboratory.

Samples of all compounds in hexane solution were loaded in a 10 µl volume onto a 0.7 x 2.5-cm filter paper strip and the solvent allowed evaporating before placing the strip into 146 mm-long Pasteur pipettes. The preparation and storage of cartridges was as
described by Baker et al. (2004) and Lee et al. (2006a). Serial dilutions of each test compound were prepared using HPLC-grade hexane.

For both *H. zea* and *H. subflexa* type-A sensilla, I used Z11-16:Ald at doses of 0, 0.1, 1, 10, and 100 µg to interrogate the co-compartmentalized secondary ORNs along with their co-resident ORNs tuned to Z11-16:Ald. In the same experiment I also used 1, (Z)-12-heptadecadiene at those same dosages to try to determine whether only the oxidation of Z11-16:Ald to its corresponding acid might be responsible for eliciting activity from the secondary neuron, or whether some other breakdown product could be doing so.

The dosages of the other heliothine pheromone components that were ordinarily used, as well as dosages of the minor constituents of heliothine pheromone glands, were 10 µg. In addition, I tested 16:Ald, Z7-16:Ald, and 14:Ald using a dosage series of 1, 10, and 100µg.

The general odorants to be tested were first segregated into 6 sets on the basis of their chemical classes and functionalities (Table 4-1). Each set, a mixture of from 8 to 11 compounds, was loaded at 10 µg per compound onto filter paper and placed into cartridges for a high-through-put screening test. The dosage used for the mixture-sets was 10 µg per compound, but sets comprised of 100 µg of each compound were also tested on six *H. subflexa* sensilla. The seven long-chain carboxylic acid compounds were loaded into individual cartridges at 100 µg of each compound.

**Single sensillum recordings**
The activities of ORNs in a sensillum were recorded using the cut-sensillum technique (Kaißling 1974, Van der Pers and Den Otter 1978, Cossé et al. 1998). The recording method was the same as reported previously (Baker et al. 2004, Lee et al. 2006a), except that in order to eliminate noise generated by muscle movement when using a whole-body preparation, these recordings were performed using an excised antenna cut at the base of the flagellum approximately 1-2 mm from the pedicel (c.f., Todd et al. 1992, Hansson and Löfstedt 1987). The base of the excised antenna was inserted into an indifferent glass electrode manipulated to have a sufficiently wide opening for receiving the antenna’s base. The recording electrode was connected to a Universal AC/DC probe assembly with pre-amplifier (Syntech, Hilversum, The Netherlands). Neuronal activity was amplified by the built-in amplifier of micromanipulator system (INR-05, Syntech), digitally processed by IDAC 4 (Syntech), and then recorded and analyzed on computer using data analysis software (Autospike32, Syntech).

Air-puffs for odorant-stimulation were generated with a flow-controller device (SFC-2, Syntech) using 100-ms pulses at 20 ml/s flow rate. Odorants in the puffs issued into an airstream flowing continuously over the antennae at a flow of 8 ml/s during the stimulation. The velocity of each puff was set to two times lower than previously used in order to reduce the possibility of electrical noise that can be generated by the mechanical movement of antenna during higher velocity puffs.

**Data analysis**
To assess the responsiveness of each ORN spike frequencies after stimulation were compared to spontaneous spike frequencies during the pre-stimulation period. All spikes numbers were automatically counted using Autospike32 (Syntech) under default setting. Both pre- and post-stimulation frequencies were counted using a 1s period preceding, and following the cessation of the stimulus puff. There were instances in which the secondary ORN’s post-stimulation action potentials in response to Z11-16:Ald and 1,(Z)-12-heptadecadiene, were obscured by the high frequency of firing by the large-spiking ORNs. In such cases the secondary ORN’s post-stimulation firing frequency was assessed using a 1s delay in counting spikes compared to normal. The tendency of the secondary ORN to fire tonically when it fires at all lends confidence to the use of this deviation from the normal protocol. Frequency histograms were generated with a 25ms bin width for spikes that had been sorted according to amplitude thresholds I selected by eye for subsequent computer sorting (Autospike) for each spike train. Histograms and graphs were produced using the spreadsheet program Microsoft ® Excel 2003 (Microsoft Corporation, Redmond, WA, USA).

Data in the histograms illustrating spike frequency changes in responses to chemical stimulants were subjected to Dunnett’s t-test for multiple comparisons to the blank (p < 0.05), except for figures 4-8 and 4-19. Data in these two figures were analyzed using a z-test in comparison to zero using Bonfferoni’s correction for multiple comparisons.

**RESULTS**
Type-A sensilla of *H. zea*

Forty-two sensilla were identified as type-A sensilla from 14 *H. zea* males. Unfortunately, 18 sensilla were not able to be used to measure spike frequencies because neuronal activities had disappeared after a few stimulations or else the spike amplitudes had diminished too much as a result of activity. I encountered several sensilla in which small-spiking secondary neuron activities were able to be observed early in the contact, but they subsequently disappeared from the records after only a few seconds. Therefore these recordings were not able to be analyzed for secondary neuron activity.

I was able to record from four sensilla that exhibited consistent spontaneous secondary neuronal activities throughout the series of dosages of the test compounds. These four ORNs were responsive to both Z11-16:Ald (Fig. 4-1) and the hydrocarbon analog (Fig. 4-2). I wanted to compare the spike frequencies of the two ORNs according to what was occurring during the 1s period just prior to stimulation with that which occurred during the first second following stimulation. However, because the high frequency of firing from the larger-spiking ORN during the 1s post-stimulation period often masked the activity of the secondary ORN when higher dosages of pheromone or hydrocarbon were used, in these instances the spike frequency of the secondary ORNs was counted for a 1s duration beginning 1s after the activity-initiating point. The spike frequencies of the large-spiking ORNs were always counted for one second without a gap from the activity-initiating point.

The dose-response series using these two compounds revealed that there was a clear correspondence between the activity of the large-spiking ORN and that of the
secondary neuron (Figs. 4-3, 4-4C, D). In most cases (22 out of 25 in this experiment) these secondary neuronal activities preceded the larger action potentials from the Z11-16:Ald-tuned ORN (Fig. 4-3). In the other three cases they occurred synchronously with the larger-spiking ORN. The latency to firing of the secondary ORN following stimulation was highly consistent, regardless of its spike frequency (Fig. 4-3), unlike the large-spiking ORN’s latency that was inversely dose-dependent (Fig. 4-3).

On the basis of the loaded dosages of Z11-16:Ald and the hydrocarbon analog, the Z11-16:Ald-responsive large-spiking ORN was less sensitive to the hydrocarbon analog than to Z11-16:Ald (Fig. 4-4A, 4-4B). On the other hand, the spike frequencies of the small-spiking ORNs in response to a given dosage of either compound were nearly identical to the aldehyde or the hydrocarbon, and as with the large spiking ORN, they increased with increasing dosage (Fig. 4-4A, 4-4B).

I challenged the ORNs from 40 type-A sensilla of 10 males with other aldehyde compounds previously found in the pheromone glands of either *H. zea*, *H. virescens*, or *H. subflexa*: hexadecanal (16:Ald), (Z)-7-hexadecenal (Z7-16:Ald), and tetradecanal (14:Ald) in a dose-response series. I also challenged these ORNs with five other heliothine pheromone-related compounds, 10 µg of Z11-16:Ald, Z9-16:Ald, Z11-16:Ac, Z11-16:OH, and Z9-14:Ald. In this series, I also recorded the neuronal activities immediately after the connection of the sensillum tip to the electrode.

In this group of 40 sensilla I encountered 25 (ca. 63 %) type-A sensilla exhibiting smaller action potentials from a secondary ORN. Out of these 25 sensilla, 13 evoked positive tonic responses at least once from the secondary ORNs in response to exposure to Z11-16:Ald. Among 25 sensilla, 15 sensilla exhibiting consistent spontaneous
activities through the series of test from both large spiking ORNs and secondary ORNs were analyzed. The two minor C16 aldehyde constituents from the \textit{H. zea} pheromone gland did not elicit any compound-specific activity from the secondary ORNs nor did the saturated C14 aldehyde (Figs. 4-5A, 4-6). There was no significant dose-response relationship of any of these compounds with the activity of the secondary ORN (Fig. 4-5B).

