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Experience-dependent plasticity in the chemosensory system of the Egyptian cotton leafworm *Spodoptera littoralis*

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Verfasserin / Verfasser:	Isabella Katharina Kauer
Matrikel-Nummer:	0402495
Studienrichtung /Studienzweig (lt. Studienblatt):	Biologie – Zoologie A 439
Betreuer:	Doz. Dr. Sylvia Anton Institut National de la Recherche Agronomique (INRA) Physiologie de l'Insecte - Signalisation et Communication Centre de Recherche de Versailles, Frankreich
	Ao. Univ.-Prof. Dr. Harald Tichy Fakultät für Lebenswissenschaften Department für Neurobiologie und Kognitionsforschung Universität Wien, Österreich

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List of abbreviations

ACT	Antenno-cerebral tract
AL	Antennal lobe
AMMC	Antennal mechanosensory and motor centre
AN	Antennal nerve
AP	Action potential
CNS	Central nervous system
FE	Female equivalent
GRN	Gustatory receptor neuron
KC	Kenyon cell
LPR	Lateral protocerebrum
MB	Mushroom bodies
MGC	Macroglomerular complex
OBP	Odorant binding protein
ORN	Olfactory receptor neuron
PBP	Pheromone binding protein
PC	Protocerebrum
PE	Pre-exposed
PER	Proboscis extension reflex
PN	Projection neuron
PNS	Peripheral nervous system
SOG	Suboesophageal ganglion

ABSTRACT

Male moths innately have a high sensitivity for female–produced sex pheromones. In the noctuid moth species *Spodoptera littoralis*, behavioural responses to the pheromone can nevertheless still be increased by previous brief experience with the sex pheromone, through neuronal plasticity (Anderson et al. 2003, 2007). This increase in sensitivity could be observed in two time windows after exposure: a short-term effect occurred within 15 minutes and a long-term effect lasted up to 51 hours. In parallel, an increase in sensitivity of neurons within the primary olfactory centre, the antennal lobe (AL), was observed 27 h after pre-exposure with pheromone.

In the present study gustatory stimuli were used for pre-exposure and test: the sugar sensitivity of antennal gustatory receptor neurons (GRNs) as a function of pre-exposure with sucrose or quinine, was measured by means of extracellular electrophysiological recording techniques to study the neurobiological mechanisms underlying the behavioural plasticity that had been observed in a prior study.

In a second series of experiments the sensitivity for the female-emitted sex pheromone was tested after brief pre-exposure with gustatory stimuli: the sensitivity of central olfactory neurons within the antennal lobe for the sex pheromone was compared in sucrose-exposed and naïve males using intracellular recording techniques. Results of a behavioural study conducted in parallel, had revealed a positive effect of brief gustatory pre-exposure onto pheromone sensitivity.

The results of the present study indicate neuronal plasticity within the gustatory pathway not to occur at the peripheral sensory level, since no difference in GRN sensitivity for sucrose was observed between naïve and pre-exposed males. Thus, the neuronal basis of the "intra-modal" plasticity must be located at some level within the central nervous system, but we do not know by now if central gustatory neurons even change their response characteristics after pre-exposure, or if the behavioural effect is caused differently.

In addition, the neuronal basis of the "cross-modal" effect of pre-exposure does not seem to be located at the antennal lobe level, since the sensitivity of AL neurons for the female sex pheromone did not differ in naïve and gustatory pre-exposed males. The non-associative learning processes caused by taste pre-exposure are more likely located at higher processing levels within the protocerebrum, like the mushroom bodies and the lateral protocerebrum, where multi-modal sensory input is integrated and processed together.

1. INTRODUCTION

1.1. The model insect

Spodoptera littoralis Boisduval (Lepidoptera: Noctuidae), the Egyptian cotton leafworm (Fig.1), is a severe agricultural pest that originates from Egypt. This generalist moth species feeds on various plant families, including economically important crops such as cotton, corn, rice and tobacco, and therefore causes important damage to industrial production in North Africa. It is distributed especially over Africa and Western Asia (Fig.1C) (EPPO).

