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# Neurophysiological Recording Techniques Applied to Insect Chemosensory Systems

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## 1. Introduction

The aim of this chapter is to discuss current electrophysiological recording techniques used to study the processing of olfactory and gustatory information in insects. More specifically, we will describe methods employed (a) to determine the physiological properties of gustatory (GRCs) and olfactory receptor cells (ORCs) in the peripheral nervous systems and (b) to physiologically characterize identifiable olfactory neurons in the central nervous system.

In studying the structural and functional organization of the nervous system, it is at times advantageous to use animal models, such as insects, for experimentation. Key factors that make insect nervous systems excellent models for analyzing gustatory and olfactory mechanisms are that they possess a relatively simple peripheral and central nervous system compared with their vertebrate counterparts. They also bear easily accessible sensory organs (sensilla) and individually identifiable neural structures. The number of receptor cells mediating mechanisms involved in olfaction and gustation is relatively small. Interestingly, some brain regions in invertebrates and vertebrates show remarkable morphological and physiological similarity and, therefore, the insect nervous system provides insights into general principles underlying taste and odor coding that occur in higher vertebrates.

Gustatory and olfactory cues play vital roles in shaping insect behavior. Insects rely on these senses in the sampling and selection of food sources, avoidance of noxious or toxic compounds, mating, and locating egg-laying sites. These chemical cues are detected by GRCs and ORCs housed in sensilla located mainly on the mouthparts, legs, and antennae and are converted into a neural code of action potentials, which is sent to the central nervous system for processing. GRCs and ORCs constitute sensory filters for environmental taste and smell signals in insects. They form the first layer of a decision making process and transfer information directly to centers in the brain.

## 2. Insect chemosensory organs

Insects are ideal models for both olfactory and gustatory studies. They bear numerous sensory organs or sensilla, which allow them to constantly monitor and respond to changes in their internal and external environments so as to maintain themselves under the most

favorable conditions for survival. Sensilla are used in the sensory perception for smell, taste, sound, touch, vision, proprioception, and geo-, thermo-, and hygroreception. These specialized cuticular structures vary in size and shape and act as their first level of environmental perception. Receptor cells that innervate these sensilla are designed to detect environmental status and change and transmit information regarding the nature of the change to the central nervous system. In insects, it is specifically taste (gustatory) and smell (olfactory) stimuli in the environment that control the behavior of these animals.

Gustatory and olfactory sensilla are typically innervated by more than one bipolar sensory neuron. These neurons bear dendrites which are wrapped by accessory or sheath cells. Sensory information is transduced by receptor cells into an electrical signal, resulting in the generation of nerve signals comprised of action potentials. The absolute frequency and temporal distribution of action potentials in a spike train contain information about the stimulus. This information is transmitted by axons of sensory neurons to modality-specific brain centers. Unraveling the sensory code can be achieved by stimulating specific sensilla and electrophysiologically quantifying the trains of action potentials (input), as well as quantifying the behavior (output).

## 2.1 Peripheral olfactory processing

Olfactory stimuli play an important role in the orientation of many animals in their environment. Moths detect odor cues with their main olfactory organs, paired antennae. The antennae detect diverse mixtures of volatiles by means of ORCs residing in various types of sensilla. These sensilla are the crucial interface between the outer world and the central nervous system of the moth. The olfactory systems of both invertebrates and vertebrates share many similarities (see Hildebrand and Shepherd, 1997) and are capable of detecting and discriminating among a large number of odorants that differ in size, shape, and complexity. The olfactory organs of invertebrates (e.g., paired antennae of insects) and vertebrates (e.g., nose in mammals) are adapted to detect a vast array of odorants by means of receptors that are located on ORCs. These ORCs are associated with various types of sensilla in invertebrates (e.g., insects) or the olfactory epithelium lining a portion of the nasal cavity of vertebrates (e.g., mammals). Clyne et al. (1999) and Vosshall et al. (1999) identified a novel family of seven-transmembrane-domain proteins, which are encoded by 100-200 genes and are likely to function as *Drosophila melanogaster* olfactory receptors. An individual ORC in the antenna of *D. melanogaster* is thought to express one or a few of the candidate olfactory receptor genes and therefore, each ORC is functionally distinct (Vosshall, 2001).

Because of the fundamental morphological and physiological similarities between invertebrate and vertebrate olfactory systems and since current evidence indicates that basic olfactory processing is similar across all phyla (Hildebrand and Shepherd, 1997), it is feasible to use insects, such as the sphinx moth *Manduca sexta*, as a model system and thereby gain insights into the neural mechanisms of odor recognition and discrimination common to insects and to other animals (Hildebrand, 1995; 1996; Hildebrand and Shepherd, 1997). Insects are ideal experimental models because they possess readily accessible olfactory sensilla, have relatively simple peripheral and central nervous systems, possess individually identifiable neural structures, and have a relatively small number of sensory cells mediating olfactory mechanisms. In moths, the olfactory system comprises two parallel subsystems. One system processes information about plant-associated volatile odors and is

very similar to the main olfactory pathway in vertebrates. The other system is narrowly specialized to detect and respond to information regarding the sex pheromone emitted by conspecific females. Many researchers have classified ORCs based on their individual response profiles and have used terms such as “specialist” and “generalist” to describe those ORCs. Specialist ORCs have been described as those responding with a high sensitivity and selectivity to a single type of odor molecule, such as a pheromone component or a narrow range of related odorants, whereas generalist ORCs have been classified as those responding with relatively lower sensitivity to a broad range of odorants, such as plant-associated volatiles (Schneider et al., 1964). Interestingly, several researchers have found, that some insect ORCs respond to plant-associated volatiles and exhibit relatively high selectivity and sensitivity for effective stimulus molecules (e.g., Priesner, 1979; Dickens, 1990; Anderson et al., 1995; Heinbockel and Kaissling, 1996; Pophof, 1997; Hansson et al., 1999; Shields and Hildebrand, 2001a).

## 2.2 Antennal morphology

The antennae of both sexes of *M. sexta* comprise three segments, two small, basal segments (scape and pedicel) and a long distal segment (flagellum) (Fig. 1A, B). In adult *M. sexta*, the antennal flagellum is about 2 cm long, comprises approximately 80 subsegments called annuli (or flagellomeres) (Fig. 1C), and is sexually dimorphic (Sanes and Hildebrand, 1976). Each male and female antenna has approximately  $4 \times 10^5$  ORCs (Sanes and Hildebrand, 1976) and  $3.0 \times 10^5$  -  $3.4 \times 10^5$  ORCs (Oland and Tolbert, 1988), respectively. The antenna of each sex is associated with about  $10^5$  sensilla (Sanes and Hildebrand, 1976; Keil, 1989; Lee and Strausfeld, 1990; Shields and Hildebrand, 1999a; 1999b) and each annulus may bear approximately 2100-2200 sensilla (Fig. 1C, D) (Lee and Strausfeld, 1990; Shields and Hildebrand, 1999b). Male flagella possess long and hair-like (trichoid) male-specific sensilla that house ORCs specialized to detect components of the conspecific female's sex pheromone (Sanes and Hildebrand, 1976; Kaissling et al., 1989). Females also possess trichoid sensilla (i.e., trichoid type-A) (Fig. 1C, D). They are much shorter than in males and respond to plant volatiles. In addition, both male and female antennae carry several other types of olfactory sensilla, some of which resemble short pegs, thought to contribute to the detection of plant-associated odorants. In total, there are five types present in males (Sanes and Hildebrand, 1976; Lee and Strausfeld, 1990) and six types present in females (Shields and Hildebrand, 1999a; 1999b). In the case of trichoid type-A sensilla, circumferential cuticular ridges are present which form a helical pattern (Fig. 1E). The cuticular shaft of all olfactory sensilla is pierced by a multitude of pores (Fig. 1E). These pores extend through the entire thickness of the shaft. It is generally believed that olfactory molecules gain entry through these pores and, in order to reach the dendritic receptor sites of an ORC, the small volatile lipid-soluble odor molecules must traverse an aqueous phase, perhaps with the aid of odorant-binding proteins. The binding of these odorant ligands to odor-specific receptors coupled to G-proteins initiates a cascade of intracellular second messengers (e.g. cyclic AMP,  $IP_3$ ) that ultimately activate cyclic nucleotide-gated cation-permeable channels (e.g.  $Ca^{2+}$ ,  $Cl^-$ ,  $K^+$ ) (reviewed by e.g. Firestein, 1992; Shepherd, 1994). These events result in the generation of action potentials in temporal patterns (in each ORC axon) and spatial patterns (across the array of ORC axons) that represent features of the stimulus and travel along the ORC axons to the primary olfactory center in the brain (i.e., the antennal lobe, AL, of insects or the olfactory bulb of vertebrates). The axons of antennal ORCs project to and terminate in

compartments of condensed synaptic neuropil (glomeruli) in the AL (Hildebrand and Shepherd, 1997). Mounting evidence indicates that glomeruli are discrete anatomical and functional units, each dedicated to collecting and processing olfactory information about a subset of odor molecules (see below). Information about the odor stimulus is conveyed to a particular glomerulus by the axons of ORCs that express a particular olfactory receptor protein (Buck, 1996; Hildebrand and Shepherd, 1997; Mombaerts, 1996).

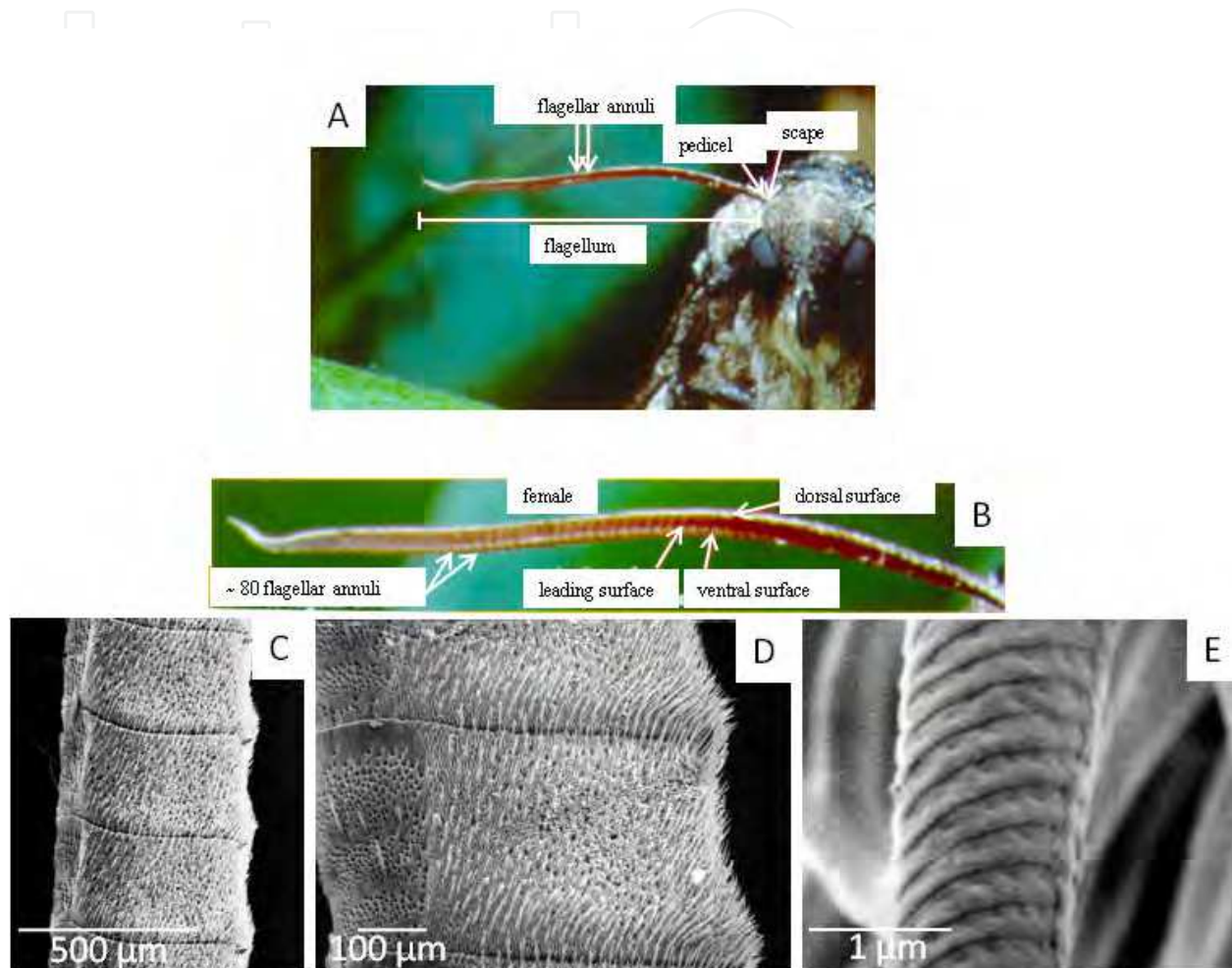


Fig. 1. Female *Manduca sexta* antenna. (A) Light micrographs showing a female *M. sexta* moth and its antenna. The antenna comprises three segments, two small, basal segments (scape and pedicel) and a long distal segment (flagellum). The antennal flagellum is about 2 cm long and comprises approximately 80 subsegments called annuli (or flagellomeres). (B) Higher magnification view of (A) showing the multitude of annuli. (C) Scanning electron micrograph of a portion of an adult female antennal flagellum showing a higher magnification of three annuli. (D) Higher magnification view of a single annulus. Long, hair-like sensory organs (sensilla) called trichoid sensilla are abundant on the surface of an annulus. (E) Higher magnification view of a single trichoid type-A sensillum showing the cuticular shaft and pores that extend through its entire thickness. The shaft bears circumferential cuticular ridges which form a helical pattern. Odorant molecules diffuse through these pores and interact with the underlying dendrites.

### 2.3 Peripheral gustatory processing

Insect larvae depend largely on their sense of taste and smell to find food. These larvae possess elaborate sensory organs (i.e., sensilla) located on the antennae and mouthparts that serve to gather olfactory and gustatory information on the chemical composition of the food plant. The feeding response in lepidopterous larvae is controlled by input from gustatory sensilla located on the mouthparts (Schoonhoven and Dethier, 1966; Shields, 1994). Food plant recognition is thought to be primarily mediated by the input from a bilateral pair of styloconic sensilla (Schoonhoven and Dethier, 1966; de Boer et al., 1977; de Boer and Hanson, 1987) located on the mouthparts. Each sensillum houses four GRCs (Figs. 2, 6) that are thought to play a primary role in hostplant discrimination. They have been referred to as the salt- sugar-, inositol-, and deterrent-sensitive cells (Schoonhoven, 1972; Schoonhoven et al., 1992), since they typically respond to salt, sweet, inositol, and bitter compounds, respectively (e.g. Schoonhoven, 1972; Frazier, 1986; Shields and Mitchell, 1995; Bernays et al., 1998; Glendinning et al., 1999). During feeding, the sensilla are in continuous contact with the sap liberated from the plant leaf and are capable of detecting different chemicals (i.e., phytochemicals) present in the plant. This gustatory sensory input is encoded as patterns of nerve impulses by GRCs and this information is then transferred to taste centers in the brain of the insect. Therefore, GRCs form the first layer of the decision-making process that ultimately determines whether food is acceptable or should be rejected. Thus, the insect faces the task to decipher individual tastants in a complex multimolecular mixture and to make appropriate feeding choices.