Analyzing the spike frequencies from 15 recordings at a dosage of 10 µg, only the compounds having a double bond five carbons from the methyl end of the chain showed any hint of heightened activity in the secondary ORN, with the exception of Z9-16:Ald (Fig. 4-5A).

In order to search for other possible odorants activating the secondary ORNs, I challenged another set of these ORNs from the Type-A sensilla with 58 “general” odorant compounds, including series of plant- as well as animal-related volatiles. I also tested 7 carboxylic acid compounds having 12C, 14C, 16C, and 18C aliphatic groups: dodecanoic acid (lauric acid, 12:COOH), tetradecanoic acid (myristic acid, 14:COOH), (Z)-9-tetradecenoic acid (Z9-14:COOH), hexadecanoic acid (palmitic acid, 16:COOH), (Z)-9-hexadecenoic acid (Z9-16:COOH), (Z)-11-hexadecenoic acid (Z11-16:COOH), (Z)-9-octadecenoic acid (oleic acid, Z9-18:COOH). Due to the relatively short longevity of ORNs when using the cut-tip sensillum recording technique, a high through-put test was devised in order to carry out the testing of general odorant compounds. Test compounds were categorized into 6 sets in relation to their functional groups (Table 4-1), and mixtures of between 8 and 11 compounds were loaded in each set at a dose of 10 µg per compound onto one filter paper in individual cartridges.
Figure 4-1

A. Spike trains of ORNs in a *H. zea* type-A sensillum in response to a puff from a cartridge containing 10 µg Z11-16:Ald. Tonic secondary neuronal action potentials are elicited along with more phasic large amplitude action potentials. Horizontal gray bar denotes 100ms stimulation. 

B. Same spike train as in (A), but simplified to show the secondary action potentials filtered from the noise (small light-toned spikes) and the large amplitude action potentials as well (black spikes). 

C. Action potential frequency histograms (Hz) and the superimposed wave forms of the large (upper) and secondary (lower) ORN spikes that were counted. Black bars represent frequencies of large-amplitude action potentials and gray bars represent frequencies of secondary ORN spikes. Small spikes maintain higher frequencies to the end of train. Bin width is 25ms. Frequency scales are shown at the left.
Figure 4-2
A. Spike trains of ORNs in a *H. zea* type-A sensillum in response to a puff from a cartridge containing 10 µg of the 1, Z12-heptadecadiene hydrocarbon analog to Z11-16:Ald. Fewer spikes were elicited in comparison to stimulation with a 10 µg cartridge of Z11-16:Ald. Horizontal gray bar denotes 100ms stimulation. B. Simplified spike train of (A), with spike filtering used to highlight the secondary ORN’s activity above the noise, as well as that of the large-spiking ORN. Light-toned spikes are from the secondary ORN. C. Action potential frequency histograms (Hz; scale level at left) using 25ms bin widths to count the spikes sorted by amplitude having the corresponding wave forms at right. Black bars represent frequencies of large amplitude action potentials and gray bars represent secondary spikes.
Figure 4-3
Spike activities of ORNs in a *H. zea* type-A sensillum in responses to different dosages of Z11-16:Ald and the 1, Z12-heptadecadienal hydrocarbon analog. Gray (light-toned) spikes are large action potentials, and the red (dark-toned) spikes are those that were elicited from the secondary ORN. The perpendicular dotted line represents the initiation time point of the secondary ORN spikes. Action potential frequencies of secondary ORNs increased with higher dosages of both stimuli. Horizontal gray bar denotes 100ms stimulation.
A-B. Mean (± SEM) spike frequencies of large-spiking and secondary ORNs from *H. zea* type-A sensilla from a dose-response series of Z11-16:Ald and the 1, Z12-heptadecadiene analogue. C-D. Relationship between spike frequencies of the large-spiking and secondary ORNs in response to a dosage series of exposures to Z11-16:Ald and the hydrocarbon analogue. Action potential frequency of the secondary ORN was correlated with that of the large-spiking ORN.
Figure 4-5  
A Mean spike frequency changes (±SEM), compared to pre-stimulus levels, of secondary ORNs in 15 *H. zea* type-A sensilla exposed to minor constituents of pheromone glands and to minor pheromone components of three heliothine moths. Spike frequencies are significantly increased in response to Z11-16:Ald and Z9-14:Ald. Spike frequency changes of the secondary ORNs in response to other compounds are not significantly different from blank puffs. Statistical analysis was performed using Dunnett’s t-test for multiple comparisons to the blank (* p < 0.05). Sample size (N = tested sensilla) for each compound ranged from 11 to 15. **B.** Relationship between the change in spike frequency of the small- and the large-spiking ORNs to in 15 type-A sensilla, excluding data for Z11-16:Ald.
Figure 4-6
Secondary (small-spiking) ORN activity (in red) in a *H. zea* type-A sensillum in response to synthetic minor constituents of pheromone glands and minor pheromone components of three heliothine moths. Only Z11-16:Ald evoked a specific increase in activity in both large- and small-spiking (secondary) ORNs. Horizontal gray bars denote 100ms stimulation.
Table 4-1
Chemical sets utilized in cartridges for a high-throughput survey of the responsiveness of secondary ORNs in the type-A sensilla of *H. zea* and *H. subflexa*, and in the *H. subflexa* type-B sensilla to possible ligands. The dosage loaded in each cartridge was 10 µg per compound except for a series of follow-up tests using *H. subflexa* sensilla in which 100 µg per compound was used (see Figs. 4-16, 4-19, 4-20).

<table>
<thead>
<tr>
<th>Category</th>
<th>Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical Set #1</strong>: Alcohol</td>
<td>1-Hexanol, 1-Heptanol, 1-Octanol, 1-Decanol, Z3-Hexen-1-ol, 1-Octen-3-ol, m-Cresol (1-hydroxy-3-methylbenzene), Eugenol (4-allyl-2-methoxyphenol), Phenethyl alcohol, Phenethyl alcohol (phenylethyl alcohol, Benzyl carbinol, 2-Phenylethanol, Mellol )</td>
</tr>
<tr>
<td><strong>Chemical Set #2</strong>: Aldehydes &amp; Carboxylic acids</td>
<td>Heptanal (1-heptanal), Octanal (1-octanal), Decanal (1-decanal), E2-Hexenal, Phenylacetaldehyde, Benzaldehyde, Butanoic acid (Butyric acid), Hexanoic acid (Caproic acid), Octanoic acid (Caprylic acid), Benzoic acid</td>
</tr>
<tr>
<td><strong>Chemical Set #3</strong>: Esters</td>
<td>Butyl butyrate (butyl butanoate), Ethyl acetate, Hexyl butanoate, E2-Hexenyl acetate, Z3-Hexenyl acetate, Methyl jasmonate, Benzyl acetate, Phenethyl acetate (phenylethyl acetate, 2-phenylethyl acetate), Ethyl benzoate, Methyl salicylate</td>
</tr>
<tr>
<td><strong>Chemical Set #4</strong>: Heterocycles, Hydrocarbons, Ketones, S- and N-containing compounds</td>
<td>1-Heptene, Tridecane, Ethylbenzene, 2-Hexanone, 2-Octanone, Acetophenone, Thujone (3-Thujanone), Hedione (methyl dihydrojasmonate)</td>
</tr>
<tr>
<td><strong>Chemical Set #5</strong>: Terpenes</td>
<td>Geraniol, Linalool, α-Terpineol, Limonene, (E)-β-Ocimene, α-Pinene, (-)-(E)-Caryophyllene, Nerolidol, (E)-β-Farnesene</td>
</tr>
<tr>
<td><strong>Chemical Set #6</strong>: Others</td>
<td>Indole, Acetic acid, Putrecine, Dimethyldisulfide, Dipropyldisulfide, Allyl isothiocyanate, Nerol, Citral, Citronellal, β-Myrcene, Ammonia (NH₃)</td>
</tr>
</tbody>
</table>
Figure 4-7
Responses of a secondary (small-spiking) ORN (in red above) in a *H. zea* type-A sensillum to stimulation with long-chain carboxylic acids and 6 general odorant chemical sets. Only Z11-16:Ald activated the small-spiking secondary ORN, which was accompanied by a strong firing increase by the large-spiking ORN. Horizontal bars denote 100ms stimulation.
A. Mean changes in action potential frequency (±SEM) of the small-spiking secondary ORNs and large-spiking ORNs in *H. zea* type-A sensilla in responses to carboxylic acids (100 µg) and general odorant sets (10 µg/compound). Large-spiking ORN activities in response to Z11-16:Ald were significantly increased (p < 0.05) and small-spiking ORNs also showed significant frequency increases (p < 0.1) in response to Z11-16:Ald and Z9-16:COOH compared to zero. Stimulation with 14:COOH increased spike frequency significantly from zero apparently because of the small variance (SEM = 1.0); however, the average spike increment was 3 spikes/s, which is less than average spike increase using blank puffs (Figs. 4-4, 4-5). A Z-test was used for comparisons to zero with Bonferroni’s correction for multiple comparisons (* p < 0.05, ** p < 0.1).

