Mechanisms of gustatory coding in Spodoptera littoralis

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THÈSE

pour obtenir le grade de

Docteur

de

l'Institut des Sciences et Industries du Vivant et de l'Environnement (Agro Paris Tech)

présentée et soutenue publiquement
par

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Le 2 décembre 2008

Mechanisms of gustatory coding in *Spodoptera littoralis*

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English summary

Taste is one of the fundamental senses by which animals can detect food sources (sugars, salts, lipids, amino acids) but also noxious compounds dissolved in aqueous solution or adsorbed on surfaces (leaf, cuticle). Unlike olfaction, where only cephalic organs are involved in the detection of volatile compounds, in insects, gustatory sensilla are located on different parts of the insect body (mouthparts, legs, wings, ovipositor) which results in the precise spatial location of the stimuli which excite them. These sensilla are involved in different behaviours and might therefore be tuned to different types of contact chemosensory stimuli. These functional constraints imply a different organisation of the nervous centres processing the information received from gustatory receptor neurons. Whereas projections from olfactory receptor neurons are clearly chemotopic, comparatively little is known on how gustatory neurons project to the central nervous system and how signals are encoded and processed by central neurons. In different insect species, including Lepidoptera, responses of gustatory receptor neurons situated on the tarsae and the abdomen have been described. However, physiological characteristics of antennal gustatory sensilla and the behavioural context in which they are used are only starting to be investigated.

The objectives of this thesis were to study the gustatory neurons of contact chemosensory sensilla present on the antennae of adult *Spodoptera littoralis* using two different approaches: an electrophysiological approach of testing soluble chemicals and recording the firing pattern of these neurons; and a neuroanatomical approach of staining their pathways and target regions in the brain.

Our electrophysiological observations show that taste sensilla possess neurons that respond to sugars like sucrose, fructose and glucose and to NaCl. We could not identify a gustatory receptor neuron responding to bitter compounds or amino acids, but the range of tested substances was limited and nothing is known on the behavioural significance of such compounds. We were able to test the sensitivity along the antenna of the sensilla located on the lateral side of the antenna but no differences were noticed. However, sensilla in males and females differed in sensitivity. In females, the intensity of responses was found to be weaker for the sensilla on the dorsal side of the antennae than for those on the ventral side.

Antennation is a behaviour frequently described before mating or egg laying. The precise role of contact chemoreceptors in this kind of behaviours is however, unknown. For a conclusive interpretation of our data on the neuronal coding and central representation of taste

information from the antennae, the involvement of antennal gustatory receptors in mating behaviour, host-plant detection and oviposition and their possible interactions with olfactory receptor neurons remains to be investigated.

A scanning electron microscopic study showed no sexual dimorphism in the distribution of taste sensilla on the antennae. Mass fills of antennal afferents and backfills of individual contact chemoreceptive sensilla using Neurobiotin revealed 4 distinct projection areas of antennal gustatory sensilla. Two areas are within the deutocerebrum: the antennal motor and mechanosensory centre (AMMC) and a region situated posteriorly to the antennal lobes. The two other areas are in the tritocerebrum/suboesophageal ganglion complex. As our electrophysiological investigations showed that different neurons in the same sensillum respond to different stimuli, including mechanical stimuli for one of the neurons, it can be hypothesized that the projection areas are functionally distinct. No evidence for somatotopy of sensillar afferents originating from different parts of the antenna was found, with the methods used. A more detailed analysis of branching patterns within each target zone might reveal some form of somatotopy, however.

Résumé en français

La gustation est un sens essentiel à tous les animaux, leur permettant de détecter aussi bien des substances à valeur alimentaire (sucres, sels, lipides, acides aminés) que des substances potentiellement toxiques dissoutes en solution aqueuse ou adsorbées sur des surfaces (feuille, cuticule). Contrairement à l'olfaction qui détecte des substances volatiles diffusées dans l'air ambiant, la gustation est étroitement liée à une localisation spatiale des stimuli, impliquant un positionnement très précis de l'organe sensitif. Ces contraintes fonctionnelles impliquent une structuration complètement différente des centres nerveux traitant les informations issues de ces récepteurs chimiques. Dans le cas de l'olfaction, les projections des récepteurs olfactifs sont clairement chimiotopiques, les afférences olfactives se regroupant sur des zones de convergence en fonction des récepteurs membranaires exprimés dans les neurones olfactifs. Dans le cas de la gustation, les projections des récepteurs gustatifs seraient étroitement associées aux projections des mécanorécepteurs, selon une organisation somatotopique.

Par rapport au système olfactif, les connaissances sur le fonctionnement du système nerveux gustatif chez les insectes sont restées en retrait, essentiellement à cause des difficultés inhérentes à la caractérisation des projections et à la difficulté de déterminer si à la somatotopie se superpose une chimiotopie.

Nous avons étudie le système gustatif associé aux antennes de lépidoptères, en prenant pour modèle la noctuelle du coton *Spodoptera littoralis*. Les récepteurs gustatifs des antennes sont impliqués dans différents comportements, comme l'ont montré notamment des protocoles d'apprentissage associatif. Cet organe est remarquable chez les lépidoptères car il est dépourvu de muscles (à l'exception de la base) et caractérisé par la duplication de segments homologues (les segments antennaires) portant une distribution d'organes olfactifs et gustatifs identique de segment à segment, avec néanmoins des différences progressives en allant de la base vers l'extrémité.

Nous avons abordé d'abord l'aspect fonctionnel des neurones en caractérisant par des techniques électrophysiologiques le spectre de réponse de ces neurones gustatifs à l'aide de stimuli simples (sucres, sels, acides aminés, composés amers).

Dans une deuxième phase du travail, la structuration du système antennaire a été abordée par des marquages cellulaires à la neurobiotine de sensilles gustatives individuelles. Ce travail a été précédé par une cartographie précise de la localisation des sensilles gustatives à la

surface de l'antenne, par microscopie électronique à balayage. La caractérisation des projections a nécessité l'utilisation d'outils de reconstruction 3D de manière à pouvoir estimer la constance des projections issues de récepteurs homologues entre différents insectes et à estimer la distribution spatiale des projections issues de neurones différents chez le même insecte.

L'antenne de l'espèce *S. littoralis* est constituée de 3 parties : le scape, le pédicelle et le flagelle. Seul le flagelle, qui est constitué d'environ 70 segments, porte des sensilles gustatives. Chaque segment a une partie ventrale et une partie dorsale. Au niveau de la partie ventrale on trouve 4 sensilles gustatives: 2 latérales et 2 médiales. Les premiers segments situés à la base de l'antenne sont dépourvus de sensilles médiales. On commence à les observer à partir des $6^{ème}$-$7^{ème}$ segments à la base de l'antenne. Au niveau du dernier segment situé à l'extrémité, on observe une couronne de sensilles gustatives (6 ou 7). La partie dorsale de l'antenne est couverte d'écailles, mais on peut observer 2 sensilles gustatives médiales au niveau de chaque segment sauf pour les segments situés à la base de l'antenne. Chaque sensille contient 4 neurones gustatifs et un mécanorécepteur.

Les males et les femelles présentent des neurones gustatifs qui sont stimulés par les sucres et le sel. Il n'y a pas de différences de sensibilité au long de l'antenne. Chez les femelles, les sensilles gustatives présentes sur la partie dorsale de l'antenne répondent de façon plus faible aux stimuli que celles présentes sur la partie ventrale. Les réponses sont généralement plus faibles chez les mâles que chez les femelles.

Les marquages neuronaux nous ont permis d'identifier 4 zones de projection. Deux zones sont dans le deutocérébron: le centre moteur et mécanosensoriel de l'antenne (CMMA) et une zone située postérieurement par rapport aux lobes antennaires. Les deux autres zones sont dans le complexe tritocérébron/ganglion sous-oesophagien. La zone de projection deutocérébrale postérieure aux lobes antennaires n'a jamais été décrite auparavant**.**

Etant donné qu'une sensille gustative contient plusieurs neurones, dont un mécanorécepteur, et que chacun de ces neurones répond à des substances différentes, on présume que les zones de projection décrites présentent des différences fonctionnelles. Les méthodes d'analyse utilisées ne nous ont pas permis de déceler de projections somatotopiques des neurones provenant des sensilles gustatives situées a des endroits différents sur l'antenne. Cependant, on ne peut pas exclure qu'une analyse plus fine de ces projections pourrait révéler une forme de somatotopie.

L'antennation est un comportement fréquent avant l'accouplement ou la ponte ; le rôle des récepteurs gustatifs dans ce type de comportement reste cependant mal connu. Pour une meilleure interprétation de nos résultats sur le codage neuronal et les projections au niveau du système nerveux central des sensilles gustatives situées au niveau des antennes, il faudra étudier plus précisément le rôle des antennes dans l'accouplement, la détection de la plante hôte et la ponte.

Acknowledgements

I would like to warmly thank all the persons who participated in the achievement of this work.

My sincere thanks go to Prof. Frédéric Marion-Poll, for giving me the chance to conduct my PhD theses and for his encouragements.

I am grateful to Dr. Sylvia Anton for coaching me in my work as well as for supporting me in the difficult moments.

I would like to warmly thank Dr. Jean-Pierre Rospars for all the rich discussions that we had during my thesis.

My thanks go also to the members of my jury who kindly accepted to judge my work.

I thank the lab members of PISC at INRA Versailles for their collaboration and their kindness: Michel Renou, Philippe Lucas, Christophe Gadenne, Didier Rochat, Emanuelle Jacquin Joly, Dominique Von Hort, Philippe Touton.

I am very thankful to all my friends from INRA: Delphine, Makoto, Romina, Christophe, Cyril, Marie Jeanne, Katie, Ali, Seb, Ene, David, Louise, Isabelle, Shereen and Antoine.

I would like to thank Prof. Tor Jorgen Almaas for his help with the staining technique and the tungsten recordings and also for being so cheerful and friendly. I would also like to thank all the members of Prof. Hanna Mustaparta's lab for their kindness during my stay in Norway!

I would like to express my acknowledgement to the scientific director from AgroParisTech, Francoise Launay for her support during these years!

Finally, I thank my family and friends who encouraged me all this time!

Abbreviations

OBP	Odorant binding proteins
Gr	Gustatory receptor
Or	Olfactory receptor
GRN (ORN)	Gustatory (Olfactory) receptor neuron
CNS	Central Nervous system
AL	Antennal lobe
AMMC	Antennal motor and mechanosensory center
SOG	Subesophageal ganglion
GFP	Green fluorescent protein
ATP	Adenosine triphosphate
IP3	Inositol triphosphate

Contents

CHAPTER 1
THE INSECT CHEMOSENSORY SYSTEM

Taste is one of the fundamental senses an insect uses to monitor chemicals present in the environment through contact chemoreceptor cells. The information received by the gustatory receptor cells together with olfactory, tactile and visual cues allows insects to find food sources, mates and oviposition sites and to avoid noxious compounds or predators. For herbivorous insects, taste plays a crucial role in assessing a host plant for egg-laying or feeding, together with other senses as exemplified below.

Host plant selection in insects

One of the major tasks for herbivore insects is to find a proper host plant. Host finding is a process that may be categorized into host-habitat location, host selection and host acceptance (Ramaswamy, 1988). Host location and host selection in phytophagous insects consists of a sequence of behavioural responses to an array of stimuli associated with host and non-host plants (Backman, 1997; Pouzat, 1980; Renwick, 1989; Renwick and Chew, 1994; Visser, 1986).(Fig 1)

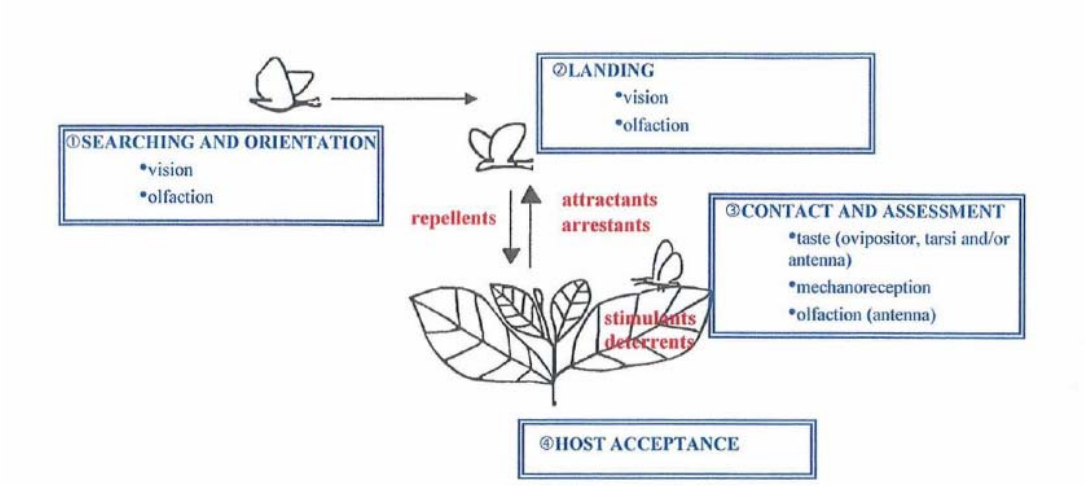

Figure 1. Behavioural steps of the host finding process and the chemical cues leading to oviposition (modified after Renwick, 1989 by Ingwild Masante-Roca (2004).

Insects detect physical and chemical signals emitted by plants in their environment and perform elaborate behaviours in order to select plants which can support their development

and reproduction. The first pieces of information collected remotely during the searching and orientation step are provided by olfactory and visual cues (Fig 1.1). The volatiles involved in the orientation towards host plants can be grouped into two different categories according to their effects on insects: attractants and repellents.

Host-acceptance is defined by an encounter with the odorant source followed by landing (Fig 1.2). This step involves a contact between the insect and a plant, which permits contact receptors of the insect to detect chemicals on the surface of their substrate (Fig 1.3). The detected chemicals involve a surface evaluation and the acceptance of the site if the stimulus is an attractant (Backman, 1997; Renwick, 1989) (Fig 1.4). The chemicals allowing a short range orientation (and oviposition) on the host-plant act as attractants and lead to oviposition or as deterrents if the plant is not suitable for oviposition.

Chemical detection and integration

Chemical detection at the antennal level

Each animal has a plethora of peripheral sensors that enable the detection of different sensory stimuli, including light, chemicals, sound and vibration, temperature, and humidity. Multiple sensors offer many functional advantages to an animal's ability to perceive and respond to environmental signals. Advantages include extending the ability to detect and determine the spatial distribution of stimuli, improving the range and accuracy of discrimination among stimuli of different types and intensities, increasing behavioural sensitivity to stimuli, ensuring continued sensory capabilities when the probability of damage or other loss of function to some sensors is high, maintaining sensory function over the entire sensory surface during development and growth, and increasing the richness of behavioural output to sensory stimulation (Derby and Steullet, 2001).

In insects, taste receptors are found on different parts of the body (like tarsi, mouthparts, antenna, ovipositor and wings) while olfactory receptors are grouped on the head of the insect, especially on antennae. The antennae (Fig 2) are appendages carrying non-visual sense organs, though in rare instances, they have become adapted for other purposes such as seizing prey items (*i.e.* the larva of the fly *Chaoborus sp.*) or holding females during mating (*i.e.* the males of the beetle *Meloe sp.*) (Duhr, 1955; Lückmann, 2005)

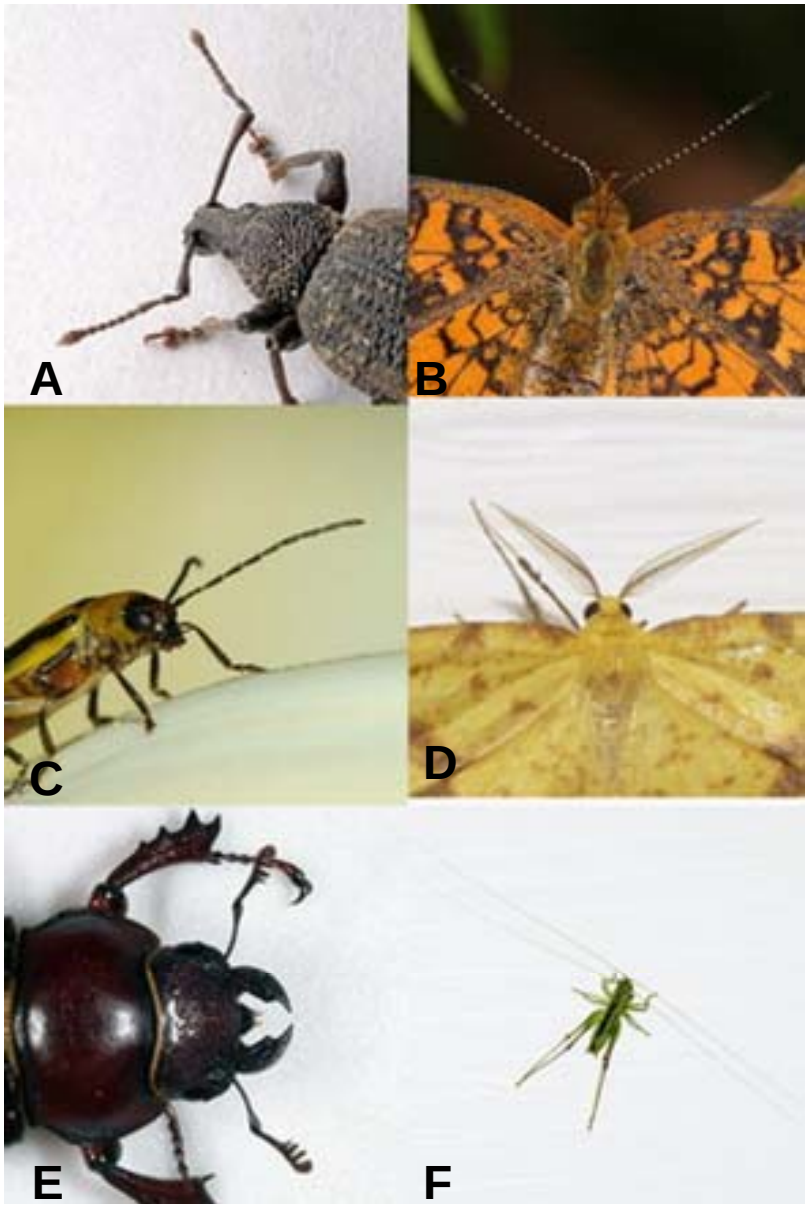

Figure 2. Different types of antennae (Chris Huh, http://commons.wikimedia.org) A. elbowed antennae; B clubbed antennae; C. serrate antennae; D. feathery antennae; E. lamellate antennae; F. threadlike antennae.

Antennae come in a wide variety of shapes and sizes (Fig. 2). The first segment is known as the scape, the second segment as the pedicel and the remaining part as the flagellum. In species using sex pheromone communication, a sexual dimorphism in antennal structure is often observed. In order to detect low concentrations of the female sex pheromone, males of many Lepidoptera species present highly branched antennae (Fig. 2bis). The greater the surface area of the antennae, the more they are able to capture highly distributed odour

molecules in space. Thus male insects with large, highly branched antennae, equipped with large numbers of pheromone-sensitive receptor neurons, are far more sensitive than the filiform antennae of crickets and cockroaches, thus allowing detection of female pheromone over large distances.

Figure 2bis. Highly branched antennae in a male moth

Chemosensory neurons are housed in a hair-like structure called a sensillum. Different types of sensilla have been described: *s. styloconica, s. chaetica, s. coeloconica, s. auricillica, s. basiconica* and *s. trichodea* (Schneider, 1964). Ulterior classifications are based on the structure of the cuticular wall (Altner and Prillinger, 1980). Each type has structural characteristics related to its function (Fig 3). Olfactory sensilla have cuticular wall with multiple pores through which odour molecules can enter; they generally contain the dendrites of one to five olfactory receptor neurons. The taste sensilla are uniporous and usually contain 2-4 chemosensory and 1 mechanosensory cell. This basic pattern undergoes many variations, depending on the species and their biological adaptations. For example, olfactory sensilla in the honeybee house about 5-35 neurons (Esslen and Kaissling, 1976), while parasitic wasps (Hymenoptera) show multiporous taste sensilla on their antennae (Nunzio Isidoro, 2001).

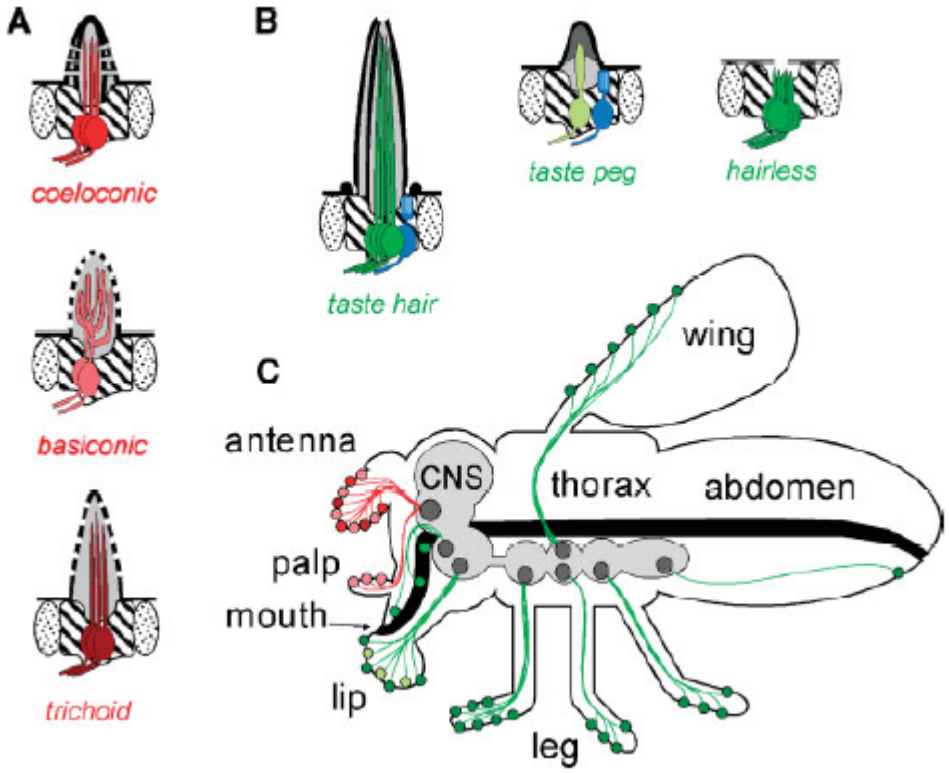

Figure 3. Location of chemosensory neurons in *Drosophila*. **A**. Three different morphologies of olfactory sensilla. In each sensilla olfactory neurons (in red) and accessory cells (hatched) are present. Pores in the cuticle allow odorants to dissolve in the sensillum lymph (grey) and reach the dendrites. **B** Two different morphologies of gustatory sensilla housing taste receptor neurons (in green) associated with a mechanoreceptor neuron (in blue). **C**. Schematic outline of the anatomy of a fly indicating the location of chemosensory sensilla (olfactory sensilla in red and gustatory sensilla in green) (de Bruyne and Warr, 2006)

Contrary to the olfactory organs which are generally grouped on the antenna and on the maxilary palps, the contact chemosensilla are distributed over the mouthparts, tarsi, ovipositor, antennae and wing margins (Boer and Hanson, 1987; Chapman, 2003; Ramaswamy, 1988; Städler and Schöni, 1991) (Fig 3). In insects that are not using their antennae to touch the substrate (Diptera), the antennae bear only olfactory sensilla, while in other insects like Lepidoptera and Coleoptera, taste sensilla are also present on the antennae.

Contact chemosensilla are commonly described as thick-walled hairs, pegs or pits where dendrites of several sensory neurons are exposed to the environment through a single opening in the cuticle. A contact chemosensory sensillum typically contains two to four chemosensory

neurons and one mechanosensory neuron attached to the hair base (Hallberg, 1981; Koh et al., 1995). The contact chemosensilla are commonly known under the term of uniporous sensilla (Fig 4) to distinguish them from the multiporous olfactory sensilla. This basic pattern varies according to the body parts and the specialisation. For example, taste sensilla located on the ovipositor of parasitoid insects and more generally in the first part of the digestive tract (pharynx), are reduced to a pore in the cuticule and are devoid of an hair shaft (Stocker, 1994).

Taste stimuli reach the dendrites through the terminal pore. The neurons respond to chemicals in solution in a similar way as taste receptors in the mouths of vertebrates. But while mammals seem unable to distinguish different chemicals within each taste category, insects seem to differentiate between a wider variety of tastants, including substances within the same category. When feeding, the sensilla on the mouthparts of an insect will often be bathed in fluid released from the food. But in many situations chemicals from dry surfaces stimulate insect contact chemoreceptors and this is probably the normal way in which these receptors operate when an insect first encounters a leaf surface (Chapman and Bernays, 1989; Städler, 2002).

It is assumed that in order to detect chemicals on a dry surface, the compound is taken up by the material surrounding the tips of the dendrites that reaches and somehow exudes from the terminal pore of the sensillum (Zacharuk, 1980). If the compounds are water soluble, they may dissolve in the material, but lipophilic compounds are probably transported by carrier proteins. Even though there is no conclusive evidence of this process, putative transport proteins have been described (Angeli et al., 1999; Nagnan-Le Meillour et al., 2000).