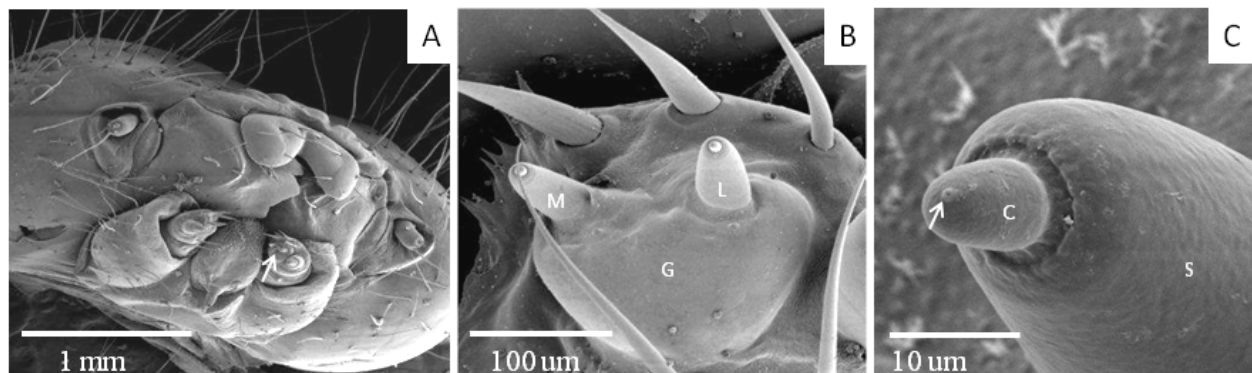


Fig. 2. Scanning electron micrographs showing the (A) whole head of a gypsy moth, *Lymantria dispar*, larva. The arrow denotes the location of the styloconic sensilla. (B) Higher magnification view of the lateral (L) and medial (M) styloconic sensilla located on the galea (G). (C) Higher magnification view of a lateral styloconic sensillum. The sensillum is comprised of a cone (C) or peg inserted into a style (S) or column. A terminal pore (arrow) is visible at the apex of the cone.

Insect GRCs transduce the quality and quantity of the complex plant chemistry into a neural code of action potentials. Complex stimuli resulting from e.g. plant saps often evoke spike trains in several receptor cells innervating one or more sensilla. The frequency of action potentials and the temporal distribution of action potentials in a spike train contain information about the stimulus. The axons of GRCs travel to, and converge in, the first relay station, the subesophageal ganglion (SOG), without intermittent synapses. Unraveling the sensory code occurs by analyzing “input-output” relationships (Schoonhoven and van Loon, 2002) and can be achieved by stimulating specific sensilla and quantifying

electrophysiologically the trains of action potentials (input), as well as quantifying the behavior (output) on the basis of how much food is consumed (Bernays and Chapman, 1994).

To better understand the neural communication between the chemosensory organs and the central nervous system that results in acceptance or rejection behavior, sensory responses have been categorized as (1) labeled line system, (2) across-fiber patterning, and (3) temporal patterning. The first theory suggests that the more important a single compound is for controlling or modifying behavior, the more likely its detection will be coded by a single cell (Stadler, 1984). This “labeled line” (i.e., line or axon along which information is transferred to the brain) to the central nervous system would only carry information from cells with a narrow and well defined sensitivity spectrum of a specific chemical (or family of chemicals) and would be directly linked to a specific behavioral response (Schoonhoven and Blom, 1988). Such chemosensory cells seem to be quite unique for specialized herbivorous insects and have not been documented for other animal groups, such as vertebrates. The second theory suggests that the nervous system bases its decision for behavioral output by evaluating the responses from many individual sensory cells with different but overlapping response spectra and the central nervous system extracts meaningful information by reading and processing the simultaneous inputs across all afferent sensory fibers (axons) (across-fiber patterning) (Dethier and Crnjar, 1982), also known to occur in vertebrates (Dethier, 1982). The third theory suggests that temporal patterning may be superimposed on across-fiber patterning, suggesting that the ratios of firing across different cells changes with time and can modify a particular message (Schoonhoven, 1982). Most importantly, it should be noted that all three theories (code types) are not mutually exclusive and can be amalgamated into one model (Schoonhoven et al., 1992).

Sensory codes mediating acceptance can: (i) stimulate specific sugar cells coding for acceptance profile; (ii) stimulate broad spectrum sugar cells that the CNS recognizes as an acceptance profile (Schoonhoven, 1982; 1987), and (iii) inhibit specific phagodeterrent receptors; this contributes to the neural coding of acceptance (Schoonhoven et al., 1998). Feeding deterrents may alter sensory input by: (i) stimulating specific deterrent receptors; (ii) stimulating broad spectrum receptors; (iii) stimulating some cells and inhibiting others, thereby changing complex and subtle codes; (iv) inhibiting specific phagostimulant receptors; this contributes to the neural coding of deterrence, and (v) evoking highly unnatural impulse patterns, often at high frequency (Schoonhoven et al., 1998). The ability of a deterrent neuron to respond to a wide range of chemicals is due to it having a diverse range of receptor sites, each with its own structure-function specificity, or due to the active chemicals having common features making them able to interact with a single receptor site (Blaney et al., 1988).

Deterrent cells possess a number of unique characteristics: (i) they generally adapt more slowly than cells which respond to phagostimulatory compounds; (ii) the tonic activity of the deterrent receptor stabilizes at a higher level than in other cell types; (iii) there may be a relatively long latency period prior to the tonic response; (iv) there may be a slow increase in spike frequency following stimulus application, and (v) there may be an increase in spike amplitude with stimulus concentration (Schoonhoven, 1982); Hanson and Peterson, 1990). Schoonhoven (1982) used differential adaptation rates to explain that the sensory code changes with time, with the result that the deterrent receptor activity gradually becomes more pronounced in the sensory message sent to the brain. Food, which at the beginning of

a meal may be acceptable, soon becomes unacceptable because of the more prominent share of the deterrent in the total sensory impression.

Recently, work by Wanner and Robertson (2008) revealed a family of 65 gustatory receptor (Gr) genes from the silkworm moth, *Bombyx mori*, genome. These authors revealed Gr genes for sugar, as well as those for cuticular hydrocarbons and carbon dioxide. Interestingly, they also found 55 Gr genes that are predominantly bitter receptors involved in the detection of a large variety of secondary plant chemicals and suggested that these Gr genes mediate food choice and avoidance, as well as oviposition site preference. This finding may provide new tools for controlling pest damage and lead to better understanding of the peripheral taste system and is noteworthy since about 99% of the 150,000 described species of lepidopterous insects are phytophagous feeders (Grimaldi and Engel, 2005). Functional characterization of GRCs may also provide a better understanding of the molecular and cellular basis of taste coding. Interestingly, Clyne et al. (2000) found a large Gr gene family and characterized the GRCs expressing divergent GR genes in the fruit fly, *D. melanogaster*. In the sequenced *D. melanogaster* genome, 68 receptors, encoded by 60 genes, were identified and were predicted to encode G protein-coupled receptors (Robertson et al., 2003). Expression and behavioral studies of two *D. melanogaster* Gr genes, *Gr5a* and *Gr66a*, revealed that inactivation of *Gr5a*-positive neurons resulted in a diminished behavioral responses to sugars and low concentrations of salt, whereas inactivation of *Gr66a*-positive neurons lowered behavioral responses to some bitter compounds (Thorne et al., 2004; Wang et al., 2004). Interestingly, molecular studies revealed that these “sugar” and “bitter” neurons also project to distinct and non-overlapping regions within the SOG (Dunipace et al., 2001; Scott et al., 2001; Thorne et al., 2004; Wang et al., 2004; Dahanukar et al., 2007).

### 3. Olfactory extracellular recording methods

A powerful tool for studying olfactory ORCs lies in physiologically recording from individual sensilla using a recording technique termed single-unit or single-sensillum recording (Figs. 3, 4). This technique monitors the electrical events elicited by ORCs when stimulated by different odor stimuli. Despite the fact that multiple GRCs are present within a single sensillum, the distinct electrophysiological responses of each ORC can be distinguished using specialized computer-aided software for spike sorting by differing spike amplitudes. Knowledge of the number of ORCs present within a particular sensillum, as visualized with transmission electron microscopy, can greatly contribute to a better interpretation and verification of electrophysiological results.

To record from olfactory ORCs to test the effect of volatile compounds, female *M. sexta* moths reared on artificial diet (17h light: 7h dark; ca., 60% relative humidity), 1-2 days post-eclosion were used for these studies (Bell and Joachim, 1976; Sanes and Hildebrand, 1976). Recordings were carried out at ambient temperatures (24-26°C). To record extracellularly from ORCs, the cut-tip recording technique was used (Van der Pers and Den Otter, 1978; Kaissling, 1995) (Fig. 4B). This technique involved restraining each moth in a plastic tube (5.2 x 1.2 cm i.d.) so that the head protruded from one end (Fig. 3). One of the moth's antennae was stabilized with a minimal amount of low melting point paraffin wax to allow for easier manipulation of the sensilla. Alternatively, double-sided tape can be used. The tip of a trichoid type-A sensillum, positioned on the distal or proximal margins of the dorsal, ventral, and leading surfaces of an annulus, was cut between two sharpened glass knives (2-



mm diameter, Corning Inc., Horseheads, NY) positioned at 90 degrees with respect to each other (micro knife-beveling device; type EG-03, Syntech, Hilversum, The Netherlands) (Van der Pers and Den Otter, 1978; Kaissling, 1995). This method is typically feasible for sensilla less than 50  $\mu\text{m}$  in length. For longer sensilla, an alternative method can be used, whereby the sensillum is cut by pinching off the tip using sharpened forceps (Fig. 4A), thus preventing the opening and subsequent damage of the ORC dendrites due their depolarization. The flat side of the forceps is coated with a thin film of Vaseline to prevent desiccation of the sensillum (i.e., loss of the sensillum lymph surrounding the dendrites) (Kaissling, 1995).

Recording and ground electrodes (tip diameters: 2.5-3.5  $\mu\text{m}$  and 4-5  $\mu\text{m}$ , respectively) were made from borosilicate glass tubing (1.5 mm o.d., 1.10 mm i.d., Sutter Instrument Co., Novato, CA) on a Flaming-Brown micropipette puller (model P-97, Sutter, Novato, CA). The recording electrode was filled with sensillum-lymph saline solution (Kaissling and Thorson, 1980) and positioned over the tip of a trichoid type-A sensillum with the aid of a micromanipulator (Leitz) and a Wild M3Z Kombistereo microscope (Leica, Germany; total magnification, 640X) (Figs. 3, 4). The ground electrode was filled with hemolymph saline solution (Kaissling and Thorson, 1980) and inserted into the moth's eye or body. Both ground and recording electrodes were held in place by mounting them in electrode holders (1.5 mm i.d., Syntech) containing 0.25 mm silver wire (>99.99 %, Aldrich Chemical Co., Milwaukee, WI) (Fig. 3). Difficulties in cutting the tip of olfactory sensilla can arise if they are very short and small. In this case, a modification of the tip-cutting technique can be used called sidewall recording. In this method, a sharpened tungsten electrode is plunged into the base of the sensillum to establish contact with the underlying olfactory ORCs. In either case, these electrophysiological recording techniques record the contribution of one or all of the ORCs present within a sensillum and preparations can remain stable for several hours. The electrophysiological components of the set-up were placed on a vibration-free air table (Figs. 3).

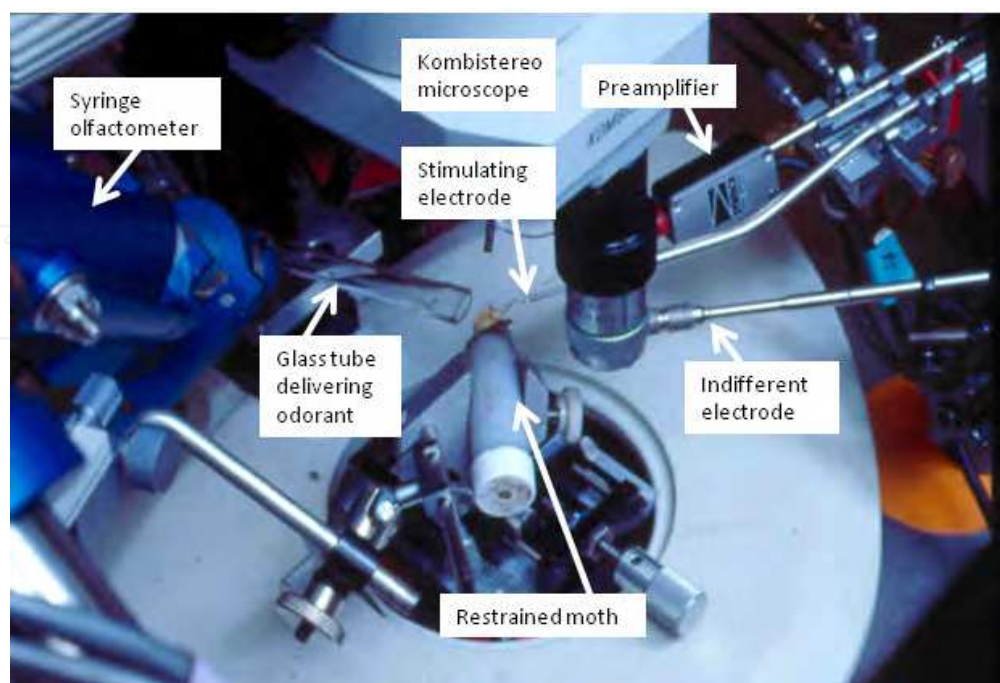


Fig. 3. Typical olfactory extracellular recording set-up.