B. Relationship between changes in spike frequency of the secondary and large-spiking ORNs in type-A sensilla following stimulation with long-chain carboxylic acids.

C. Relationship between changes in spike frequency of the secondary and large-spiking ORNs in type-A sensilla following stimulation with general odorants sets. There was no correlation between the activities of the two compartmentalized ORNs in response to either the acids or the general odorants.

Figure 4-8
A set of 30 type-A sensilla was tested on 10 animals and these revealed that none of my test compounds, including the 7 carboxylic acids, reliably activated the secondary ORN (Fig. 4-7, 4-8). Among 30 sensilla, six were found to show spontaneous secondary neuronal activity. Other sensilla exhibited only large-spiking action potentials responding specifically only to Z11-16:Ald, and none of my test compounds elicited activity from the secondary ORN. Small-spiking ORNs in four out of the six sensilla showed tonic responses only when stimulated with Z11-16:Ald, and the spontaneous spikes of the small-spiking ORNs of the two other sensilla were not indicative of responsiveness to any compounds.

Type-A sensilla of *H. subflexa*

I challenged 17 type-A sensilla from 5 moths with Z11-16:Ald and its hydrocarbon analog in order to determine whether or not a possible relationship exists between the activity of the large-spiking ORN and the secondary ORN in type-A sensilla. Nine of the secondary ORNs exhibited spikes that were large enough to discern above the background noise, and five of these exhibited a tonic response to puffs of Z11-16:Ald and the hydrocarbon analog (Figs. 4-9, 4-10, and 4-11). The initiation of the spike trains from secondary ORNs always occurred either earlier than, or simultaneous with, the onset of spiking from the larger-spiking ORN, just as was the case for the secondary ORNs of *H. zea* in type-A sensilla (Fig. 4-11). Dose-response experiments on the five secondary ORNs that maintained their activity throughout the complete series of exposures to the Z11-16:Ald hydrocarbon stimuli revealed that the spike frequency of the secondary ORN
was highest in response to Z11-16:Ald at a dosage of 10 µg (Fig. 4-12A). The intensity of its firing in response to the highest dosages (10 and 100 µg) reduced its spike size and may have been part of the reason that the frequency appeared to diminish at this dose (Figs. 4-9, 4-11, 4-12); the spikes became too small to be discriminated from the base line in these instances. The firing frequency of the secondary ORN appeared to correspond with that of the large-spiking ORN in response to Z11-16:Ald (Fig. 4-12C).

In response to the hydrocarbon analog, the secondary ORN did not exhibit a consistent increase in firing frequency from sensillum to sensillum with increasing dosages (4-12B), although in some sensilla the relationship was strong (Fig. 4-10). The large-spiking ORN did, however, exhibit such an increase (Fig. 4-12B). The poor correlation between firing frequency of the secondary ORN with that of the large-spiking ORN when the hydrocarbon was used as the stimulus is evident from the linear regression plot (Fig. 4-12D).

When I searched for secondary ORNs in 30 type-A sensilla in order to challenge them with the minor constituents of the three heliothine moths’ pheromone glands, I encountered 22 type-A sensilla exhibiting spontaneous activities from the secondary ORN. The activity of these ORNs in ten of the sensilla disappeared in the middle of testing the series of compounds, and thus I could not assess their relative activities to my entire series. Five out of the twelve remaining secondary ORNs exhibited only phasic spike trains in response to almost every test compound, and the other seven secondary ORNs displayed more tonic responses, especially to Z11-16:Ald (Fig. 4-13). The chemical dosage I applied was usually 10 µg, with other dosages of 1 and 100 µg being used for the three minor constituents of heliothine pheromone glands: 16:Ald, Z7-16:Ald,
and 14:Ald. In both groups, test compounds other than Z11-16:Ald did not cause a significant increase in firing frequency compared to that evoked by stimulation by the blank puff (Figs. 4-13, 4-14A). However, the firing increase in response to stimulation with Z11-16:Ald by the tonic-firing group was significantly greater than the blank (Figs. 4-13, 4-14A). There was little correspondence between the frequency of firing of the large-spiking ORN and the secondary ORN (Fig. 4-14B).

In the test in which a variety of general odorants from various chemical classes was tested, plus a series of long-chain carboxylic acids related to pheromone compounds, I examined 35 type-A sensilla from 11 male *H. subflexa*. I encountered 13 sensilla having a secondary ORN exhibiting spontaneous activity. Five out of the 13 ORNs exhibited tonic firing at least once in response to stimulation with 10 µg Z11-16:Ald (Fig. 4-15), but one of them lost spontaneous activity after a few puffs and thus could not be analyzed. The other 8 secondary ORNs did not exhibit tonic responses, or else their spike frequencies decreased in response to stimulation with Z11-16:Ald. As illustrated in Fig. 4-15, the secondary ORNs’ activities were highly phasic, and were sometimes evoked by nearly all the odor cartridges and sometimes even by the blank cartridge. Tonic spike trains by the secondary ORN were elicited only with stimulation with Z11-16:Ald (Fig. 4-15). No significant activity from the secondary ORN could be evoked by any of the general odorants or by the long-chain acids and general odorants (Fig. 4-16A). The lack of a correspondence between the activity of the large-spiking ORN and the secondary ORN in this test is displayed in Fig. 4-16B.
Figure 4-9
A. Spike trains of ORNs in a *H. subflexa* type-A sensillum in response to 10 µg Z11-16:Ald. Horizontal gray bar denotes 100ms stimulation. B. Simplified spike train of (A) following spike-amplitude-related filtering. Intermediate-sized gray spikes represent the activity of the secondary ORN I was monitoring. C. A spike frequency (Hz) histogram (25ms Bin-width) with corresponding superimposed wave forms of the two ORNs. Black bars represent the frequencies of the large-amplitude ORN’s action potentials, and gray bars represent those of the secondary ORN.
Figure 4-10
A. Spike trains of secondary and large-spiking ORNs in a *H. subflexa* type A sensillum in response to 10 µg of the 1, Z12-heptadecadiene analog of Z11-16:Ald. Horizontal gray bar denotes 100ms stimulation. B. Simplified spike train of (A) following computer filtering according to amplitude. Gray spikes represent activities of the intermediate-spike-sized secondary ORN I was monitoring. C. A spike frequency histogram (Hz scale indicated at left; 25ms bin-width) with corresponding overlapping wave-forms of the two ORNs shown as well. Black bars represent the spike frequencies of the large-spiking ORN and gray bars represent those of the secondary ORN.
Figure 4-11
Spike activities of the large-spiking and secondary ORN in a *H. subflexa* type-A sensillum in responses to different dosages of Z11-16:Ald and its 1, Z12-heptadecadiene hydrocarbon analogue. Gray (light-toned) spikes are action potentials from the large-spiking ORN, and the red (dark-toned) spikes are those elicited from the secondary ORN I was monitoring. The perpendicular dotted line represents the initiation time of the secondary ORN’s spikes following each stimulus. In this sensillum the action potential frequency of the secondary ORN increased in response to higher dosages of both compounds. Horizontal gray bars denote 100ms stimulation.
**Figure 4-12**