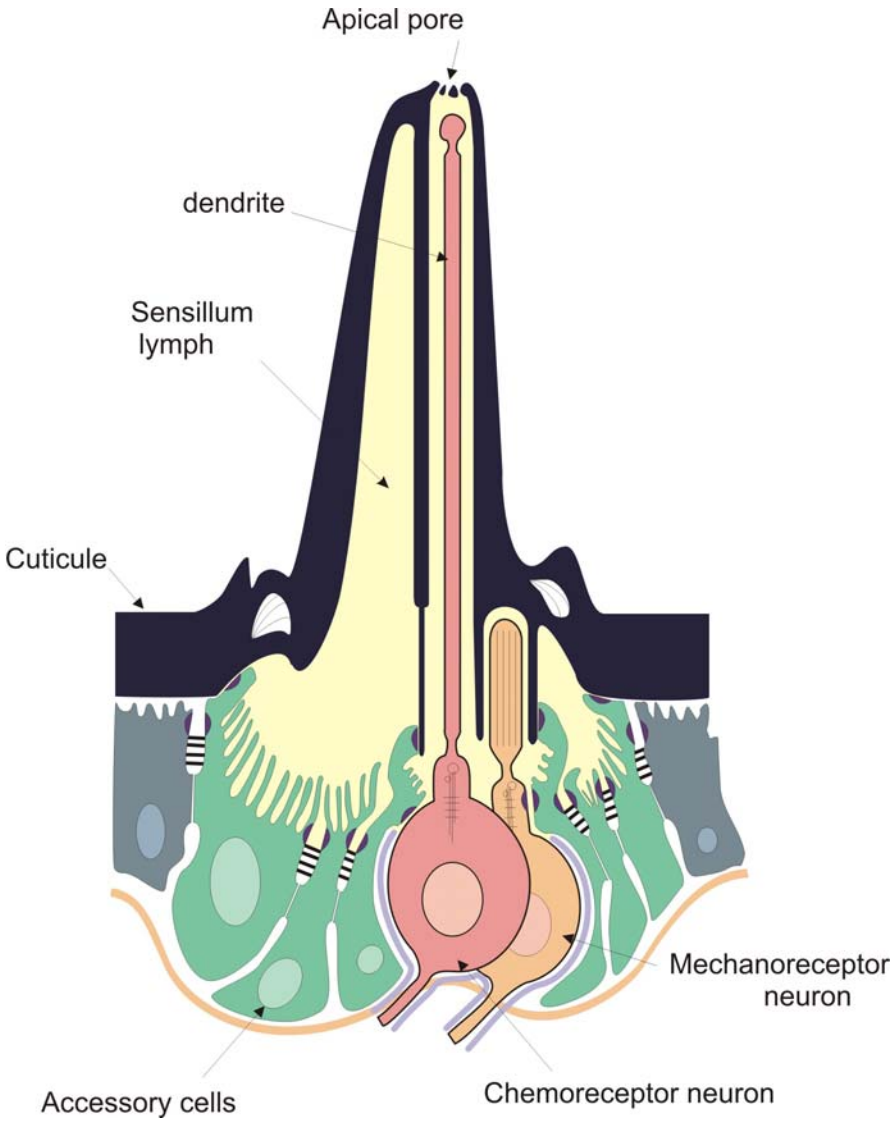

Figure 4. Taste sensillum showing only one chemoreceptor neuron and the mechanoreceptor neuron surrounded by accessory cells (Frederic Marion Poll after K. Hansen, 1978).

Olfactory sensilla (Fig 5) show a large diversification of sensillum types even in the same species. We find them as long slender hairs, pore plates or pit pegs. Their cuticular surface is perforated with wall pores, which can have different shapes and densities. In many insects the olfactory sensilla are present on the antennae and the maxillary palps. The demand for extreme sensitivity in moth pheromone communication supported the evolution of long sensilla trichodea with high efficiency of capturing odor molecules (Steinbrecht, 1996).

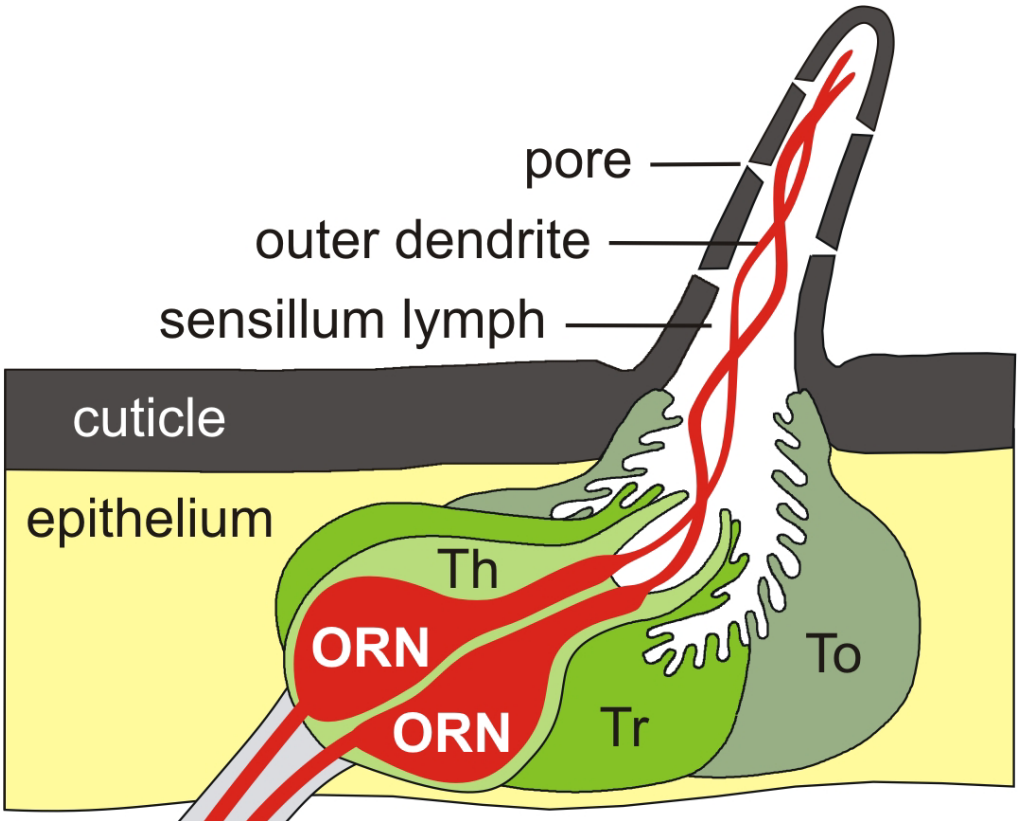

Figure 5. Olfactory sensillum with two olfactory neurons (ORN) and accessory cells (thecogen cell Th, trichogen cell Tr, tormogen cell To). Odour molecules diffuse through the cuticular pores into the sensillum lymph where they are bound to specific odorant binding proteins (OBP) and transported to receptor sites on the membrane of the olfactory neurons.(Jacquin-Joly and Lucas, 2005)

Odour molecules striking an antenna adsorb to the waxy surfaces of its sensilla and diffuse through narrow pores in their cuticular walls to their lumen (Kanaujia and Kaissling,

1985; Steinbrecht and Kasang, 1972). Because the odour substances are usually hydrophobic, special mechanisms are needed to transport the odour molecules through the sensillum lymph to receptive sites on the membrane of the olfactory receptor neurons (Steinbrecht and Kasang, 1972; Vogt, 1987). Vogt and Riddiford (1981), working with the giant silkmoth *Antheraea polyphemus*, showed that antennae of male moths contain a highly abundant 15 k-Da protein that specifically binds components of the sex pheromone released by conspecific female moths, which they named pheromone-binding protein (PBP). They found that the lymph also contains a pheromone-specific sensillar esterase, and, thus, postulated both that PBP binds pheromone molecules and transports them to the neuronal membrane and that the esterase degrades and thus inactivates the pheromone. Subsequent research has demonstrated a family of odorant-binding proteins (OBP's) in several species of moths (Gyorgi et al., 1988; Krieger et al., 1993; Krieger et al., 1991) as well as in *Drosophila melanogaster* (McKenna et al., 1994; Pikielny et al., 1994).

Identification of chemosensory receptor genes

A decade after the identification of the first odour receptors (Buck and Axel, 1991), taste receptors were identified in both mammals (Adler et al., 2000; Hoon et al., 1999) and insects (Clyne et al., 2000). Two families of gustatory receptor genes have been identified in mammals, coding for the receptors T1R and T2R (Hoon et al., 1999; Adler et al., 2000). Natural sugars and artificial sweeteners are detected by the dimer of T1R2 and T1R3, whereas the dimer of T1R1 and T1R3 detects umami (Chandrashekar et al, 2006). For the coding of bitter substances, 25 T2R receptor types are involved in humans and 35 types in mice, and multiple bitter receptors are expressed in the same gustatory cells (Adler et al., 2000).

The *D. melanogaster* gustatory receptor (*Gr*) gene family includes 68 receptors encoded by 60 genes (Clyne et al., 2000; Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001), comparable to the number of genes in the olfactory receptor (*Or*) family. These genes share no sequence similarity with the mammalian T1R or T2R receptors. Gustatory and olfactory systems in insects are very closely linked. Their receptors belong to the same family of G-protein coupled receptors. Phylogenetic analysis suggests that the *Gr* gene family is an ancient chemoreceptor family from which a branch of *Or* genes subsequently evolved (Python and Stocker, 2002; Scott et al., 2001) (fig 6). Or and Gr genes are dispersed throughout the genome and several are present in clusters of two to six genes (Robertson et al., 2003).

The Gr5a receptor in *Drosophila* is a candidate sugar receptor; genetic ablation causing behavioural taste deficits to trehalose, sucrose and glucose (Wang et al., 2004). Another receptor gene, Gr66a, which is never co-expressed with the Gr5a gene, is believed to code for a bitter receptor (Thorne et al., 2004; Wang et al., 2004). Genetic ablation of Gr66a results in behavioural taste deficits to bitter substances.

After the first identification on *D. melanogaster*, a family of 76 gustatory receptor genes (*AgGr* genes) was subsequently identified in *Anopheles gambiae* (Hill et al., 2002). Comparison of the sequences of the *DmGr* and *AgGr* genes confirms the ancient origins of the insect chemoreceptor superfamily, but also indicates that species-specific expansions have occurred within some subfamilies (Hill et al., 2002). There are seven possible orthologous pairs of *A. gambiae* and *D. melanogaster* Gr receptors, some of which are relatively well conserved – for example, DmGr21a and its mosquito ortholog AgGr22 share 68% identity.

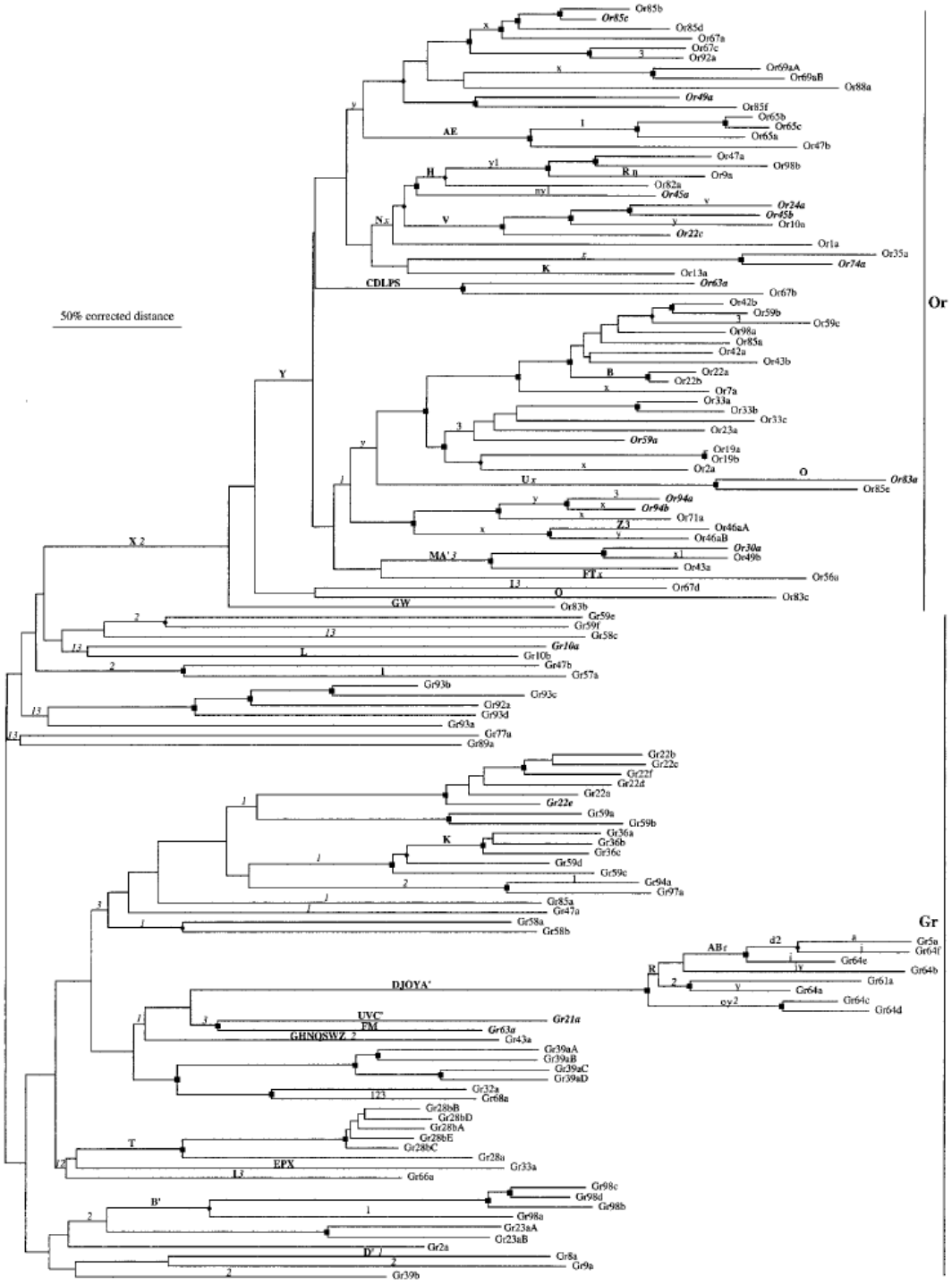

Figure 6. The phylogenetic tree of the chemoreceptor superfamily reveals deep branches connecting multiple highly divergent clades within the *Gr* family, and the *Or* family appears to be a single highly expanded lineage within the superfamily.(Robertson et al., 2003)

Transduction mechanisms

The transduction mechanisms for sweet, umami and bitter compounds have recently been elucidated in molecular biological studies in mammals. Salts and sour substances are detected by ligand-gated ion channels that open in the presence of cations. The cations pass through the channels and directly depolarise the cell membrane. The transduction pathways for bitter, sweet and umami, all seem to be G-protein coupled (Chandrashekar et al., 2006). The same phospholipase C/IP_3 second messenger pathway and cation channel (TRPM5) expressed selectively in gustatory cells seem to be involved in the transduction of all three modalities (Zhang et al., 2003). The neurotransmitter is suggested to be ATP (Finger et al., 2005).

Even if the transduction mechanisms for taste in insects are not completely elucidated, there seem to be a lot of similarities with the olfactory transduction pathway (Fig 7). Like the ORNs, GRNs are bipolar and connected to the brain without peripheral synapse. The olfactory receptor molecules are seven-transmembrane proteins, and the binding of the odorant molecule triggers a second messenger cascade. In insects, this cascade involves the formation of inositol 1,4,5,-triphosphate (IP3) that elicits an influx of calcium ions into the dendrite. The calcium influx in turn activates non-specific cation channels and causes a depolarisation of the membrane (Stengl, 1999). Recent studies indicate, however, that in addition to the second messenger pathway, membrane receptor molecules dimerized with the ubiquitous receptor Or83b might have a direct ion channel function (Sato et al., 2008; Wicher et al., 2008). If the membrane potential exceeds a certain threshold, an action potential is generated near the soma. The action potential is transmitted along the axon into the primary olfactory centre of the brain, the antennal lobes (AL). The frequency of the action potentials in a neuron is often an hyperbolic function of the concentration of the stimulus, thus coding the odour quantity.

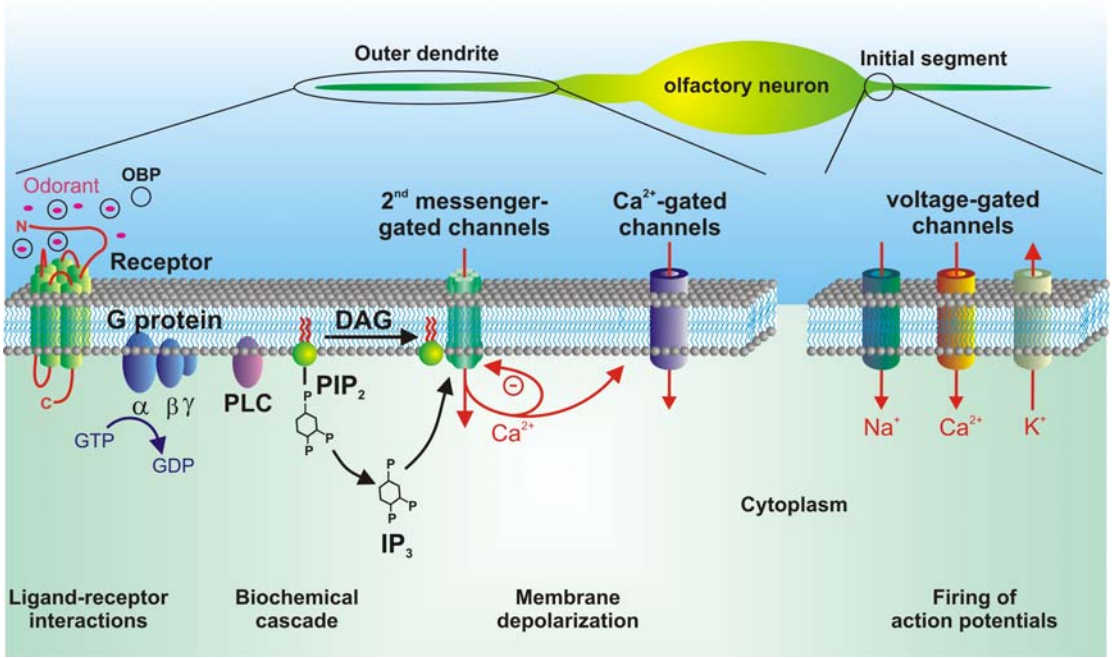

Figure 7: Putative signaling cascade involved in moth pheromone transduction. Pheromone compounds interact with pheromone binding proteines (PBPs) and bind to olfactory receptors (Ors) localised in the outer dendritic membrane. G-proteins link OR binding and phospholipase C-β (PLCβ) stimulation, generating the formation of inositol triphosphate (IP_3) and diacylglicerol (DAG). IP_3 gates Ca^{2+} channels and DAG opens Ca^{2+}-permeable cationic chanels. Both channel types are modulated by intracellular Ca^{2+}. Openings of IP_3- and DAG-gated channels result in membrane depolarisation and in an increase in intracellular Ca^{2+} concentration. Ca^{2+} opens at least 2 different types of cationic channels which amplify the initial membrane depolarisation. The resulting receptor potential passively propagates towards the initial segment where voltage-gated channels encode it in trains of action potentials (Jacquin-Joly and Lucas, 2005).

Functional characterisation of olfactory and gustatory neurons

Insect ORNs have been studied extensively by extracellular recording techniques, to examine the responses of single ORNs to odours (Kaissling et al., 1978). Extracellular recordings from olfactory sensilla in many insect species including moths, honey bees, mosquitoes and flies have revealed that different ORNs respond to different odours and that they also differ in response properties such as signaling mode (whether the response is excitatory or inhibitory) and response dynamics (Fig 8) (de Bruyne et al., 1999; Heinbockel and Kaissling, 1996; Laurent et al., 2002; Meijerink and van Loon, 1999; Shields and Hildebrand, 2000).

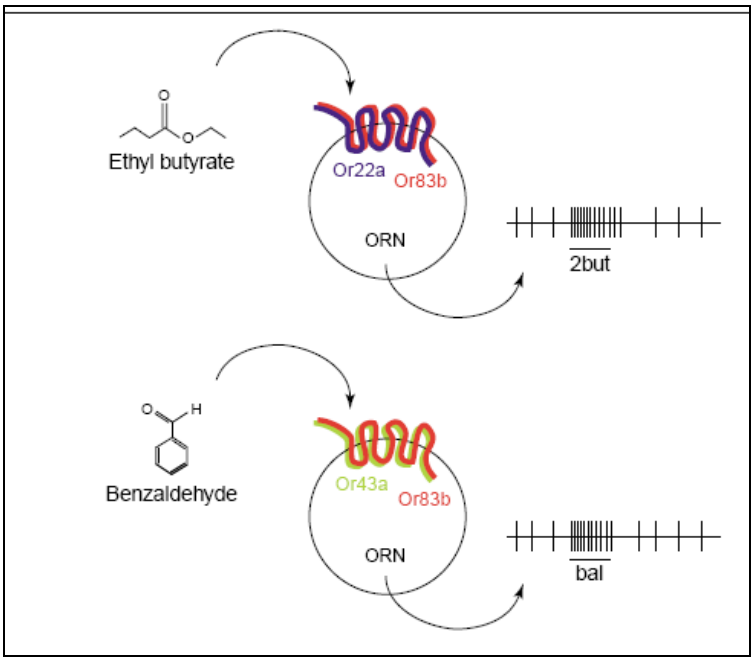

Figure 8. Odorant receptor function in *Drosophila*. Odorant receptor Or22a (blue) for ethyl buthyrate (2but) and Or43a (green) for benzaldehyde (bal) and the excitatory action potential response. These receptors are co-expressed with the non-canonical receptor Or83b (red). (Dahanukar et al., 2005)

The gustatory receptor neurons (GRNs) of insects have been analysed mostly by the tip-recording technique, in which the pore at the tip of the sensillum is put in electrical contact with a solution containing an electrolyte and the taste stimulus (Hodgson et al., 1955). Experiments with various taste stimuli have revealed in flies the presence of four types of neurons: a sugar-sensitive neuron (S), a water-sensitive neuron (W), a neuron sensitive to low concentrations of salts (L1) and a neuron sensitive to high concentrations of salts (L2) (Dethier, 1976; Falk and Atidia, 1975; Hiroi et al., 2004; Wieczoreck and Wolff, 1989).

Gustatory receptor neurons respond to more than one compound within the same or different categories. In larger flies such as the flesh fly *Boettcherisca peregrina,* S cells respond to at least five different types of sugars and sugar derivates: pyranose, furanose, nucleotides, sugars with an aryl group and sugars with an alkyl group (Furuyama et al., 1999; Shimada et al., 1985). In *D. melanogaster,* S cells respond to sucrose, pyranose, fructose, trehalose and glycerol (Koseki et al., 2004; Tanimura et al., 1982; Tanimura and Shimada, 1981; Wieczoreck and Wolff, 1989); responses to amino acids and fatty acids have not been described. Quantitative differences in sugar sensitivity among S cells have been described

(Hiroi et al., 2002; Meunier et al., 2000), but qualitative differences have not yet been found. In addition, some S cells also respond to low concentrations of salt (Hiroi et al., 2004).

In *Drosophila*, the L2 cell was recently found to respond to bitter stimuli in addition to salt stimuli (Hiroi et al., 2004; Liscia and Solari, 2000; Meunier et al., 2003). Recordings of responses to bitter compounds show that there is some degree of functional heterogeneity among sensilla (Meunier et al., 2003).

Salt reception in *Drosophila* is controlled by different genes (Pickpocket11 and Pickpocket19) and disruption of these genes results in a diminished behavioural response to salt but not to sucrose (Liu et al., 2003).

While it is important to describe the responses of taste neurons to individual compounds, we must not forget that insect sensilla encounter mixtures of compounds and therefore the responses of the neurons within a sensillum are not necessarily reflected in their responses to the individual compounds alone. Interactions between stimulating molecules may occur before any cell is stimulated, and there may also be some interaction between the neurons after electrical events have been initiated. Binary mixtures of compounds that stimulate the same cell usually produce additive effects, although limited by the maximum firing rate of the cell. This is the case for both phagostimulants and deterrents (Bernays and Chapman, 2001a; Glendinning et al., 1999; Glendinning and Hills, 1997). However, complex mixtures of amino acids, resembling the free amino acid composition of the host plants, are markedly less stimulating than expected from the effects of the individual components (Bernays and Chapman, 2001a).

Mixtures of nutrient compounds that stimulate separate cells in the sensillum usually cause the cells to fire independently of each other, but sometimes produce synergy (Dethier and Kuch, 1971). This is also sometimes true of mixtures of nutrient and deterrent compounds (Dethier and Kuch, 1971; Ishikawa et al., 1969). Typically, however, the interaction between a phagostimulant and a deterrent results in some concentration-dependent inhibition of the response (Schoonhoven et al., 1992). Although the effects of inhibition are widespread, the concentration at which they occur often seems too high to have relevance in natural situations. However, there are interactions also occurring at relevant concentrations (Schoonhoven and van Loon, 2002).

The mechanisms involved in these interactions between chemicals at the peripheral level are not completely understood, but several different ones are almost certainly present. Mitchell & Harrison (1985) consider the inhibition of the responses to nutrients in *Leptinotarsa decemlineata* results from a damage of the cells. This certainly occurs in the case

where sensilla have been exposed to a chemical for extended periods (1 minute or more). In most cases, however, the recovery of the responses occurs without any delay, suggesting that there is no damage of the cells. Non-stimulating nutrients may affect the activity of phagostimulatory cells by altering the cell environment. This appears to be the case with organic acids. Their effect in *Manduca sexta* is to alter the activity of other cells through their effects on pH (Bernays et al., 1998), and in mixtures with glucose or inositol, ascorbic acid causes a reduction in activity of the phagostimulatory cells responding to these compounds. Other interactions probably involve the receptor sites on the neuronal membrane (Bernays and Chapman, 2001b).

The insect brain and central chemosensory processing

Anatomy of the insect central nervous system

The insect brain (Fig. 9) is a complex of six fused ganglia (three pairs) located dorsally within the head capsule: the protocerebrum, the deutocerebrum and the tritocerebrum. The protocerebrum is the first pair of ganglia largely associated with vision; they are innervated by the compound eyes and ocelli and contain important "higher" integrating centres, i.e. the mushroom bodies, the central complex and the lateral accessory lobes, serving multimodal integration among other functions. The deutocerebrum**,** the second pair of ganglia, processes sensory information collected by the antennae and contains the antennal lobes (ALs) and the antennal mechanosensory and motor centres (also called dorsal lobes) (Homberg et al., 1989) The tritocerebrum is the third pair of ganglia that innervates the labrum and integrates sensory inputs from the protocerebrum and the deutocerebrum and serves as a relay station to transmit highly integrated sensory information between the protocerebrum and deutocerebrum and the thoracic ganglia. They also link the brain with the rest of the ventral nerve cord and the stomodaeal nervous system that controls the internal organs. The tritocerebrum represent a transmitting area intercalated between sensory input and motor output. The commissure of the tritocerebrum loops around the digestive system, suggesting that these ganglia were originally located behind the mouth and migrated forward (around the oesophagus) during evolution.

Located ventrally in the head capsule (just below the brain and the oesophagus) is another complex of fused ganglia (maxillar, mandibular and labial) jointly called the suboesophageal ganglion (SOG). A pair of circumoesophageal connectives loop around the digestive system to link the brain and suboesophageal complex. In adult Lepidoptera the SOG has merged with the tritocerebrum due to condensation of the circumoesophageal connectives and no borders can be defined between the two ganglionic masses (fig. 9) (Yack and Homberg, 2003).

In the thorax, three pairs of thoracic ganglia (sometimes fused) receive sensory input from and control locomotion of the legs and wings. Thoracic muscles and sensory receptors are also associated with these ganglia. Similarly, abdominal ganglia receive sensory information from the abdomen and control movements of abdominal muscles.