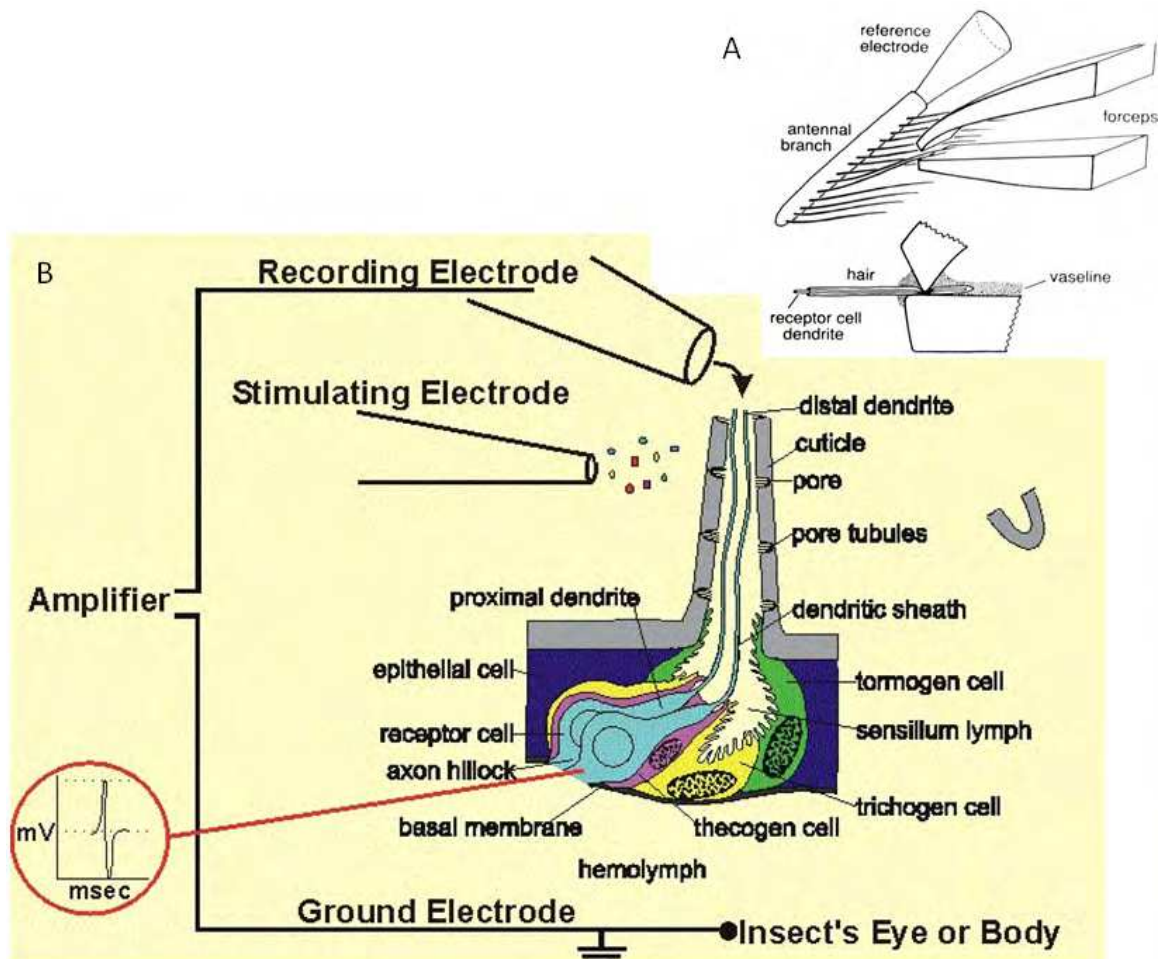


Fig. 4. Extracellular recording technique. (A) After the sensillum is cut, shown here using sharpened forceps, the recording electrode (B) is placed over the tip of the sensillum. The stimulating electrode, containing the odorant, is used to deliver the odorant to the sensillum. Odor stimuli are presented randomly to the sensillum. Action potentials are amplified, recorded, and relayed to a computer for further analyses. Fig. (A) was taken from Kaissling, 1995.

#### 4. Gustatory extracellular recording methods

Randomly selected, fifth instar, 12-24 hours post-molt, *Lymantria dispar* larvae, reared on artificial diet (12h light: 12h dark; ca., 60% relative humidity) (Shields et al., 2006) were used for all experiments. The larvae were food deprived at least 12 hours prior to the experiments and were naive to the test compounds. Recordings were carried out at ambient temperatures (24-26°C). Each larva was transected just behind the head and a blunt-tipped saline-filled glass electrode was inserted into the head with sufficient force to cause the eversion of the lateral and medial styloconic sensilla. This pipette was filled with an electrically conductive solution (typically KCl or NaCl) and served as the ground or indifferent electrode (Figs. 5, 6). The cut end of the head was sealed with a minimal amount of melted bee's wax (Fig. 5A). The stimulating or recording electrode was filled with a test solution and was positioned over the tip of a single styloconic sensillum (Figs. 5A, 6) with the aid of a micromanipulator (Leitz) under visual control on a vibration-free air table (Fig. 5B). The preparation lasted, on average, one to two hours.

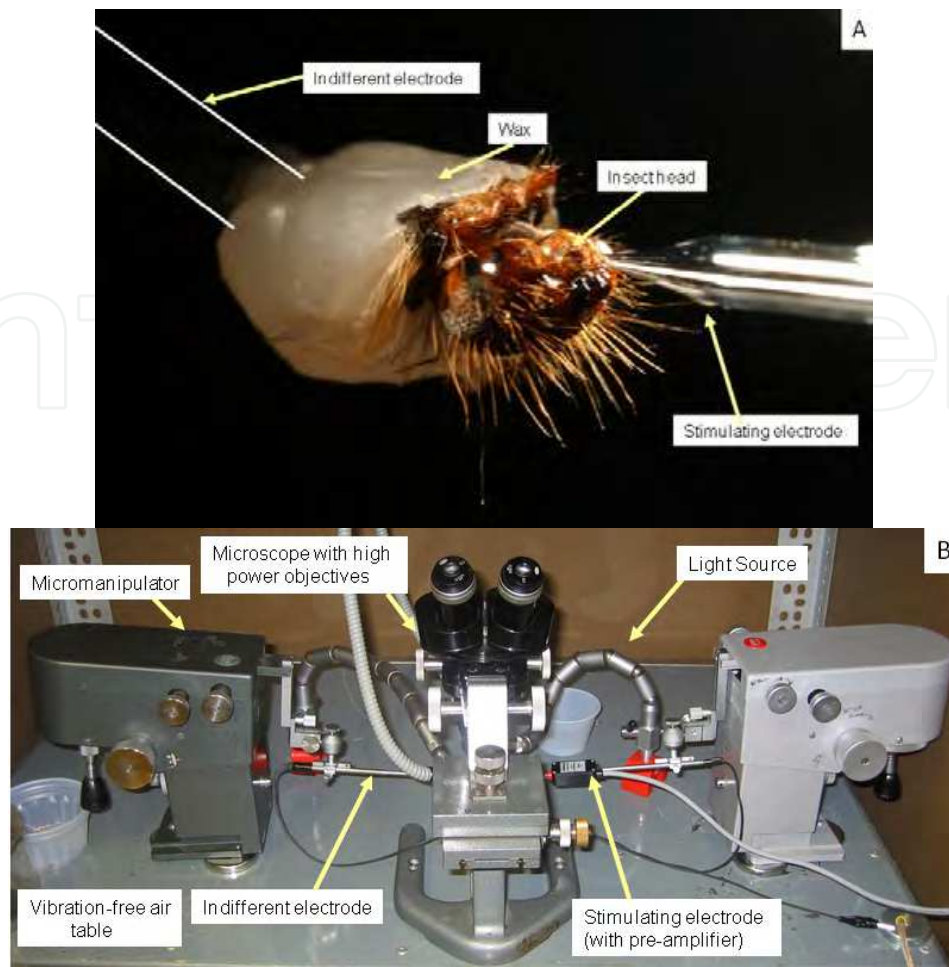


Fig. 5. (A) Higher magnification view of the insect preparation. (B) Gustatory extracellular recording set-up. Fig. (A) was modified from Shields and Martin, 2012.

## 5. Olfactory stimuli and odor delivery

Olfaction is the principal sensory modality through which insects locate their food sources, mates, and oviposition sites. Over the past three decades, or so, moth olfaction has focused primarily on mechanisms through which male moths detect, process sensory information about, and respond behaviorally to the sex pheromones emitted by conspecific females. In contrast, relatively little is known about similar mechanisms with respect to non-pheromonal odors, such as plant volatiles and the mechanisms by which female moths detect and discriminate plant-associated volatiles (odorants) for foraging and oviposition purposes and how this information is processed by the olfactory system.

Previous studies have indicated that scent emitted by sphinx moth-pollinated flowers (Knudsen and Tollsten, 1993; Raguso and Willis, 1997; Raguso and Light, 1998), as well as floral and vegetative volatiles of tobacco, tomato, and other hostplants (Andersen et al., 1988; Buttery et al., 1987a; 1987b) attract adult *M. sexta* for feeding (Morgan and Lyon, 1928; Yamamoto et al., 1969; Raguso et al., 1996; Raguso and Light, 1998). Flowers having a “white floral” scent, such as that associated with many night-blooming moth-pollinated flowers, possess acyclic terpene alcohols (e.g. linalool, nerolidol, and farnesol), as well as the corresponding hydrocarbons, aromatic alcohols, and esters derived from them, in addition to

esters of salicylic acid (Knudsen and Tollsten, 1993). Also thought to play a significant role in the attraction of insects to, and their recognition of, their hostplants are various plant-associated odorants, such as green-leaf volatiles (mainly saturated and unsaturated C<sub>6</sub> alcohols and aldehydes), terpenoids, and benzenoid compounds (e.g. Boeckh, 1974; Visser and Avé, 1978; Renwick, 1989; Heath et al., 1992; Knudsen and Tollsten, 1993; Raguso and Willis, 1997).

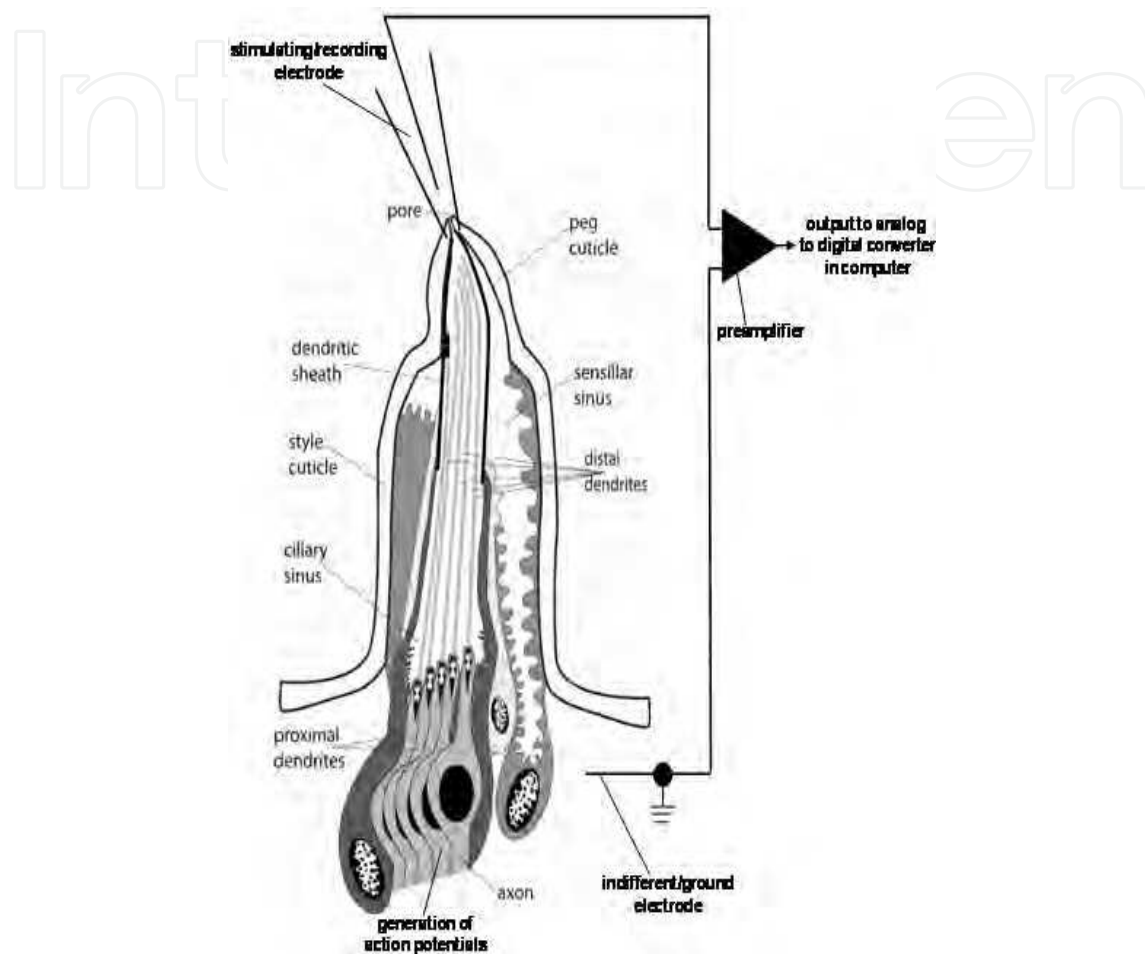


Fig. 6. Schematic diagram showing extracellular recording from a single gustatory styloconic sensillum showing the stimulating electrode containing the stimulus positioned over the tip of the sensillum. This figure was taken from Shields and Martin, 2010.

The majority of the selected, purified, and synthetic odorants tested (chemical purity of 95-99.9%) (Shields and Hildebrand, 2001a) represented floral headspace volatiles of native, night-blooming flowers, such as *Datura wrightii* (jimson weed), *Hymenocallis sonorensis* (spider lily), and *Oenothera caespitosa* (evening primrose), to which *M. sexta* and other sphinx moths are attracted for nectar-feeding and were available from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI). Complex blends of volatiles emitted by the headspace inflorescence from *D. wrightii* (i.e., jimsonweed) and the foliage of two solanaceous plants (i.e., *Lycopersicon* (tomato) and *Nicotiana* (tobacco) species) were also tested. This was carried out by placing the entire inflorescence or two to three undamaged leaves in a 20 ml capped syringe, which was allowed to equilibrate at room temperature for 24 h prior to use. Tomato and tobacco foliage are preferred by female *M. sexta* for oviposition (Yamamoto et al., 1969), while jimsonweed provides a nectar feeding source.

For testing individual compounds, each odorant was dissolved in odorless mineral oil (light white oil, Sigma, St. Louis, MO) at a dilution of 1:10 (v:v). A 30- $\mu$ l aliquot was pipetted onto a 1.5-cm<sup>2</sup> piece of grade-1 filter paper (Whatman, Kent, ME) and inserted into a disposable 20 ml syringe. Control syringes, loaded with 30  $\mu$ l of mineral oil, were also prepared. All syringes were capped and allowed to equilibrate, similar to that described above. After each experimental session, the capped odorant syringes were stored at 4°C. Some odorants eliciting the strongest responses were tested in dose-response experiments. For these experiments, dilutions were prepared in decadic steps (v:v) over at least four log units from stock solutions. Stimulus loads ranged from  $3 \times 10^{-4}$  to 3  $\mu$ l of the test compound (approximately 0.3  $\mu$ g to 3 mg). Odorants were tested in order of increasing concentration to prevent adaptation of the ORCs. Also included among the odorants, were two key components of the female moth's sex pheromone, E10,E12-hexadecadienal (bombykal) and E11,Z13-pentadecadienal ("C15"), a relatively stable mimic of E10,E12,Z14-hexadecatrienal (see below) (Kaissling et al., 1989). Each compound was dissolved in *n*-hexane and applied directly (without mineral oil) at 1 ng· $\mu$ l<sup>-1</sup> and 10 ng· $\mu$ l<sup>-1</sup> to the filter paper.

The odor was delivered to the female antenna using a stimulating device (type CS-01, Syntech) (Shields and Hildebrand, 2001a). The tip of the syringe containing the odorant was introduced into a 20 cm long, L-shaped glass tube (1 cm i.d.) whose open end was positioned 5-7 mm from the test antenna (Fig. 3). The end of the tube was flattened to 0.5 cm to correspond with the length of the antenna. The antenna was continuously flushed with a 5 cm·s<sup>-1</sup> stream of charcoal-filtered, humidified air. To deliver a stimulus, 2 ml of odor-laden air was injected at a constant rate over 200 ms from the syringe into the airflow using a motor-driven syringe olfactometer, resulting in increased airflow of 20 cm s<sup>-1</sup> (measured by a thermo-anemometer) (Fig. 3). A glass funnel (3.5 cm i.d.) attached to an air evacuation line was positioned near the preparation to draw away any odor-bearing air following stimulus delivery. Odor stimuli were presented randomly to the sensilla to be tested. Blank controls were tested repeatedly, but never elicited a response from the ORCs. An interstimulus interval of at least 60 s, or longer, if necessary for spontaneous activity of the ORC to return to their initial value, was allowed between stimulations.