A-B. Mean (± SEM) action potential frequencies of large-spiking and secondary ORNs in *H. subflexa* type-A sensilla in response to a dosage series of Z11-16:Ald and its hydrocarbon analog. C-D. Relationship between the spike frequencies of the large- and secondary ORNs in response to Z11-16:Ald and the hydrocarbon analog at dosages of 0.1, 1, and 10 µg.
Fig. 4-13
Responses of the large-spiking (light gray) and secondary (red) ORNs in *H. subflexa* type-A sensilla to synthetic heliothine pheromone gland constituents and pheromone-related compounds. In this sensillum, the secondary ORN showed a tonic response only to Z11-16:Ald and phasic responses to every stimulus applied, even to the blank cartridge. The large-spiking ORN responded only to the stimulation of Z11-16:Ald. Horizontal gray bars denote 100ms stimulation.
Figure 4-14
A. Mean changes in spike frequency (±SEM) compared to pre-stimulation, of secondary ORNs in *H. subflexa* type-A sensilla following exposure to synthetic minor constituents (1, 10, 100 µg) of heliothine pheromone glands and pheromone-related compounds of three heliothine moths (10 µg). Action potential frequencies increased significantly only in response to Z11-16:Ald. Data were analyzed using Dunnett’s t-test for multiple comparisons to the blank (* p < 0.05). B. Relationship between the spike frequency changes of the large-spiking and secondary ORNs in response to the stimuli shown in (A), excluding data for Z11-16:Ald.
Figure 4-15
Responses of the large-spiking (gray) and small-spiking secondary ORNs (red) in a *H. subflexa* type-A sensilla following stimulation with long-chain carboxylic acids and the 6 sets of general odorants. In this sensillum, tonic action potential activity was elicited from the small-spiking secondary ORNs only following stimulation with Z11-16:Ald, and weak phasic activity occurred in response to every other stimulus, including the blank (red or dark spikes). Horizontal bars denote 100ms stimulation.
A

Figure 4-16

A. Mean changes in spike frequency (± SE) of the large-spiking and secondary ORNs in *H. subflexa* type-A sensilla following exposure to long-chain carboxylic acids (100 µg, n=7) and general odorant sets (10 µg, n=8; 100 µg, n=4). There were no significant frequency differences in response to any of the compounds compared to the blank (n=6), except for the large-spiking ORN responding to Z11-16:Ald. Data were analyzed using Dunnett’s t-test for multiple comparison to the blank (* p < 0.05).

B-C. Relationship between spike frequency of the large-spiking and secondary ORN in response to the carboxylic acids (B) and the general odorant sets (C). Secondary-ORN-activities are not correlated with the activities of the large-spiking ORN in either series.
Figure 4-17
Spike trains of large-spiking and secondary ORNs in H. subflexa type-B sensilla following stimulation with synthetic heliothine pheromone-related compounds and minor constituents found in pheromone glands of three heliothine species.

A. The large-spiking ORN is responsive to Z9-16:Ald and Z9-14:Ald. The spontaneous smaller-spiking secondary ORN was not responsive to any of the stimuli. B. Superimposition of a collection of the two action potential waveforms generated from the two co-compartmentalized ORNs in this type-B sensillum. Large spikes (gray) were evoked by Z9-16:Ald/Z9-14:Ald. The secondary ORN’s wave forms (black) are more asymmetric than those of the Z9-16:Ald/Z9-14:Ald-responsive ORN.
Figure 4-18
A. Mean (±SEM) changes in spike frequency of the secondary ORN in *H. subflexa* type-B sensilla following exposure to heliothine pheromone-related compounds. Data were acquired from the test for minor constituents and test for general odorants/acids, and then combined for analysis. Z9-14:Ald evoked the highest spike frequency among the test compounds, but it was not significantly different from the blank. Statistical analysis was performed using Dunnett’s t-test for multiple comparisons to the blank (p < 0.05). B. Relationship between spike frequency of the secondary ORN and the larger-spiking Z9-16:Ald/Z9-14:Ald-responsive ORN. There was no correlation between the activities of the two co-compartmentalized ORNs in response to the heliothine pheromone-related compounds.
Figure 4-19
Mean changes in spike frequency (±SEM) of the secondary ORN in *H. subflexa* type-B sensilla following exposure to various long-chain carboxylic acids (100 µg) and the six general odorant sets. The secondary ORNs displayed no significant increases in spike frequency in response to the carboxylic acids or general odorant sets at 10 µg per compound. However, exposure to the chemical sets at a dosage of 100 µg per compound in each set significantly increased the secondary ORNs’ spike frequencies. Only two sensilla were tested with 100 µg dosage. One sensillum showed responsiveness of the secondary ORN to every one of the 6 chemical sets (Fig. 4-20), and the secondary ORN in the other sensillum responded to 5 out of the 6 chemical sets, being unresponsive to set# 2. A Z-test was used in comparison to zero using Bonferroni’s correction for multiple comparisons (* p < 0.05).
Figure 4-20
Responses of the large-spiking and secondary ORN in a *H. subflexa* type-B sensillum. Neither ORN in this sensillum responded to any of the stimuli (fig. 4-19) at 10 µg. They did respond to the 100 µg stimulus of 14:COOH and the 6 general odorant chemical sets at 100µg per compound. The secondary ORN was not responsive to Z9-16:Ald and Z9-14:Ald, but as usual the large-spiking ORN was. Horizontal bar denotes 100ms stimulation.
Type-B sensilla of *H. subflexa*

The secondary ORNs of type-B sensilla were challenged with 8 pheromone-related compounds of three heliothine species, with 7 long-chain carboxylic acids related to the pheromone compounds, and with the high-throughput mixtures of 58 general odorants.

For tests of the pheromone-related aldehydes, two sensilla were located and identified as type-B after searching 35 sensilla of 6 moths. Both of these sensilla had a secondary ORN exhibiting spontaneous firing, with a typical asymmetric waveform approaching a unipolar form (Fig. 4-17). Data analyzed for the pheromone-related compounds were combined from the data acquired from the test series for minor constituents with (2 sensilla) and the test for general odorants/acids with (6 sensilla). The only two compounds that activated the large-spiking ORN were Z9-16:Ald and, to a lesser degree, Z9-14:Ald (Figs. 4-17, 4-20), as has been reported before (Baker et al., 2004). None of the other compounds in this set elicited specific activity from the secondary ORN (Figs. 4-17, 4-18), and there was no correspondence between the activity of the larger-spiking ORN and that of the secondary ORN (Fig. 4-18B).

For testing the other compounds, 8 sensilla were identified as type-B out of 45 sensilla tested from 11 moths. The spontaneous activities of the secondary ORNs in two sensilla dwindled to nothing shortly after establishing contact with recording electrode. I challenged the other six type-B secondary ORNs with the 58 general odorants and 7 acids.
Four of the ORNs remained active through the whole series, but some of test compounds were not able to be applied to two of the sensilla before the ORN expired, unfortunately. The secondary ORNs’ firing frequencies did not increase in response to any of the 6 sets of test compounds at the 10µg loading (Fig. 4-19). The only responses I saw to single odorants was one ORN showing increased activity following stimulation with 100 µg Z9-14:COOH. In addition to these test compounds, I also exposed two of these type-B secondary ORNs to the series of general odorant sets, but this time at dosages of 100 µg per compound in the cartridge mixtures. The secondary ORN in one of the sensilla responded to 5 chemical sets except to set# 2 (the aldehydes and carboxylic acids; Fig. 4-19), but the ORN in the other sensillum responded to all six 6 sets including set# 2 and also 14:COOH (Figs. 4-19, 4-20).