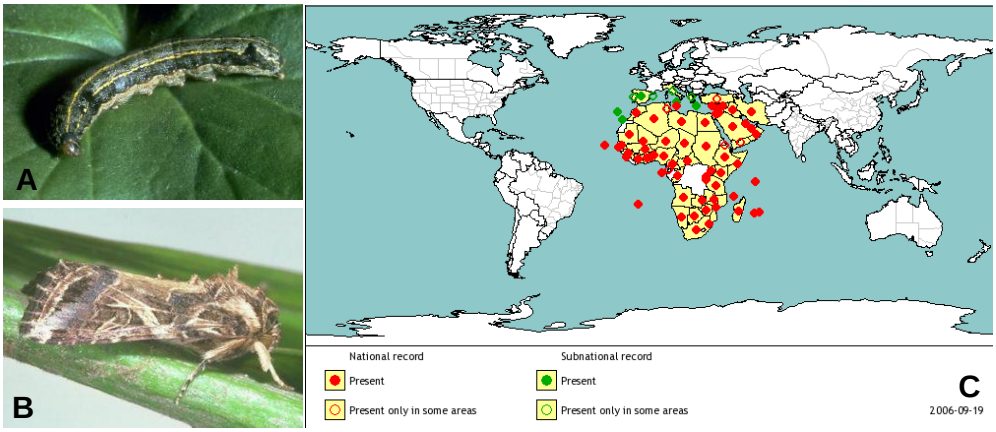

Figure 1 A-C: *Spodoptera littoralis* is a polyphagous moth species which is distributed mainly over Africa and Western Asia. **A**: larva (source: http://photos.eppo.org), **B**: adult (source: http://www-physiologie-insecte.versailles.inra.fr), **C**: Distribution map of *S. littoralis* (source: http://pqr.eppo.org)

Between 2 and 5 days after emerging, females lay clusters of eggs on the lower leaf surface of the host plant. The eggs hatch in about 4 days in warm conditions. The larvae pass through six instars in 15-23 days and reach a length of approximately 40 mm (Fig.1A). They feed on leaves and roots of the host plants. The pupal period, which is spent in earthen cells in the soil, lasts about 11-13 days. The life span of adults is about 4-10 days, thus, the life cycle can be completed in about 5 weeks. In the humid tropics there can be eight annual generations (EPPO).

Its well-documented olfactory system using pheromones for intraspecific communication as well as its short reproductive cycle make this species an ideal model insect for the present study.

1.2. Olfaction in insects

Olfaction plays a crucial role in the life of most insect species, such as night-active Lepidoptera. It allows selecting host plants, finding oviposition sites and communicating with conspecific individuals. Males of *Spodoptera littoralis*, as many other moth species, find their mating partner through very low doses of highly specific female-emitted sex pheromones. (Baker 1989). An adult male moth has to be able to detect conspecific females over great distances and discriminate them from other species' females. Although very similar pheromone blends are used among moth species, the proportion of the chemical compounds in each species is unique (Klun et al. 1975). The male must therefore be able to detect components as well as the proportions in which they are emitted with high sensitivity.

In *Spodoptera littoralis* a high number of components are produced in the female pheromone gland, but a blend of only two components is sufficient to trigger upwind flight of the male towards the female. These two components are (Z,E)-9-11-tetradecadienylacetate (Z9,E11-14:OAc) and (Z,E)-9-12-tetradecadienylacetate (Z9,E12- 14:OAc) in a 100:1 ratio (Kehat and Dunkelblum 1993).

The main olfactory organ of Lepidoptera is the antenna, on which olfactory sensilla containing receptor neurons to detect odours are present (Hansson 1995). In order to detect very low pheromone concentrations over wide distances, the male antennae in many moth species are highly branched, leading to a sexual dimorphism. The closer the sensilla are arranged on the antenna and the longer they are, the greater the surface area and thus the better the ability to capture highly distributed pheromone molecules (Steinbrecht 1987).

The olfactory sensilla are hair-like structures (sensilla tichodea) and usually contain between 1 and 3 olfactory receptor neurons (ORNs) that send their outer dendritic segments into the hair lumen (Zacharuk 1980; Steinbrecht and Gnatzy 1984).