## 6. Olfactory responses from trichoid type-a sensilla

Alternating current (AC) signals were recorded for 6 s, starting 1 s before stimulation (olfactory experiments) and for 10 s, beginning 0 s before stimulation. In either case, these signals were pre-amplified 10X using a Syntech Probe run through a 16-bit analog-to-digital interface (IDAC-02, olfactory, or IDAC-4, gustatory) (Syntech), and then analyzed off-line with Autospike software (Syntech). This software allowed the user to more easily interpret how many cells were responding to specific compounds, as determined by their size, shape, and firing frequency of the biphasic action potentials. The net number of spikes within the first 500 ms after stimulus delivery was considered to be a measure of the strength of excitation and was calculated as the mean number of spikes within 500 ms after the termination of stimulus delivery minus the mean number of spikes within 500 ms before stimulus onset. The responses of ORCs were classified as excitatory (Figs. 7A-F, 7H-M, 8A-N, 8P, 8Q) or inhibitory (Figs. 7G, 8O) if there was cessation in spiking activity for more than 500 ms after stimulation. Dose-response experiments were conducted after the general screening of the odorants was completed and only if the response of an ORC remained constant throughout the experimental session. In order to increase the accuracy of the data analyses, vapor pressure of a particular odorant was taken into consideration when analyzing the sensitivities of selected ORCs.

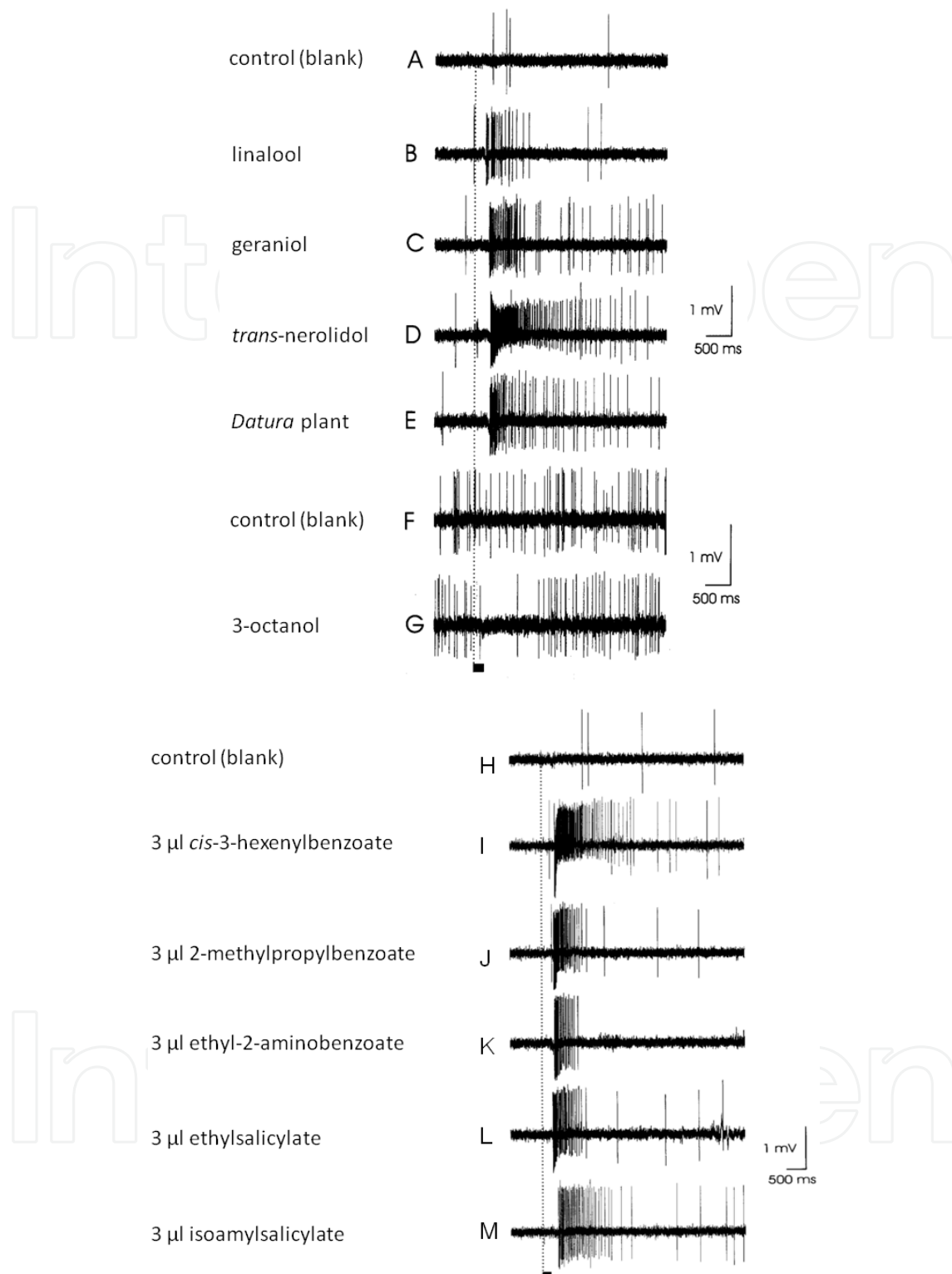


Fig. 7. Representative extracellular olfactory electrophysiological recordings of from three different olfactory receptor cells (ORCs) from female *Manduca sexta* trichoid type-A sensilla showing excitatory (A-F, H-M) and inhibitory (G) responses. The responses in (F) and (G) are from a different ORC than those in (A-E) and (H-M). The stimulus bar is represented by a filled rectangle and represents 200 ms and represents the onset of odor delivery. This figure from modified from Shields and Hildebrand, 2001b.

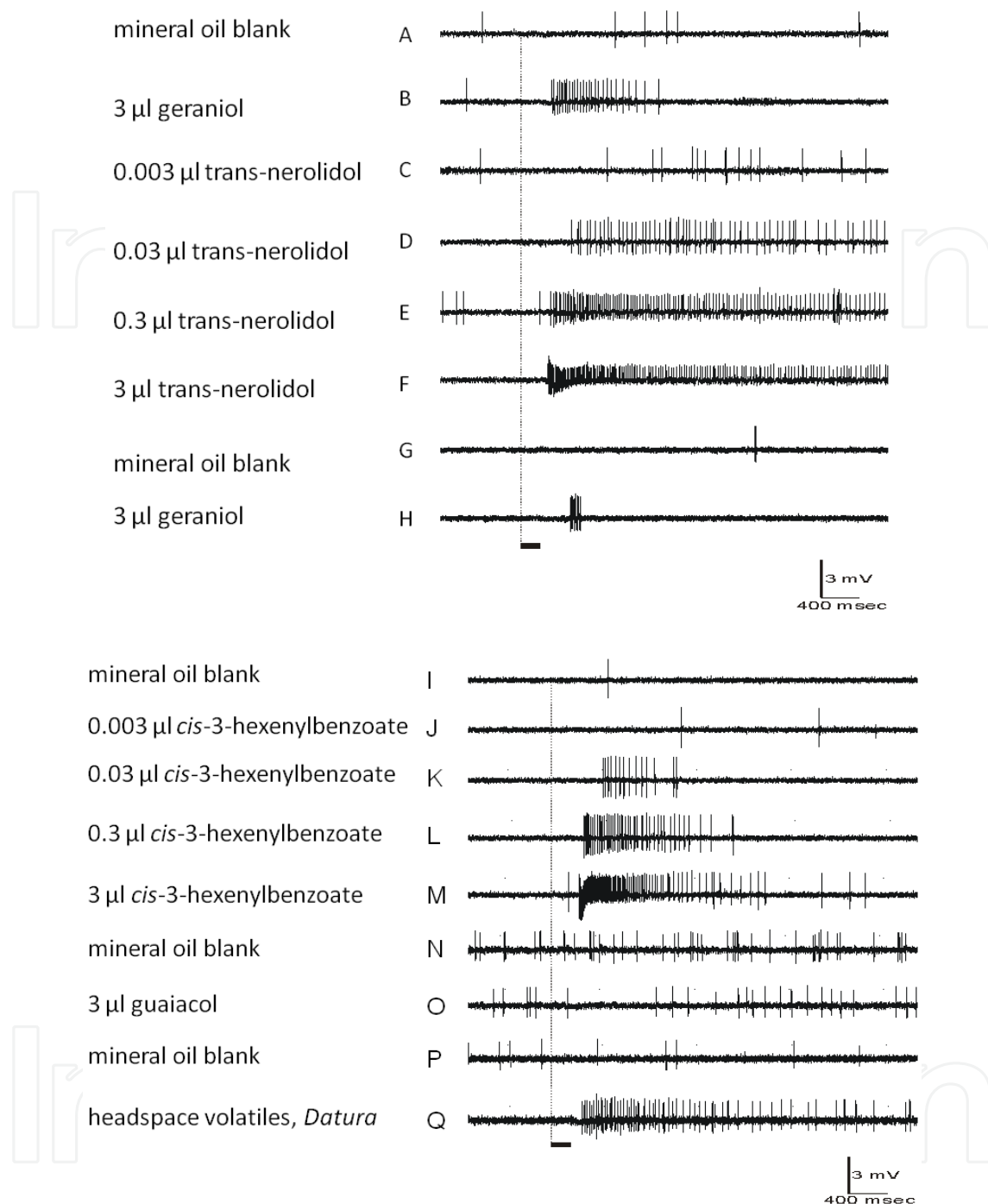


Fig. 8. Representative extracellular olfactory electrophysiological recordings from five different olfactory receptor cells (ORCs) from female *Manduca sexta* trichoid type-A sensilla showing excitatory responses (A-N, P, Q) and inhibitory (O) responses. Note the increasing excitatory activity of the ORC to increasing concentrations of trans-nerolidol (C-F) and *cis*-3-hexenylbenzoate (J-M). Note the two different types of excitatory responses to geraniol (B and H). The stimulus bar is represented by a filled rectangle and represents 200 ms and represents the onset of odor delivery. This figure from modified from Shields and Hildebrand, 2001a.

## 7. Staining and mapping of ORC axonal projections

The axons of ORCs project to and terminate in compartments of condensed synaptic neuropil (i.e., glomeruli) in the primary olfactory centers in the CNS of invertebrates and vertebrates (Hildebrand and Shepherd, 1997). Each olfactory glomerulus is a discrete anatomical and functional unit and represents a specific anatomical "address" dedicated to collecting and processing specific molecular features about the olfactory environment, conveyed to it by ORC axons expressing specific olfactory receptor proteins (Buck, 1996; Buonviso and Chaput, 1990; Christensen et al., 1996; Hildebrand and Shepherd, 1997; Mombaerts, 1996). Over approximately the past two decades, there has been mounting evidence that the arrays of glomeruli in the ALs of insects and the olfactory bulbs of vertebrates are organized chemotopically (e.g., Sharp et al., 1975; Rodrigues and Buchner, 1984; Hansson et al., 1992; Mombaerts, 1996; Friedrich and Korsching, 1997; 1998; Galizia et al., 1999), analogous to visuotopy, in visual systems, and tonotopy, of auditory systems.

The axons of antennal ORCs project via the antennal nerve to the ipsilateral AL in *M. sexta*. In the ALs, they form synapses with processes of a subset of the approximately 1,200 central neurons (Homborg et al., 1988; Christensen et al., 1995; Rössler et al., 1998; 1999). In both sexes of *M. sexta*, each AL bears 60 ordinary, sexually isomorphic glomeruli and three sexually dimorphic glomeruli (Rospars and Hildebrand, 2000). Male *M. sexta* moths bear three prominent glomeruli (cumulus, toroid 1, toroid 2), which constitutes the male-specific macroglomerular complex (MGC). This complex processes information about the conspecific female's sex pheromone (Hansson et al., 1991; Heinbockel et al., 1999; Rospars and Hildebrand, 2000) (discussed in more detail, below). Interestingly, females also bear two homologous, sexually dimorphic glomeruli (i.e., large female glomeruli or LFG, lateral and medial) (Rössler et al., 1998; 1999; Rospars and Hildebrand, 2000). Central neurons with arborizations leading to the lateral LFG were found to display a preferential response to linalool and certain other monoterpenoids (King et al., 2000). LFGs have been implicated in being involved in olfactory information attributed to the interactions of females with hostplants or with courting males.

To determine where the axons of ORCs of trichoid type-A sensilla project in the AL of female *M. sexta*, we performed anterograde labeling using dextran-tetramethylrhodamine of ORCs in groups of 5-10 sensilla on various surfaces of a single annulus in the middle of an antennal flagellum (Fig. 1C, D). This was carried out by restraining an adult female moth in a plastic tube (Fig. 4) and cutting the tips of 5-10 trichoid type-A sensilla (Fig 4); (see Shields and Hildebrand, 1999a; Shields and Hildebrand, 2001) using the method outlined, above. In order to stain the ORCs associated with these cut sensilla, we created a small well from melted paraffin wax around the selected annulus and filled it with a small amount of 1% solution of dextran-tetramethylrhodamine (3000 MW, anionic, lysine-fixable, D-3308, Molecular Probes, Eugene, OR) until the annulus became completely submerged. The area was then covered with a small amount of petroleum jelly. We transferred the preparation to a humid chamber and kept it in darkness for 2-3 days. Following this period, we excised the brain and fixed it for 24-48 h in 2.5% formaldehyde solution in 0.1 M sodium phosphate buffer (pH 7.4) containing 3% sucrose. Following this, the tissue was dehydrated in a graded ethanol series, cleared in methyl salicylate, and viewed as a temporary whole mount in a laser-scanning confocal microscope.



We found that a majority of axonal projections from these ORCs terminated in the two LFG located in the dorsolateral region of the ipsilateral (AL) (Fig. 9A), more specifically, near the entrance of the antennal nerve into the AL. We also found that in addition to the LFGs, a subset of the other 60 spheroidal, ordinary, sexually isomorphic glomeruli received sparse projections of a subset of ORC afferent axons (Fig. 9). The results of these anatomical studies and our electrophysiological results that some trichoid type-A sensilla are tuned mainly to terpenoids and aromatic esters, provide a basis to hypothesize that information about odorants belonging to those chemical classes is processed in the LFGs (King et al., 2000; Shields and Hildebrand, 2001a; b). To improve visualization (i.e., resolution) of successfully labeled preparations, samples were embedded in Spurr's low-viscosity embedding medium (Electron Microscopy Sciences, Fort Washington, PA) and sectioned at 48  $\mu\text{m}$ . The preparations were then viewed in a laser scanning confocal microscope (Bio-Rad MRC-600; Cambridge, MA) equipped with a Nikon Optiphot-2 microscope and both 15-mW krypton-argon and 100-mW argon laser light sources and YHS filter cube (excitation wavelength 568 nm). Serial optical sections were collected at 3- $\mu\text{m}$  intervals through the whole mount or 2  $\mu\text{m}$  intervals from the embedded sectioned preparations (Fig. 9). Image processing and analysis were performed using Confocal Assistant 4.02 (copyrighted by Todd Brelje, distributed by Bio Rad, Cambridge, MA), Corel Photopaint 8, and Corel Draw 8 (Corel Corporation, Ottawa, Ontario, Canada).