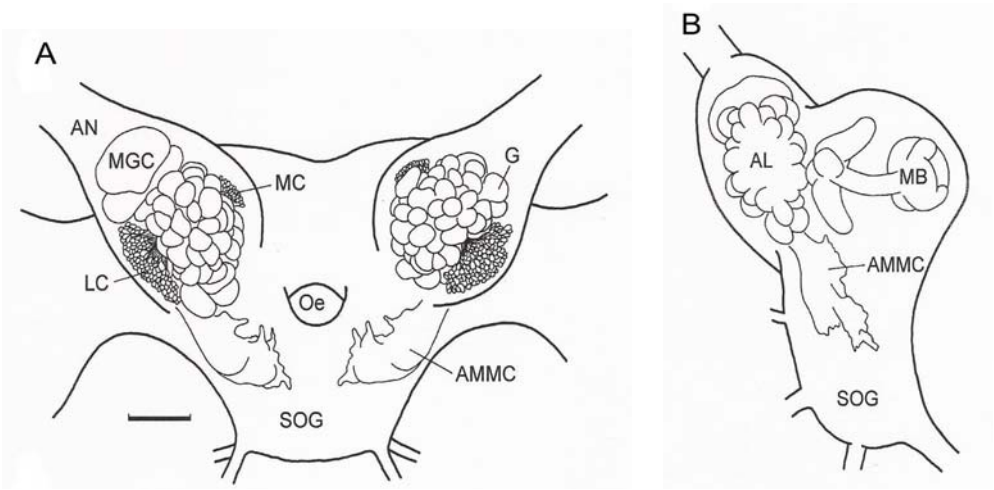

Figure 9. Frontal (A) and sagittal (B) view of the brain of the sphinx moth *Manduca sexta*, showing the two neuropils of the deutocerebrum, the antennal lobe (*AL*) and the antennal mechanosensory and motor centre (*AMMC*). A) AL of a male (*left*) and a female (*right*) moth. The macroglomelular complex (*MGC*) is only present in males. Most cell bodies of AL interneurons are concentrated in two cell groups, a medial (*MC*) and a large lateral cluster (*LC*). *AN* antennal nerve, *G* glomerulus, *SOG* suboesophageal ganglion, *Oe* oesophageal canal. B) *MB* mushroom body, situated posterior to the AL, Scale bar: 200µm (Anton and Homberg, 1999).

Chemosensory input to primary olfactory and taste centres: functional maps

The nervous system of many animals throughout the animal kingdom is characterized by maps formed by the consistent and orderly projections of sensory neurons. These maps may represent the location of sensory receptors on the body and/or coding properties of the sensory neurons (Newland et al., 2000). Insects are used as a model to study these maps, because their nervous system has a limited degree of complexity as compared to vertebrates. Information from insect receptor neurons goes to the brain or to the local ganglia, depending on their functional specificity and their location on the body parts. Central projections of mechanosensory neurons form somatotopic maps in which the spatial location of the receptor on the body part is preserved (Murphey et al., 1989). Central projections of sensory neurons from the eyes are arranged retinotopically (Strausfeld, 1976) while central projections of auditory neurons form a tonotopic map (Oldfield, 1982; Römer et al., 1988).

In the olfactory systems of both invertebrates and vertebrates there is an odotopic mapping of sensory neurons into compartments within the primary olfactory neuropils of the brain (Hildebrand and Shepherd, 1997). The primary olfactory centre in insects consists of the antennal lobes (AL) that are situated in the deutocerebrum. The axons of ORNs from both the

antenna and the maxillary palps project to processing units called glomeruli, forming a chemotopic map (fig. 10). Glomeruli correspond to neuropilar islets that are in many species arranged in a single layer around a central fibre core. All synaptic interactions in the AL happen within the glomeruli. The number of glomeruli is species specific and ranges from about 50 in Diptera, around 60 in moths, 160 in honeybees to more than 1000 in locusts (Anton and Homberg, 1999; Ignell et al., 2005; Rospars, 1988; Vosshall et al., 2000). In general, each ORN projects to a single glomerulus except for Diptera species where some ORNs project to one glomerulus in each lobe (Strausfeld, 1976) and locusts where several small glomeruli contain arborisations from a single ORN (Ignell et al., 2001).

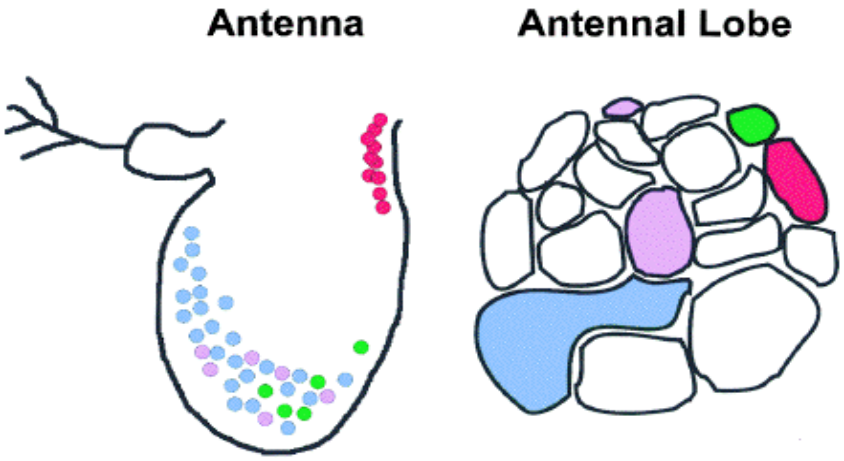

Figure 10. Spatial maps in peripheral and central olfactory tissues in schematic drawings of *Drosophila* odorant receptor (DmOR) gene expression in the antenna (left) and corresponding glomerular convergence in the antennal lobe (right). The relative position and number of cells expressing four DmOR genes is indicated by the following color code: Or22a (red), Or47b (light blue), Or47a (green), and Or23a (lavender). In the right panel, a schematic drawing of an antennal lobe showing the relative dorsal (top) and medial (right) positions of glomeruli receiving projections from these four populations of neurons. Glomerular color codes match those in the antennal schematic: Or22a (red), Or47b (light blue), Or47a (green), and Or23a (lavender) (Vosshall et al., 2000).

In contrast to the above mentioned sensory systems, comparatively little is known about the gustatory chemosensory system concerning the organization of the chemosensory afferents and the coding and processing of their signals by central neurons. The fact that gustatory sensilla are bimodal, containing both mechanosensory and gustatory neurons has led to the hypothesis that projections might be organized both in a somatotopic and chemotopic manner.

Until now, the most relevant results concerning the taste system have been obtained in *Drosophila*. The taste system in *Drosophila* is an attractive model to study the gustatory system because taste-related behaviours are robust, simple to assay and carried out by a nervous system that is amenable to molecular genetic and functional studies. *Drosophila* sample their local chemical environment with taste bristles on the proboscis, internal mouthpart organs, legs, wings and ovipositor (Dethier, 1976; Singh, 1997; Stocker, 1994). Accordingly, taste neurons project to different regions of the CNS such as the suboesophageal ganglion, the thoracic ganglion or the abdominal ganglion. Cobalt and Neurobiotin filling studies in *Drosophila* and other insects have shown that GRNs from different organs from the head project to different regions of the suboesophageal ganglion (SOG) and tritocerebrum in the brain (Edgecomb and Murdock, 1992; Kent and Hildebrand, 1987; Rehder, 1989; Stocker and Schorderet, 1981) whereas GRNs from the tarsi project to the thoracic ganglion (Murphey et al., 1989; Newland et al., 2000) and GRNs from the genitalia project to the abdominal ganglia (Tousson and Hustert, 2000). Sometimes putative chemosensory afferents project through the SOG to the thoracic ganglion (Kent and Hildebrand, 1987) and from the thoracic ganglia to the SOG (Newland et al., 2000).

In *Drosophila*, different populations of GRNs from the same part of the body also have distinct projection patterns. Backfills of labellar GRNs have shown that their arborizations in the SOG are varied, even though the SOG lacks glomerular organisation as compared to the antennal lobe (Nayak and Singh, 1985; Stocker and Schorderet, 1981). Moreover, the activity-dependent staining of single neurons revealed that different GRNs within the same sensillum have different projection patterns (Shanbhag and Singh, 1992).

More recent molecular studies in *D. melanogaster* have revealed that different classes of projections to the SOG correspond to different populations of neurons which suggests a chemotopic organisation of the gustatory projections: the two GRN populations defined by either *Gr5a* or *Gr66a* expression project to non-overlapping regions of the SOG (Thorne et al., 2004; Wang et al., 2004), suggesting the existence of an anatomical map of different taste modalities (fig. 11). Genetic ablation of these sets of neurons demonstrated that *Gr5a* defines a population of sugar-sensitive neurons and that *Gr66a* defines a population of bitter-sensitive neurons, essential for sugar acceptance behaviour and for avoidance of bitter components, respectively (Thorne et al., 2004; Wang et al., 2004).

Projections from different peripheral tissues are also segregated in the brain, even when the neurons express the same receptors, forming somatotopic maps. Examination of transgenic *D. melanogaster* in which only *Gr32a*-positive neurons were labelled with GFP showed that such neurons in the labellum project to a medial region of the SOG, those in the internal mouthparts project to a more anterior region of the SOG, and those in the leg project through the thoracic ganglion and terminate in a region posterior to the SOG (Wang et al., 2004).

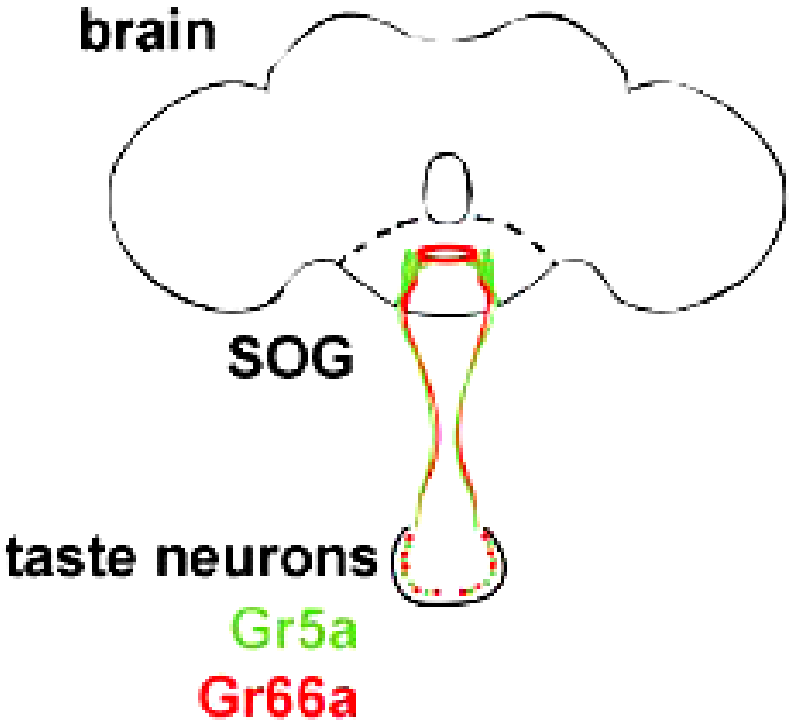

Figure 11. Schematic representation of taste neurons in the proboscis and their projections to the fly brain. Neurons expressing the Gr5a receptor (green) send axons through the labial nerve that project to the anterior SOG and do not cross the midline. Gr66a-expressing neurons (red) send axons through the labial nerve and terminate in the medial SOG in a ring-shaped way (Marella et al., 2006).

In locusts, the central projections of the sensory neurons from bimodal contact chemoreceptors (basiconic sensilla) were compared with those from mechanosensory tactile hairs (trichoid sensilla) located on similar regions of the middle leg of the locust (Newland et al., 2000). The projections of the basiconic and trichoid sensilla in the thoracic ganglion formed parallel overlapping maps (Fig 12).

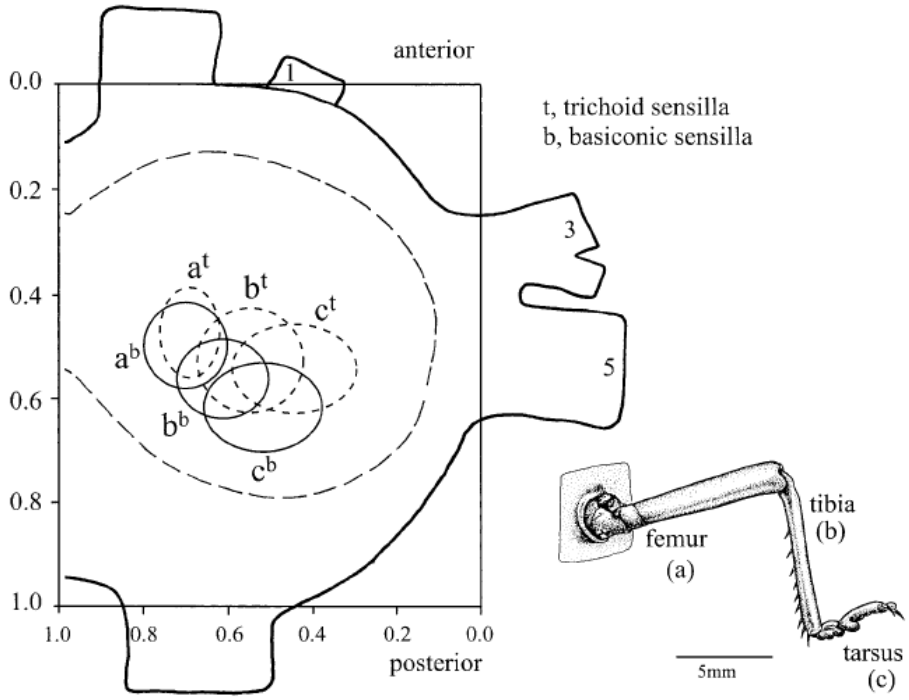

Figure 12. Parallel overlapping maps from tactile hairs (trichoid sensilla) and basiconic sensilla on the leg of the locust. (Newland et al., 2000). Both tactile hairs (mechanosensory) and basiconic sensilla (chemosensory and mechanosensory) project in an ordered way within the mesothoracic ganglion forming a somatopic map. Sensilla from the femur are projecting more medial than the ones from the tibia and the projections from tarsal sensilla are the most lateral (there is however some overlap between the three regions).

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CHAPTER 2
CONTRIBUTION TO THE STUDY OF THE ANTENNAL GUSTATORY SYSTEM OF *SPODOPTERA LITTORALIS*

The model insect

The model used for my studies is the Egyptian cotton leafworm *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) which is a severe pest on a large variety of crops in large parts of the world. It is mainly found in Africa (across the entire continent), the Middle East and Southern Europe, and the larvae feed on a wide variety of hosts, *e.g*. cotton, maize, tomato etc.

Fig 1 Adult male *Spodoptera littoralis*.
(http://extension.entm.purdue.edu/CAPS/pestInfo/egyptLeafworm.htm)

Adult *Spodoptera littoralis* (fig 1) have flagellar antennae (annulated antennae) with two basal segments, scape and pedicel, and a flagellum composed of similarly shaped segments. The particularity of the flagellum is that each segment bears the same chemosensory sensilla along the antennae with a small difference in number at the base and at the tip of the antennae.

Objectives

S. littoralis is an important model in insect chemosensory research. Orientation behaviour of this species is well studied concerning both, host-plant search and identification and intraspecific communication. For both kinds of behaviour, long- and short-range cues play an

important role. It is evident that the different stimuli interact and that information on both must be integrated in the central nervous system to result in adequate behaviour. Whereas central representation and processing of olfactory stimuli has been extensively studied, very little is known on central processing of contact stimuli and on multimodal integration of the two chemical modalities. The antennae of Lepidoptera are the ideal system to investigate this type of question, because both olfactory and gustatory sensilla are distributed in a regular way and they are accessible for electrophysiological and staining approaches.

The objectives of the present thesis were to study the central nervous representation patterns and physiological characterization of contact chemosensory sensilla present on the antennae of adult *S. littoralis*. Given the highly repeated structure of the antenna in Lepidoptera, we addressed the question whether the peripheral distribution pattern of the gustatory sensilla is spatially represented within the central nervous system and/or whether some chemotopic projection pattern exists. An electrophysiological approach, using extracellular recording methods and testing different categories of tastants served to characterize the GRNs present within the antennal gustatory sensilla.

Results

In the **third chapter** of this thesis I describe the anatomy of the antennal gustatory system. I aimed to characterize the responses of gustatory neurons to simple compounds like sugars (sucrose, glucose and fructose) and salts (NaCl, KCl). I chose to test these sugars because the main food source of adult moths is floral nectar. The nectar contains mostly sucrose, glucose and fructose, and as such, are biologically meaningful for the moths. The role of salts in the moth's life hystory is not well understood, but generally, salts are important for homeostatic balance.

I wanted to know if gustatory neurons along the antenna present different sensitivities or specificities. So, I divided each flagellum in three parts: the base, the middle and the tip. I also examined whether the detection abilities of the sensilla might depend on their location on the ventral, dorsal or lateral side of each segment.

I used the “tip recording” technique (Fig 2) to record the electrical activity of gustatory neurons (Hodgson et al., 1955). The technique consists in recording the variations of the electric potential between a recording electrode (a silver wire within a saline –filled glass

capillary) and a reference electrode situated in the insect's body. The recording electrode contained the stimulating solution. In this way, the activity of all gustatory neurons, as well as the mechanosensory neuron present in one sensillum, was recorded.

Individual neurons can be identified by the amplitude and shape of the action potentials they generate. The size of action potentials in a recording depends on the spatial relation between the recording electrode and the neurons. In some cases it might depend on the diameter of the neurons (Hansson et al., 1994).

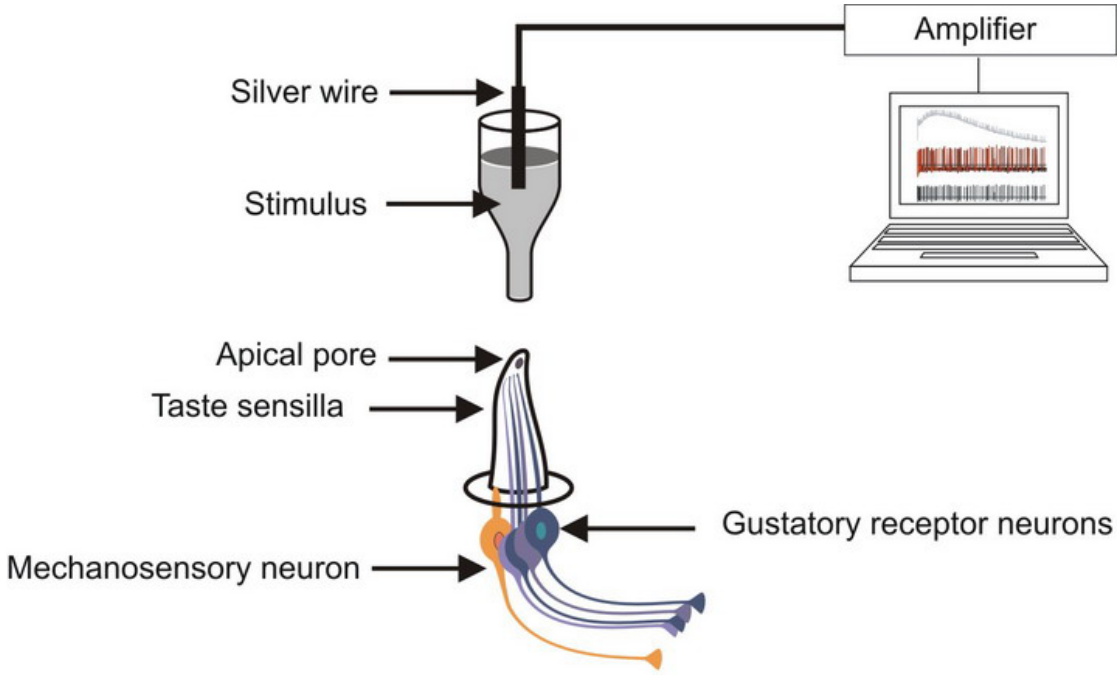

Figure 2. "Tip recording" technique. The tip of the sensillum is capped with a glass capillary filled with a stimulating solution. The electrical signals are amplified and recorded.

The **fourth chapter** describes preliminary experiments aimed at examining the detection of other compounds (mainly bitter compounds and amino acids) by gustatory chemosensilla on the antennae. I also tried to separate and identify the neurons responding to salts and sugars by cross-adaptation tests and the use of mixtures of salts and sugars.

Another recording technique using a tungsten electrode (Fig. 3) was used in additional experiments in order to record spontaneous activity of gustatory neurons and to compare the quality of the recordings between the two techniques.

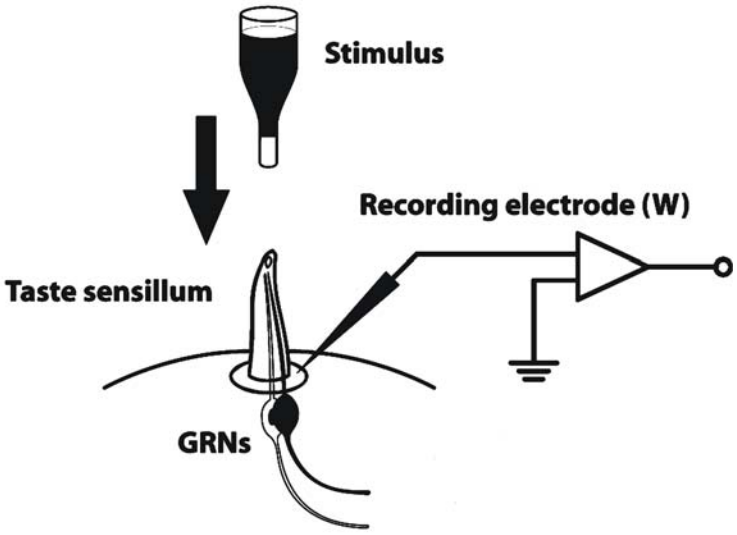

Figure 3. Schematic diagram of the recording setup for tungsten electrodes. Electrical signals were recorded with an electrolytically sharpened tungsten electrode inserted through the cuticle at the base of a taste sensillum.

The **fifth chapter** is focused on the central projections of the receptor neurons in the contact chemosensilla on the antennae. There are at least two different potential methods of characterizing single neuron types. One possibility is to use fluorescent dyes directly or neurobiotin coupled with a fluorescent dye. Neurobiotin is likely to be taken up preferentially by physiologically active neurons. In this case wholemounts can be visualized using a confocal microscope and 3D reconstructions can be built. Resolution of fine branches deep in the brain is, however, limited by the limited reflection of fluorescent light while passing through the tissue.

Another technique is the cobalt-lysine technique, which is also thought to stain active neurons specifically. The staining needs to be silver-intensified to give a good resolution of fine branches within the CNS, and therefore brains must be sectioned physically to observe stainings. This makes 3D reconstructions more difficult than with techniques using fluorescent dyes and confocal microscopy. In addition, cobalt is toxic for neurons and therefore results in sub-optimal tissue preservation especially if long diffusion times are required.

The ideal would be to use both methods, which are complementary. During my thesis, I was, however, not able to use both techniques because of the limited time available. In order

to show the gustatory pathways we chose Neurobiotin coupled with a fluorescent dye and confocal laser scanning microscopy. Projections of individual neurons and massfills were analysed and the target areas were shown in three-dimensional reconstructions.

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CHAPTER 3

SUGAR AND SALT DETECTION BY SENSILLA CHAETICA ON THE ANTENNAE OF THE EGYPTIAN COTTON LEAFWORM, *SPODOPTERA LITTORALIS* (LEPIDOPTERA: NOCTUIDAE)

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Abstract

Adult Lepidoptera use their antennae to probe their distant and proximate environment with sensory organs which detect volatile and contact chemicals. In this work, we have investigated the role and the sensitivity of antennal contact chemoreceptors of *Spodoptera littoralis*. Adults of both sexes have long and filiform antennae composed of about 70 flagellomeres. Each flagellomere bears six taste sensilla, except on the basal segments 1-5 where two sensilla are missing and on the terminal segment which has an additional crown of six sensilla. This represents a population of about 420 sensilla which provide taste and mechanosensory information. By mean of tip-recordings, we recorded the responses of these sensilla to aversive stimuli (KCl, NaCl) and to appetitive stimuli (sucrose, fructose, glucose). The questions we addressed were whether the sensitivity of these sensilla depends on (i) their position along the antenna, (ii) their position on each flagellomere, and (iii) sex. In all responsive sensilla, sucrose, fructose and NaCl elicited dose-dependent phasic responses while KCl and glucose did not elicit dose-dependent responses. In some sensilla, we could detect the activity of up to two neurones with different spike amplitudes in response to sucrose and KCl. No differences could be detected between sensilla located at the basal, medial and terminal regions of the antenna. No differences could be detected either between the lateral, dorsal and ventral sensilla in males; in females, however, the dorsal sensilla responded less than ventral and lateral sensilla but with the same response profile. No obvious differences were noted between sexes. These observations suggest that antennal contact sensilla serve as a warning and proprioreceptive system rather than as an elaborate taste system as found on the mouthparts or on the legs.

Introduction

In adult Lepidoptera, antennae are complex multimodal sensory structures providing physical and chemical information about the environment. At close range, most Lepidopterae actively explore the substrate by vibrating the antennae and by touching it repeatedly with the terminal segments. This behaviour is usually called “antennation”. It is regularly observed in the context of sexual encounters, before laying eggs and before feeding. It may help the insects to sample odorants which remain within the boundary layer and is analogous to “sniffing” in vertebrates (Koehl, 2006). Antennation also helps to detect chemicals by contact, such as, for example during nectar feeding. Touching the antennae with a sugar solution elicits the extension of the proboscis and subsequent ingestion activities: this reflex can be used to

condition during associative olfactory learning (Daly and Smith, 2000; Jørgensen et al., 2007b; Skiri et al., 2005) as observed in honeybees *Apis mellifera* (Menzel and Müller, 1996).

The most conspicuous part of the antenna is the flagellum which is formed by the duplication of "segments" or flagellomeres that develop synchronously during ontogenesis (Sanes and Hildebrand, 1976). Each flagellomere bears sensilla of different type that are involved mostly in olfaction, but also in mechanoreception, contact chemoreception, thermo- and hygroreception (Altner and Prillinger, 1980). Flagellomeres vary in size along the length of the antenna and their organization varies continuously from the base to the tip. This affects the number of olfactory sensilla per segment, which is generally larger at the base than at the tip in both sexes (Calatayud et al., 2006; Cornford et al., 1973). Changes in the organization of the flagellomeres may be correlated with functional differences. In cockroaches, which have long and flexible antennae like Lepidoptera, the hypothesis that all flagellomeres are functionally equivalent has been tested using local olfactory stimulation. Electroantennogram responses show differences between sexes and between the three different parts (basal, medial, distal) of the antenna (Nishino and Takayanagi, 1979).