DISCUSSION

Type-A sensilla of *H. zea* and *H. subflexa*

The only consistent activation of the small-spiking, secondary ORN in type-A sensilla of *H. zea* occurred whenever the large-spiking ORN was activated. One possibility for this correspondence is consistent with the idea that the secondary ORN is a sensor fortuned to some kind of a degradation product of Z11-16:Ald. In many noctuid moth species, the smaller-spiking ORN that is co-compartmentalized with the larger-spiking ORN that is tuned to that species’ major pheromone component is tuned to the
degradation product of that component (c.f., Trichoplusia ni, O’Connell et al. 1983; Todd et al. 1992; Agrotis segetum, Van der Pers and Löfstedt 1986, Hansson et al. 1992; Pseudoplusia includens Grant et al. 1988). This tendency in the noctuids was the rationale for challenging these ORNs with various pheromone-component-related acids, because both an aldehyde dehydrogenase and an aldehyde oxidase that convert Z11-16:Ald to Z11-16:COOH have been isolated from the antennae of H. zea, H. subflexa, and H. virescens (Tasayco J and Prestwich 1990a,b).

Interestingly, the results showed that none of the pheromone-component-related acids that I puffed over the sensilla reliably evoked significant activity from the secondary ORN. The inactivity of these externally puffed acids might be explained by the acids not issuing at sufficiently high rates from the odor cartridges to evoke action potentials. It is also possible that the acids from the ambient environment might not be able to traverse the pore tubules to enter the sensillar lymph. It is thus entirely possible that the acids need to be generated internally, in the sensillar lymph itself, in order to excite the secondary ORNs.

However, the co-activity of the smaller-spiking ORN along with the larger spiking ORN in response to 1,(Z)-12-heptadecadiene, the hydrocarbon analog of Z11-16:Ald does not support this idea, because there is no obvious way that an acid breakdown product can be made from this hydrocarbon. In noctuids, it is not unusual for the smaller-spiking, secondary ORN co-compartmentalized with the larger, pheromone-component-tuned ORN to be co-activated when the pheromone component is used as the stimulus, especially at the higher dosages that are more likely to generate more degradation product (c.f. Todd et al. 1992; Van der Pers and Löfstedt 1986; Hansson et al.
In my study the secondary ORN was co-activated when either the pheromone component or the hydrocarbon was used.

Thus, the data from these experiments are not consistent with the secondary ORN being tuned to the acid degradation product, but rather to a breakdown product that I had not expected, and that is the compound, E11-16:Ald. This product would require a cis-trans isomerase, a degradative enzyme that has not heretofore been found in moth trichoid sensilla. It is possible that there is, even more specifically, a Δ-11-cis-trans isomerase that is involved.

The possibility that such an enzyme is at work in these sensilla is supported, at least in *H. zeae*, by the response of the secondary ORN to both the hydrocarbon mimic of Z11-16:Ald and to Z11-16:Ald itself. The correlation of activity in dose-response series of both these compounds is indicative of there being more E-isomer ligand available when there is more Z-isomer available for degradation. The slight increase in secondary ORN response to Z11-16:Ac, Z11-16:OH and Z9-14:Ald, all having five carbons from the terminal methyl group to the Z-double bond also supports the possibility of a Δ-11 cis-trans isomerase. The heightened activity sometimes occurring in response to Z9-16:Ald does not. I am working to obtain samples of E11-16:Ald to directly test this idea.

The inconclusive relationship in *H. subflexa* between the activity of the large-spiking ORN and the secondary ORN in the type-A sensillum in response to both Z11-16:Ald and the hydrocarbon analog may be due to the small sample size of only five ORNs for this study. This deserves further study in both these species, using E-isomers of many of the pheromone component aldehydes.
A *cis-trans* isomerase would not be the degradative enzyme “of choice” in the most lepidopterous pheromones, because a large percentage of the pheromone communication systems that have been elucidated require a precise blend ratio of the *cis* isomer major component and the corresponding *trans* isomer minor component for there to be optimum behavioral response. ORNs tuned to these Z and E components are routinely co-compartmentalized within the same sensillum (examples in Todd and Baker 1999; Hansson et al. 1992). A *cis-trans* isomerase used in this situation would destroy the ability of the ORNs in each sensillum to accurately report the precise ratio of E and Z isomers received by the male from the pheromone plume, destroying communication ability (Todd and Baker 1999). Perhaps the large sub-group of noctuids using aliphatic straight-chain compounds as pheromone components has avoided this limitation by using only the Z isomers as pheromone components, allowing them to use this particular isomerase. Perhaps it was the other way around; early evolutionary use of a cis-trans isomerase prevented these moths from ever using an E isomer in a blend. In any case, degradation of the Z, into the E isomer would not impair the ability of the ORNs of these species to report the relative abundances of the (all-Z) pheromone components to the glomeruli of the macroglomerular complex in the antennal lobe.

I do not have any explanation for why the activity of the secondary ORN always precedes, or at least never occurs after, the activity of the larger-spiking ORN. One possibility is that it is actually the smaller-spiking ORN that is the one that is tuned to Z11-16:Ald and the large-spiking ORN is tuned to the breakdown product. However, this would run against precedent, because in the vast majority of cases in moths, it is the larger-spiking ORN in co-compartmentalized ORNs that responds to the major
pheromone component (Hansson 1995). If it is not a breakdown product of Z11-16:Ald or its analog that explains the smaller-spiking ORN’s activity, then another possibility is that an ion current or ion imbalance suddenly resulting from the flows from the smaller-spiking ORN’s ion channels is needed to trigger the activity of the large-spiking ORN. This would seem to be a laborious, cumbersome mechanism, and one that has not been seen before in insect pheromone component transduction. The lack of correlation between the large- and the small-spiking ORNs’ activities when they were challenged with a wide variety of odorants, none of which had a Z11 double bond, is corroborative evidence that the secondary ORN is tuned to an odorant related to Z11-16:Ald.

This argument notwithstanding, the sensillum lymph space is known to be isolated electrically from an outer lymph space by the tight contact zone between the apical membrane of tormogen cell and the sensillum cuticle base (Keil 1984). It therefore cannot be entirely ruled out that electrical signals generated from ORNs in neighboring sensilla were detected from the electrode connected to the target sensillum by means of a loosened contact zone between the sensilla during the tip-recording process. However, this possibility cannot explain completely the following two questions. Why do these (exogenous) signals represented by the small-spiking cell always temporally precede the signals generated from large-spiking ORNs inside the target recording sensillum? In addition, why are the action potentials of the small-spiking ORNs always tonic when the large-spiking action potentials are phasic?

All these uncertainties notwithstanding, the biological function of the secondary ORN in type-A sensilla is still in question. It projects with 100% fidelity to one specific glomerulus in a newly described cluster of glomeruli, the Posterior Complex (PCx), that
resides immediately posterior to the macroglomerular complex (Lee et al. 2006a). The projection of an ORN tuned to a pheromone-related compound, if the activity of this secondary ORN is indeed caused by a breakdown product of Z11-16:Ald, would be a first for moths. In all cases thus far, the secondary ORNs project to a glomerulus residing in the macroglomerular complex, the sexually dimorphic sub-cluster having all-sex-pheromone-related inputs (Berg et al. 1998; Berg et al. 2005; Lee et al. 2006a,b; Ochieng et al. 1995).

The results with *H. subflexa* for the secondary ORN in type-A sensilla are similar to those of *H. zea*, but are less conclusive, perhaps due to the smaller sample sizes I obtained. The one result thus far that is common to *H. subflexa* and *H. zea* is that when Z11-16:Ald was used as the stimulus, the increase in activity of the secondary ORN corresponded with, at least a small degree, an increase in activity of the ORN tuned to Z11-16:Ald. The time-course of the activity was similar to that seen in *H. zea* as well, with the onset of activity from the secondary ORN always preceding, or at least not lagging behind, that of the Z11-16:Ald-tuned ORN.