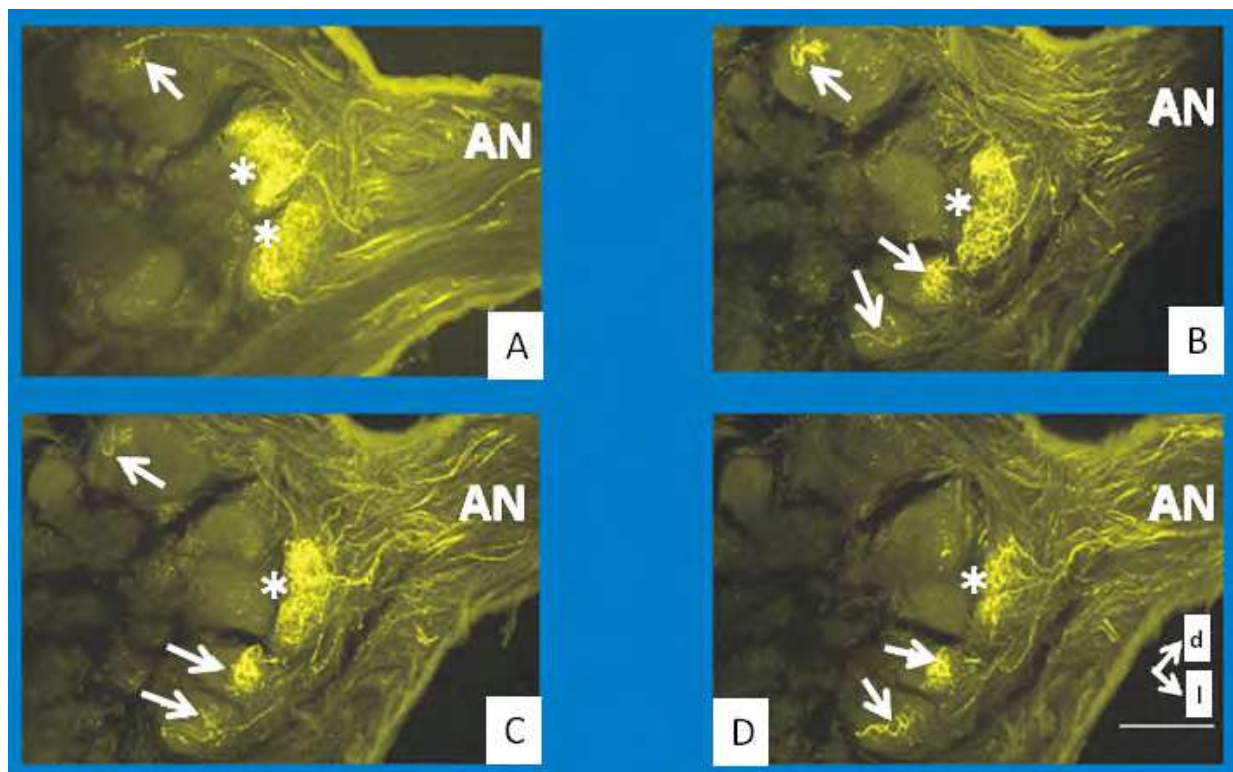


Fig. 9. Confocal microscopic images (A-D) showing serial optical sections of female *Manduca sexta* specimens embedded in plastic and sectioned to improve resolution taken at different depths through the antennal lobe. Images show the central projections of axons from olfactory receptor cells from trichoid type-A sensilla stained with the fluorescent dye dextran-tetramethylrhodamine. Asterisks indicate the sexually dimorphic large female glomeruli located in the dorsolateral region of the antennal lobe, near the site of entry of the antennal nerve (AN). The arrows indicate ordinary sexually isomorphic glomeruli. d, dorsal; l, lateral. Scale bars = 100  $\mu\text{m}$ . Figs. (A) and (B) were modified from Shields and Hildebrand, 2001a.

### 8. Gustatory stimuli and responses from the medial styloconic sensilla

For all gustatory experiments, stimulus compounds were dissolved in 30 mM potassium chloride (KCl) (control) (Fisher Scientific, Fair Lawn, New Jersey) in distilled water to enhance the electrical conduction of the recording electrode and to improve the signal-to-noise ratio. This inorganic salt was chosen since the hemolymph of plant-feeding (i.e., phytophagous) feeders typically shows high  $K^+$  and low  $Na^+$  concentrations (Kaissling, 1995). KCl was also used to fill the indifferent electrode (Figs. 5, 6). Selected carbohydrates (i.e., sucrose and inositol), as well as alkaloids (Fig. 10) were tested in this study to observe

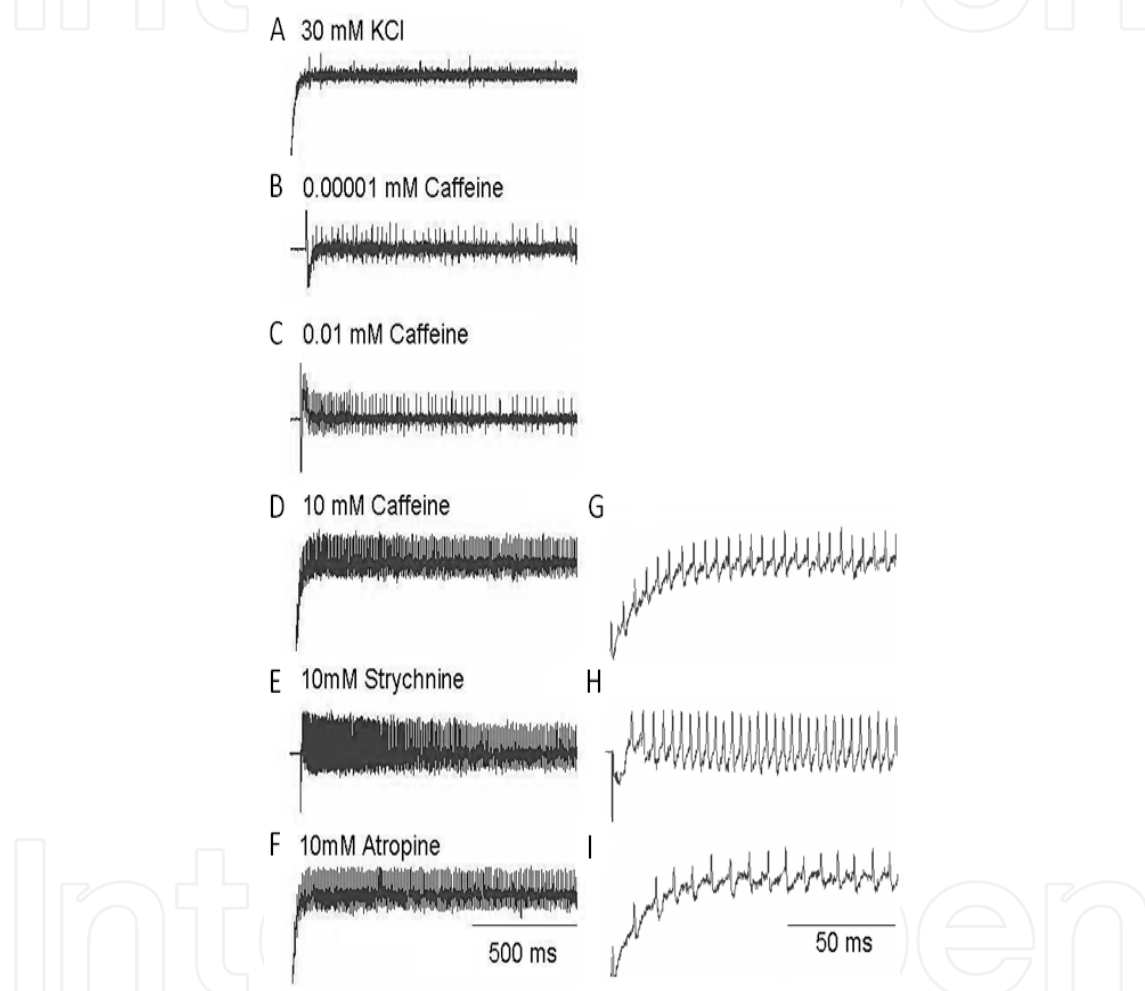


Fig. 10. Representative extracellular gustatory electrophysiological recordings from the medial styloconic sensillum of *Lymantria dispar*. The deterrent-sensitive cell responds to (A) potassium chloride (control) and to the alkaloids, (B-D) caffeine, (E) strychnine, and (F) atropine. The recordings in G, H, and I are higher magnifications of the action potentials from the deterrent-sensitive cell to (D) caffeine, (E) strychnine, and (F), atropine, respectively. The deterrent-sensitive cell displays a typical phasic-tonic response (i.e., a transient pattern of firing changing to that of a more sustained pattern of firing) to these alkaloids. This feature is more apparent at higher concentrations (compare (C) and (D) with (B)) and is shown for caffeine. A weaker concentration of caffeine (B) elicits fewer spikes from the deterrent-sensitive cell. This figure was modified from Shields and Martin, 2010.

the responses of the deterrent-sensitive, inositol-sensitive, sucrose-sensitive, and salt-sensitive cells (see also Shields and Martin, 2010; 2012). The alkaloids were diluted in 10% ethanol solution, which was added to the electrolyte solution. Ethanol, at this concentration, was found to have no discernible effect on the activity of cell(s) (referred to as KCl-sensitive cell(s)) (see below) responding to the control (i.e., KCl) alone. Each stimulus to be tested was applied to the tip of the styloconic sensillum by way of the stimulating electrode (Figs. 5, 6).

Electrical activity from individual styloconic sensilla was amplified and conditioned (bandpass filter set at 100-1200 Hz) prior to being digitized by a 16-bit analog-to-digital interface (IDAC-4, Syntech). For each electrophysiological recording, we stimulated a sensillum for total of 10 sec. Action potentials generated 50 ms after contact with the sensillum were analyzed off-line using a computer equipped with Autospike (Syntech). A pause of at least 2 minutes was allowed between successive stimulations to minimize any carry-over effects. For dose-response experiments, only those phytochemicals that elicited a robust response were tested. Test stimuli were presented to the sensillum from lowest to highest concentration. To ensure a reproducible response to a particular concentration, 1-2 replications of each were made. The number of action potentials generated 0.05 s after the contact artifact was quantified in 100 ms increments.

We found two cells (i.e., one large spike amplitude and one small spike amplitude) in the medial styloconic sensillum that responded to KCl (Fig. 10A). Another cell in the same sensillum responded robustly to various alkaloids (i.e., deterrent-sensitive cell) (Fig. 10B-F). This cell displayed a typical phasic-tonic response (i.e., the initial high firing frequency of the cell changed rapidly to a more sustained pattern of firing). The ability of the deterrent-sensitive cell to respond to more than one alkaloid may be due to either the presence of multiple receptor sites, each with their own structure-function specificity, or a broadly tuned single receptor site (Blaney et al., 1988). These results correlated well with feeding behavioral studies using gypsy moth larvae (Shields et al., 2006), where it was shown that an increase in feeding detergency (decrease in consumption) occurred with increasing alkaloid concentration.

## 9. Intracellular recording methods

ORCs in the antenna send olfactory information as trains of action potentials to the ipsilateral ALs of the insect brain where the axon terminals of ORCs synapse onto central neurons in neural structures known as olfactory glomeruli. Central neurons in the ALs, such as projection neurons and local interneurons, can be characterized by intracellular recordings with sharp electrodes. These glass electrodes are filled with a physiological solution that mimics the intracellular fluid of the recorded neuron. In addition, the electrodes can contain intracellular markers such as fluorescent dyes. The development of new intracellular markers provides the basis for rapid and complete reconstruction of individual neurons with little or no toxicity to the neuron. For examples, central neurons are injected iontophoretically with Lucifer yellow, neurobiotin, or biocytin. Brains are then dissected and fixed overnight in formaldehyde with sucrose in phosphate buffer. To visualize biocytin-injected neurons, brains are incubated with, e.g., Cy3-conjugated streptavidin. After subsequent histological processing, neurons are further investigated by laser-scanning confocal microscopy. This approach allows the study of both the physiological as well as morphological properties of the recorded neuron in a relatively undisturbed *in vivo* preparation as described below.

### 9.1 Olfactory neurobiology

Female moths release sex pheromones that attract conspecific males over long distances (Kaissling, 1987; Hildebrand, 1996). An outstanding challenge in olfactory neurobiology is to understand how a male moth is able to locate a mate, namely, a conspecific female releasing sex-pheromone. The olfactory brain of a male moth must integrate information about qualitative, quantitative, and spatiotemporal features of an attractive blend of volatile compounds, the sex pheromone, released by a conspecific female (Hildebrand, 1995; 1996; Christensen et al., 1996). Central processing of this information occurs in a sexually dimorphic cluster of olfactory glomeruli, the MGC, in the male moth's AL. Since information about the sex-pheromone is primarily processed in the MGC, it serves as a model for studies of the functional architecture of glomeruli, as well as the physiological relationships between glomeruli in the olfactory system (Hansson and Christensen, 1999; Christensen and White, 2000; Christensen and Hildebrand, 2002; Reisenman et al., 2008; Lei et al., 2010).

Several species of moths use the same chemical compounds in their sex-pheromone blend, but the attractant signal produced by each species is nevertheless unique because each blend has characteristic proportions of the components (Arn et al., 1992; Kaissling, 1996). Chemical studies revealed that in the sphinx moth *M. sexta*, the sex-pheromone blend comprises eight components. Behavioral data shows that two of the eight components are required to evoke olfaction-modulated flight in males (Tumlinson et al., 1989). The antennae of male *M. sexta* are covered by different types of sensilla (see above). The long trichoid sensilla on the male's antennae house highly selective and sensitive ORCs that detect one or the other of the two key pheromone components (Kaissling et al., 1989). Each of these ORC populations sends their axons to a different glomerulus in the MGC (Christensen et al., 1995). Projection neurons (PNs) that arborize in one of these glomeruli, the toroid 1 [T1] (Strausfeld, 1989; Homberg et al., 1995) respond to antennal stimulation with *E,Z*-10,12-hexadecadienal (bombykal or BAL, one of the two key components of the female's sex pheromone). PNs that arborize in a neighboring glomerulus (the cumulus) respond selectively to *E,E,Z*-10,12,14-hexadecatrienal (the second key component) or its more stable mimic, *E,Z*-11,13-pentadecadienal (C15; Kaissling et al., 1989; Hansson et al., 1991). An MGC-PN with arborizations in both glomeruli is activated by both BAL and C15 (Hansson et al., 1991; Heinbockel et al., 2004).

For a number of moth species it has been demonstrated in behavioral studies in the field and in laboratory wind tunnels that pheromone-modulated upwind flight is regulated in a dose-dependent manner (Baker, 1989). Similarly, electrophysiological studies of AL neurons in male *M. sexta* have shown how the responses of MGC-PNs to pheromone components and extracts of the female's pheromone gland are influenced by stimulus intensity (Christensen and Hildebrand, 1990). In a technological advance, neural-ensemble recordings have revealed concentration-dependent patterns of synchronous firing between MGC-PNs responding to the two key pheromone components (Christensen et al., 2000). A growing body of evidence suggests that glomeruli can receive converging excitatory and inhibitory input. Subpopulations of MGC-PNs receive excitatory input driven by BAL and inhibitory input driven by C15 (or vice versa) (Christensen and Hildebrand, 1987; Hansson et al., 1991; Heinbockel et al., 1999; Lei et al., 2002). Input from both excitatory and inhibitory pathways enhances the ability of MGC-PNs to resolve multiple pulses of pheromone such as intermittent odor stimuli found in natural stimulus situations (Christensen and Hildebrand,

1997; Christensen et al., 1998; Heinbockel et al., 1999). Here, we describe how this processing function is optimized at particular stimulus concentrations or ratios in different PNs (Heinbockel et al., 2004). By using intracellular recording and staining methods, we examined the effect of changing these quantitative attributes of the pheromonal stimulus on the responses of identified PNs innervating different glomeruli in the MGC.