Taste sensilla, which are involved in antennation and palpation, represent only a fraction of the sensory equipment of the antenna. In *Ostrinia nubilalis*, there are about 300 *sensilla chaetica* (presumably contact chemoreceptors) compared to 7500 olfactory sensilla trichodea in males and 4000 in females; these sensilla chaetica are regularly disposed on the antenna except the first and last segments which are slightly different (Cornford et al., 1973). Each of these sensilla have a terminal pore and host five sensory processes, four of which are chemosensory neurons which extend their dendrites to the distal pore of the hair while the fifth neuron is mechanosensory and has its dendrite inserted at the base of the hair (Anderson and Hallberg, 1990; Gaffal, 1979; Hallberg, 1981). While taste sensilla are distributed evenly along the antenna, they might have different sensitivities according to their location, especially those located on the terminal flagellomeres which come regularly in contact with the substrates during palpation.

We studied the distribution and physiology of antennal contact chemoreceptors of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae). This insect is polyphagous and is considered as a serious pest of crops throughout warm-temperate and subtropical countries in the Old World (Brown and Dewhurst, 1975). In the adults of both sexes, the antennae are filiform and bear numerous olfactory basiconic and trichoid sensilla (Ljungberg et al., 1993).

Males bear more trichoid sensilla per flagellomere (75-100) than females (50-60), a difference that is due to a greater number of sensilla trichodea of type I (pheromone-sensitive sensilla) in the males (Ljungberg et al., 1993). They are equipped with taste sensilla that elicit a proboscis extension reflex when stimulated with a solution containing sugars (Fan et al., 1997; Hartlieb et al., 1999).

In this work, we examined the distribution of these taste sensilla and studied with electrophysiological techniques their responses to a dilution series of a set of aversive (NaCl, KCl) and appetitive (sucrose, fructose, glucose) stimuli. By sampling hairs systematically on a given flagellomere and along the antenna, we investigated whether all hairs of a given flagellomere have the same responses and whether the responses change quantitatively and qualitatively across the antenna among the sensilla of the same flagellomere and whether differences occur between males and females. Despite some differences observed between sensilla according to their location on the flagellomere, along the antenna and to the sex, our observations support the conclusion that antennal taste sensilla are functionally homogenous with respect to the stimuli tested.

Materials and methods

Insects

S. littoralis moths were reared on a semi-artificial medium in our laboratory (Poitout and Bues, 1974). From the third to last instar, larvae were reared individually. Male and female pupae were kept separately in groups of 20-30 at 20-24°C, 55-75 % relative humidity, and under a 16:8 h light:dark cycle. Adult moths were kept in plastic containers and provided with a 10 % sucrose solution to feed *ad libitum*. Electrophysiological recordings were performed at room temperature on adults, 12-36 hours after emergence.

Chemicals

Sucrose, glucose, fructose, potassium chloride and sodium chloride were purchased from Sigma-Aldrich (France). Compounds were diluted in ultra pure water and 1 mM KCL was added in the sugar solutions to ensure the conductivity of the stimulating solution. All solutions were kept at 4°C and used at concentrations ranging from 0.1 mM to 100 mM.

Scanning electron microscopic study

The antennae were cut and fixed in paraformaldehyde. After rinsing in phosphate buffer they were dehydrated in ethanol 70° and air dried. At the end they were mounted with glue on stubs and coated with gold/palladium before examination using a scanning electron microscope (JEOL JSM 840).

Electrophysiology

Moths were secured into a holder carved from thermal isolation plates used in construction with the head protruding over a flat surface. Each antenna was tightened to the support with tungsten hooks, wax and sticky tape. The insect was grounded using electrocardiogram gel deposited on the antenna (Redux ® Gel, Parker Laboratories Inc., Fairfield, USA), which served as a bridge to a silver wire connected to the ground. To stimulate a sensillum, the tip of the hair was covered during 2 seconds with an electrode containing both an electrolyte (1 mM KCl) and the stimulus (Hodgson et al., 1955). Electrodes were made from borosilicate glass capillary tubes (GC-100 T, Clark, USA) pulled in two steps so that the tip had a diameter of about 40-60 µm (PC-10 vertical puller, Narishige, Japan). This electrode was directly fitted on a silver wire connected to a probe (Syntech, DE) mounted on a micromanipulator (Leitz), and advanced under visual control (MZ12, Leica, France). The probe was connected to an amplifier (TasteProbe DT-02, Syntech, DE) with an automatic compensation of the offset (Marion-Poll and Van der Pers, 1996). Electric signals were further amplified and filtered (CyberAmp 320, Axon Instrument, USA; gain 1000; 8^{th} order Bessel pass-band filter: 1 Hz – 2800 Hz). Contacting a taste hair with the stimulus electrode triggered data acquisition and storage on a disk, under the control of a custom software, dbWave (Marion-Poll, 1995).

We tested three to five homologous sensilla per preparation. As a control, we used 1mM KCl. Each sensillum was stimulated by presenting an increasing order of concentrations (0.1 mM, 1 mM, 10 mM and 100 mM) of each substance. We waited at least 1 min between consecutive stimulations of the same hair. Sensilla were sampled along the antenna at the base (segments 1 to 10), in the middle (segments 20-40) and the tip (the 10 terminal segments). We sampled for this study 70 males and 110 females, which yielded 8800 recordings.

For each recording, spikes were detected and analyzed using dbWave. We evaluated the responses by counting spikes during the first second of recording. Whenever possible, we sorted the spikes according to their amplitude and shape, as well as by taking into account

spike superposition (Meunier et al., 2003a). The results were statistically analyzed by running Poisson regressions using SAS software.

Results

Distribution of contact chemoreceptive sensilla

The antennal flagellum of *S. littoralis* is composed of about 70 flagellomeres with a similar topographic organization. Taste sensilla project from the antennal surface and extend above the other sensilla except on basal flagellomeres. Each segment bears 6 *sensilla chaetica* except at both ends of the antenna. The last flagellomere presents an additional crown of 7-8 sensilla chaetica at the very tip (Fig 1 C). The six proximal flagellomeres house only 2 sensilla, one on each side at the border between the dorsal side filled with scales and the ventral side, densely packed with sensilla trichodea.

The six sensilla are regularly disposed around the flagellomere approximately in the middle of its length. Two sensilla chaetica are found on the ventral side covered with sensilla trichodea and other sensillum types (Fig 1 A,B), two are on the dorsal side covered with scales (Fig 1 B) and one sensillum is found on each side, at the border between the ventral and the dorsal fields (Fig 1 A,B). All these sensilla look similar with SEM observations and occasionally a terminal pore could be observed at high magnification. No sexual dimorphism was detected in the number and disposition of these sensilla chaetica.

Electrophysiology

When the tip was capped with an electrode, each sensillum chaeticum yielded an electrical contact, confirming that they are contact chemoreceptors. When neurons were active, we could record action potentials with amplitudes ranging between 0.8 mV and 2.6 mV on a baseline noise of about 0.05 mV peak-to-peak.

In most sensilla, the responses to NaCl, sucrose, and fructose were strongly phasic with an initial burst of spikes of 100-200 ms followed by a gradually declining activity (Figs. 2, 3, 4). This initial burst was different from the activation of the mechanoreceptors, which fired smaller amplitude action potentials when lateral movements were imposed on the recording electrode. Glucose elicited only occasional responses in a few sensilla (mainly in dorsal sensilla). KCl did not elicit any increase of the firing rate and in some cases, an inhibition of the spiking activity was observed with increasing concentrations (fig 5).

It was usually difficult to determine how many cells were active in a given recording because the amplitudes and shapes of the spikes were very similar (fig 6). However, we could monitor spike superposition (indicating that spikes originated from two neurons) and in some sensilla, the spike amplitudes were different so that we could discriminate them without ambiguity. Using these criteria, two distinct neurons were found responding to the sugars, sucrose, fructose, and glucose (fig 3). For NaCl, we usually observed responses from only one cell (fig 4), but in few recordings (about 3%), a second cell could be identified at high concentration (0.1 M).

Sensitivity of taste sensilla from the same flagellomere

To assess if the sensilla from the same flagellomere had similar sensitivities, we examined the sensilla chaetica located on the tip of the antenna. Ventral and dorsal sensilla at the base and the middle of the antenna were difficult to record because of the size of the neighboring olfactory sensilla, which prevented us from establishing a clean contact with them.

In all sensillum types at the tip of the antennae of females (fig 7A), we observed increasing dose-responses curves to sucrose, fructose and NaCl and to a lesser extent to glucose (Table 1). The effect of concentration is significant for the responses to sucrose, fructose, NaCl ($p < 0.005$) but less clear for glucose ($p < 0.04$). In males (fig 7B, table 1), concentration has a significant effect on the responses to sucrose, fructose, NaCl ($p < 0.005$) and to a lesser extent to KCl ($p < 0.05$).

Table 1. Effect of concentration on sensilla of the last flagellomeres[1]

	Sucrose	Fructose	Glucose	KCl	NaCl
Males	p = 0.0003	p = 0.0020	p = 0.0786	p = 0.0334	p = 0.0001
Females	p = 0.0037	p = 0.0026	p = 0.0382	p = 0.3348	p = 0.0006

In females, the responses of the dorsal sensilla were lower than those of the lateral and ventral sensilla (fig 7A). The dose-response curves for sucrose, fructose, glucose and NaCl showed significant differences according to the location of the sensillum on the flagellomere

[1] All sensillum types from the tip of the antenna were pooled. The responses at different concentrations were compared using a Poisson regression test. P-values are tabulated

(Table 2). In males (fig 7B), only the dose-response curve to NaCl showed significant differences between ventral sensilla and the other types (Table 1).

Table 2. Effect of the position (ventral, lateral, dorsal) of sensilla on the last flagellomeres[2]

	Sucrose	Fructose	Glucose	KCl	NaCl
Males	p = 0.1304	p = 0.1866	p = 0.4595	p = 0.2506	p = 0.0159
Females	p = 0.0026	p = 0.0126	p = 0.0007	p = 0.1035	p = 0.0007

Sensitivity of taste sensilla across the antenna

To assess the effect of the position along the antenna, we tested the responses of lateral sensilla sampled at the base, at the middle and at the tip of the antenna. We found no significant differences in the dose-response curves of these lateral sensilla regarding their position along the antenna in males and in females (Table 3; Poisson regression test). When all lateral sensilla were pooled, we found that responses in females and males (fig 8) changed significantly with increasing concentrations of sucrose, fructose and NaCl (Table 4). In females, KCl inhibited the activity of one cell (Figure 8 A) while no statistically significant effect was observed in males.

Table 3. Effects of the position along the antenna on the responses of lateral sensilla to a series of chemicals[3]

	Sucrose	Fructose	Glucose	KCl	NaCl
Males	p = 0.3945	p = 0.1101	p = 0.0883	p = 0.1416	p = 0.1213
Females	p = 0.1431	p = 0.3888	p = 0.3933	p = 0.2219	p = 0.5771

Table 4. Effects of the concentration of the stimulus[4]

	Sucrose	Fructose	Glucose	KCl	NaCl

[2] Comparison of dose-response curves as a function of sensillum position on the flagellomeres at the tip of the antenna tested by Poisson regression (p-values) at least 20 observations per category in females and at least 6-10 observations per category in males)
[3] Same test and presentation as in Table 2
[4] Same test and presentation as in Table 1

Males	$p = 0.0127$	$p = 0.0389$	$p = 0.3961$	$p = 0.6279$	$p = 0.0067$
Females	$p < 0.0001$	$p = 0.0009$	$p = 0.1266$	$p = 0.2521$	$p = 0.0015$

Differences between males and females

Males and females responded to the same compounds but with some variation in the intensity of the responses (7 A, B, 8 A, B). When the responses are compared across the antenna using the recording performed on the lateral sensilla, dose-response curves for fructose ($p < 0.05$) and NaCl ($p < 0.001$) differed with respect to sex (Table 5, top row). We also compared the responses of all sensilla of the tip of the antenna and there is a marked difference between sexes for dose-response curves for all chemicals ($p < 0.01$ to $p < 0.001$) except for KCl, with the responses of males being much lower than those of females (Table 5, bottom row).

Table 5. Effect of the sex on the responses of sensilla[5]

	Sucrose	Fructose	Glucose	KCl	NaCl
Lateral sensilla[6]	$p = 0.0622$	$p = 0.0228$	$p = 0.0516$	$p = 0.9776$	$p = 0.0006$
Sensilla on tip of antenna[7]	$p = 0.0096$	$p = 0.0008$	$p = 0.0123$	$p = 0.2589$	$p < 0.0001$

[5] Poisson regression test. P-values are given (at least 20 observations per category in females and at least 6-10 observations in males).
[6] Comparison of dose-response curves of lateral sensilla along the flagellum.
[7] Comparison of dose-response curves of all sensillum types (ventral, lateral, dorsal) recorded at the tip of the antenna.

Discussion

In *S. littoralis* we found that a small number of sensilla chaetica were distributed evenly along the antenna, forming a crown of six sensilla on each flagellomere inserted roughly in the middle of the segment. An additional crown of taste sensilla was present on the most distal flagellomere and only two taste sensilla were found on the flagellomeres at the base of the antenna. Similar figures have been found in other Lepidoptera with filiform antennae like *O. nubilalis* (Cornford et al., 1973) and *H. virescens*.(Jørgensen et al., 2006) In this work, we found that these sensilla chaetica are contact receptors that responded to some sugars and salts. We excluded dorsal and ventral sensilla at the base of the antenna from our investigations: these sensilla were difficult to stimulate because they did not stand out the field of scales and sensilla trichodea. Their specifical study would necessitate a different approach, for example the removal of the scales and sensilla trichodea which surround them.

Our main objective was to test if sensilla from different flagellomeres could be considered as functionally identical or if the sensilla located at the tip would have different sensitivities under the rationale that terminal sensilla are more likely to be involved in sampling tastants than those at the base of the antenna. Our current data support the first hypothesis because we did not find any compound-specific sensillum or gradient of sensitivity along the antenna. However, some differences were noted. In females, dorsal sensilla of the terminal segments were less responsive to the test stimuli than the ventral and lateral ones. In males, the responses to all stimuli were less marked than in females but their responses were qualitatively similar to that of the females, with the most stimulatory sugars being sucrose and fructose.

Although our study indicates that the responses of the taste sensilla do not change much along the antenna, in *H. virescens*, it was reported that the number of sensilla chaetica responding to sucrose increases significantly from the base to the tip of the flagellum (Jørgensen et al., 2007a). Our conclusions are based on recordings made from the lateral sensilla and it is possible that variations may occur within the population of ventral or dorsal sensilla not sampled here. The second difference lies in the sample size: our observations are based on sampling 900 hairs over 180 insects while the study of *H. virescens* relied on 132 sensilla from 11 moths. Further studies are needed to resolve this discrepancy.

Sensilla chaetica of insects house typically four taste neurons and one mechanoreceptor. The mechanoreceptive function is consistent with our SEM observations that sensilla chaetica

have a basal articulating socket. It is also consistent with our electrophysiological recordings which show an additional neuron when the hairs are moved laterally by the recording electrode. So far, it is still uncertain if *S. littoralis* sensilla chaetica house four taste neurons. My ad hoc observations suggest that up to 5 axons are stained with neurobiotin when the projections from individual sensilla chaetica are marked (Popescu, unpublished observations; see Chapter 5). In *H. virescens* as well, one to five neurons were stained within individual sensilla (Jørgensen et al., 2006). Ultrastructural observations in other moths like *Ephestia kuehniella* (Anderson and Hallberg, 1990; Chaika and Sinitsina, 1997) or in *Yponomeuta* sp.(Cuperus, 1985), show that sensilla chaetica house five neurons, including one mechanoreceptor.

In the present recordings, we could not discriminate if the responses originated from one neuron or from several neurons. Although it was usually not possible to resolve individual spike trains from each neuron, co-occurrence of spikes from different neurons induced visible superposition of spikes. This criterion leads us to propose that sugars elicit action potentials in two neurons at most concentrations and in two neurons for NaCl at a high concentration. In *Drosophila melanogaster*, each gustatory neuron encodes different categories of tastants; the neurons are called sugar, water and salt cells according to their best stimulus.(Singh, 1997)

Our observations are consistent with this encoding system if one considers that one cell corresponds to a water cell and the other, to a sugar or a salt cell. This water cell is probably active at low concentrations of KCl and is inhibited at higher concentrations. A water cell was initially described in Diptera (Evans and Mellon, 1962; Inoshita and Tanimura, 2006; Meunier et al., 2000; Meunier et al., 2003b) and was found in several orders of insects, including in the larvae (Schoonhoven and van Loon, 2002) and adults (Chapman, 2003) of phytophagous Lepidoptera. This hypothesis does not explain, however, why some sensilla keep firing at about the same level over the whole range of KCl concentrations. We propose that in these sensilla, either a water cell is missing or a salt cell is activated by salt, compensating the decrease of activity of the water cell as in *D. melanogaster* (Fujishiro et al., 1984).

Fructose, sucrose and glucose are encountered when moths are foraging on flowers for nectar and these three sugars are the most common (Gottsberger et al., 1984). In *S. littoralis*, these three sugars are detected also by tarsal taste sensilla (Blaney and Simmonds, 1990). Stimulating the legs or the antenna with sucrose triggers proboscis extension and associative

learning in *S. littoralis* (Fan et al., 1997; Fan and Hansson, 2001) and in other Lepidoptera (Daly and Smith, 2000; Hartlieb et al., 1999; Romeis and Wackers, 2000; Skiri et al., 2005). While slight differences exist in the respective sensitivities of antennal and tarsal taste sensilla, the antennal input is likely to be most important because it is the first appendage used to explore food located in the immediate vicinity of the animal. In support of this hypothesis, recent experiments in honeybee showed that although associative learning is possible when tarsal input is used, learning is more difficult than when using the antennal input (de Brito Sanchez et al., 2008).

Salts are important in maintaining the homeostatic balance in all organisms. If their presence in small quantities is necessary, high concentrations of salt are harmful and have been proposed as a deterrent chemical to protect crops (Loni and Lucchi, 2001). In *Drosophila* as well, elevated concentrations of NaCl are deterrent while low concentrations are attractive (Hiroi et al., 2004; Meunier et al., 2003b). Male Lepidoptera have special needs for sodium chloride as the developpement of their spermatophores requires elevated intake of sodium (Pivnick and McNeil, 1987; Smedley and Eisner, 1996). Males of several species of Lepidoptera are known to visit stands of water, a behaviour known as puddling, which is thought to provide them with salts (Boggs and Dau, 2004; Watanabe and Kamikubo, 2005). These observations are consistent with our electrophysiological observations that NaCl is detected by one cell at low concentration, presumably mediating appetitive behaviour, while another cell is active at high concentration, possibly mediating aversive behaviour. Sensitivity to salts might be especially important in Lepidoptera or beetles (Merivee et al., 2004) when foraging for food, in contrast to cockroaches which seem to lack taste receptors on their antennae (Hansen-Delkeskamp, 1992).

In summary, the sensilla chaetica present on the antenna of *S. littoralis* adults are remarkably homogenous in their morphology, distribution and sensitivity across the length of the antenna. The absence of compound-specific sensillum types is possibly related to the limited number of substances we have used in this study. In a recent study, Jørgensen et al. have found several sensillum types responding to antifeedant molecules in *H. virescens* (Jørgensen et al., 2007a). Pilot experiments performed on *S. littoralis* did not allow us to find similar responses (Popescu, unpublished observations). While these experiments focused on food-related molecules, one should also consider non-volatile lipophilic molecules which are important either to assess the quality of a host-plant,(Grant et al., 2000; Müller and Riederer,

2005; Powell et al., 1999; Steinbauer et al., 2004; Udayagiri and Mason, 1997) or which are important in the context of mating, like cuticular molecules which can be either inhibitory (Lacaille et al., 2007) or excitatory aphrodisiacs.

While olfactory sensilla outnumber taste sensilla by a factor of 10 or 100 in *S. littoralis*, the number of taste sensilla on the antenna is not negligible. It represents a total of about 400 sensilla chaetica. It remains now to be seen how insects really use these sensilla and if the sensilla provide them enough information to discriminate many chemicals. That taste sensilla from each flagellomere are functionally almost identical militates against the hypothesis that this organ is used to discriminate tastants. Buccal appendages and even legs stand in sharp contrast with the antennal taste system because (i) the density of their taste sensilla is much higher and because (ii) more variation has been found in the responses of sensilla from the same sensillar field to sugars,(Hiroi et al., 2002; Liscia et al., 1998) salts (Maes and Den Otter, 1976) and antifeedant molecules (Meunier et al., 2003b). We hypothesize that the antennal taste system is relatively primitive and is used as a warning system, complementing other sensory modalities to spatially localize objects (Okada and Toh, 2006), passing the relay to other taste organs when finer discrimination tasks are necessary.

Figures and legends for Chapter 3

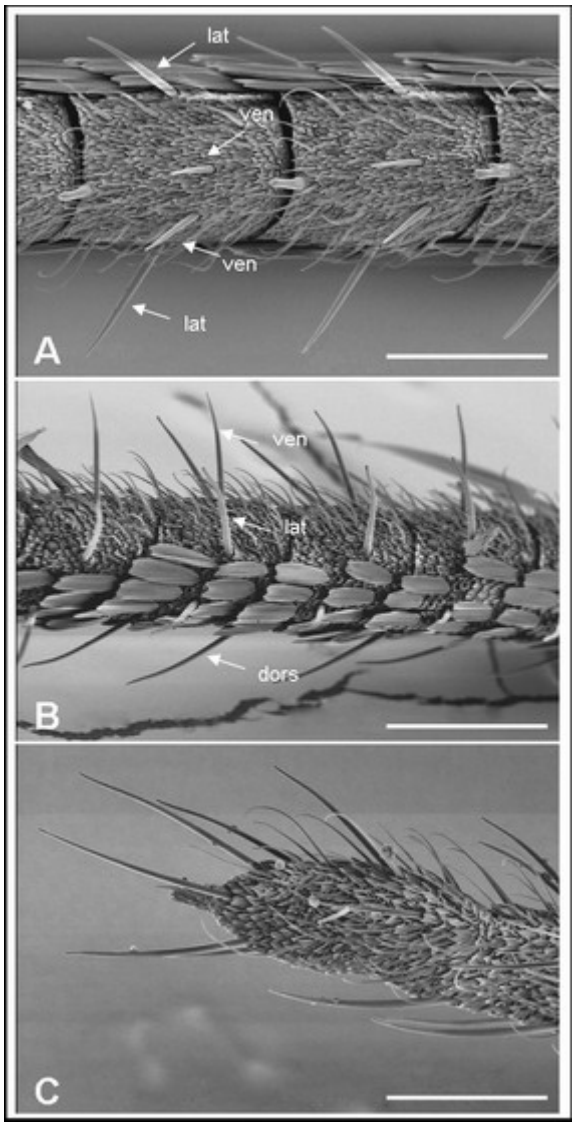

Figure 1. Different views of the flagellum of the moth *S. littoralis*. A. Ventral view showing four sensilla chaetica: two ventral and one lateral on each side. B. Lateral view showing lateral, dorsal and ventral taste sensilla. C. Tip of the antenna showing a crown of six sensilla chaetica. Scale bars= 100 μm.

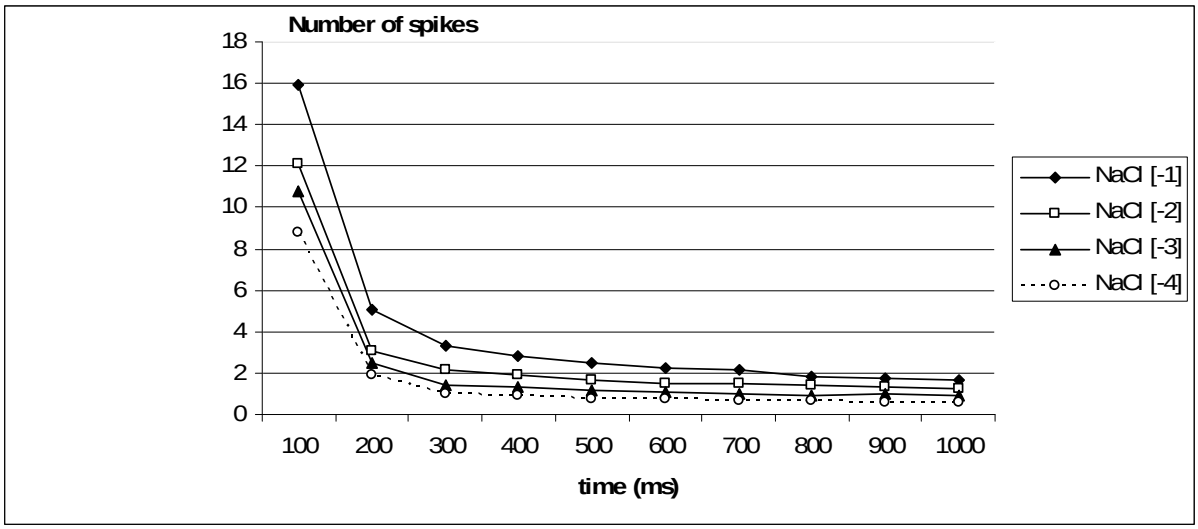

Figure 2. Temporal response pattern for NaCl stimulation in lateral sensilla in females –phasic response. Concentrations expressed in $\log_{10}$M

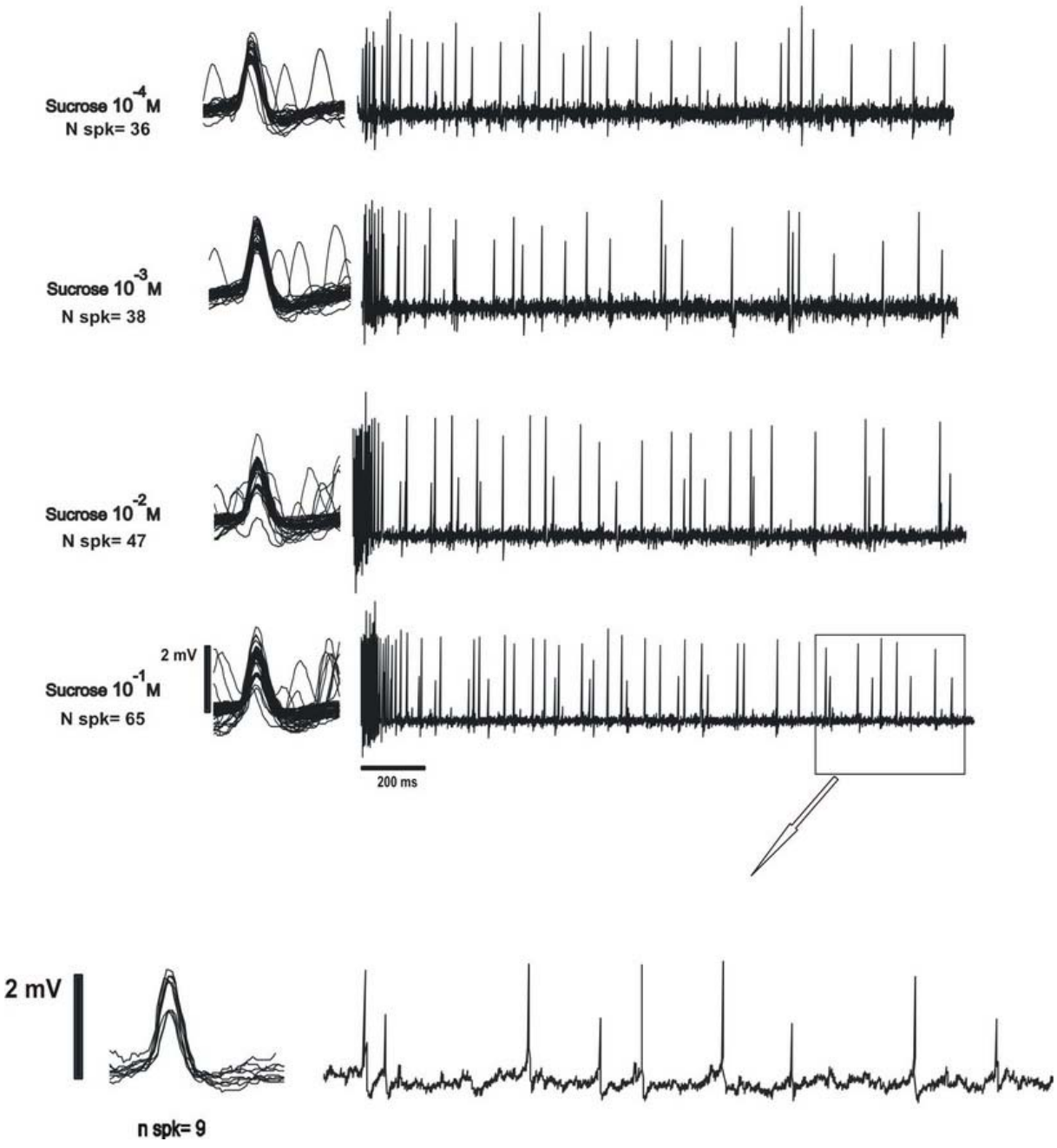

Figure 3. Electrophysiological recordings of GRNs from one lateral sensilla in response to increasing concentrations of sucrose.- Two GRNs are firing as shown by the different sizes of the spikes.