Transduction is initiated on the olfactory pathway when hydrophobic air-borne odour molecules reach the antennae and enter the lymph which surrounds the ORNs through the perforated cuticular wall of the sensilla. There they are bound by odorant-binding proteins (OBPs), which can be divided into two classes depending on the substance: Pheromone binding proteins (PBP) are present in pheromone detecting sensilla, while general odorant binding proteins (GOBP) were found in sensilla detecting e.g. host odours (Vogt and Riddiford 1981; Krieger et al. 1993). These proteins allow the hydrophobic odour molecules to cross the aqueous sensillum lymph and reach the ORN membrane (Stengl et al. 1999).

In the dendritic membrane of the ORNs, odour molecules interact with specific receptors, which are seven-transmembrane proteins. This interaction leads to the opening of ion channels, triggered by a second messenger transduction cascade, which involves the formation of inositol 1,4,5-triphosphate (IP3) that elicits an influx of Ca^{2+} ions into the dendrite. This influx opens non-specific cation-channels, which leads to depolarization of the membrane (Stengl et al. 1999). Recent studies show that in addition to this second messenger pathway, membrane receptor molecules dimerized with the receptor OR83b might have a direct ion channel function (Sato et al. 2008; Wicher et al. 2008).

Signal transduction triggers the generation of action potentials. They move along the axon of the ORN into the ipsilateral primary olfactory centre of the central nervous system, which is the antennal lobe (AL) of the deutocerebrum. Here, receptor neurons branch into a spheroidal neuropil, the glomeruli, which are separated from each other by glial processes (Anton and Homberg 1999). Each glomerulus receives olfactory information from ORNs expressing the same receptor protein (Vosshall et al. 1999). ORN axons project into single glomeruli of the ipsilateral AL, where they make intense synaptic interaction with other neuron types, which either distribute the information within the antennal lobe (local interneurons) or carry it to higher brain centres (projection neurons, PNs) (Tolbert and Hildebrand 1981; Anton and Homberg 1999). Cell bodies of these AL neurons are situated in two clusters located laterally and medially of each AL (Fig. 2) (Anton and Homberg 1999).

In males of species utilising female sex pheromone for searching over long distances, a distinct part of the antennal lobe has been transformed into the macroglomerular complex (MGC), an array of enlarged glomeruli situated close to the entrance of the olfactory nerve (Christensen and Hildebrand 1987) (Fig. 2).

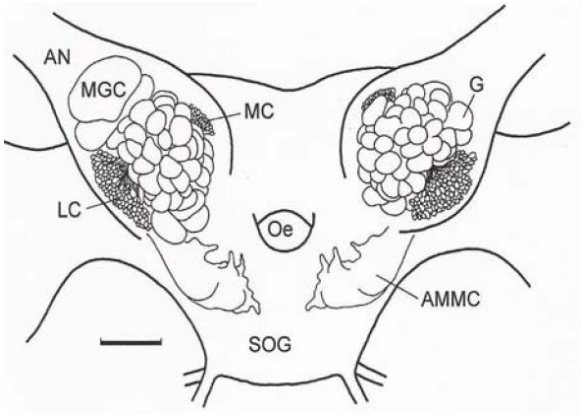

Figure 2: Frontal view of a moth brain showing a female (right) and a male (left) antennal lobe. Scale bar: 200µm
AMMC Antennal mechanosensory and motor centre, AN antennal nerve, G glomeruli, LC lateral and MC medial cluster of cell bodies, MGC macroglomerular complex, Oe oesophageal canal, SOG suboesophageal ganglion.
From: B.S. Hansson (Ed.) Insect Olfaction. Springer 1999

The central pathways processing pheromone and non-pheromone information in the insect brain are spatially separated (Hildebrand 1996). ORNs responding to female sex pheromone only arborise in the MGC, whereas ORNs responding to plant odours arborise in ordinary glomeruli (Anton and Homberg 1999).

In *S. littoralis* males, sex pheromone information is received by specifically tuned ORNs and transmitted to the MGC, which consists of four large glomeruli (Ljungberg et al. 1993; Ochieng et al. 1995; Carlsson et al. 2002).