## 9.2 Experimental preparation

Male *M. sexta* were reared on an artificial diet (modified from that of Bell and Joachim, 1976) at 25°C and 50-60% relative humidity under a long-day photoperiod regimen (17h light : 7h dark) as described previously (Sanes and Hildebrand, 1976; Prescott et al., 1977). Adult moths (1-3 days post-eclosion) were immobilized and prepared by standard methods (Christensen and Hildebrand, 1987). With the antennae intact, the head was separated from the rest of the body and pinned in a Sylgard-coated (Dow Corning, Midland, Michigan) recording chamber (volume <0.5 ml) (Heinbockel and Hildebrand, 1998; Heinbockel et al., 1998). Isolation of the head from the rest of the thorax had no detectable adverse effect on neural responses (Christensen and Hildebrand, 1987). Part of the AL was then desheathed with fine forceps to facilitate insertion of the recording electrode. The brain was superfused constantly with physiological saline solution (modified from that of Pichon et al., 1972; ca. 2 ml/min) containing 150 mM NaCl, 3 mM KCl, 3 mM CaCl<sub>2</sub>, 10 mM TES buffer (pH 6.9), and 25 mM sucrose to balance osmolarity with that of the extracellular fluid

## 9.3 Electrophysiological recordings and data analysis

Sharp glass microelectrodes for intracellular recording were produced from borosilicate tubing (o.d. 1.0 mm, i.d. 0.5 mm, World Precision Instruments, Sarasota, Florida) with a Flaming-Brown Puller (P-2000, Sutter Instrument Co., Novato, California). The tip of each electrode was filled with a solution of an intracellular stain (see below), and the shaft was filled with filtered (0.2 µm pore size) 2.5 M KCl. Electrode resistance ranged from 60-100 MΩ, measured in the tissue. Movements of microelectrodes were controlled with a Burleigh Inchworm (Model 6000/ULN; Burleigh Instruments Inc., Fishers, New York) attached to a Leitz micromanipulator (Leitz, Wetzlar, Germany). Recordings were made from neurites in the synaptic neuropil of the MGC. Because the site of the electrode impalement in a neuron can affect the amplitude of postsynaptic potentials, impalements targeted the same area of neuropil in all preparations. Typically, one neuron was recorded per animal, except when a second recorded neuron had different pheromone response patterns (excited by BAL vs. excited by C15). Intracellular recording and current injection were carried out in bridge mode with an Axoclamp-2A amplifier (Axon Instruments - Molecular Devices, Sunnyvale, California), and data were initially stored on magnetic tape (Hewlett Packard Instrumentation Tape Recorder 3968A, Palo Alto, California) and subsequently transferred to a computer and analyzed with Experimenter's Workbench (Datawave Technologies Co., Longmont, Colorado) or Autospike (Syntech, Kirchzarten, Germany).

The data presented below were analyzed statistically for differences using one-way analysis of variance (Kruskal-Wallis One-Way Analysis of Variance by Ranks, ANOVA) (Zar, 1984). A multiple-comparison procedure was used to isolate groups from each other (All Pairwise Multiple-Comparison Procedures, Dunn's Method, or Student-Newman-Keuls Method;  $P < 0.05$ ) (Sigma Stat, Jandel Scientific Software, Richmond, California, Version 1.0, Statistical Software).

#### 9.4 Intracellular staining and confocal microscopy

Neurons were injected iontophoretically with either Neurobiotin (Vector Laboratories, Burlingame, California; 3-5% in 2 M KCl with 0.05 M Tris buffer, pH 7.4) or biocytin (Sigma, St. Louis, Missouri; 3-5% in 2M KCl with 0.05 M Tris buffer, pH 7.4). Alternating hyperpolarizing and depolarizing current pulses (30 sec, 1 nA) for about 10 min were used to inject either tracer. Brains were then dissected and fixed overnight in 2.5% formaldehyde with 3% sucrose in 0.1 M sodium phosphate buffer. To visualize injected neurons, brains were incubated with Cy3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, Pennsylvania; diluted 1:100 with 0.2 M sodium phosphate buffer containing 0.3% Triton X-100) for 3 days on a shaker at 4°C. The brains were then dehydrated with increasing concentrations of ethanol and cleared in methyl salicylate. Neurons were further investigated by laser-scanning confocal microscopy (Fig. 11) using a BioRad MRC 600 (BioRad, Cambridge, Massachusetts) with a Nikon Optiphot-2 microscope, a Krypton/Argon (15 mW) laser light source, and appropriate dichromatic filter cubes (Sun et al., 1993). Serial 2- $\mu$ m optical sections were imaged through whole mounts and saved as series of images on disks.

#### 9.5 Olfactory stimulation

We used synthetic pheromone compounds kindly provided by Dr. J.H. Tumlinson (USDA, Gainesville, FL). The following olfactory stimuli were applied: (1) *E,Z*-10,12-hexadecadienal (bombykal or BAL); (2) *E,Z*-11,13-pentadecadienal (C15), a synthetic mimic of the second pheromone component, *E,E,Z*-10,12,14-hexadecatrienal, and hereinafter referred to as the second component; and (3) mixtures of BAL and C15 with various blend ratios. These compounds were dissolved in *n*-hexane and applied to a piece of filter paper (1 x 2 cm), which was inserted into a glass cartridge (acid-cleaned glass syringe barrel), as described previously (Christensen and Hildebrand, 1987). The stimulus load on the filter paper was reported as the number of ng of compound applied in hexane solution. A pulse of charcoal-filtered, humidified air (1,000 ml/min) moving through the cartridge carried the stimulus, roughly proportional to the loading on the filter paper, to the proximal half of the antenna ipsilateral to the impaled AL. The antenna typically was stimulated with five 50-ms pulses from a cartridge at 5 pulses sec<sup>-1</sup>.

The two key pheromone components used for sexual communication in *M. sexta* are found in a 2:1 ratio in solvent rinses of intact pheromone glands of calling virgin females (Tumlinson et al., 1989). Because the mimic (C15) of the second key component is less potent than the natural component itself, a 1:1 BAL-to-C15 ratio was used to elicit physiological responses comparable to those recorded in response to the pheromone-gland extract, as found in earlier electrophysiological studies (Christensen and Hildebrand, 1987; Kaissling et al., 1989). In our experiments, different blend ratios above and below 1:1 were tested in sequence at intervals of 1 min. For one series of blend ratios, the BAL stimulus load was held constant (1 ng) while the C15 stimulus load was varied (0.01, 0.1, 1.0, and 10 ng). For the second series of blend ratios, the C15 stimulus load was held constant while the BAL stimulus load was varied in a similar fashion. In addition to these stimulus series, an elevated stimulus level (10 ng) of either BAL or C15 and a blend of BAL and C15 (10 ng of each) were also tested.

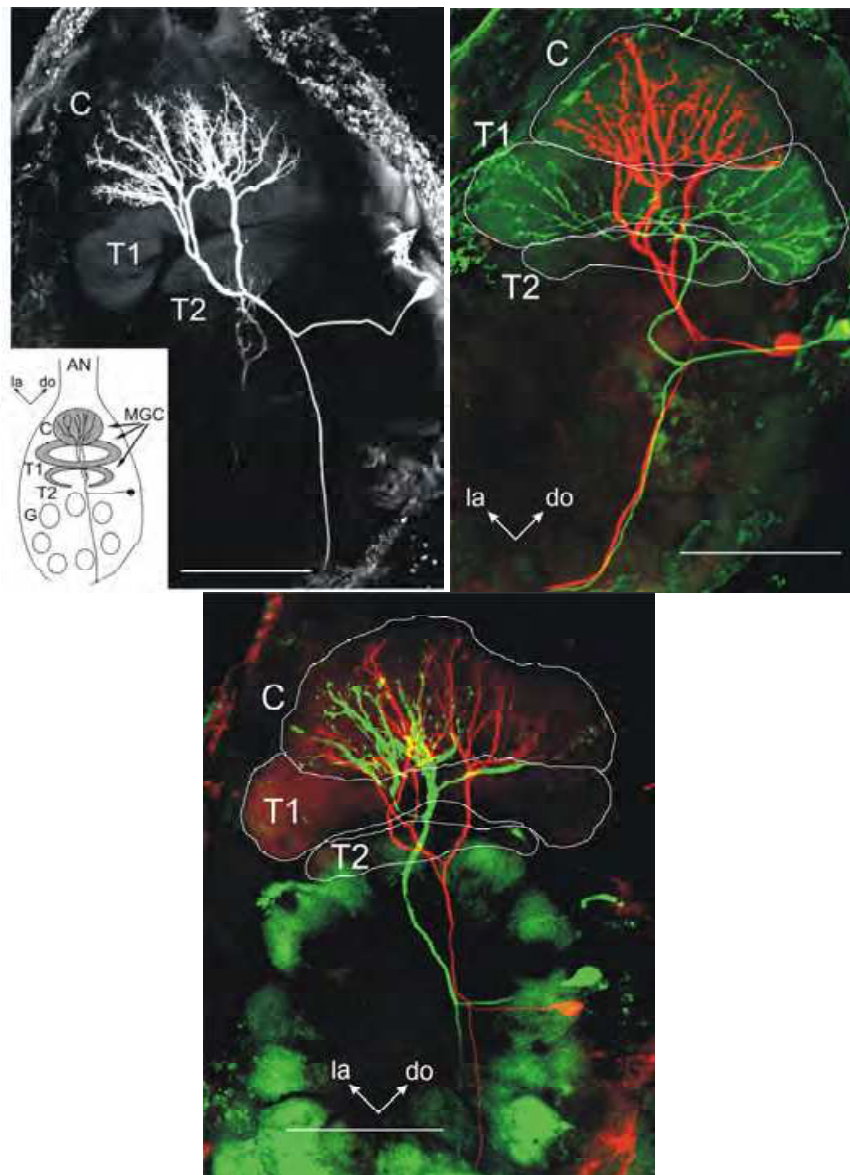


Fig. 11. Laser-scanning confocal micrographs of antennal lobe projection neurons in the moth antenna of male *Manduca sexta*. Left panel: Image of a C15-specialist MGC-PN with arborizations confined to the cumulus. The inset illustrates the organization of the antennal lobe with the macroglomerular complex (MGC) and other glomeruli (G). Right panel: Two specialist MGC-PNs, one neuron, stained with Lucifer Yellow (colored red here), had arborizations confined to the cumulus (C), and the other neuron, stained with biocytin (colored green here) had arborizations confined to the toroid (T1). Areas of apparent overlap between the 2 neurons are shown in yellow and are possible sites of synaptic contact. Bottom panel: Morphological diversity in cumulus neurons. Image of two C15-specialist MGC-PNs with arborizations confined to the cumulus. While the branches of the two neurons apparently overlapped in certain parts of the cumulus (indicated in yellow), other parts were innervated by just one of the two neurons. The green neuron was stained with Lucifer Yellow and the red neuron with biocytin. C - cumulus, T1 - toroid 1, T2 - toroid 2; do, dorsal; la, lateral. Scale bar = 100  $\mu\text{m}$ . Modified from Heinbockel and Hildebrand, 1998 (left panel); Heinbockel et al., 1999 (right and bottom panels).

## 9.6 Calculating stimulus "tracking" in MGC-PNs

We applied several criteria to assess the ability of PNs to register each odor pulse of a series of pulses with a discrete burst of action potentials. In most cases, a stimulus pulse was scored as "tracked" if the evoked burst comprised at least five action potentials and was separated from the next burst by a period of inactivity of at least 50 ms, which corresponded to the inter-stimulus interval. Alternatively, in cases where PNs showed a higher level of spontaneous activity, the instantaneous spike frequency (ISF) was calculated for each spike train. A given stimulus pulse was scored as "tracked" if (a) the ISF showed a two-fold increase in response to the odor pulse and (b) there was a clear decrease in ISF before the next odor pulse (<50% of the maximum ISF observed during the previous pulse). The "tracking index" thus ranged from 1 to 5 and was defined as the number of pulses tracked in response to the 5-pulse stimulus train. Essentially identical results were obtained with both methods.

## 9.7 Olfactory processing

Changes in stimulus intensity can modulate the patterns of glomerular activity and output from the olfactory bulb and AL as has been shown for olfactory systems of vertebrates and invertebrates alike (Anton et al., 1997; Hildebrand and Shepherd, 1997; Christensen and White, 2000; Keller and Vosshall, 2003; Leon and Johnson, 2003; Sachse and Galizia, 2003; Stopfer et al., 2003). Until recently, little was known about how the responses of uniglomerular PNs can be influenced by specific stimulus blends (Wu et al., 1996). Below, we present direct evidence for PNs in the AL of *M. sexta* that the temporal responses of some PNs are optimized for a particular ratio of stimulus compounds in a blend and that altering this ratio dramatically changes the central representation of the blend at the first stage of processing in the brain.

### 9.7.1 MGC-PNs have different dynamic ranges and response thresholds

When we changed the intensity of the pheromone stimulus blend, we observed pronounced effects on the strength of the responses (IPSPs, EPSPs, number of impulses) in two major types of MGC-PNs: the BAL-selective (*BAL<sup>+</sup> PNs*) and C15-selective cells (*C15<sup>+</sup> PNs*) (Fig. 12). However, not all of these neurons exhibited classical concentration-dependent responses in the range of concentrations used in this study. A subset of *BAL<sup>+</sup> PNs* innervating the T1 glomerulus and *C15<sup>+</sup> PNs* innervating the cumulus were extremely sensitive at all concentrations tested (Fig. 12, left panel), while others with similar morphology failed to respond except at the highest stimulus concentration (Fig. 12, right panel). Such functional diversity is not an exclusive property of the male MGC as shown in a study in female *M. sexta* (Reisenman et al., 2004). Similar diversity exists in female *M. sexta* among PNs that innervate a single glomerulus and respond selectively to one enantiomer of linalool, a common plant volatile. The results present us with evidence from both male and female moths to clearly demonstrate functional heterogeneity with respect to threshold sensitivity and concentration-response characteristics among PNs innervating a single glomerulus.