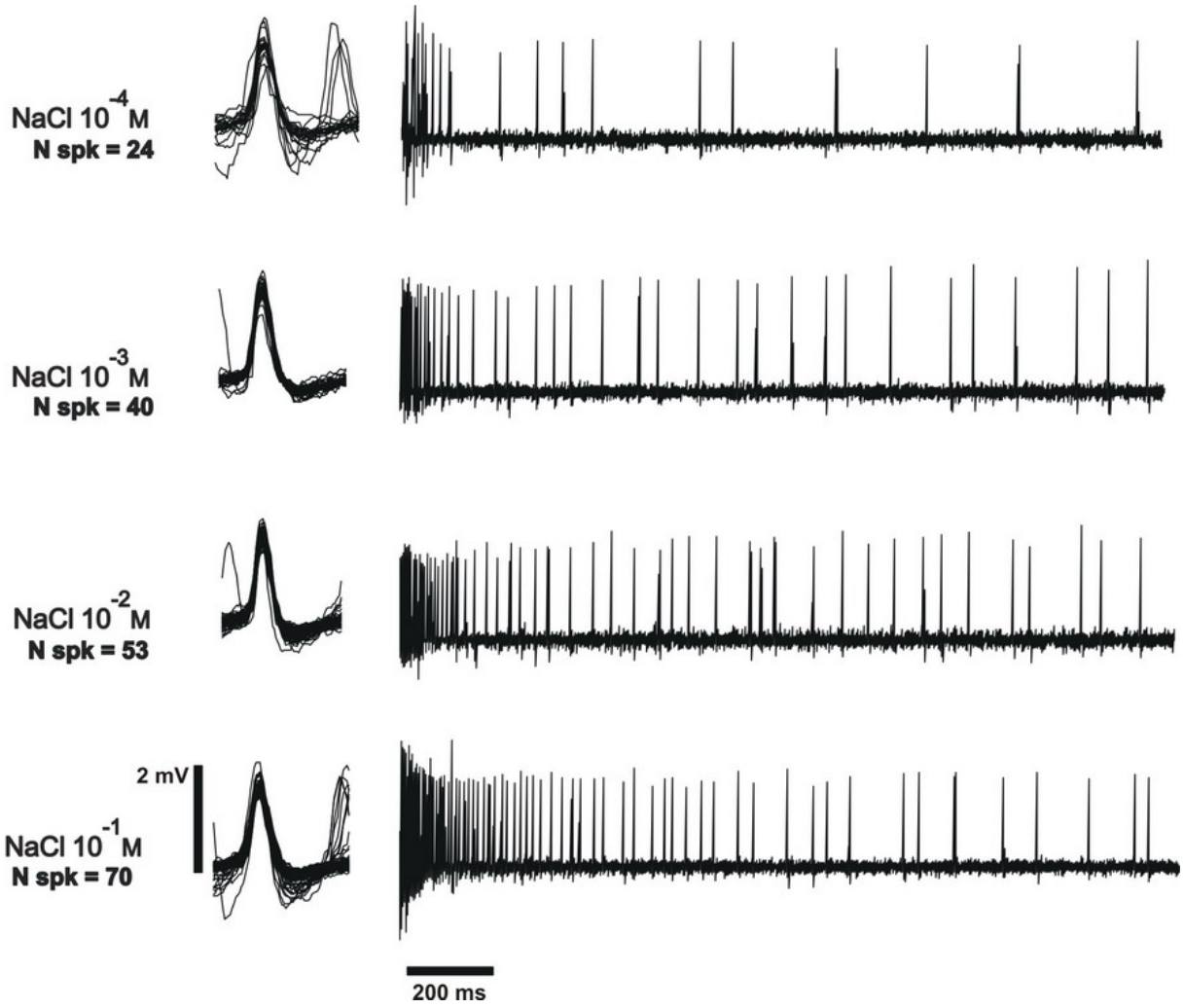

Figure 4. Electrophysiological recordings of GRN from one lateral sensilla in response to increasing concentrations of NaCl. A single GRN is firing.

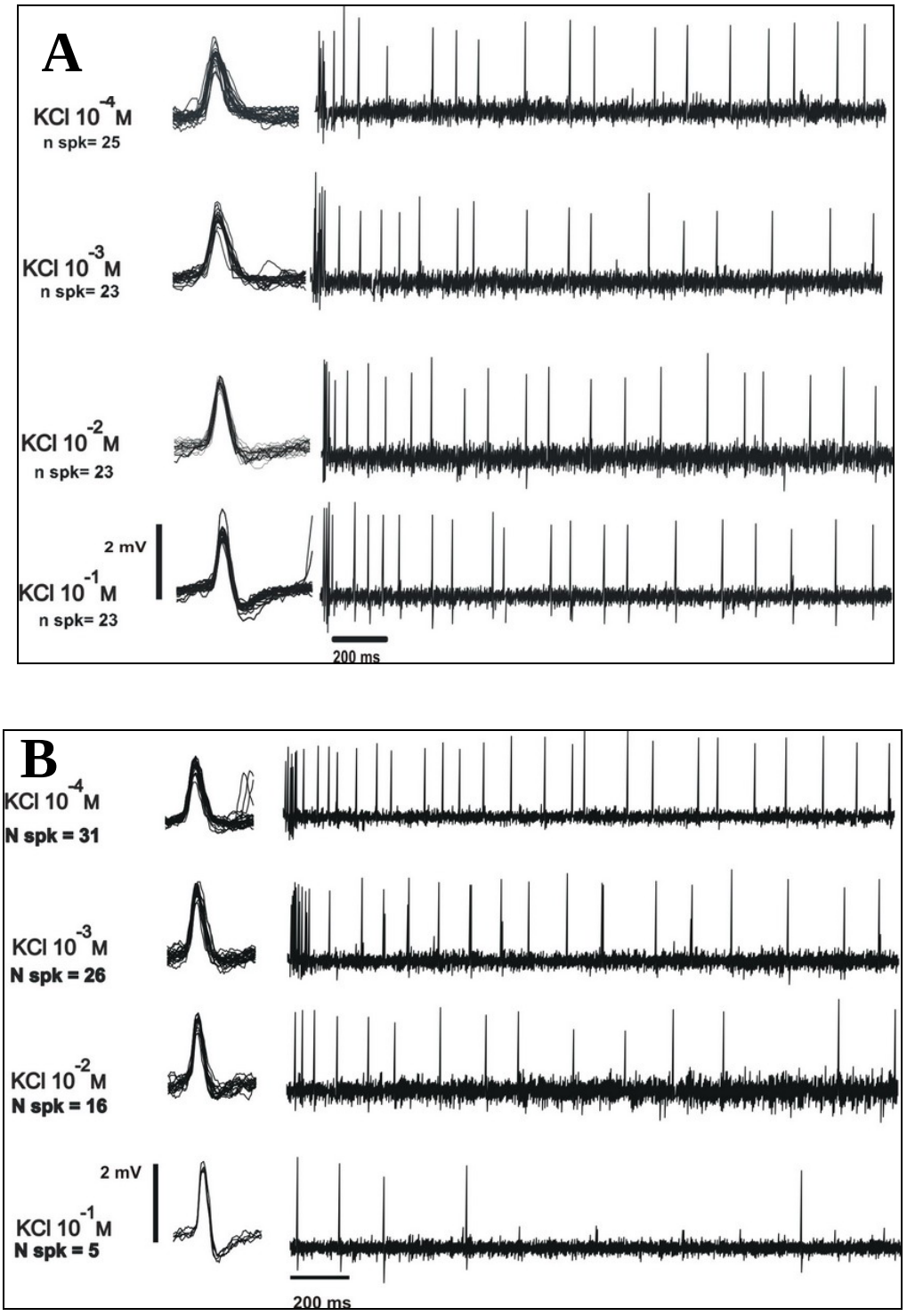

Figure 5. Electrophysiological recordings of GRN in response to increasing concentrations of KCl. A. No variation of the firing rate while the concentration of the tested solution is increased. B. Inhibition of the firing cell when the KCL concentration is increased.

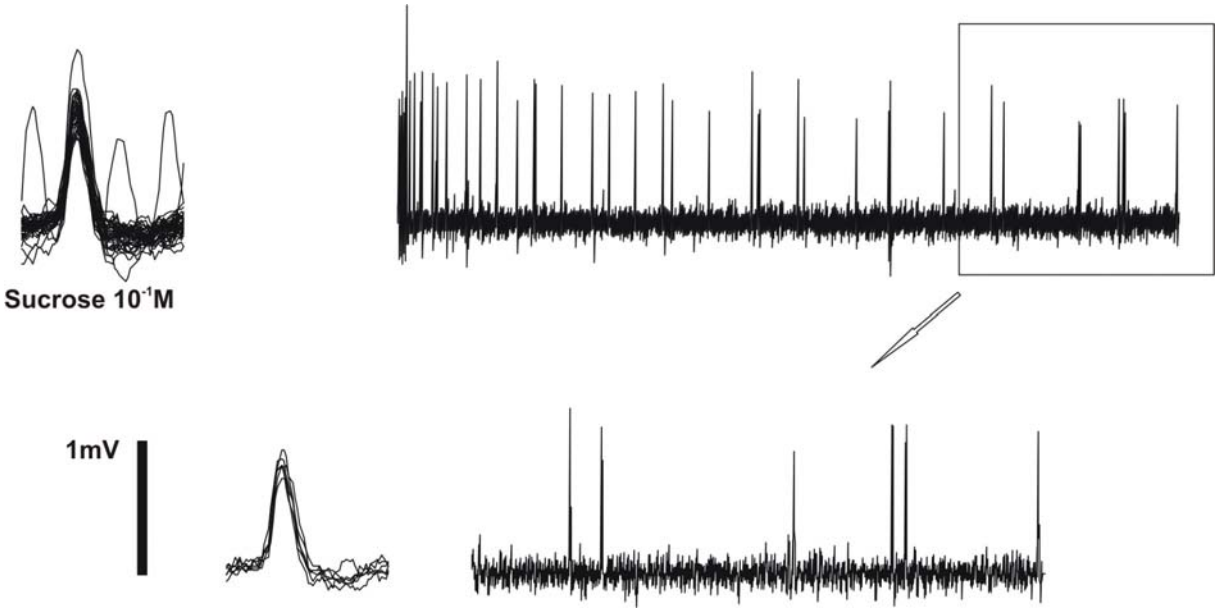

Figure 6. Electrophysiological response to sucrose stimulation showing neurons of similar amplitudes.

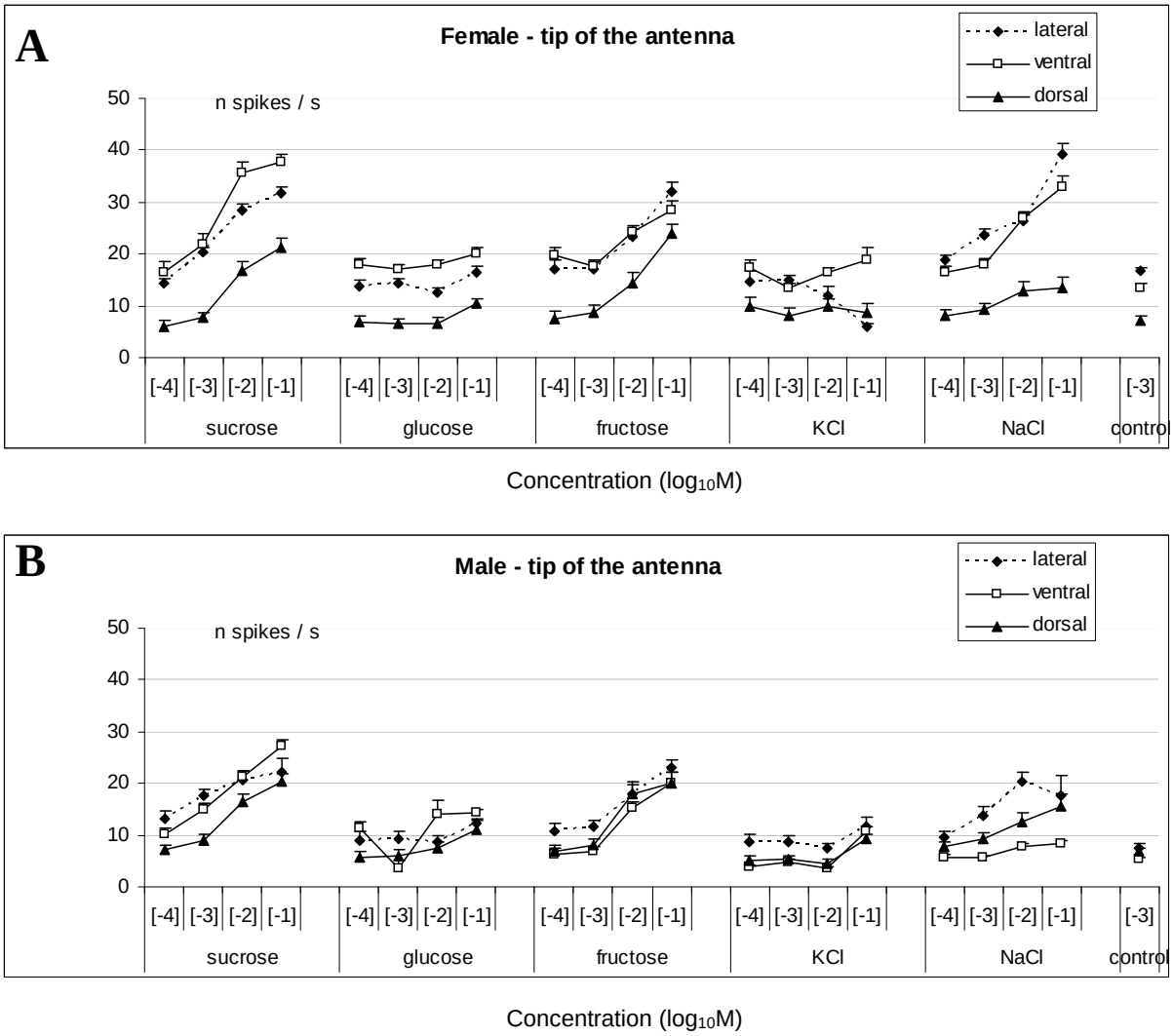

Figure 7. Dose-response curves from lateral, ventral and dorsal sensilla in response to sucrose , fructose, glucose, KCl and NaCl A. In females (N sucrose= 25-70, N fructose = 20-40, N glucose = 35-65, N KCl= 20-40, N NaCl = 50-80, N control = 80-100). B. In males (N sucrose= 20-45, N fructose = 20-35, N glucose = 6-20, N KCl= 25-30, N NaCl = 40-85, N control = 20-70). Error bar indicates the standard error.

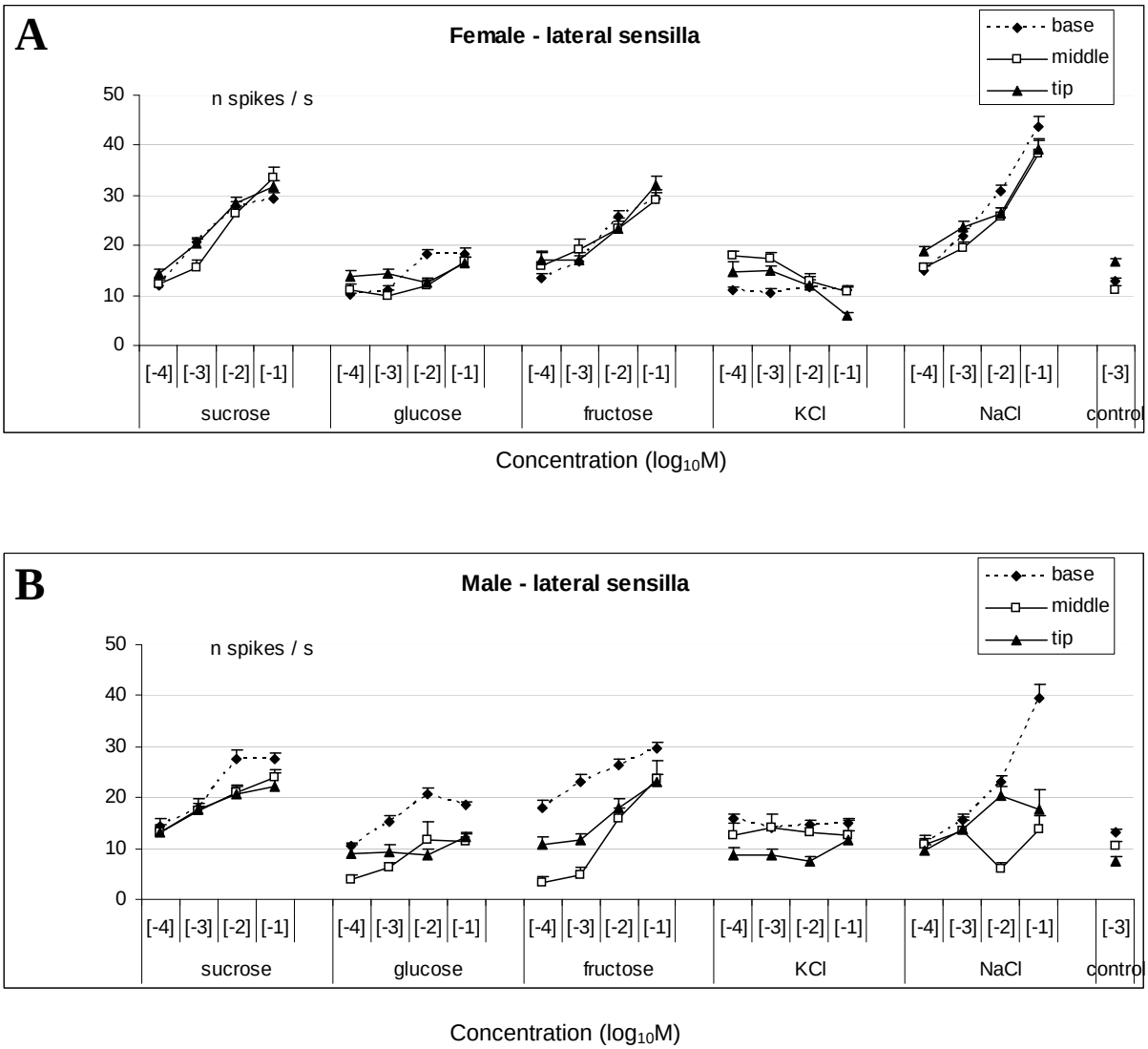

Figure 8. Dose response curves from lateral sensilla in response to sucrose, fructose, glucose, KCl and NaCl A. In females (N sucrose= 35-70, N fructose = 30-40, N glucose = 20-70, N KCl= 20-40, N NaCl = 35-80, N control = 40-100). B. In males (N sucrose= 20-25, N fructose = 10-40, N glucose = 10-25, N KCl= 16-30, N NaCl = 22-45, N control = 20-70). Error bar indicates the standard error.

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CHAPTER 4
ELECTROPHYSIOLOGICAL RESPONSES OF ANTENNAL GUSTATORY RECEPTOR NEURONS TO APPETITIVE AND AVERSIVE COMPOUNDS

Abstract

The survival of all animals depends on the discrimination between beneficial and noxious compounds. From our previous work, we know that taste neurons from the antennae of the moth *Spodoptera littoralis* are activated by sugar and salts. We examined here whether these neurons respond to a series of 14 compounds, including amino-acids found in the nectar of plants, and several alkaloids. We did not record any excitatory response with this series of 14 compounds, most of them inducing rather a dose-dependent inhibition of the firing activity. We could identify a "water neuron" and one taste neuron that might respond to both salt and sugars. The absence of excitatory responses to alkaloids and amino acids is striking because some of these substances are biologically active. This might suggest that antennal inputs play a minor role in detecting appetitive or aversive molecules in this insect.

Introduction

The antennae are complex sensory structures helping the insect to find out more about its environment. They bear many sensilla of different sensory modalities. While the main function of the antenna is olfactory, it carries also numerous sensilla with receptor neurons sensitive to other stimuli, such as touch, taste, temperature and humidity. These stimuli are likely involved in the so-called antennation behaviour, in which contact with a substrate is established. Antennation has been shown to be involved in diverse activities such as spatial orientation, egg laying, food finding and mate recognition in several insects like Lepidoptera, crickets and cockroaches (Balakrishnan and Pollack, 1997; Okada and Toh, 2004; Parra-Pedrazzoli and Leal, 2006).

Detection of tastants varies between different species as a function of the diet breadth and habitat. Bitter compounds have been reported as stimulants of gustatory receptor neurons (GRNs) in insects (Hiroi et al., 2004; Meunier et al., 2003). On the antennae, GRNs responding to bitter compounds are present in the moth *Heliothis virescens* (Jørgensen et al., 2007). In *S. littoralis* we found that most taste sensilla on the antennae are stimulated by sugars (sucrose and fructose) and by salts (NaCl), although in some cases high doses were necessary to elicit a response, and responses to sugars and salts were not always clearly dose-dependent (see chapter 3). For these reasons we wondered if some of the antennal taste sensilla might be tuned to detect other compounds. Other substances besides sugars and salts might be important for survival, such as toxic compounds, appetitive or aversive substances present in food or contact pheromones used for sexual and social communications. Amino acids are a category of substances that can be important for insects; they are abundant in nectar, and the food source of moths. The amino-acids valine, proline and serine are among the most prevalent amino acids in nectars (Baker and Baker, 1973). Alkaloids are another potentially important category which elicits aversive behaviour (Dethier and Bowdan, 1989). We also included 20-hydroxyecdysone (20E) in this test, because this molecule had been shown to be detected as a bitter molecule by *S. littoralis* larvae (Marion-Poll & Descoins).

The main limitation we encountered in our previous study (see chapter 3) was that we could not sort the spikes within the recordings. Sorting spikes is a difficult task because of the anatomy of taste sensilla and of the limitations of the electrophysiological techniques available. Each taste bristle houses up to four gustatory receptor neurons that respond to specific stimuli (Koh et al., 1995; Ozaki and Tominaga, 1999). In some insects, the spikes elicited from these four neurons are of different sizes and shapes, which makes possible to separate them. This is the case for many taste sensilla of *Drosophila*, where each gustatory receptor neurons has been classified according to its best stimulus: S for sugar, W for water, L1 for low concentrations of salts and L2 for high concentrations of salts and for bitter compounds (Hiroi et al., 2004; Meunier et al., 2003; Singh, 1997). This is also the case for taste receptors on the antenna of *H. virescens*, where 4 gustatory receptor neurons have been described although their best stimulus has not been identified.(Jørgensen et al., 2007)

In order to cope with the difficulty of sorting spikes recorded from the sensilla chaetica on the antenna of *S. littoralis*, we tried two approaches: stimulating with a mixture of stimuli and performing cross-adaptation experiments. In the first approach we applied the test compounds singly at different concentrations in order to discover whether some of them elicit

excitatory responses. After, we applied them as binary mixtures. If two cells responded to the mixture while only one cell responded to the individual compounds previously, this would indicate that the cells detecting these two compounds are distinct. Conversely, if only one cell responded to the mixture, than these two compounds must be detected by the same cell. In the second approach we performed cross-adaptation experiments. During this protocol, a compound "A" was applied to the sensillum at a fairly high concentration for about a minute or more: this adapts the neuron responding to compound A, because if we stimulate the sensillum with A within minutes after the exposure, we should observe no response or a strongly reduced response. If A is replaced with a second compound B and if B is detected by the same neuron, no response to B can be recorded; otherwise, if A and B are detected by separate neurons, the response to B will be unaffected by the stimulation with A.