Most mechanoreceptive neurons of the antennae pass by the AL and project to a separate neuropil in the deutocerebrum, the antennal mechanosensory and motor centre (AMMC) (Anton and Homberg 1999) (Fig.2). However, some mechanorecepive neurons project into the AL (Han et al. 2005).

Several fibre tracts (antennocerebral tracts, ACTs) make connections between the AL and the protocerebrum, the suboesophageal ganglion (SOG) and the contralateral AL. Most axonal fibres of projection neurons of the ACTs project to the calyces of the mushroom bodies (MBs) and the lateral protocerebrum (LPR). Here, integration of input from peripheral sensory centres and co-ordination of appropriate output takes place (Anton and Homberg 1999; De Belle and Kanzaki 1999).

The inner ACT (iACT) sends a main branch of PNs from the AL into the LPR and the MB calyces. The LPR receives input from visual, mechanosensory and olfactory centres. Extrinsic output and feedback neurons of the MBs arborise in the LPR, which is supposed to have an important role in olfactory signal processing.

The MBs consist of Kenyon cells (KC) and are thought to participate in memory formation and other complex behaviour. Their size correlates with the complexity of social behaviour. They receive input from the ACTs and from visual centres and feed output mainly into the LPR.

An enormous number of possible cell-contacts between Projection neurons and Kenyon cells within each MB calyx provide a neural matrix for spatial and temporal integration of olfactory and other sensory input (De Belle and Kanzaki and references therein 1999).

1.3. Taste in insects

Olfaction allows insects to find food items, but to prove its quality and discriminate edible from noxious food, the sense of taste is crucial. Contrary to olfactory receptors, gustatory receptors in insects are not only found on the antennae but also on other parts of the body like mouth parts, wings, tarsi or the ovipositor (Dethier 1976; Dahanukar et al. 2005).

The most obvious difference between olfactory and gustatory sensilla is the distance over which a stimulus can be detected. In contrast to olfactory sensilla which are able to receive odorant information over long distances, taste receptors are contact chemoreceptors (Ozaki and Tominaga 1999). Taste sensilla are thick walled hairs (often sensilla chaetica) that do not feature multiple pores in their cuticular wall but only a single pore at the hair tip (Mitchell et al. 1999). They contain two to four taste neurons with dendrites that extend towards the tip of the sensillum hair and one mechanosensory cell attached to the hair base (Ozaki and Tominaga 1999; Jørgensen et al. 2006; Kvello et al. 2006).

Gustatory receptor neurons (GRNs) have been studied mostly by the tip-recording technique, which involves making contact with the pore at the tip of the sensillum with a solution containing both an electrolyte and the taste stimulus (Hodgson et al. 1955). In *Drosophila*, experiments with different taste stimuli revealed the presence of four types of neurons: a sugar-sensitive neuron, a water-sensitive neuron, a neuron responding to low salt concentrations and a neuron responding to high salt concentrations as well as to bitter compounds (Thorne et al. 2004; Hiroi et al. 2004; Inoshita and Tanimura 2006; Hallem et al. 2006).

In moths, taste receptors are present on the antennae (sensilla chaetica), and on the proboscis (sensilla styloconica) and tarsi (Blaney and Simmonds 1990; Jørgensen et al. 2006; Kvello et al. 2006). In *Spodoptera littoralis* the flagellum of the antenna in both sexes is composed of about 70 flagellomeres, each of them bearing six taste sensilla (Fig.3).