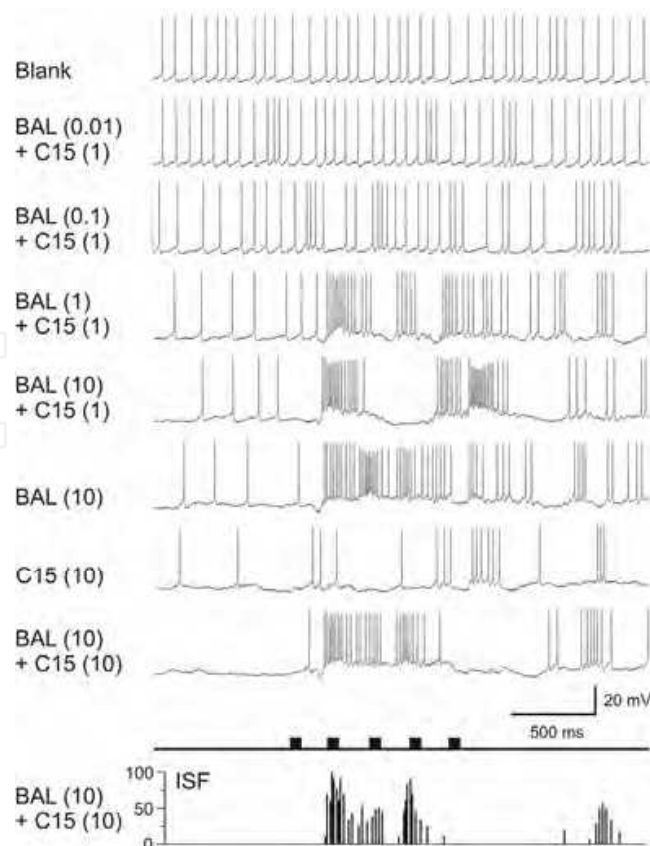
The observed diversity in the PN population of a single glomerulus could reflect distinct functional roles for different PNs under diverse environmental conditions, as previously proposed (Christensen et al., 2000). In this scenario, PNs that are little affected by a change in pheromone concentration might signal the presence of the pheromone without regard to its intensity, whereas the PNs with higher response thresholds, recruited into the coding



ensemble only at relatively high pheromone concentrations, might therefore function in source location (Murlis, 1997).

Concentration changes may be encoded in two distinct ways by PNs: (1) by individual neurons that give incremental responses to increasing concentrations and/or (2) by recruitment of different populations of PNs at different concentrations. Our data presented evidence for such a dual coding strategy in MGC-PNs (*BAL*<sup>+</sup> PNs and *C15*<sup>+</sup> PNs) in that some displayed a monotonic response across all concentrations, whereas others showed concentration-dependent responses over several orders of magnitude of stimulus concentration. The representation of sex-pheromonal information in the AL of *M. sexta* is sparse at low stimulus intensities because only a subset of MGC-PNs is active under these conditions, whereas the representation becomes increasingly combinatorial and complex as pheromone concentration increases because it involves a greater number and functional variety of types of MGC-PNs. This is similar to the neural coding of general olfactory stimuli in AL glomeruli in fruit flies and honey bees (Galizia et al., 1999, Ng et al., 2002; Wang et al., 2003).

A large number of pheromone-responsive afferent neurons converges onto many fewer central neurons. Therefore, threshold values are expected to be lower for MGC-PNs than for ORCs. Kaissling et al. (1989) determined a 1-ng threshold stimulus load in the stimulus-delivery cartridge for *BAL*-specific ORCs and a 100-ng load for *C15*-specific ORCs for *M. sexta*. The threshold stimulus loads found in our experiments are 0.01 ng for *BAL*<sup>+</sup> PNs and 0.1 ng for *C15*<sup>+</sup> PNs. These >100-fold lower corresponding thresholds of MGC-PNs are thought to overcome the critical signal-to-noise ratio, when pheromone molecules activate only a few ORCs. Similar response threshold relationships between ORCs and AL neurons have been observed in other insect species (Boeckh, 1984).



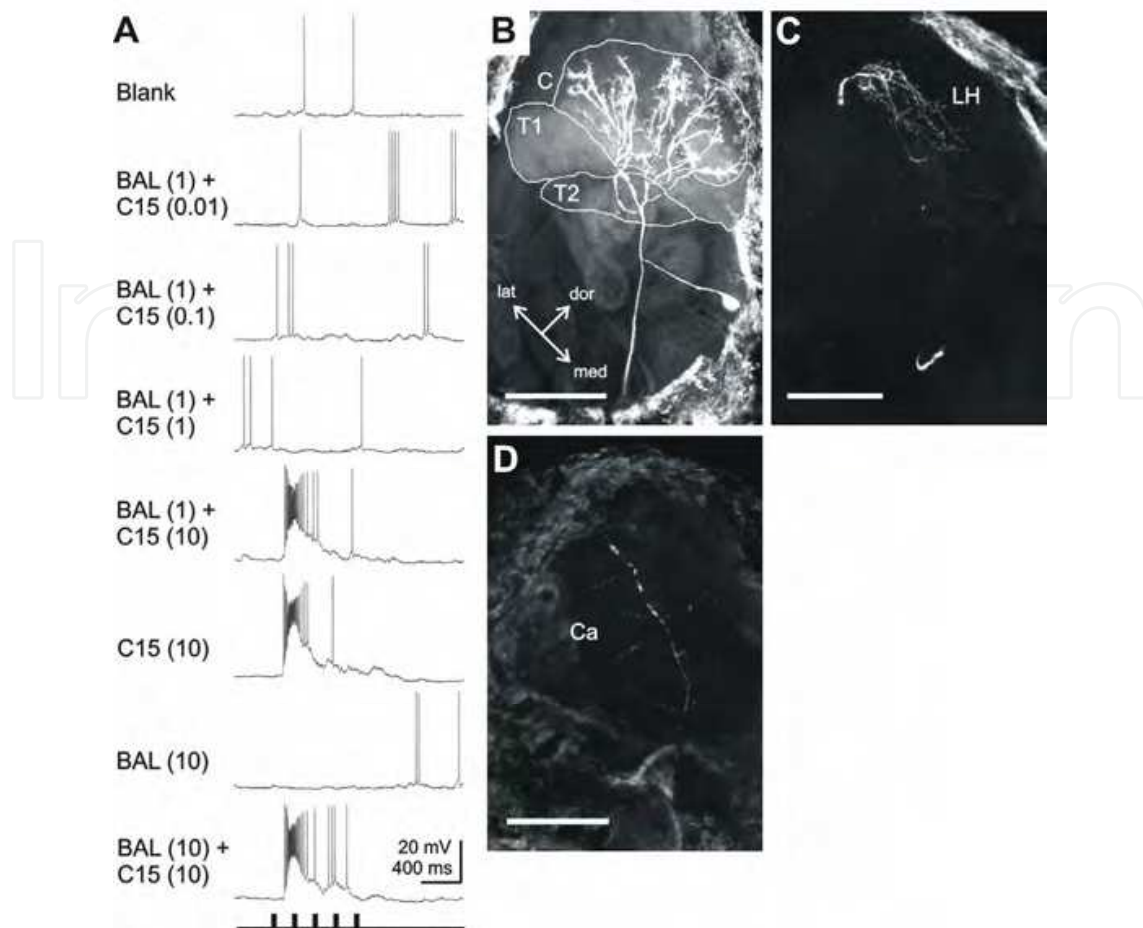


Fig. 12. Panel on previous page: Intracellular recordings from male *Manduca sexta* showing one class of  $BAL^+$  PN that was exclusively responsive to BAL and thus classified as a  $BAL^+$ -specialist PN. This type of PN responded to BAL in a dose-dependent manner but is unresponsive to C15, and thus did not show a blend effect. Small BAL stimulus loads evoked only a membrane depolarization (EPSP) accompanied by spiking, but elevated dosages also triggered a brief IPSP preceding the EPSP. In each case, five identical stimulus pulses were delivered to the ipsilateral antenna at a frequency of  $5 \text{ sec}^{-1}$  (stimulus markers for the 50-ms pulses are shown beneath the records). A plot of instantaneous spike frequency (ISF) calculated from the last record illustrates that the firing dynamics of this type of MGC-PN could not accurately track all pulses of a stimulus train, thus leading to a low tracking index as defined in the text. Panels on this page: (A-D): Olfactory responses and morphology (frontal view) from one class of  $C15^+$  PN of male *M. sexta* that was solely responsive to C15 ( $C15^+$ -specialist PN), and therefore did not exhibit a blend effect. (A) This neuron was strongly depolarized by the first pulse of any stimulus that contained 10 ng of C15, but it did not respond to lower stimulus intensities. The neuron showed no clear response to BAL even at the elevated 10-ng load. In each trace, the ipsilateral antenna received five 50-ms stimulus pulses at  $5 \text{ sec}^{-1}$  (stimulus markers are shown beneath the records). (B) The branches of this PN were confined to the cumulus (C) of the MGC. *dor*, dorsal; *lat*, lateral; *med*, medial; *T1*, toroid 1; *T2*, toroid 2. (C) Frontal view of the axon's ramifications in the lateral horn (LH) of the protocerebrum. (D) Collateral branches innervating the calyces (Ca) of the mushroom body in the posterior protocerebrum. Scale bar =  $100 \mu\text{m}$ . From Heinbockel et al., 2004.

### 9.7.2 Effects of blend ratio on temporal response dynamics in PNs

In subpopulations of *BAL*<sup>+</sup> PNs and *C15*<sup>+</sup> PNs, a change in the ratio of BAL and C15 clearly affected pulse tracking of these PNs (Figs. 13, 14).

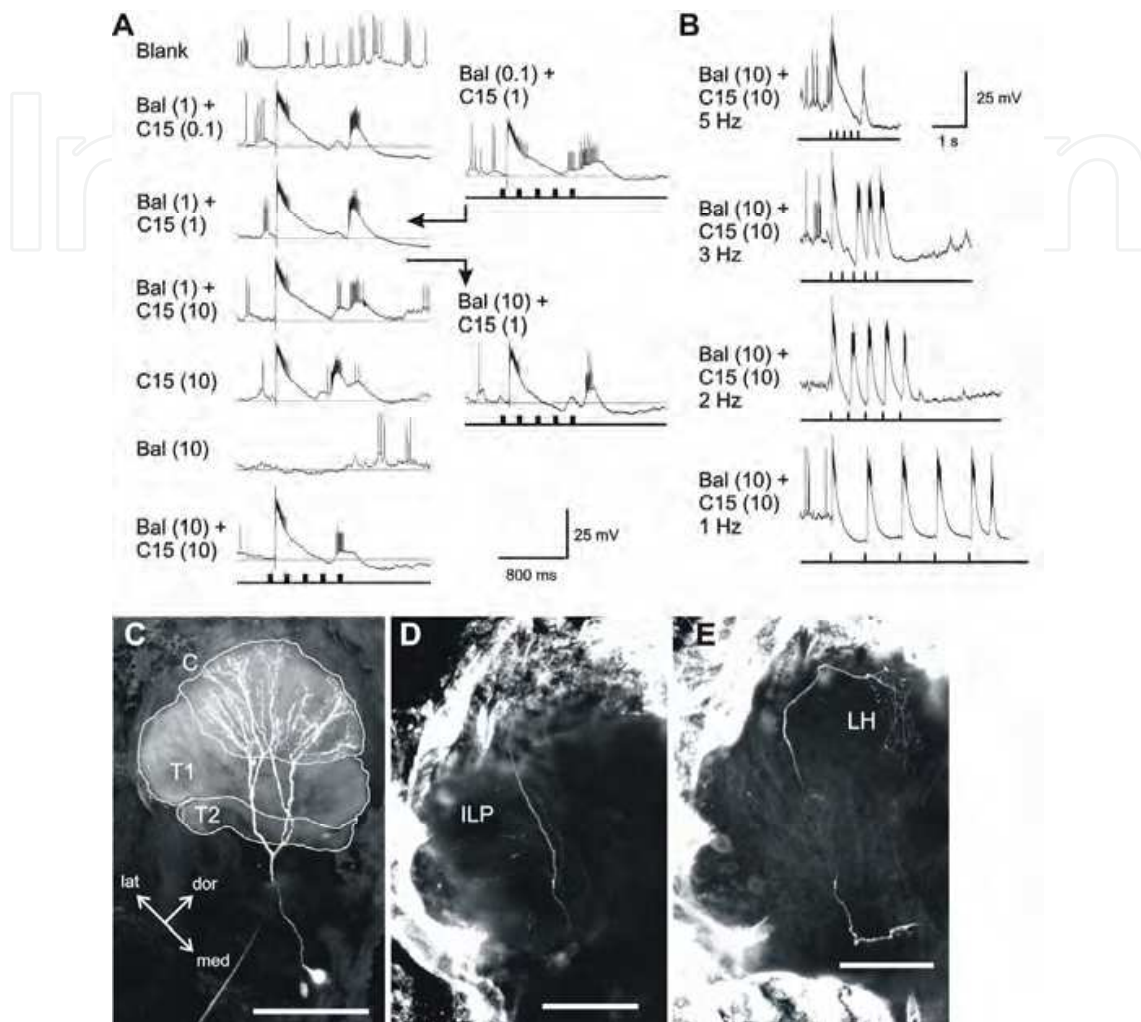


Fig. 13. Responses and anatomy of a *C15*<sup>+</sup> PN from male *Manduca sexta* that gave depolarizing responses to C15 and inhibitory responses to BAL (*C15*<sup>+</sup> / *BAL*<sup>-</sup> PN). (A) Responses from left to right are to blends containing 1 ng C15 plus increasing amounts of BAL. The ipsilateral antenna received five 50-ms stimulus pulses at 5 pulses sec<sup>-1</sup> (stimulus markers are shown beneath the records). This neuron had a low threshold for excitation and gave strong responses to stimuli containing only 0.1 ng of C15. The horizontal lines in the records indicate the membrane potential observed prior to stimulation. Notice that BAL alone hyperpolarized the PN and suppressed spiking activity (*C15*<sup>+</sup> / *BAL*<sup>-</sup> PN). (B) Responses of the same PN to different pulse rates of antennal stimulation (BAL, C15, 10 ng each) ranging from 1 to 5 sec<sup>-1</sup>. At higher frequencies, the PN was unable to repolarize sufficiently to track each of the five stimulus pulses. The tracking index improved dramatically at lower pulse frequencies. (C) The neuron in (A) branched in the cumulus and not in the toroid 1 of the MGC (frontal view). C, cumulus; do, dorsal; la, lateral; me, medial; T1, toroid; T2, toroid 2. (D) More anterior view of the ramifications of the axon in the protocerebrum. ILP, inferior lateral protocerebrum. (E) More posterior aspect of protocerebral ramifications of the axon. LH, lateral horn. Scale bar = 100 μm. From Heinbockel et al., 2004.