One difference in the behavior of the *H. subflexa* secondary ORNs was that they responded poorly to the hydrocarbon analog of Z11-16:Ald even though the Z11-16:Ald-tuned ORN responded moderately well to the higher dosages of the hydrocarbon. Whether or not this represents a real difference between the species or just a small sample size from *H. subflexa* remains to be determined with further experiments. As in the case of the secondary ORN of *H. zea*, the lack of activity of the *H. subflexa* secondary ORN in response to the wide variety of other possible odorants to which it might be tuned is corroborative evidence that its ligand is related somehow to Z11-16:Ald degradation.
Type-B sensilla of *H. subflexa*

I did not find any consistent stimulus for the secondary ORN in the type-B sensilla of *H. subflexa*. Its behavior was dissimilar to type-A secondary ORNs of both *H. zea* and *H. subflexa*. For *H. subflexa*, unlike the secondary ORNs in type-A sensilla, the secondary ORN in type-B sensilla did not become activated when the large-spiking ORN sensitive to a pheromone component, Z9-16:Ald, became active. As in an earlier study (Baker et al. 2004) there was good activity from the large-spiking ORN when Z9-16:Ald was puffed, and slightly less to Z9-14:Ald. However, there was no corresponding increase in activity from the secondary ORN. Baker et al. (2004) did not note, or monitor, any activity from the secondary ORN.

The inability to find a consistently active odorant that excites the secondary ORN in type-B sensilla does not mean that this ORN is unresponsive (Lee et al. 2006a). However, at present, given my results, I might consider this ORN as being “non-functional” for olfaction, because it thus far does not show itself to be a reliable reporter for any particular odorant, and it takes large dosages to get it to fire.

It appears as though this ORN from type-B sensilla is somewhat more responsive to general odorants than is the secondary ORN from type-A sensilla. This may be due to the fact that there are many instances in *H. subflexa* of a third, or even a fourth ORN co-compartmentalized in these type-B sensilla, as revealed during cobalt staining studies of *H. subflexa* ORNs (Chapter 3). Because these third and fourth ORNs project to a small, consistent set of ordinary glomeruli, one being called ‘water-drop-shaped, they are most likely tuned to general odorants. Ordinary glomeruli in moths are known to receive inputs
from general-odorant-tuned ORNs (Carlsson and Hansson 2003; Galizia 2000; Skiri et al. 2004). In such cases any activity of the secondary ORN that always arborizes in glomerulus PCx4 of the Posterior Complex may have been obscured by these ORNs’ activities.

I do not regard this survey for possible ligands for the secondary ORNs in type-A and -B ORNs in these two heliothine species to be by any means exhaustive. On the contrary, it is but a first attempt to prove or disprove that some of these ORNs are non-functional with respect to contributing to odor quality discrimination. I do believe that if a logical approach to trying possible ligands such as pheromone degradation products continues to be followed, then a ligand will be more likely to be found for these ORNs. Considering for instance just the secondary ORN in the type-A sensillum of *H. zea*, once it can be stimulated reliably by a synthetic ligand without the large-spiking ORN being activated, then at that point the search for a ligand will have ended. Once that happens, it will be interesting to see whether or not the same ligand will work on the type-A secondary ORN of *H. subflexa*. I would guess that the answer will be ‘yes’, due to the fact that in both species the type-A sensillum’s secondary ORN projects to the PCx1 in the Posterior Complex. Structure quite often teaches us function.

**REFERENCES**


Tasayco J ML, Prestwich GD (1990a) Aldehyde oxidases and dehydrogenases in antennae of five moth species. Insect Biochem 20:691-700


CHAPTER 5

SUMMARY AND CONCLUSION

In this dissertation, I have demonstrated the primary neuronal pathways of pheromonal olfaction that result in intraspecific male attraction in, and prevent interspecific attraction between, two sympatric species of heliothine moths, *Helicoverpa zea* and *Heliothis subflexa*. I have described the glomerular structure of a previously undescribed sub-cluster of glomeruli that I named the Posterior Complex (PCx), showed that specific glomeruli in the PCx are the projection targets of ORNs that are co-compartmentalized in particular sensilla trichodea with pheromone-component-responding ORNs. I also scrutinized the functional characteristics of these secondary ORNs using the single-sensillum recording technique while challenging the ORNs with a vast array of odorants.

**Linear pathways of pheromone-related ORNs**

My research showed that each type of ORN that responds to a particular pheromone-related compound in the specific sensillar types projects with 100% fidelity to a particular glomerulus in the MGC in both species (Chapter 2, 3). This consistency explains how the previously reported electrophysiological characteristics of the ORNs in
the three sensillar types correspond with the tuning and glomerular targets of interneurons to produce intraspecific male attraction while preventing interspecific attraction among three sympatric species of heliothine moths.

These findings are important in that they show that in these noctuid species, the pheromone-component-specific olfactory pathways are linear, i.e., travel from ORN to glomerulus and directly out through that same glomerulus via a projection interneuron to higher centers (mushroom body and lateral protocerebrum). In some other noctuid species it has been found that the pathways can sometimes involve a jump from the glomerulus targeted by an ORN over to a different glomerulus (even an ordinary glomerulus) and thence out via projection interneuron to higher centers (Anton and Hansson 1994, 1995; Anton and Homberg 1999). These glomerular shifts from input sites to different output locations would involve the inhibition and disinhibition imposed by local interneuron circuits that could scramble the otherwise direct lines. Thus my results add to the evidence in the heliothines that there are only linear pathways into and out of each pheromone-component-specific glomerulus and on to higher centers.

The ORNs responding to the major pheromone component (Z11-16:Ald) of both species projected their axons to a specific glomerulus, the cumulus, with no exceptions for either H. zea or H. subflexa. Each of the two co-compartmentalized ORNs in the type-C sensilla of H. zea always projected to one of two smaller glomeruli, the DMA and DMP glomeruli of the MGC. The projection patterns of the two successful stainings of type-B sensilla ORNs were the same as for type-C sensilla, indicating for the first time that type-B sensilla are actually type-C with the ORNs having become less responsive to the pheromone components. The calcium imaging results for H. zea that were performed
by Dr. Mikael Carlsson in Professor Bill Hansson’s laboratory (Swedish Agricultural University at Alnarp, Sweden) confirmed that the foci of glomerular activity within the MGC in response to populations of specifically tuned ORNs responding to pheromone-related compounds were the same as what I found with individually cobalt-stained ORNs. Combined with intracellular stainings of PNs (Vickers et al. 1998) my staining results clarified that the pathway of each ORN tuned to a pheromone-related compound in male *H. zea* is indeed linear into and out of a particular glomerulus. Z11-16:Ald-specific ORNs converged on the cumulus, Z9-16:Ald-specific information converged on the DMP glomerulus, and behaviorally antagonistic excitation from Z11-16:Ac/Z11-16:OH/Z9-14:Ald-tuned ORNs only projected into the DMA glomerulus.

Stainings of the type-B sensilla ORNs of *H. subflexa* showed that at least one ORN stained the DM glomerulus of the MGC with the exception of one occasion in 28 successful stains. The co-compartmentalized two ORNs in type-C sensilla of *H. subflexa* consistently arborized in two smaller MGC glomeruli, the AM and VM glomeruli. Combined again with the study of component-specific PNs projecting out of the MGC from specific glomeruli (Vickers and Christensen 2003), my ORN stainings clarified there is a linear pheromone-component-specific pathway into and out of each glomerulus in the MGC of *H. subflexa*. The cumulus is the primary olfactory processing center for the major pheromone component. Olfactory information about the minor pheromone component Z9-16:Ald is conveyed through the DM glomerulus, and information about the other minor pheromone component Z11-16:OH travels through the AM glomerulus. Finally, the Z11-16:Ac-responsive ORN that is co-compartmentalized with the Z11-16:OH-responsive ORN sends its excitation into the VM glomerulus, which is where this
information exits to higher centers via Z11-16:OH-tuned PNs (Vickers and Christensen, 2003).