This chapter reports on a series of attempts to find excitatory responses to some compounds (water, sugar, salts, alkaloids and amino acids) and reports our attempts to better characterize the number of cells active in response to a few stimuli. The chemicals tested have been chosen because of their presence in the diet of adult moths or their use in similar studies done on other insects. The amino acids valine, proline and serine are among the most prevalent amino acids in nectars (Baker and Baker, 1973). The alkaloids tested were frequently used in behavioural and electrophysiological experiments done in our laboratory (Meunier et al., 2003).

Materials and methods

Insects

Larvae of *S. littoralis* (Lepidoptera: Noctuidae) were reared on a semi-artificial medium in our laboratory (Poitout and Bues, 1974). From the third to last instar, larvae were reared individually. Males and female pupae were kept separately in groups of 20-30 at 20-24° C, 55-75% relative humidity, 16:8 h light: dark cycle. Adult moths were kept in plastic containers and provided with a 10% sucrose solution to feed *ad libitum*. Electrophysiological recordings were performed at room temperature on females 12-36 hours after emergence.

Chemicals

Valine, serine, proline, maltose, choline chloride, caffeine, aristolochic acid sinigrin monohydrate and denatonium benzoate were purchased from Sigma-Aldrich Corp. (France). Strychnine nitrate, salicin, and berberine sulfate trihydrate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Quinine hydrochloride was purchased from Tokyo Kasei Chemicals Co. (Tokyo, Japan). 20-Hydroxyecdysone was obtained from SciTech (Praha, CZ). All compounds were dissolved in 1 mM KCl solution prepared using distilled water and stored at -20° C. Solutions for stimulation were stored at 4°C for less than one week.

Electrophysiology

Recordings were performed on taste sensilla located on the last 20 segments at the tip of the antenna of *S. littoralis* females. Adults were fixed with tape on a polystyrene block. The antennae were fixed with tungsten hooks on wax. A reference electrode was placed at the base of one antenna in electrically conductive gel. Taste sensilla were stimulated by capping them with a capillary tube filled with the test solution. We waited 2 minutes between stimulations of the same sensillum.

The number of stimulations performed for each compound varied from 5 to 10 for aristolochic acid, 20-hydroxyecdysone (20E), serine, valine and salicin, 20 to 40 for choline chloride, maltose, proline, strychnine, salicin, berberine, denatonium benzoate and more than 40 for sinigrin, caffeine and quinine. The compounds were used as an ascending concentration series in order to avoid adaptation of the cells. As a control experiment, recordings were made in parallel from tarsal sensilla, stimulated with 20E. These observations were done on 60 individuals.

Electrical activities were recorded using a TasteProbe amplifier DTP-02 (Syntech, DE) and further amplified and filtered (Cyberamp, 320, Axon Instruments USA; 8-poles Bessel band-pass filter: 0.1-30 Hz to 2800 Hz). Data were recorded and stored on a computer with a 16 bits A/D conversion card (DT9803; Data Translation, USA) under the control of a custom software, dbWave. Each recording lasted two seconds and was triggered by a pulse delivered by the amplifier on the initial contact of the electrode with the sensillum. Recordings were then analyzed using dbWave, in order to detect and sort spikes according to their amplitude

and shape using interactive procedures. Responses to the different stimuli were evaluated by counting the total number of spikes during the first second of the recording.

The separation of the different GRNs within a sensillum proved difficult because the amplitude of spikes varied with the concentration. As an attempt to solve this problem we tested a different recording technique using a tungsten electrode inserted at the base of the sensilla. This technique did not allow a better discrimination than the tip recording technique of the different GRNs. All results presented below are based on the tip-recording technique.

Cross-adaptation test

In order to verify if the same taste neuron responds both to sugars and salts we performed cross-adaptation tests as follows. The response to a first stimulus (100 mM NaCl) was evaluated over a group of lateral sensilla. Five minutes later, one of these sensilla was capped with 100 mM sucrose for 5 minutes. Immediately after adaptation to sucrose, we recorded the response of this sensillum to sucrose (100 mM) and then to the initial stimulus (100 mM NaCl).

Results

Responses to single amino acids and alkaloids

None of the amino acids and alkaloid and aversive molecules like 20E elicited responses like previously shown for sodium chloride and sugars where the firing rate increases with concentration (Fig. 1). In all of our recordings, we detected the spiking activity of a single GRN. The activity of this GRN was inhibited by increasing concentrations of choline chloride, aristolochic acid, berberine, denatonium benzoate, serine, sinigrine, strychnine, quinine and caffeine (Figs. 1, 2). Proline, valine, salicin, maltose and 20E did not affect its firing rate over the concentration range tested (Figs. 1, 3). When high concentrations (10 mM) of quinine were used, we observed an erratic baseline and an irregular firing of action potentials, otherwise the number of spikes increased with decreasing concentrations (Figs. 1, 4).

In order to test if this response pattern was specific to antennal sensilla, we performed a few recording from taste sensilla on the tarsi, which were described earlier in the literature.

Contrary to the antenna where 20E did not induce any response, we found sensilla on the tarsi, which showed an excitatory and dose-dependent response to 20E (fig 5).

Responses to mixtures of salts and sugars

In order to identify the individual neurons stimulated by salts and sugars, two approaches were tried. First, we used a mixture of salts and sugars of different concentrations along with single compounds with the aim of determining the number of GRNs stimulated by each compound. When using increasing concentrations of NaCl, sucrose and a mixture containing equal concentrations of NaCl and sucrose, the number of spikes elicited by NaCl, sucrose, or the mixture at 0.1 mM and 1 mM is similar for the three stimuli. At 10 mM and 100 mM the number of spikes elicited by the mixture is higher than the number elicited by each of the 2 components alone, but is smaller than the sum of the number of spikes elicited by NaCl and sucrose alone (Fig. 6). Even in the mixture experiments, we were not able to unambiguously identify the number of neurons firing in all recordings. In the recordings that we could analyse (72 out of 187), we observed one neuron firing when the sensilla were stimulated with NaCl, two neurons firing when stimulated with sucrose and at least two neurons firing when the mixture was used.

Responses to sodium chloride after adaptation to sucrose

The second approach was a cross adaptation test in which GRNs were adapted to sucrose in order to see if the response to salts was modified. The adaptation of GRNs within individual sensilla with sucrose did not change the firing frequency observed in response to NaCl (Fig. 7). This result indicates that sucrose and NaCl are detected by different GRNs within the same sensillum.

Discussion

Responsiveness of antennal taste neurons to amino acids and bitter compounds

In all of our recordings, one neuron was active when using low stimulus concentrations. None of the substances seemed to induce an increase of the firing rate when the concentration of the compounds was increased, contrary to what was shown for NaCl (chapter 3).

All alkaloids tested (except salicin) and 20E inhibited the firing cell with increasing concentrations. Alkaloids and antifeedant molecules are of considerable interest because of their supposed key role in food selection, especially for phytophagous insects (Glendinning et al., 2001). Lepidopteran caterpillars posses a neuron responding to bitter substances, which is correlated with feeding inhibition and thus called "deterrent cell" (Glendinning and Hills, 1997; Marion-Poll and Descoins, 2002; Schoonhoven and van Loon, 2002). In adult *S. littoralis* we did not find such GRNs on the antennae, but the presence of GRNs responding to sinigrin and quinine has been reported recently on the antennae of the moth *H. virescens*(Jørgensen et al., 2007). In our case both quinine and sinigrin inhibited the firing cell.

20-hydroxyecdysone (20E) is one of the most common phytoecdysteroids. Phytoecdysteroids are secondary metabolites that affect the growth and development of insects and therefore their detection can be useful to phytophagous insects. They are known to inhibit feeding behaviour in some larvae of Lepidoptera like *Pieris brasicae* (Ma, 1969) and *Bombyx mori* (Tanaka et al., 1994). In *Ostrinia nubilalis* larvae 20E stimulates the deterrent cell (Marion-Poll and Descoins, 2002). In *S. littoralis*, we did not notice any change in the firing rate when the antennae were stimulated with increasing concentrations of 20E. GRNs on the tarsi of *S. littoralis* moths, however, were excited by 20E, suggesting that 20E might play an inhibitory role either for feeding or oviposition and that tarsal and antennal sensilla have clearly different response spectra in *S. littoralis*.

Most adult Lepidoptera feed on flower nectars which contain sugars, various levels of free amino acids, proteins, lipids, antioxidants, organic acids and other non-nutritive substances (Gardener and Gillman, 2002). We have shown in a previous work that antennal GRNs respond to sucrose, fructose and in a lesser extent to glucose. In this work we did not find any GRNs responding to maltose. Even if neurons responding to some amino acids have been previously described in the literature, we found no specific responses to the amino acids tested (proline, serine and valine).

Interpretation of the inhibitory effects

Neurons with a firing activity when stimulated with water and inhibited by an increase in osmolarity have been described as "water" cells (W cells) (Evans and Mellon, 1962). If the cell that fires at low concentration is the W cell, then the inhibition of firing can be due to the osmolarity of the substances tested or to a direct interaction with the transduction

pathway.The activity of the W cell was tested using choline chloride, which is not a stimulant by itself. Choline chloride provoked an inhibition of the cell supporting the hypothesis that the active cell was a W cell. Most likely, the decreasing firing with increasing concentrations of aristolochic acid, berberine, denatonium benzoate, serine, sinigrine, strychnine, quinine and caffeine reflects responses of the W cell to increasing osmotic pressure and indicates a lack of response to the tested substances.

The "flat" dose-response curves of GRNs stimulated with certain amino acids might reflect, however, a combined inhibition of the W cell and excitation of another neuron, resulting in a constant firing rate over a large range of concentrations. Although we cannot confirm any clear response to the tested substances in this study, there might be “hidden” responses to some of them because the corresponding cell(s) may have been masked by the activity of the water cell. In addition, the spectrum of tested substances was limited and other compounds within these categories might play a more important role for adult *S. littoralis*.

We observed erratic baseline and an irregular firing of action potentials following a contact with quinine at high concentrations. Similar effects were described in the blowfly (Dethier and Bowdan, 1992), in the fruit fly (Meunier et al., 2003), *H. virescens* (Jørgensen et al., 2007) and in other insects (Schoonhoven and van Loon, 2002). Amphiphilic molecules such as bitter compounds are known to cross the membrane. Previous studies on bitter taste transduction on vertebrates suggest that bitter compounds could directly interact with the transduction pathway of taste neurons by inhibiting a phosphodiesterase (Rosenzweig et al., 1999) or by activating G proteins (Naim et al., 1994). Our observations are in line with the hypothesis that the irregular bursting activity could be due to a direct action of some bitter compounds on the transduction pathway.

Responses from individual neurons within a sensillum

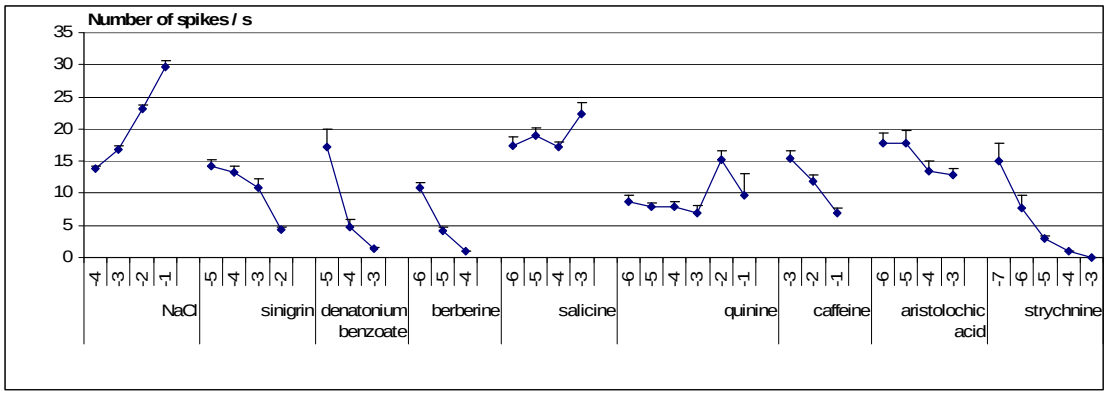
The second goal of this study was to identify responses from individual GRNs. In our previous study we showed that salts and sugars elicited a response from the GRNs on the antennae but we didn’t identify the GRN responding to these substances. Because no new GRN was identified, we tried to better characterise the cells responding to NaCl and sucrose. We tested the tungsten recording technique for this purpose but found that it did not facilitate the GRNs identification because of a high variability of the shape and amplitude of the spikes (fig 8). Using mixtures of both stimulants and a cross adaptation test proved more informative. The cross adaptation test has shown that by adapting the neurons to sucrose the

response to NaCl was not affected. This suggests that the two substances are detected by different GRNs. However, the stimulation with mixtures of the two compounds have shown that the number of neurons firing when stimulated with the mixture is equal to the number of neurons stimulated by sucrose (2) and the total number of spikes elicited by the mixture of NaCl and sucrose is lower then the sum of the responses elicited by the compounds alone. These observations suggest that the same neuron responds to both NaCl and sucrose. However, the existence of two neurons generating action potentials of similar amplitude cannot be excluded. According to our observations, even the two neurons responding to sucrose can have similar amplitudes and are difficult to separate in some recordings. The conclusion is that either the same neuron is stimulated by both substances, but the detection involves separate transduction pathways for sucrose and NaCl, or that there are two different neurons generating action potentials of similar amplitude, one for NaCl and one for sucrose.

A neuron responding to NaCl and to sucrose has been described in *Drosophila* in one type of sensilla on the labellum (Hiroi et al., 2004) and in grasshoppers (White and Chapman, 1990). A GRN stimulated by NaCl can be a "salt neuron" but can also be a "Na^+" neuron. The sugar chemoreceptor of the cherry fruit fly is specifically sensitive to Na^+ and the cross adaptation with sucrose gave no evidence of a reduced response to NaCl (Städler and Schöni, 1991).

In conclusion, based on our observations we could identify in the antennal taste sensilla of *S. littoralis* one "water neuron", one neuron responding to sucrose and NaCl using two different transduction pathways and a third neuron responding mainly to sucrose. We expect that the fourth neuron is stimulated by toxic compounds, contact pheromones or plant cuticular compounds. This hypothesis will have to be tested in further studies.

Figures and legends for Chapter 4



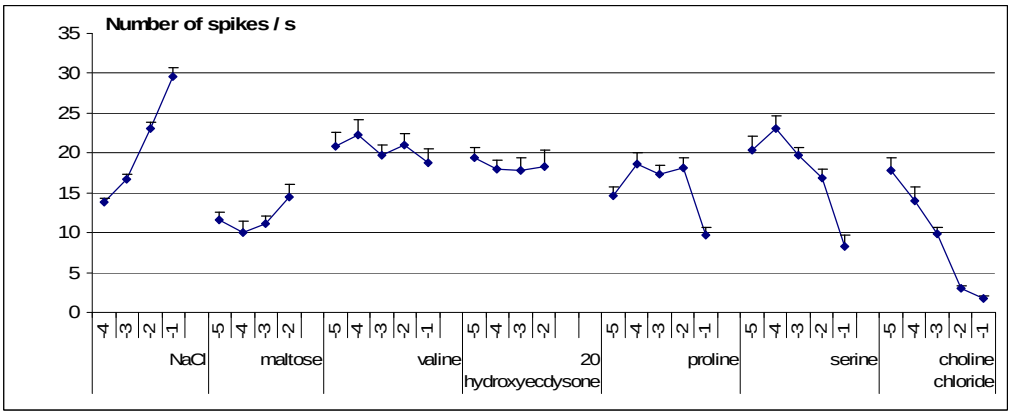


Figure 1. Dose-response curves for different compounds. Concentrations tested from 10^{-5} to 10^{-1} M. N = 5-10 (for aristolochic acid, 20-hydroxyecdysone (20E), serine, valine and salicin), N = 20-40 (for choline chloride, maltose, proline, strychnine, salicin, berberine, denatonium benzoate), N = 40 (for sinigrin, caffeine and quinine). The error bars indicate the standard errors.

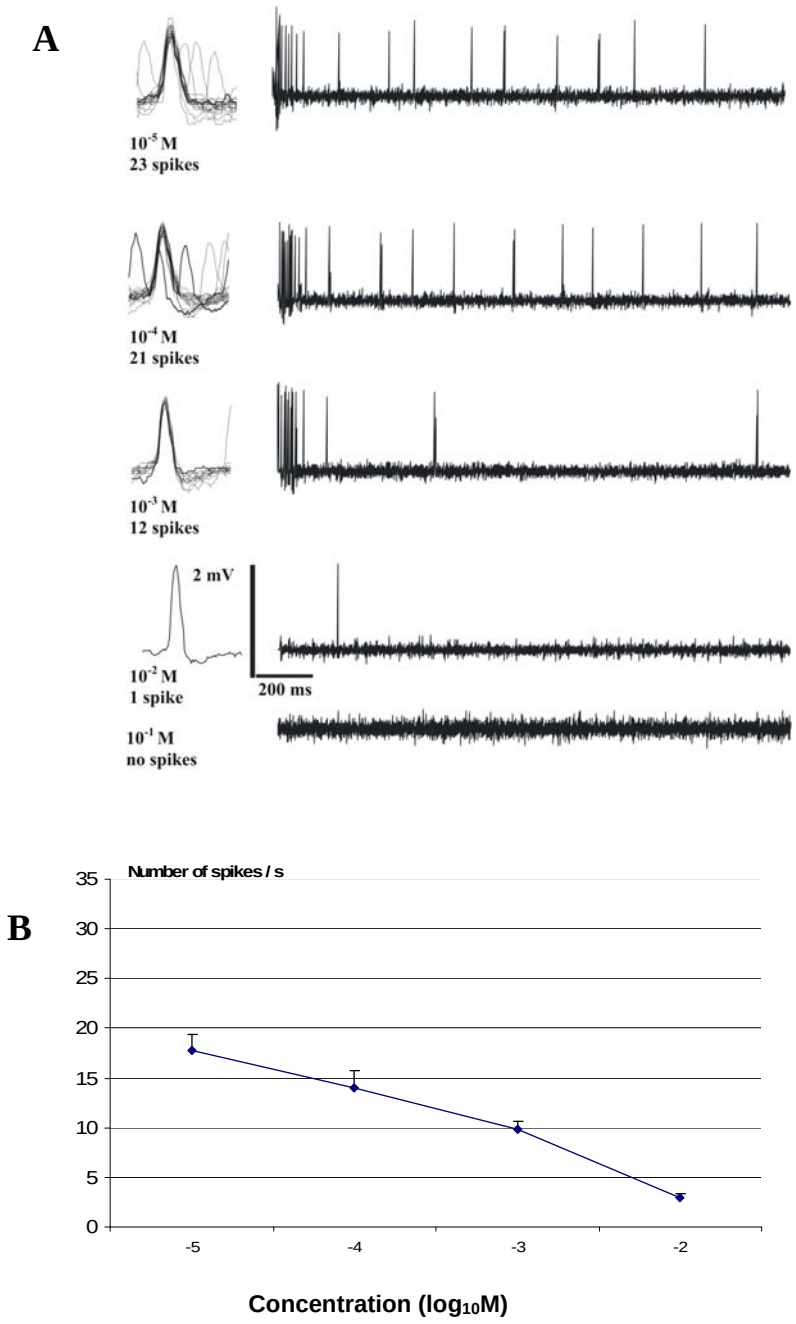
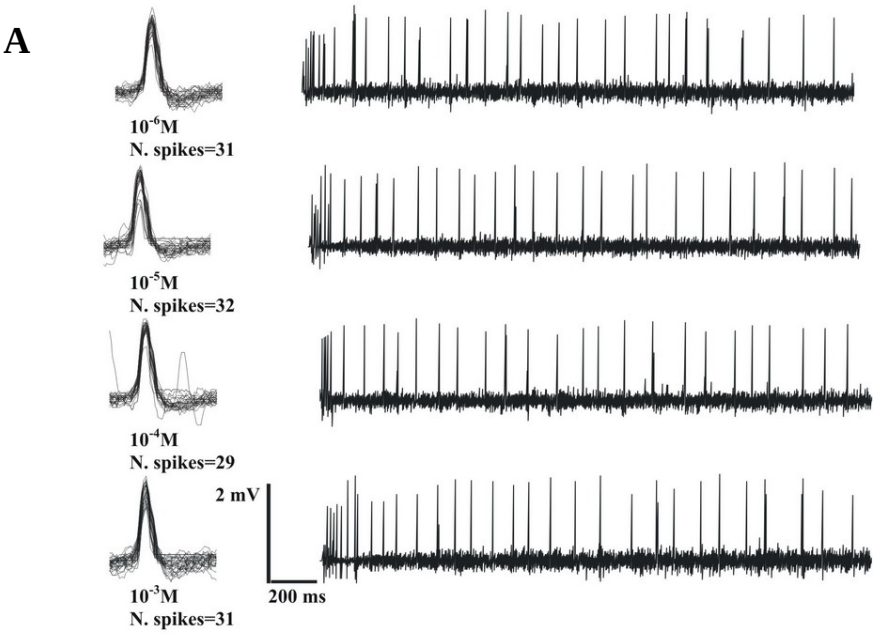


Figure 2. Responses of lateral taste sensilla from the antennae to choline chloride. A. Recordings of GRNs during 2 s in response to increasing concentrations of choline chloride. B. Dose-response curves to choline chloride (N= 25). Error bar indicates the standard error.



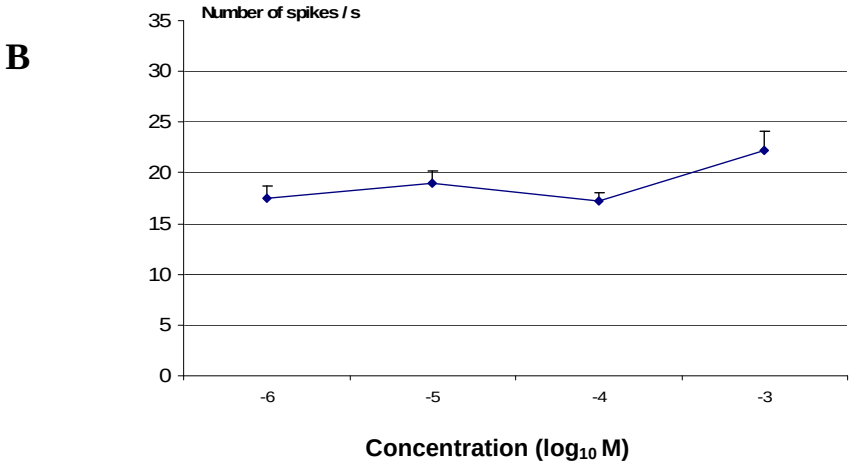


Figure. 3. Responses of lateral taste sensilla from the antennae to salicin. A. Recordings of GRNs in response to increasing concentrations of salicin. B. Dose-response curves to salicin (N= 25). Error bar indicates the standard error.

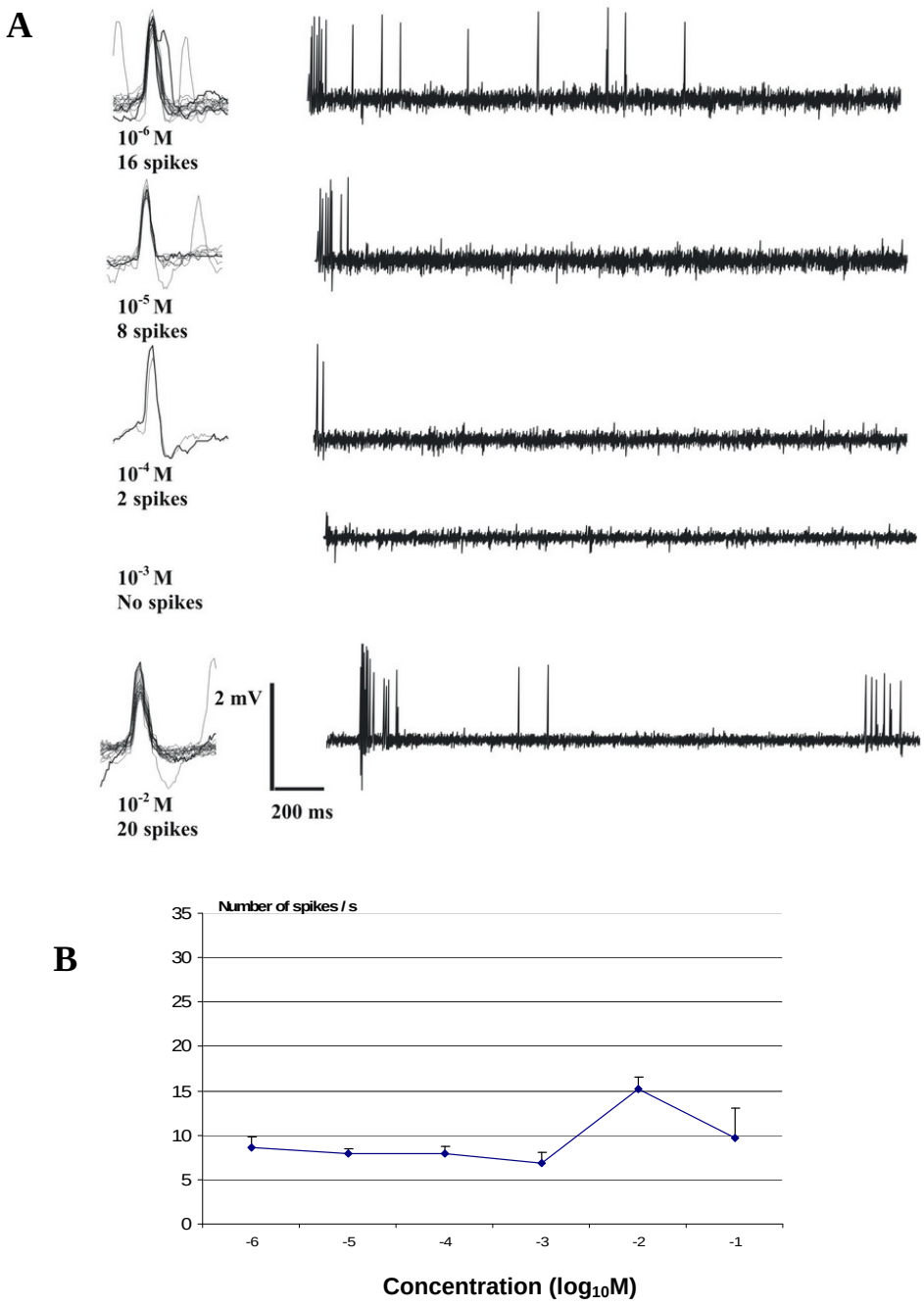


Figure 4. Responses of lateral taste sensilla from the antennae to quinine. A. Recordings of GRNs in response to increasing concentrations of quinine. B. Dose-response curves to quinine (N= 50). Error bar indicates the standard error.