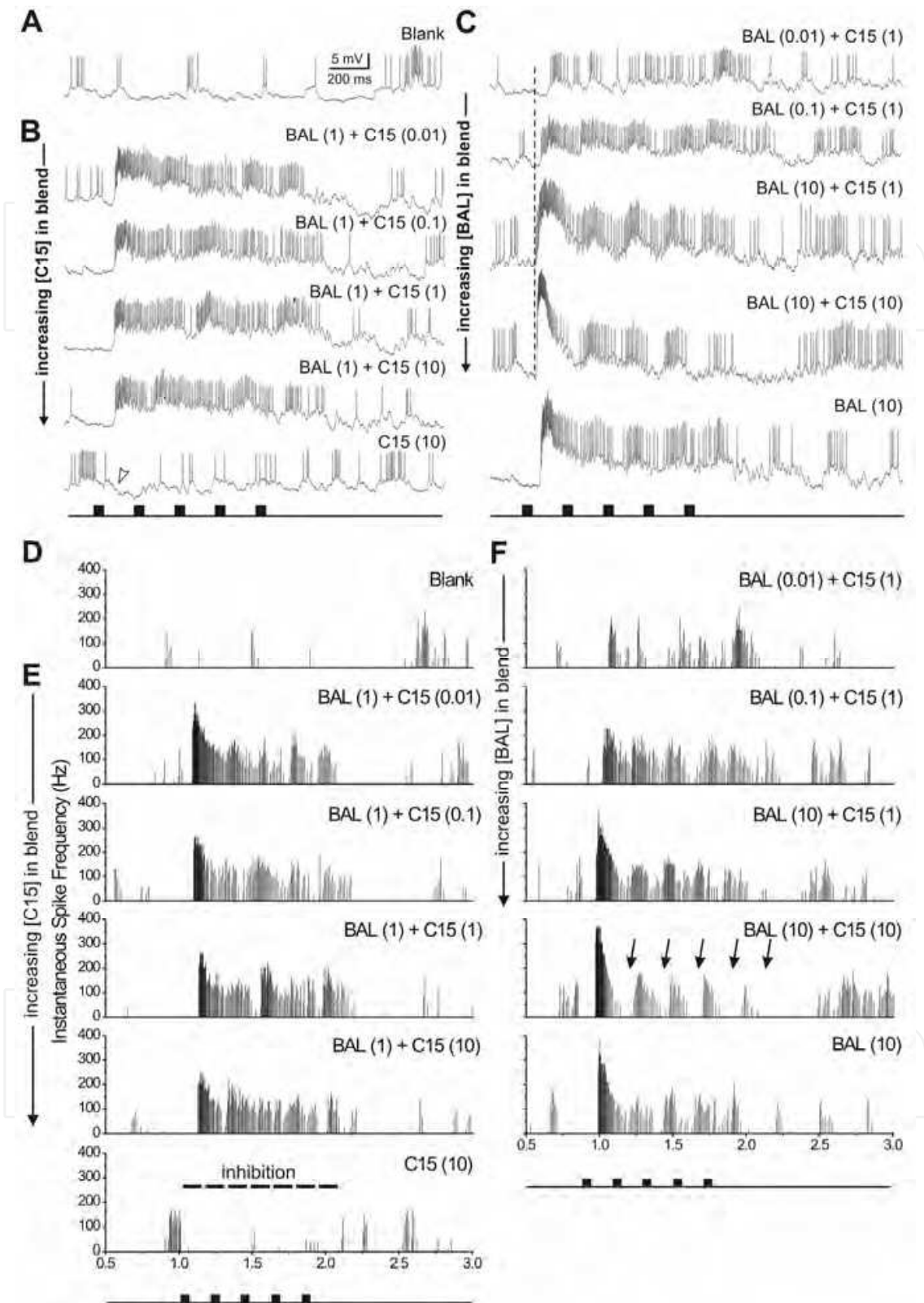


Fig. 14. Intracellular recordings and ISF plots from one *BAL*<sup>+</sup> PN from male *Manduca sexta* that gave depolarizing responses to BAL and an inhibitory response to C15 (*BAL*<sup>+</sup> / *C15*<sup>-</sup> PN). Responses to blanks are shown in (A) and (D). (B), (E) Intracellularly recorded responses and corresponding ISF plots to blends containing 1 ng BAL plus increasing

amounts of C15. Repetitive stimulus pulses were delivered to the ipsilateral antenna at a frequency of 5 pulses  $\text{sec}^{-1}$  (stimulus markers are shown beneath the records). Note the modulations of PN firing with each stimulus pulse. C15 at 10 ng led to a hyperpolarization and suppression of firing in the PN (*dashed line*). (C, F) Responses (intracellular records and ISF plots) of the same neuron to blends containing 1 ng C15 plus increasing amounts of BAL. The tracking index of this PN improved at a ratio of 10 ng BAL to 1 ng C15, but was optimized at a 1:1 ratio with 10 ng of each component. Note the distinct periods of inactivity (*arrows*) between spike bursts evoked by consecutive stimulus pulses. The concentration-dependency of this neuron was also indicated by a marked reduction in response latency as the amount of BAL in the blend was increased. From Heinbockel et al., 2004.

These MGC-PNs integrated convergent excitatory and inhibitory afferent input and better resolved intermittent olfactory signals. This processing function was optimized at particular blend ratios for different PNs.  $C15^+ / BAL^-$  PNs and, particularly,  $BAL^+ / C15^-$  PNs were best suited to convey the temporal structure of the stimulus to higher brain centers, whereas responses of  $BAL^+$ - and  $C15^-$ -specialist PNs often lacked the temporal precision to represent rapid changes in pheromone concentration. These results show that information about individual components and stimulus timing is transmitted from the MGC in different output channels.

We also observed blend effects in  $BAL^+ / C15^+$  PNs and the  $C15^+ / BAL^+$  PNs (Fig. 15). These blend effects were clearly ratio-dependent. These MGC-PNs are excited by stimulation with either of the two key pheromone components and, therefore, they are considered to be pheromone “generalists” (Christensen and Hildebrand, 1987; 1990; Christensen et al., 1996).

Principally, the responses of these MGC-PNs to stimulation with either pheromone component were primarily excitatory. However, the cells often received different amounts of excitatory and inhibitory input when stimulated with blends of the two components. Several PNs responded with a strong, long-lasting excitatory response to stimulation with one component and a less-intense and brief response to stimulation with the other component. Stimulation with the blend evoked a “mixed” response of intermediate character depending on the concentrations of the components. The excitatory phase of this blend response was not simply the sum of the excitatory responses elicited by each of the two components alone but the response was typically weaker than expected from adding the two responses. MGC-PNs with temporally distinct blend responses could serve specific functions in olfactory information processing. They could function as the neural substrate in the AL for rapid behavioral changes in response to the appropriate component ratio of a pheromone blend (Heinbockel et al., 2004).

### 9.7.3 Phasis vs. tonic patterning of postsynaptic response

Both key pheromone components (represented in this study by BAL and C15) must be present to elicit upwind olfaction-modulated flight in a wind tunnel in *M. sexta* (Tumlinson et al., 1989). Individual MGC-PNs gave multiphasic responses (inhibition-excitation-afterhyperpolarization) (Figs. 12-15), which are typical of PNs in the AL of *M. sexta* (Christensen and Hildebrand, 1987; Christensen et al., 1998). However,  $BAL^+$  PNs

had larger IPSPs, smaller EPSPs, weaker responses during 200 ms after stimulus onset, and stronger responses over 1000 ms after stimulus onset than did *C15<sup>+</sup>* PNs. This suggests that BAL evokes sustained responses and C15 evokes shorter, more phasic responses. Corresponding response patterns were observed among ORCs in the antenna of *M. sexta* (Kaissling et al., 1989) such that responses of BAL-specific ORCs to antennal stimulation with BAL are typically more phasic-tonic whereas the phasic part of the response is more pronounced in C15-specific receptor cells. Different ORCs with selective responses to the same stimulus compound could nevertheless exhibit different response dynamics as has been shown for pheromone-specific ORCs of moths (Almaas et al., 1991) and ORCs in *D. melanogaster* (de Bruyne et al., 2000). It is tempting to speculate that MGC-PNs with phasic olfactory responses could be synaptic targets of phasic ORCs, whereas PNs giving tonic responses could receive input largely from ORCs that exhibit tonic firing patterns. In this scenario, at least some aspects of temporal coding and coding of stimulus concentration and mixture ratio are regulated through the activation of different populations of ORCs.

If MGC-PNs respond to pheromonal stimulation with sustained firing outlasting the stimulus duration they are likely to report the onset of stimulation rather than its termination and could serve as a substrate for more temporally complex spike codes (Heinbockel and Kloppenburg, 1999; Friedrich and Stopfer, 2001; Laurent et al., 2001; Christensen and Hildebrand, 2002). If MGC-PNs respond with a more phasic spike pattern and show better pulse-following, they could encode both the beginning and the end of each stimulus pulse. These MGC-PNs are able to convey information about rapidly changing signals and by doing so transmit information about the physical dynamics of the stimulus to higher olfactory centers in addition to cues about stimulus identity (Heinbockel et al., 1999; Vickers et al., 2001).

Since each AL is innervated by fewer than 50 MGC-PNs (Homberg et al., 1989), each MGC-PN contributes significantly to information flow from the MGC to higher order olfactory centers. Different features of the olfactory stimulus are likely to be encoded by these different outputs arising from a single MGC glomerulus, i.e., functional heterogeneity exists in the population of olfactory output channels from the MGC. In the AL of male *M. sexta*, both component-specific and blend-specific MGC-PNs exist that are affected by changes in concentration and/or blend ratio, but the performance of different PNs is optimized under different ambient conditions.

Advances in intracellular recording, staining, confocal microscopy and data analysis allowed us to characterize the responses of individual MGC-PNs and their ability to encode features of the stimulus, e.g., to follow intermittent olfactory stimuli. PNs that integrated information about the two-component pheromone blend (i.e., they received excitatory input from one component and inhibitory input from the other) were particularly well suited to track a train of stimulus pulses. Stimulus-pulse tracking was furthermore optimized at a synthetic blend ratio that mimics the physiological response to an extract of the female's pheromone gland. Our results show that optimal responsiveness of a PN to repetitive stimulus pulses depends not only on stimulus intensity but also on the relative strength of the two opposing synaptic inputs that are integrated by MGC-PNs.

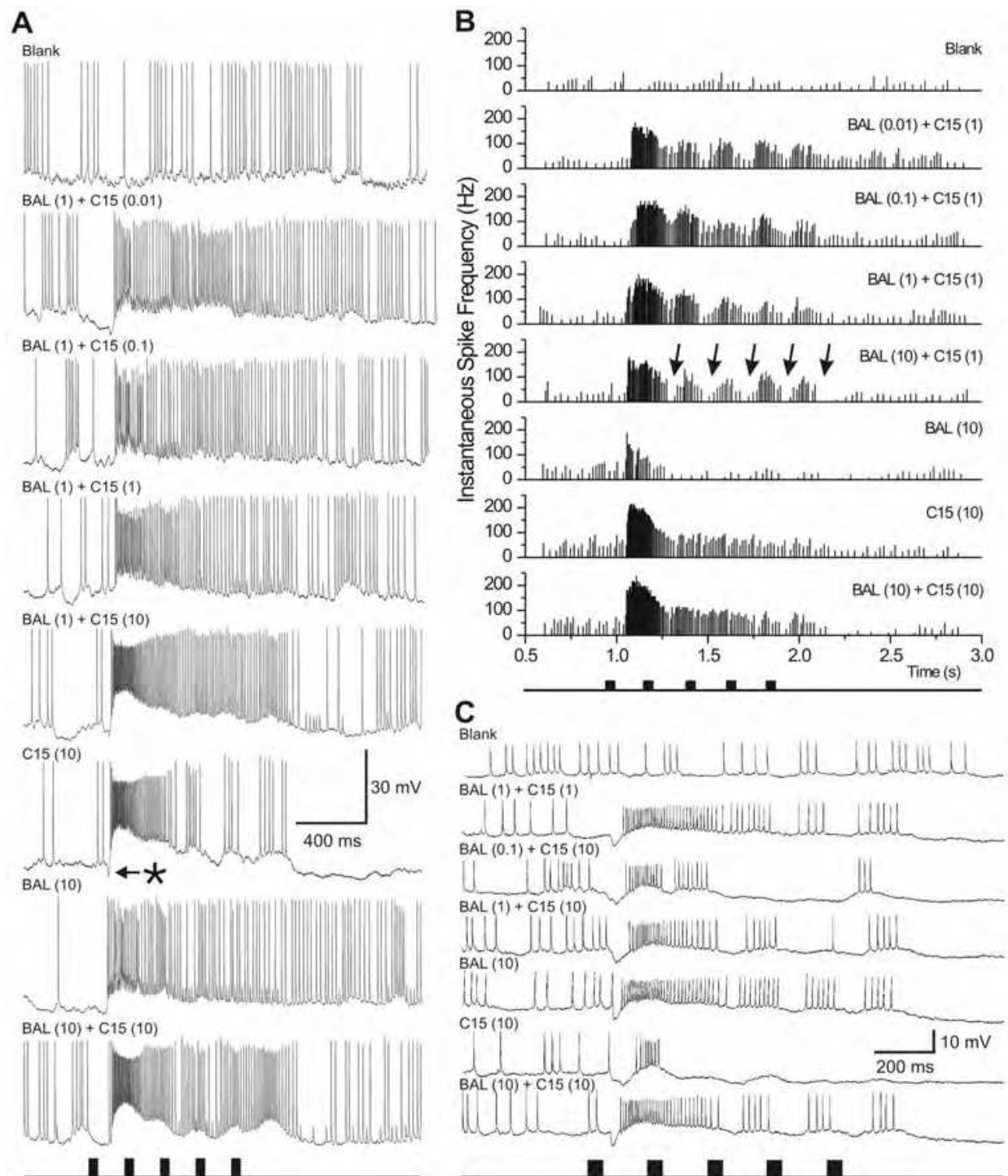


Fig. 15. Responses of  $BAL^+ / C15^+$  PNs and  $C15^+ / BAL^+$  PNs from male *Manduca sexta*. These MGC-PNs were depolarized by antennal stimulation with either BAL or C15. (A) This neuron responded with a strong response to C15 and a more sustained response to BAL ( $C15^+ / BAL^+$  PNs). Stimulation with C15 also evoked a distinct IPSP (marked by asterisk) resulting in a mixed response (inhibition / excitation / inhibition). Varying the blend ratio changed the response character to more phasic or more tonic, depending on the load in the stimulus blend. (B) ISF response plots of a  $C15^+ / BAL^+$  PN to various BAL and C15 stimulus loads and ratios revealed a sustained phasic-tonic response to C15 and a brief excitatory

response to BAL followed by inhibition. Addition of increasing amounts of BAL to the blend resulted in improved stimulus-pulse tracking. Note the distinct periods of inactivity (*arrows*) between spike bursts evoked by consecutive stimulus pulses. (C) In this neuron, stimulation with C15 evoked a brief excitatory response preceded by an IPSP and followed by a strong and prolonged inhibitory response phase. Stimulation with BAL resulted in a mixed response (inhibition / excitation / inhibition) comprising a strong IPSP and a strong excitatory response phase (*BAL<sup>+</sup>/C15<sup>+</sup> PN*). From Heinbockel et al., 2004.

## 10. Conclusions

This chapter details both extracellular and intracellular recording methods used with insect preparations in the fields of insect olfaction and/or gustation. Using one or both olfactory recording techniques contributes to a better understanding of (i) how and what olfactory information is processed in the insect brain, (ii) the chemical identification of important plant volatiles for insect-plant interactions, and (iii) which components of the female sex pheromone, as well as plant-associated volatiles, play important roles in male and female moth orientation, respectively. Complementing these recording techniques with the use of fluorescent markers allows axonal projections to be traced to the brain or central neurons to be labeled individually. Gustatory extracellular recording methods can ultimately (i) lead to a clearer understanding of the importance of gustatory cues involved in larval host-plant interactions, (ii) give us a better perception on how taste stimuli code for different behavioral responses, and (iii) provide ideas and strategies for crop protection from insect predation. Overall, either one or both of these methods (i.e., extracellular or intracellular) can contribute to an increased understanding of how and what chemosensory information is processed in the insect brain and shed more light on how nervous systems recognize, analyze, and respond to complex sensory information.

## 11. Acknowledgments

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## **Zoology**

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The present book is not a classical manual on Zoology and the reader should not expect to find the usual treatment of animal groups. As a consequence, some people may feel disappointed when consulting the index, mainly if searching for something that is considered standard. But the reader, if interested in Zoology, should not be disappointed when trying to find novelties on different topics that will help to improve the knowledge on animals. This book is a compendium of contributions to some of the many different topics related to the knowledge of animals. Individual chapters represent recent contributions to Zoology illustrating the diversity of research conducted in this discipline and providing new data to be considered in future overall publications.

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