Thus my findings show that specific glomeruli of the MGC of both species are dedicated to particular pheromone-related compounds that are critical for discriminating conspecific pheromone plumes from those emitted by females of other heliothine species. The selection pressure that would have helped shape such discrimination would likely be related to mating mistakes made by males in the past.

**Posterior Complex**

During my analysis of the cobalt-staining data, secondary neuronal stainings were commonly observed from images of ORN arborizations from *H. zea* type-A sensilla. These secondary neurons consistently targeted a particular glomerulus situated posterior to the MGC. These images resulted in my characterizing for the first time a previously unrecognized sub-cluster of glomeruli that I named the Posterior Complex, or “PCx”. I named a specific glomerulus in this cluster “PCx1” as the glomerulus that is a specific target of the secondary ORNs in type-A sensilla. I also observed that the secondary ORNs of *H. subflexa* type-A sensilla targeted a glomerulus that again resided in a previously unrecognized sub-cluster of glomeruli, a Posterior Complex for *H. subflexa*. In examining publications from other laboratories I found evidence that it is mostly probable that the projection patterns from type-A-sensillar secondary ORNs of other heliothine moths, *H. virescens* and *H. assulta* would be the same as what I found in the two species I worked with.
I found that the unresponsive ORNs paired in the type-B sensillum of *H. subflexa* with the ORN tuned to the Z9-16:Ald, the minor component, projects consistently to a another specific glomerulus in the PCx, the PCx4. The recurrence of a specific PCx arborization destination for a secondary ORN, this time co-compartmentalized with an ORN tuned to a different pheromone component from a different sensillar type caused me to perform electrophysiological recordings that interrogated these ORNs to try to see what they were tuned to in order to try to explain the reason behind the sub-clustering of glomeruli into a PCx grouping. The high consistency of secondary neuronal projection also called into question whether the secondary neurons are truly “unresponsive” to any compound.

**Evidence for effective ligands for the secondary ORNs**

The new, lower-noise recordings that I performed using excised male antennae to interrogate the small-spiking secondary type-A-sensillar ORNs of *H. zea* and *H. subflexa* showed that Z11-16:Ald was an effective stimulus for these ORNs in both species. Moreover, the hydrocarbon analogue of Z11-16:Ald, 1,Z12-hexadecadiene, also was effective in evoking tonic firing from these same ORNs in *H. zea* but was not consistently effective for these secondary ORNs in *H. subflexa*. The coincidental firing of the large-spiking ORN tuned to Z11-16:Ald in both species with that of the secondary ORN caused me to propose that the secondary ORN fires in response to a degradation product of Z11-16:Ald in the sensillum lymph. In *H. zea*, this idea is supported by the co-responsiveness of the primary and secondary ORNs to the 1,Z12-heptadecadiene analog of Z11-16:Ald.
Because of the results using this hydrocarbon analog, I raised the possibility that the pheromone-degrading-enzyme that may be at work in these sensilla might be a *cis-trans* isomerase. This will be directly tested using E11-16:Ald that I hope to obtain soon. In both species, the lack of significant activity of this secondary ORN in response to the large array of test compounds other than Z11-16:Ald is also supportive of this idea.

I did not find any effective ligand for the secondary ORN in *H. subflexa* type-B sensilla, and this ORN did not respond co-incidentally with the Z9-16:Ald-tuned ORN when Z9-16:Ald was applied. However, I cannot yet conclude that this ORN is truly “unresponsive”.

An idea presented itself that other evidence for or against the PCx being involved in pheromone-component-related olfaction (such as degradation products) might be obtained by comparing the anatomies of male PCx versus female glomeruli in the same posterior region of the antennal lobes of these two species. Recently, I was able to make use of the high-quality microscopy imaging facilities provided by the Huck Institutes of Life Sciences here at Penn State, to probe further in this direction than I had previously thought possible. I present these preliminary findings below.

**New images lend support to a sex-pheromone-related function of the PCx**

I used laser scanning confocal microscopy (LSCM) with one- or two-micrometer-thick optical sections after staining the glomeruli with fluorescent-dye-conjugated antibody against synapse-specific protein (synaptotagmin) of *Drosophila* (see Materials and Methods, Chapter 3). This gave me an opportunity to scrutinize the PCx
structure of both *H. zea* and *H. subflexa* males in higher resolution without the arbitrary reorientation needed for the reconstruction of physical microtome sections as I did for the 10 µm sections in the cobalt staining studies. After securing optical sections series from 8 antibody-stained antennal lobes of five *H. zea* males, I first of all became aware that the number of PCx glomeruli might not be seven, but rather nine in all. I found what appears to be one extra one from the PCx2 and one extra one from PCx7 of my previous classification system (Figs. 5-1, 5-5). This discrepancy was not recognized under the 10 µm thickness used in the physical sectioning because of the similar size and unclear boundary between two adjacent glomeruli in both cases. In most instances, the boundaries between the two unrecognized glomeruli and the neighboring glomeruli were somewhat obscure even under LSCM. Thus a further analysis with more animals is needed in order to confirm these additional PCx glomeruli. The LSCM image analyses of *H. subflexa* PCx glomeruli are fortunately in accord with the interpretation I made using the physical sections.

Using the LSCM imaging technique, I produced some initial antennal lobe images from 3 *H. zea* females and 1 *H. subflexa* female (Figs. 5-2 and 5-4) to compare their dorsal-most posterior regions with the corresponding male PCx structures (Figs. 5-1 and 5-3). Female glomeruli in the antennal lobes of Lepidoptera are generally known to be sexually isomorphic to the ordinary glomeruli of conspecific males (Carlsson and Hansson 2003; Galizia 2000; Skiri et al. 2004), and in my images the ordinary glomeruli of male and female antennal lobes did have distinct similarities (Figs. 5-1 to 5-4). However, full 3-dimensional reconstructions will be needed to be made in order to determine the degrees of male-female similarity in these two species.
My interpretation of the images of both *H. zea* and *H. subflexa*, female antennal lobes is that there are topographically segregated sub-clusters of glomeruli that are discernable in the posterior region of the AL similar to the region in which the PCx is situated in the male ALs (Figs. 5-1, 5-2, 5-3, and 5-4). However, as for the ordinary glomeruli, it is difficult to deduce the precise comparable structures from female dorso-posterior AL to the male PCx, because the anteriorly positioned few glomeruli of the female sub-clusters lie very close to the ordinary glomeruli. Also, there is no MGC to use as a landmark in the females. Moreover, probably due to the lack of an MGC that fills up precious space at the antennal base, the shapes of some female ordinary glomeruli do not exactly match those of the corresponding male ordinary glomeruli.

The glomerular activities within the antennal lobes of *H. virescens* males and females have been examined using 8 odorants for sexual comparison of 8 individual ordinary glomeruli located close to the anterior surface of the antennal lobe (Skiri et al. 2004). In this study only one topographically identical glomerulus in both sexes was activated to a specific odorant (linalool). Activity in a second glomerulus was likely to be related to generalist ORN inputs, and the other glomeruli did not show functional identity between males and females (Skiri et al. 2004). In light of their results, in heliothine moths it may be that even glomeruli that appear to be topographically (and therefore chemotopically) accordant in both sexes may not in fact be functionally identical. This is a new area deserving of further inquiry in heliothine moths, because it is at variance with studies in other moths.

This caveat notwithstanding, my images show that the female antennal lobes of *H. zea* and *H. subflexa* both have a glomerular cluster that appears to be topographically
separated from other ordinary glomeruli. With regard to only the general morphology and position of the PCx and corresponding female glomerular sub-clusters in the antennal lobes, the sexual identity of the antennal lobe cannot be discerned. Therefore, these clusters appear to be comparable in males and females. Regarding the more specific morphologies of male versus female posterior sub-clusters in both *H. zea* and *H. subflexa*, my conclusion thus far is that the female glomeruli these clusters are not sexually isomorphic with those in the male PCx. This would lead to the conclusion that the function of the glomeruli in the PCx of males is related to some aspects of sex pheromone olfaction. However, a general consensus about the degree of sexual isomorphism of ordinary glomeruli in heliothines needs to be reconsidered and compared with the degree of isomorphism in the posterior clusters before a more decisive conclusion about the latter’s function in sex pheromone-component-related olfaction can be made.