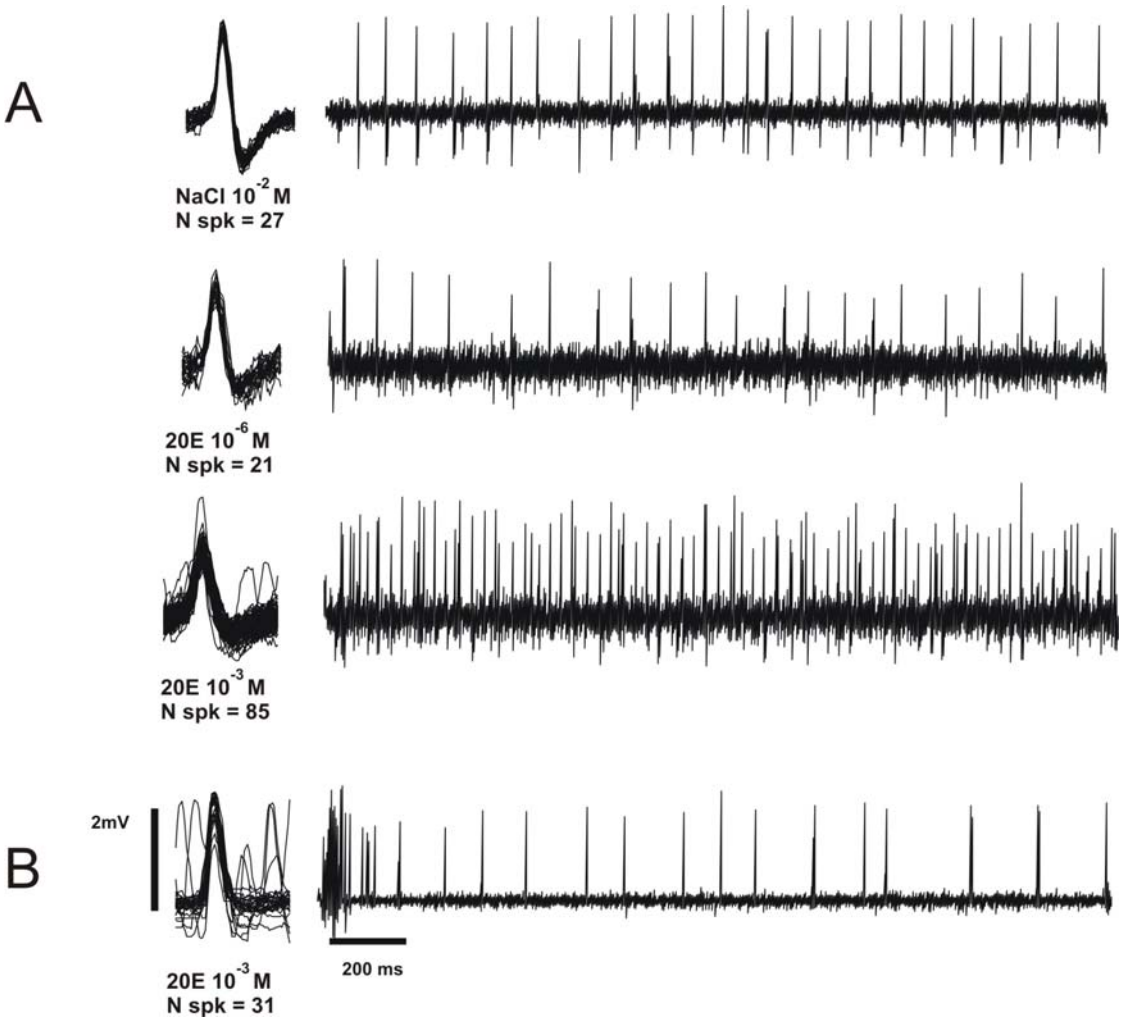


Figure 5. Electrophysiological responses to NaCl (10^{-2}M) and 20 hydroxyecdysone (10^{-6} M and 10^{-3}M) stimulation on the tarsi (A) and 20 hydroxyecdysone (10^{-3}M) stimulation on the antenna (B).

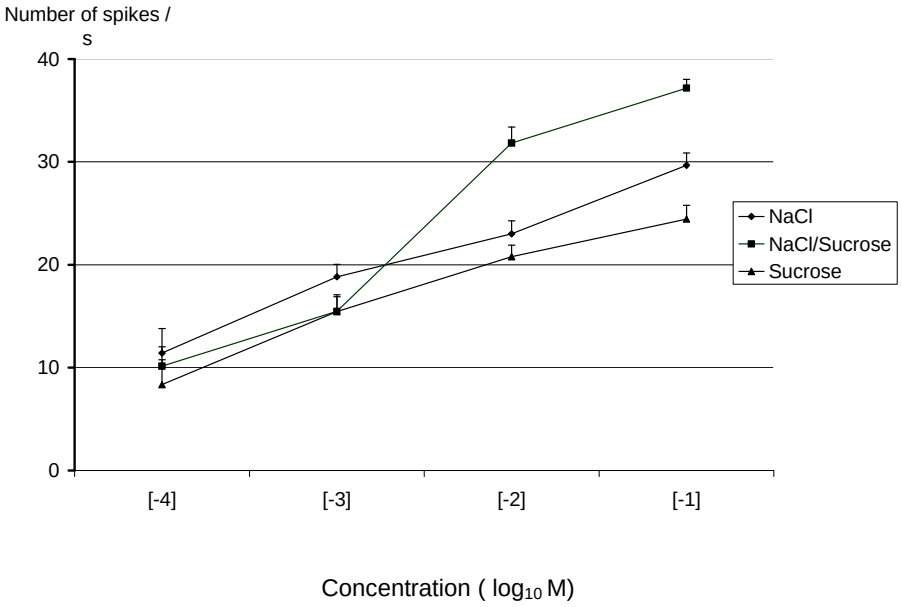


Figure 6. Dose-response curves to NaCl (diamonds), sucrose (triangles) and a mixture of sucrose and NaCl of equal concentrations (squares) (N = 15). Error bar indicates the standard error.

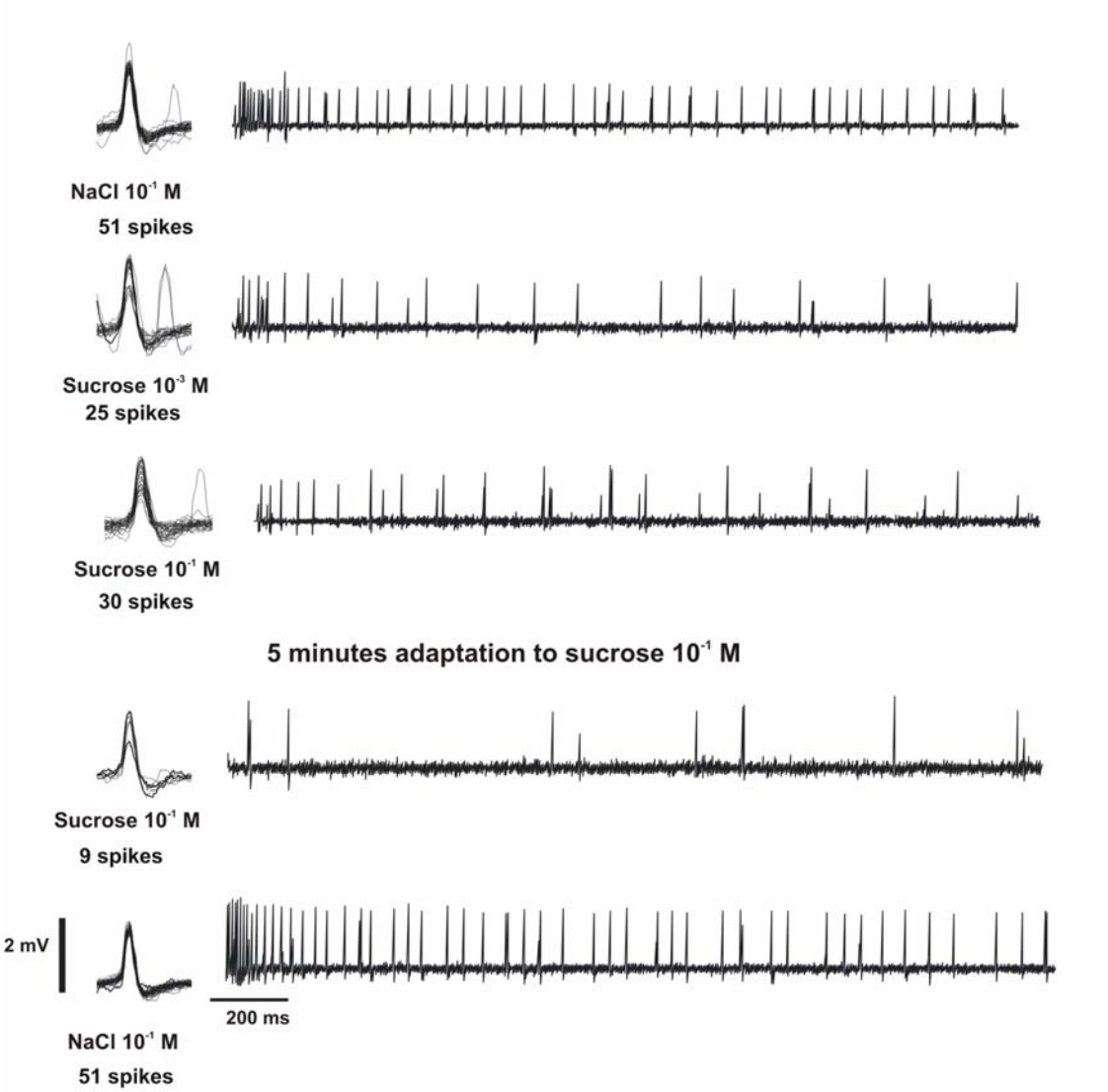


Figure 7. Cross-adaptation test between sucrose and NaCl on antennal taste sensilla.

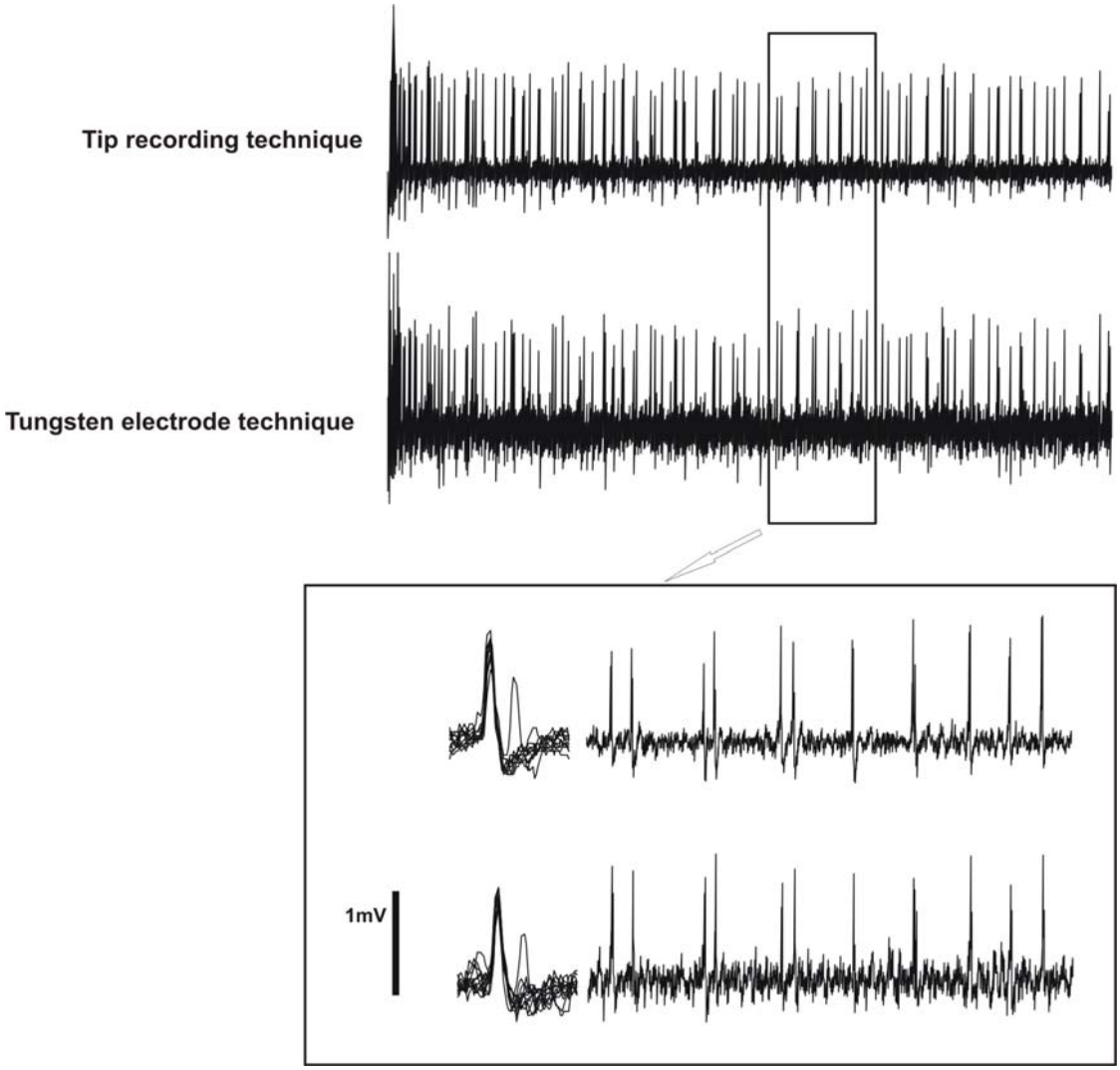


Figure 8. Electrophysiological responses to 0.1 M sucrose recorded simultaneously on the same lateral sensilla using the tip recording technique and the tungsten electrode technique.

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CHAPTER 5
ANTENNAL PROJECTIONS FROM TASTE SENSILLA IN A MOTH BRAIN

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Key words: gustatory receptor neuron, axon tracing, *Spodoptera littoralis*

Abstract

As a first step to understand multimodal integration in the brain of the moth *Spodoptera littoralis*, an important model organism for chemosensory research, we investigated the central projections of contact chemoreceptive sensilla on the antennae, containing both gustatory and mechanosensory receptor neurons. Mass fills of antennal afferents and backfills of individual contact chemoreceptive sensilla using Neurobiotin revealed four different target zones in the deutocerebrum and in the tritocerebrum/suboesophageal ganglion (TS) complex. One group of neurons project to the antennal motor and mechanosensory centre (AMMC) of the deutocerebrum; these projections are likely correspond to the mechanosensory neurons associated with each taste sensillum. The second deutocerebral projection area was identified posterior to the antennal lobe and two distinct projection areas were found in the TS complex. These three projection areas also receive gustatory afferents. Interestingly, some neurons branched both in the deutocerebrum and the TS complex, indicating that specific information from individual neurons is transmitted to different zones, possibly as part of different sensory maps. In addition, a few projections were found to extend beyond the suboesophageal ganglion towards the thoracic ganglions. We hypothesize that the distinct projection areas of gustatory neurons within individual sensilla reflect the responses of these neurons to different taste modalities.

Introduction

The antennae of moths and other insects are multimodal sensory appendages bearing several types of cuticular sensilla innervated by sensory receptor cells. The most prominent function of moth antennae is to detect odorants involved in intra-specific communication, to find host plants and to search for food. In most insects, including moths, however, receptor neurons housed in other types of sensilla also detect stimuli of other modalities, such as gustatory and mechanosensory information, as well as temperature and humidity (Altner, 1977). Moths, like many other insects, move their antennae actively to detect position, shape, mechanical texture and chemical identity of surrounding objects during various behaviours such as walking/searching for food sources, escaping from enemies, mate recognition and other intra-specific communication (Nishino et al., 2005). Taste and smell are essential for detecting food, mates and noxious stimuli in the environment. Whereas olfaction allows insects to discriminate between a large number of different odours in many different combinations, taste is more elementary allowing only the identification of a few categories of tastants.

Gustatory receptor neurons are housed in taste sensilla and their dendrites are exposed to the environment through a single opening at the hair tip. In each sensillum, up to five contact chemosensory neurons and one mechanosensory neuron can be found (Ozaki and Tominaga, 1999; Singh, 1997). In addition to the antennae, taste sensilla are broadly distributed on the body surface of insects (mouthparts, tarsi, wings and ovipositor), where they are involved in eliciting different behaviours (Chapman, 1982; Dahanukar et al., 2005; Dethier, 1976; Stocker, 1994). Gustatory neurons from the tarsi, the antennae and the mouthparts respond to sugars and their activation elicits proboscis extension (e.g. Menzel and Müller 1996, Fan et al. 1997) whereas activation of the taste neurons on the ovipositor of females with sugars may generate egg-laying behaviour. On the contrary, many secondary plant compounds (steroids, alkaloids) activate “deterrent” taste neurons, which are known to inhibit such behaviours (Schoonhoven and van Loon, 2002). This bipolar function of the taste receptors opens up the question of whether their central projections are clustered in two distinct ensembles (labeled lines) respecting somatotopy or whether the hedonic value of tastants is the result of the integration of the responses of all gustatory neurons (across fiber pattern). This question is still open, one of the reasons probably being that most taste sensilla are bi-modal, containing a mechanosensory and chemosensory neuron each with different response properties whose projections are difficult to differentiate. There are, however,

indications in both vertebrates and invertebrates that primary taste modalities might be represented in separate zones within the central nervous system (Accolla et al., 2007; Newland, 1999; Wang et al., 2004).

All antennal sensilla send their axons to the brain via the antennal nerve. The deutocerebrum receives the majority of the antennal sensory inputs (Homberg et al., 1989). There are two main parts of the deutocerebrum: the antennal lobe (AL) and ventral and adjacent to it, the antennal mechanosensory and motor center (AMMC) which in some insects has been called the "dorsal lobe" (Homberg et al., 1989).

The AL receives all olfactory information (Hansson and Anton, 2000). The neuropil of the AL includes distinct compartments called glomeruli, which have been mapped in several species and can be identified individually on the basis of size, shape and relative position (*e.g.* in moths: (Berg et al., 2002; Greiner et al., 2004; Masante-Roca et al., 2005; Rospars, 1983; Rospars and Hildebrand, 1992; Sadek et al., 2002; Skiri et al., 2005). Each glomerulus receives information from olfactory receptor neurons expressing the same receptor protein (Vosshall et al., 1999), odours thus being represented as a functional map within the AL (Heisenberg, 2003; Hildebrand and Shepherd, 1997)

The AMMC neuropil does not appear to be organized into glomeruli or other distinct subdivisions and its boundaries with the surrounding areas cannot easily be defined (Kloppenburg et al., 1997). This region receives projections of the antennal mechanosensory axons in locusts, cockroaches, honeybees and moths (Homberg et al., 1989; Kloppenburg, 1995) but also some contact chemosensory axons (Jørgensen et al., 2006; Nishino et al., 2005).

Some antennal afferents pass the AMMC region with or without giving rise to arborisations within it and project further into the suboesophageal ganglion /tritocerebrum region (Jørgensen et al., 2006; Nishino et al., 2005), and in some insects further into the thoracic ganglia (Kent and Hildebrand, 1987). In addition to receiving afferents from the antennae, the SOG receives inputs from the gustatory receptor neurons of the mouthparts (Edgecomb and Murdock, 1992; Kvello et al., 2006; Mitchell et al., 1999).

Little is known about functional mapping of chemosensory afferents in general, and about the pattern and distribution of projections of taste neurons from the antennae in the central nervous system. The projection areas described until now for presumptive gustatory afferents from the antennae are the AMMC and the suboesophageal ganglion/ tritocerebrum (Haupt, 2007; Jørgensen et al., 2006; Nishino et al., 2005). There is some evidence in moths for a central organization according to the location of the taste sensilla on different head

appendages: taste neurons from the proboscis in *Heliothis virescens* show a parallel finger-like projection pattern into the suboesophageal ganglion (SOG) anterio-medially with respect to the antennal gustatory neurons (Jørgensen et al., 2006). Lastly, the SOG also receives projections from thoracic afferents (Edgecomb and Murdock, 1992) indicating that in addition to being involved in mapping taste information from antennal and mouth appendages, the SOG plays a role in central integration of gustatory information from different parts of the body.

The present paper focuses on the projections of the receptor neurons in the contact chemosensory sensilla on the antennae of *Spodoptera littoralis*. This species is used as a model to study intra-specific communication and host plant finding. Whereas long-range orientation is well known (Anderson and Alborn, 1999; Kehat et al., 1976), close range orientation, "decision making" and multimodal integration have been very little studied. Our study is an attempt to identify possible areas in the moth brain where multimodal integration involved in intra-specific communication and host plant finding might occur. In parallel we aimed at unveiling the existence of somatotopic and/or chemotopic maps of receptor afferents from gustatory sensilla within the *S. littoralis* brain.

Materials and methods

Insects

S. littoralis moths were reared on a semi-artificial medium in our laboratory (Poitout and Bues, 1974). Larvae were reared individually from the third to last instar. Male and female pupae were kept separately in groups of 40-60 at 20-24° C, 55-75% relative humidity, 16:8 h light:dark cycle. Male and female adult moths were kept separately in plastic containers and provided with a 10% sucrose solution to feed *ad libitum*. Both male and female adults were used in the experiments and no difference was found in the projection patterns of gustatory and mechanosensory neurons from antennal sensilla between the sexes.

Staining of gustatory receptor neurons

Insect preparation

The insects were placed in a plastic tube and the head was immobilized with wax. The antennae were left free for mass staining and attached to a wax platform with tungsten hooks for single sensillum staining.

Staining

Mass staining was performed on the flagellum of *S. littoralis*. The flagellum was cut distally, medially or proximally and the cut end was covered with a glass capillary filled with a 1% Neurobiotin (SP-1120, Vector Laboratories, Inc., Burlingame, USA) solution in ultrapure water.

Staining of single sensilla was performed by cutting one sensillum in a pool made of vaseline and filled with distilled water. After 6 minutes the water was removed and replaced by a 1% solution of Neurobiotin. The preparations were placed in Petri dishes moistened with a wet piece of tissue and kept for 7-10 days in the refrigerator at 4°C to allow diffusion of the dye. The brains were dissected and fixed in 4% paraformaldehyde (over night at 4ºC) and rinsed in phosphate buffer (Millonig's) containing 0.25% Triton X. The brains were then dehydrated and rehydrated to make membranes more permeable. Subsequently they were rinsed in buffer (3 times 10 minutes) and incubated over night at 4ºC in Millonig's buffer containing 0.25% TritonX, 1% bovine serum albumine and Oregon green-avidin conjugate (Oregon Green®, Invitrogen ™, France). Finally, the preparations were rinsed in phosphate buffer and transferred in vectashield medium (Vectashield® Mounting Medium, Vector Laboratories, ABCYS, France).

Confocal laser scanning microscopy

The projections of stained receptor neurons were examined with a confocal laser-scanning microscope equipped with an Argon laser (Leica SP2 AOBS, Leica Microsystems Heidelberg, Germany) using a 10× dry objective. The brains were scanned in frontal plane with a 1.5 µm step size and 1024×1024 resolution for single staining and 3 µm step size and 512×512 resolution for mass staining.

Reconstructions

The optical sections were used for the creation of three-dimensional reconstructions using a custom-made program in Matlab (Louise Couton, Kiên Kiêu, Jean-Pierre Rospars). Some neurons were manually reconstructed from confocal stacks to visualize details of arborisation patterns. Maximum projections of optical sections were obtained through stacks transferred to ImageJ software (NIH, USA).

Results

Mass-staining of receptor neuron projections

By applying neurobiotin on the cut antennae, 39 successfully stained preparations were obtained in which axons reaching the brain through the antennal nerve were visible. Of these 39, 14 out of 25 attempts were from the base of the antenna, 19 out of 24 from the middle of the antenna and only 6 out of 24 from the tip (table 1). . The axons from olfactory neurons projected into the antennal lobes, the others bypassed the AL posterio-laterally and projected ipsilaterally in four areas.

The first area (A1) is situated in the deutocerebrum posterior to the antennal lobes and close to the oesophagus (Fig. 2A, D). These axons left the axon bundle coming from the antennal nerve before it reached the AMMC region. In most of the preparations we observed a fork-like shape of the axon branches in area A1 (Fig. 2A, D). The second projection area is the AMMC (A2), which is known to be the primary centre for the processing of mechanosensory information from the antennae (Fig. 2B, D). Some axons arborised within the AMMC and then projected postero-medially into the SOG as a finger-like projection with very few branches, targeting a third projection area (A3) (Fig. 2B, C, D).

The fourth area (A4) concerned the axons leaving the AMMC with very few or no arborisations and projecting postero-medially into the SOG/tritocebrum region. These axons gave rise to dense arborisations dorso-laterally to the endings of the "finger-like" projections in area 4 (Fig. 2C, D). In one preparation this type of neuron sent one branch to the deutocerebrum in the area situated postero-medially to the AL (data not shown). In two preparations where the proximal part of the connectives to the thoracic ganglia were present we could observe one axon going further from the SOG to the thoracic ganglia (data not shown).

We compared the mass stainings obtained by cutting the antennae at different levels (table1). No evident correlation of the projection patterns according to the location of the stained sensilla on the flagellum could be found (table1).

Table 1. Projection areas in the CNS given by stained sensilla from the antenna cut at the base, middle and tip[a].

	Area 1[b]	Area 2[c]	Area 3[d]	Area 4[e]
Base	33% (15)	90% (15)	100% (15)	40% (15)
Middle	25% (19)	100% (19)	75% (19)	60% (19)
Tip	33% (6)	100% (6)	100% (6)	66% (6)

[a] The percentage of preparations with successful staining in the respective area is given. In parentheses the total number of stained preparations analysed. Each half brain was analysed separately in case of bilateral stainings.
[b] Area 1 is situated in the deutocerebrum, dorso-medial to the antennal lobes.
[c] Area 2 is the antenal mechanosensory and motor center located in the deutocerebrum.
[d] Area 3 is located in the SOG and is characterised by a finger-like projection.
[e] Area 4 is located in the dorsal SOG/tritocerebrum.

Neurons originating from individual sensilla

Twenty-eight successful preparations resulted from 77 attempts to stain individual sensilla. Twelve of the successful preparations contained 5 or more stained neurons which means that more than one sensillum was stained, probably due to damages that occurred during the manipulations. One to four neurons could be identified in 16 preparations in which single sensilla were stained. The axons of these neurons ran tightly together when leaving the antennal nerve, bypassing the AL. In eight preparations (out of 16), one axon left the others before entering the AMMC and projected into the postero-medial part of the deutocerebrum, posterior to the AL (area A1 defined in the previous section) (Fig. 3A, 4).