A large amount of information about insect olfaction, and specifically insect sex pheromone olfaction, has been generated over the past 45 years or so since the first chemical characterization of a pheromone. As can be seen above, knowledge about heliothine sex pheromone communication systems is particularly rich with information about behavior, sensory physiology including neurophysiology, biochemistry, and molecular biology of receptors, and central nervous system integration including functional morphology of the antennal lobe. This prior knowledge provides a particularly rich landscape upon which to place new information that addresses gaps in our knowledge. I have tried to address these knowledge gaps with my research in order to further enrich this field of research into heliothine moth sex pheromone olfaction. It is my
hope that these findings will enlighten researchers in these areas to new avenues of inquiry that otherwise would have been overlooked.

REFERENCES


Figure 5-1
Confocal images (frontal view optical sections) of a male *H. zea* antennal lobe. A. A section taken 36 µm posterior to the first section in which the anterior- most glomeruli first become discernable. The three glomeruli comprising the MGC are visible. B. A second section taken 30 µm posterior to the section shown in (A). Two PCx glomeruli (2a and 2b) appear to comprise what I earlier had called “PCx2” from my 10 µm-thick physical sections. PCx 4 is also noted in this section. C. A third section taken 20 µm posterior to the section shown in (B). PCx1 is now visible. D. The most posterior three PCx glomeruli, numbers 7b, 6, and 5 are visible in this section taken 18 µm posterior to that shown in (C). I did not recognize glomeruli 7b and 7a as separate glomeruli until these higher-resolution confocal optical sections. Cu, cumulus; DMA, dorsomedial anterior; DMP, dorsomedial posterior. D dorsal, L lateral. Scale bar= 50 µm
No MGC structure was visible in any sections of the lobe. A. A section taken 38 µm posterior to the first section in which the anterior-most glomeruli first become discernable. B. A section taken 14 µm posterior to the section shown in (A). Three labeled (*) glomeruli might be analogs of the male PCx glomeruli numbered in Fig. 5-1B, but it is difficult to distinguish these from ordinary glomeruli in this section. C. A section taken 20 µm posterior section to that shown in (B). The marked glomeruli (*) may be homologs of the male PCx glomeruli numbered in Fig. 5-1C given their arrangement, although the morphology of the two marked glomeruli to the right is dissimilar to the male PCx1 shown in Fig. 5-1C. D. The most posterior three PCx glomeruli, in the section taken 22 µm posterior to that shown in (C). The three labeled glomeruli (*) might have homology with the three PCx glomeruli of a H. zea male, although their arrangement is different from that in the male. D dorsal, L lateral. Scale bar= 50 µm
Figure 5-3
Confocal images (frontal view optical sections) of a male *H. subflexa* antennal lobe. A. A section taken 53 µm posterior to the first section in which the anterior-most glomeruli first become discernable. Two smaller MCG glomeruli, the VM and AM, are visible. B. A second section taken 40 µm posterior to the section shown in (A). Two MCG glomeruli, the cumulus and DM, are visible, and the PCx4 (4) looks as if it is more associated with the MGC then with other, ordinary, glomeruli. C. A third section taken 20 µm posterior to that shown in (B). PCx1 is visible, as are PCx glomeruli numbers 2, 3, and 4. The posterior remainder of the cumulus (upper left) and the fourth MGC glomerulus, the DM (to left of PCx1) are also apparent in this section. D. A fourth section taken 20 µm posterior to that shown in (C). The number and structure of the PCx are in accordance with the typical PCx morphology of *H. subflexa* described in Chapter 3. The numbers denote the PCx glomeruli that reside toward the posterior limit of the PCx. Cu, cumulus; AM, anteromedial; VM, ventromedial; DM, dorsomedial; Ord ordinary glomeruli. D dorsal, L lateral. Scale bar= 50 µm
Figure 5-4
Confocal images (optical sections from frontal view) of a female *H. subflexa* antennal lobe. The depths of images A, B, C, and D correspond to each similar-lettered section of the *H. subflexa* male shown in Fig. 5-3. A. MGC-like glomeruli are not visible. Possible sexual isomorphism of the ordinary glomeruli in this section compared to that of the male in Fig. 5-3A is difficult to recognize. B. Some of the female glomeruli in this section exhibit a certain degree of morphological similarity to those of the male in Fig. 5-3B. C. A glomerular cluster composed of 8 glomeruli (*) is shown, which might be functional analogs of the *H. subflexa* male PCx glomeruli shown in Fig. 5-3C. However, there are still dissimilarities with regard to individual glomerular shapes, and their placement in the cluster. D. The most posterior three female antennal lobe glomeruli. The three labeled glomeruli (*) might possibly have homology with the PCx glomeruli of the *H. subflexa* male glomeruli shown in Fig. 5-3D, but their arrangement and numbers present do not obviously correspond to PCx glomeruli in the male in this image. D dorsal, L lateral. Scale bar = 50 µm
Figure 5-5
Three-dimensional reconstructions made from the confocal micrographs of the male *H. zea* MGC and PCx shown in Fig. 5-1. A. Frontal view of MGC and PCx. PCx2a and PCx2b are depicted now as separate glomeruli of the PCx2 described in Chapter 2 from physical sectioning. B. View from the posterior side. PCx7a and PCx7b were represented as a single PCx7 in Chapter 2 from the 10µm physical sections that were available. In this AL and in 6 out of nine samples that were reconstructed this way, 9 glomeruli are visible in the PCx, not seven as described in Chapter 7. However, the boundaries between PCx2a/2b and PCx7a/7b are somewhat unclear in many series of optical sections; only two preparations showed extremely clear boundaries between PCx2a and PCx2b, as well as between PCx7a and PCx7b. The other numbers denote the other PCx glomerular numbers, and DMA, DMP, and Cumulus denote the three MGC glomeruli. D, dorsal; A, anterior; L, lateral.
VITA
Seong-Gyu Lee

Education
Penn State University, 2003 fall -present
   Seeking PhD in Entomology
   Transferred with Dr. Thomas C. Baker from Iowa State University
   Adviser: Dr. Thomas C. Baker

Iowa State University, 1998-2003
   PhD program in Entomology
   Advisor: Dr. Thomas C. Baker,
   Co-advisor: Dr. Russell A. Jurenka

Korea University, 1995-1997, M.S. in Zoology,
   Thesis: “Binding characteristics of lipophorin binding protein on the ovarial
   membrane of common cutworm, Spodoptera litura”
   Advisor: Dr. Hak Ryul Kim

Korea University, 1989-1995, B.S. in Biology

Professional Experience
Graduate research assistant, Penn State University, 2003-present
Graduate research assistant, Iowa State University, 1998-2003
Graduate research assistant, Korea University, fall semester, 1996
Instructor (Animal Physiology Lab.), Korea University, spring semester, 1996
Teaching Assistant (Insect Physiology Lab.)(Animal Physiology Lab.),
   Iowa State University, spring semester, 2001, 2002
Lab manager, Korea University, 1996

Publications
   the moth, Heliothis subflexa. Chem Senses Submitted.
   organization of the antennal lobe of the moth, Helicoverpa zea. J Comp Physiol A
   192:351-363
   comparison of responses from olfactory receptor neurons of Heliothis subflexa and
   Heliothis virescens to components of their sex pheromone. J Comp Physiol A
   109:155-165
Lee S-G, Han WD, Kim HR (1997) Binding characteristics of lipophorin binding protein
   on the ovarial membrane of common cutworm, Spodoptera litura. Korean Journal of
   Entomology, 27:227-236