Three other types of axons could be identified. A first type projected only into the AMMC (area A2) The second type showed massive arborisations within the AMMC and projected further postero-medially into the SOG (area A3) (Fig. 3B, 4). Its finger-like process gives rise to very few or no arborisations (Fig 3B, 4). The third type of axons passed through the AMMC with very few or no arborisations inside and projected with widespread arborisations into the SOG/tritocerebrum covering a triangular–shaped area (A4) dorso-laterally to the finger-like projection area (Fig 3C, 4).

Discussion

Both mass-fills and staining of individual taste sensilla on the antennae of *S. littoralis* allowed us to describe 4 distinct projection areas within the central nervous system. All projections resulting from antennal staining were restricted to the ipsilateral side of the brain, as shown in other insects (*Heliothis virescens:* Jorgensen *et al*., 2006; *Periplaneta americana:* Nishino *et al*., 2005). This pattern seems specific to antennal sensilla since projections from taste sensilla on other head appendages can also be contralateral, as shown on the proboscis of *Heliothis virescens* (Jørgensen et al., 2006) and labellar hairs in *Phormia regina* (Edgecomb and Murdock, 1992).

Segregation of mechanosensory and gustatory fibres

In many insects, axons from the mechanosensory neurons have a larger diameter than the fibres from chemosensory neurons. For example, in the locust and in the fly, mechanosensory fibres from gustatory sensilla on the legs have a larger diameter than chemosensory fibres and project in a somatotopic manner into the thoracic ganglia (Murphey et al. 1989, Newland et al. 2000). In our preparations, we could not identify mechanosensory neurons based on the size of their axon diameter. However, we suppose that mechanosensory fibres in *S. littoralis* project mainly to the AMMC/dorsal lobe area, as found in other Lepidoptera and in the cockroach (Camazine and Hildebrand, 1979; Nishino et al., 2005). Mass stainings of the antennae in *Heliothis virescens* have revealed two projection areas for mechanosensory and chemosensory neurons: a fan-shaped region within the AMMC and a finger-like projection within the dorsal SOG. The mechanosensory neuron from individual sensilla in *H. virescens* could be identified because of a larger axon diametre than the gustatory fibres, and it often, but not exclusively, terminated within the AMMC (Jørgensen et al., 2006). Interestingly, in the fly, one or more axons originating from taste sensilla on the legs ascend from the thoracic ganglia towards the brain and arborize in the same region of the SOG as neurons from labellar hairs (Edgecomb and Murdock, 1992; Murphey et al., 1989) .

Separate target areas of gustatory receptor neurons

As in many other insects, in addition to the mechanosensory neuron, every taste sensillum on the antenna of *S. littoralis* contains neurons with different functional roles: one sugar-sensitive neuron, a water cell and a sugar and salt sensitive neuron (see chapters 3, 4). The separate projection areas of gustatory neurons within the same antennal sensillum found in our study

are consistent with the hypothesis that each area gathers neurons with the same response characteristics, representing a form of chemotopic map.

Gustatory neurons seem to target both a deutocerebral region and two areas within the tritocerebrum/SOG. These results indicate that antennal gustatory information is transmitted in parallel to the brain segment corresponding to the antennae, the deutocerebrum, and to the tritocerebrum/SOG, which receives also direct gustatory input from the mouthparts and from neurons passing through and branching in the thoracic ganglia. The tritocerebrum/SOG seems thus to serve not only as primary, but also as secondary integration centre for gustatory information from different parts of the body.

Segregation of the projections as a function of the quality of the taste stimulus has been shown in different insects. In *Drosophila,* neurons detecting deterrent substances, situated in sensilla on the labellum, seem to project bilaterally in the tritocerebrum/SOG and neurons responding to phagostimulants project ipsilaterally (Thorne et al., 2004; Wang et al., 2004). Two antagonistically responding taste neurons have been described in the same sensillum in *Drosophila* (Hiroi et al., 2004). One encodes the presence of attractive stimuli like sugars and salts at low concentrations while the second one responds to aversive stimuli like bitter compounds and salts at high concentration. These observations support the working hypotheses proposed by Chapman (Chapman, 2003) that phagostimulatory and deterrent neurons are the basic labelled lines of the insect taste receptor system and that these lines are represented in different areas of the CNS. These findings are confirmed by studies on central neurons processing information from contact chemoreceptive sensilla. Recordings from SOG interneurons in the fly *Sarcophaga bullata* showed that interneurons responding to NaCl stimulation of the labellar lobes did not respond to sucrose stimulation and *vice versa* (Mitchell and Itagaki, 1992). In *Locusta migratoria*, neurons from the SOG responded both to chemical and mechanical stimulation but the time course of the responses to host plant *versus* non-host-plant stimuli was different (Rogers and Simpson, 1999).

Conclusions

Our study reveals the target areas of gustatory and their associated mechanosensory neurons originating from antennal taste sensilla. Together with the broad knowledge on processing of olfactory stimuli, these data will be important for future research on integration of different sensory modalities in contexts such as host plant evaluation and appetitive or aversive

learning, where olfactory, gustatory and mechanosensory stimuli interact to elicit specific behaviours. Although the honeybee has been the main model for research on appetitive learning associating olfactory and gustatory stimuli (for review see (Menzel and Muller, 1996), both aversive and appetitive learning paradigms are now well established in moths (Fan et al., 1997; Hartlieb et al., 1999; Jørgensen et al., 2007; Skiri et al., 2005) allowing to approach peripheral and central processing of multimodal signals.

Acknowledgements

This study was supported by INRA (Jeune Equipe), a fellowship from the doctoral school ABIES and a bilateral exchange program Aurora (EGIDE).

Figures and legends for Chapter 5

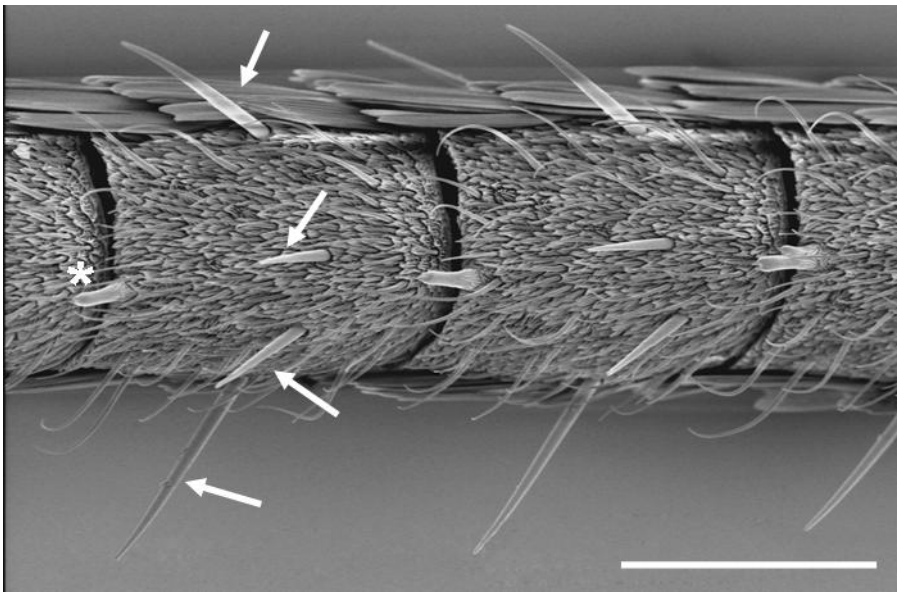

Figure 1. Ventral view of the flagellum showing four *sensilla chaetica* (arrows) on each segment and one sensillum styloconicum at the anterior rim of each segment (asterisk). Scale bar=100 μm.

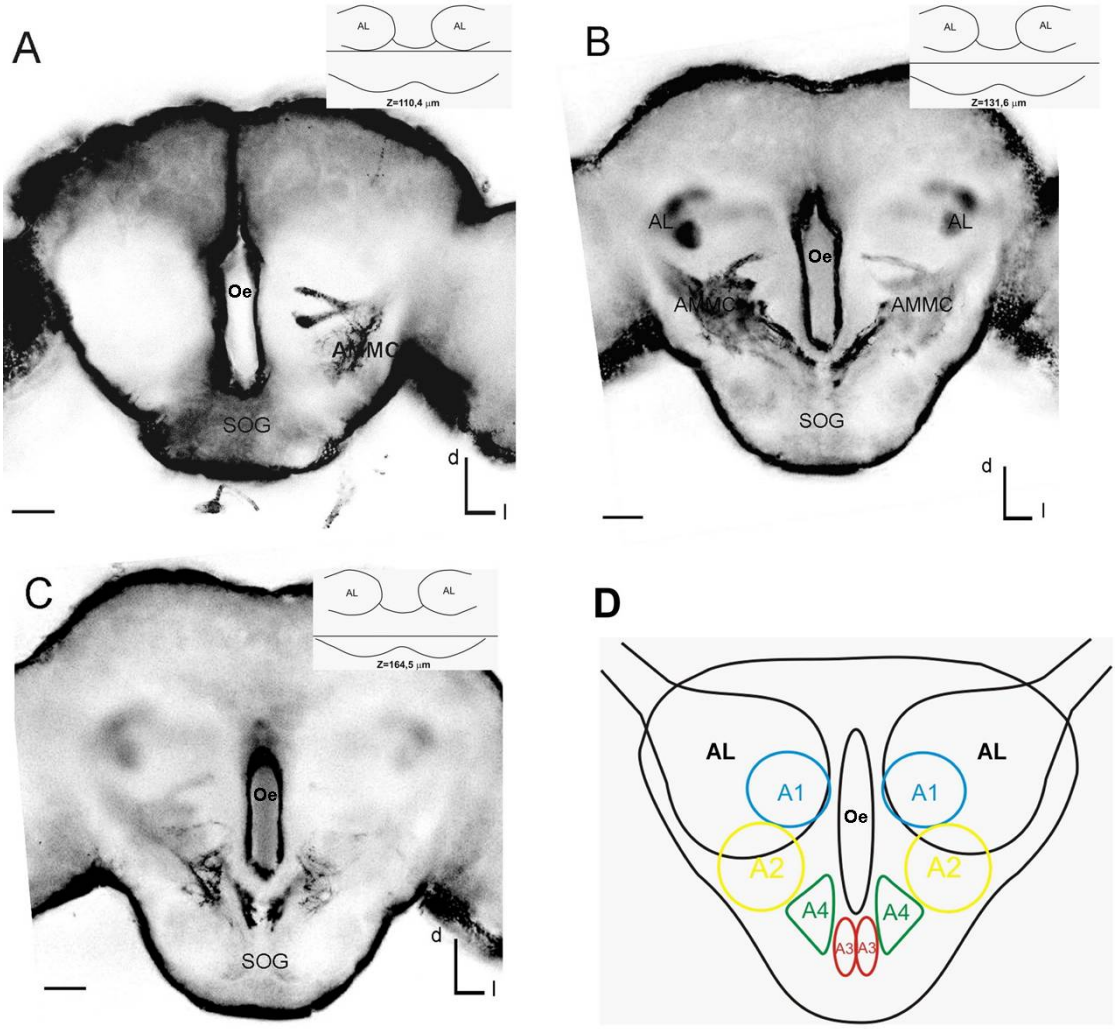

Figure 2. Optical sections and schematic representation of the *S. littoralis* brain (frontal view) with mass stained afferences from the antennal nerve. **A.** Receptor neuron projections in the posterior part of the deutocerebrum, underneath the antennal lobe (AL) (area A1) and in the antennal motor and mechanosensory centre (AMMC, area A2). **B.** Massively stained receptor neurons in the glomeruli of the antennal lobe, the AMMC, and within the medial suboesophageal ganglion (SOG, area A3). **C.** Massively stained receptor neurons, showing projections to the medial SOG and the posterior SOG/tritocerebrum (area A4). **D**. Schematic representation of all projection areas in a frontal view. d dorsal; l lateral. AL antennal lobe, AMMC antennal mechanosensory and motor centre, Oe Oesophagus, SOG suboesophageal ganglion Scale bars=100 µm. Z indicates depth of optical sections.

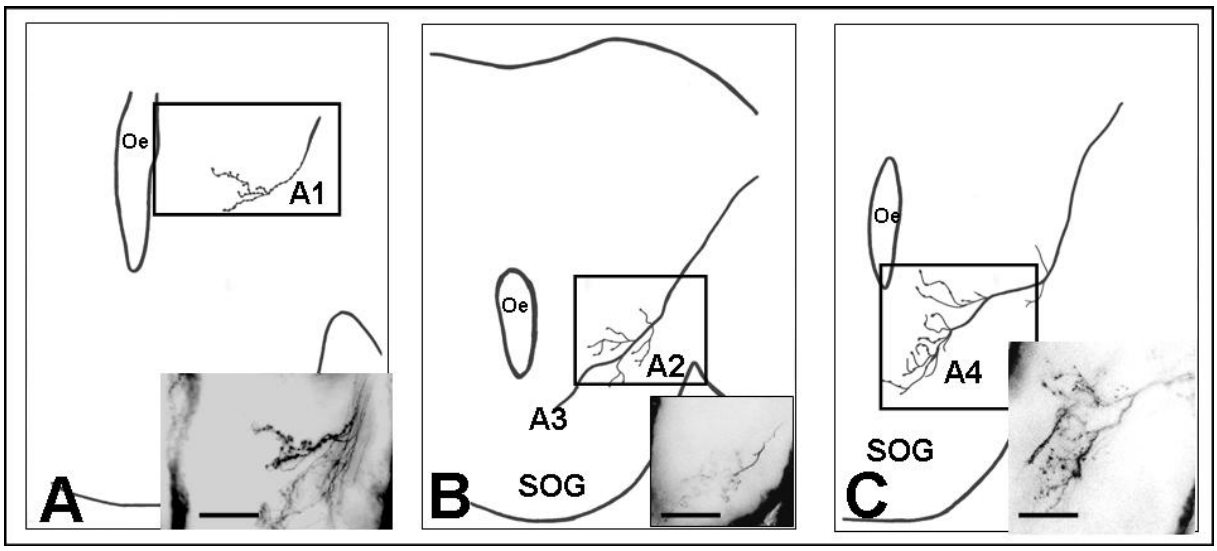

Figure 3. Manual reconstructions of the central projections of individual axons from antennal *sensillum chaeticum*. Insets show partial projections of optical sections from the area indicated in each square. **A**. Axon projecting to the deutocerebrum in an area located posterior to the antennal lobe and close to the oesophagus (A1). **B**. Axon projecting to the SOG region (A3) after giving rise to arborisations in the AMMC area (A2). **C.** Axon projecting into the SOG/tritocerebrum area (A4). Note varicosities on all axonal branches. AMMC antennal mechanosensory and motor centre, Oe Oesophagus, SOG suboesophageal ganglion. Scale bars=75 μm.

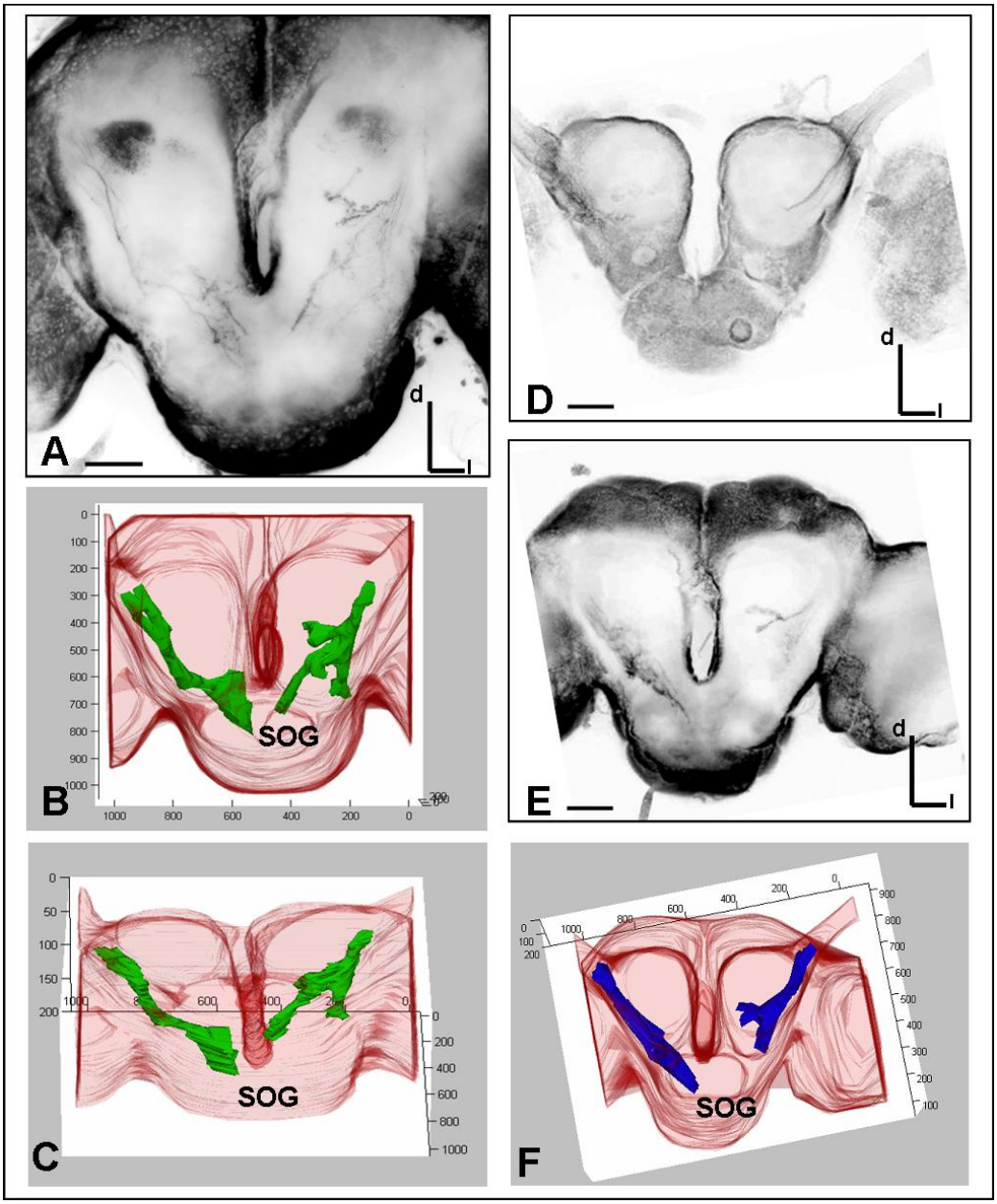

Figure 4. Confocal micrographs of individual gustatory receptor neuron axon projections in *S. littoralis* and 3D reconstructions of the target areas. **A.** Section of a brain showing projections in the AMMC, medial SOG and in the SOG/tritocerebrum (maximum projection of the sections of the posterior 150 µm of the brain). Scale bar=75 µm. **B.-C.** Three-dimensional reconstructions of the preparation in A. **B,** frontal view; **C,** ventral view. **D.** Projection of a 85 µm stack of the anterior part of a different brain, showing receptor neuron axons bypassing the antennal lobe. **E.** Maximum projection of the 150 µm slice posterior to the slice shown in D., showing projections in the medial deutocerebrum, the AMMC, and the medial SOG. F. Three-dimensional reconstruction of the preparation in D and E, frontal view. AMMC antennal mechanosensory and motor centre, d dorsal, l lateral, SOG suboesophageal ganglion. Scale bars 100µm in D and E.

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CHAPTER 6
FINAL DISCUSSION AND CONCLUSION

The present thesis is a first contribution to the analysis of the gustatory system in the moth *Spodoptera littoralis.* Our research unit aims at elucidating the mechanisms involved in the interaction between insects and their chemical environment and *S. littoralis* is one of the main model organisms selected for that purpose. Insect models used are mainly pest species of economic importance in order to contribute to the development of integrated pest management.

My work was focused on the structure and function of the antennal gustatory system. For functional aspects I studied the responses of the taste sensilla to various contact stimuli. For structural aspects I described the projection areas in the brain and the suboesophageal ganglion of the neurons from taste sensilla.

Function: Responsiveness of antennal taste sensilla to various compounds

The functional studies are the subject of chapters 3 and 4. Different compounds were studied in these two chapters:

Sugars and salts

In the third chapter, we asked whether the antennal taste sensilla of the adult moth *S. littoralis* are responding to gustatory compounds (sugars, salts) and if the responses vary as a function of the location of taste sensilla on the antennae and the moth's gender.

Scanning electron microscopic studies showed no sexual dimorphism concerning the distribution of taste sensilla on the antennae. We found 6 taste sensilla disposed symmetrically on each flagellomere: two on the ventral side (the side covered with sensilla), one on each of the lateral sides and two on the dorsal side. This distribution was constant along the antenna with two exceptions: the first 6 segments which bore only the two lateral sensilla and the last segment which has at the very tip 7-8 taste sensilla.

Our electrophysiological observations showed that taste sensilla possess neurons that respond to sugars such as sucrose, fructose and glucose and to NaCl. In the sensilla located on the lateral side of the antenna, we were able to test the sensitivity along the antenna but no differences were noticed. The intensity of the responses varied between the sensilla on the dorsal side of the antennae and the ventral side of the antennae, being weaker on the dorsal side.

Males and females did not show the same sensitivity. The firing frequency in response to sugars and NaCl was higher in females than in males. This might provide females with a more accurate information, which would be useful because females should avoid to lay eggs on substrates containing high concentrations of salt which are toxic to their eggs (Loni and Lucchi, 2001). Our observations suggest that each antennal segment bears taste sensilla with comparable sensitivities and that there are no functional specialisations across the antennae.

Amino acids and bitter compounds

In the forth chapter we attempted to identify the neurons firing in response to other gustatory compounds beside sugars and salts. A few compounds were tested (mainly amino acids and bitter compounds) but no specific neuron was firing in response to these stimulations.

We were able to identify one "water neuron" which decreases its firing with the increase of the osmolarity of the solution tested. This neuron was visible only when the compound tested didn't elicit a high number of action potentials. We could not identify the "water" neuron in the recordings with sugar (sucrose, fructose and glucose stimulation) and NaCl stimulations.

We conclude from the results obtained that taste sensilla contain one "water" neuron, one neuron responding mainly to sugars and one neuron responding to sugars and to NaCl, possibly using different transduction pathways. However, we cannot exclude the possibility of two neurons generating action potentials of the same amplitude that could have led us to a wrong conclusion by considering only one neuron.

Structure: Projection areas of gustatory receptor neurons

In the fifth chapter we have studied the projection areas of gustatory receptor neurons. We have found 4 distinct areas: two in the deutocerebrum, one in the SOG and one in the

tritocerebrum/SOG area. One of the areas is the AMMC (the antennal motor and mechanosensory center) of the deutocerebrum, which received the projections of some axons while the others went further after giving rise to some ramifications in this area or not.

Stainings of single sensilla showed up to 5 neurons that projected in different projection areas but we couldn't separate the axon of the mechanosensory neuron from the chemosensory ones. However, a detailed quantitative analysis of our data, which was not possible within the time of this thesis, might reveal different projection areas of different physiological sensillum types or of sensilla located on different parts of the antennae.

The up to 5 axons stained from an individual sensillum indicate the presence of one mechanosensory and 4 chemosensory neurons. Since electrophysiological recordings did not allow us to confirm the exact number of neurons within one sensillum, because spike discrimination was difficult, an analysis by transmission electron microscopy is required.

The actual cutting technique used in the staining experiments did not allow us to exclude artefacts caused by accidentally stained sensilla that could have been broken during the manipulation of the antenna. A technique using a current injector could facilitate the penetration of the dye and eliminate this drawback. This technique has been used successfully in intracellular stainings and could be adapted for extracellular staining.

Additional use of other staining techniques, such as cobalt lysine backfills, might result in better resolution of axon terminals. The better estimates of fibre diametres might thus allow one to differentiate between the thick mechanosensory neuron and the thin chemosensory neurons originating from the same sensillum. Introducing a specific stimulus solution with the dye in the staining capillary might lead to specific staining of the respectively activated taste neuron, as has been shown for pheromone-sensitive olfactory receptor neurons on noctuid antennae (Hansson et al., 1992).

Since a few stainings showed some projections descending from the SOG to the thoracic ganglion it would be useful to dissect the brain connected to the thoracic ganglia to investigate the target areas of these descending axons.

Future developments

Many other substances are still to be screened. Some studies have documented how contact chemicals affect the choice of host plants and oviposition sites such as sugars (Derridj and Fiala, 1983) and surface waxes (Udayagiri and Mason, 1995). Plant waxes contain a

highly variable range of lipids and their composition varies greatly according to the plant species and the site of wax deposition (leaf, flower, fruit, etc.). In future work, it would be interesting to test the electrophysiological responses of antennal sensilla using extracts of some of *S. littorais'* main host plants.

Previous studies on insects have shown that the sensitivity of taste sensilla to some substances varies according to the location of taste sensilla on the body. Depending on location, taste sensilla mediate information that elicits different behaviours related to feeding, oviposition or pheromone communication. From this point of view, it would be interesting to compare sensitivities of taste sensilla on the tarsi, proboscis, antennae and ovipositor.

In our study we did not characterize the responses of taste sensilla at the tip of the antennae. To record them, a preparation different from that used for the other antennal taste sensilla would have been necessary. These terminal sensilla seem to be longer than those distributed in rows along the antenna. Since in the antennation process the tip of the antenna is likely to be the first to touch the substrate it would be interesting to investigate their response spectra.

Electrophysiological recordings were not done under controlled conditions of humidity and temperature but rather at room temperature and humidity. Humidity can sometimes affect the electrophysiological responses of taste sensilla (Städler et al., 1987). In order to avoid any interference of temperature and humidity variations with the electrophysiological response further experiments should be done in a controlled environment.

Lastly, we noticed that the strain we have in our laboratory is inbred for many years and some signs of genetic mutations have appeared (frequently we observed individuals with distinct characters such as yellow eyed and red eyed moths, very dark coloured males and one case of a third antenna). To perform valid behavioural experiments and further neurobiological investigations, a renewal of the strain seems essential.

For a conclusive interpretation of our data on the neuronal coding and central representation of taste information from the antennae, data on the actual involvement of antennal gustatory receptors in mating behaviour, host-plant detection and oviposition should be collected. Antennation is a behaviour frequently described before mating and egg laying. Since the function of gustatory sensilla is disabled by $ZnSO_4$ (Balakrishnan and Pollack, 1997; Groh et al., 2002) it would be interesting to disable them and thus see the involvement of taste sensilla from the antennae or other location on the body on mating and egg laying.

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