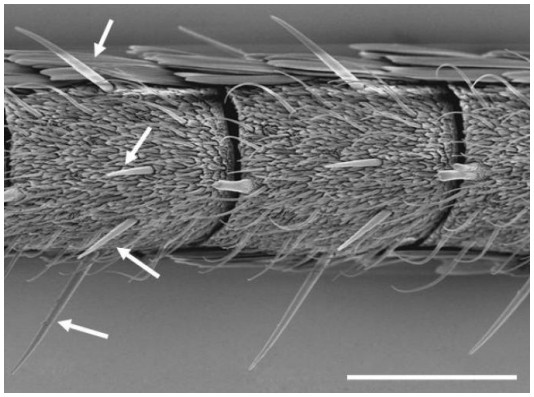

Figure 3: Electron microscope image showing the ventral view of a *Spodoptera littoralis* antenna. The arrows point at two ventral (center) and two lateral taste sensilla on one flagellomere (dorsal sensilla not shown). Scale bar: 100µm
From: Popescu 2008, modified

No differences in sensitivity have been found between taste sensilla on different parts of the flagellum when tested to sugar and salt stimuli. Similar like in *Drosophila*, four taste receptor cells could be identified by neuronal staining from individual sensilla. In electrophysiological experiments they were shown to respond to different stimuli: one cell responds to water, one to sucrose mainly, and a third one to both salt and sugar. A fourth neuron is expected to be stimulated by toxic compounds, contact pheromones or plant cuticular compounds (Popescu 2008).

When the moth antennates (e.g. the animal moves its antennae to examine its environment), taste stimuli are detected by the GRNs of the sensilla chaetica on the antennae by touching the substrate. Primary axons convey the gustatory information to different areas of the deuto- and tritocerebrum as well as the suboesophageal ganglion (SOG), while the mechanosensory neurons mainly project to the antennal mechanosensory and motor center (AMMC) (Jørgensen et al. 2006; Popescu 2008).

1.4. Neuronal plasticity in the chemosensory system of insects

Neuronal plasticity is the ability of the nervous system to adapt to varying conditions by changing its neuronal organization. It is indispensable for animals living in a constantly varying environment to adapt their behaviour to ever-changing conditions. Environmental factors, physiological state, or previous experience might influence the behaviour of an animal via mechanisms induced by neuronal plasticity, like neurophysiological, molecular or anatomical changes (Anton et al. 2007).

Two types of behavioural plasticity have mainly been studied so far: plasticity induced by physiological changes and experience. Adaptations through neuronal plasticity can either be short-term modifications leading to changes in neuronal activity, or long-term modifications leading to changes in gene expression and neuronal structure (Anton et al. 2007).

Research in moths has mainly been done at the level of physiologically induced neuronal plasticity: In males of the noctuid species *Agrotis ipsilon*, the level of juvenile hormone has been shown to control the sensitivity for the female-emitted sex pheromone (Gadenne et al. 1993; Anton and Gadenne 1999). The mating status also has an effect on the olfactory system in both sexes of moths, inducing higher sensitivity for host plants in females and temporarily low sensitivity for the female pheromone in males (Gadenne et al. 2001; Masante-Roca et al. 2007).

The finding of adult neurogenesis in the mushroom bodies of both male and female *Agrotis ipsilon* moths and changes in the sensitivity of AL neurons indicate that the observed behavioural changes might be rather due to changes in the sensitivity of central olfactory neurons and higher brain centres than to changes in the peripheral sense organs (Anton and Gadenne 1999; Dufour and Gadenne 2006).

Another type of behavioural plasticity is experience-dependent plasticity, where sensory input induces modifications in the nervous system leading to learning processes and therefore modification in behaviour. These learning processes can be divided into associative learning like classical and operant conditioning, and non-associative learning like habituation and sensitization respectively.

Experiments on moth associative learning using geraniol as conditioned stimulus and sucrose solution as unconditioned stimulus showed that *Spodoptera littoralis* both males and females have a good capability to associate a flower odour with a food reward (Fan et al. 1997). Further investigation showed that they are even able to learn to associate single pheromone components with a food reward (Hartlieb et al. 1999; Hartlieb and Hansson 1999).

However, the present study will not deal with associative learning featuring repeated presentations of a conditioned stimulus, but focus on neuronal long-term effects induced by short experience as a form of non-associative learning caused by plasticity within the chemosensory system of moths.

1.4.1. Intra-modal effects of short pre-exposure in olfaction

Concerning the olfactory system, brief pre-exposure to female-emitted sex pheromone has been shown to increase the subsequent behavioural response of *S. littoralis* males to the pheromone in two different time windows (3 h and 27 h later) (Anderson et al. 2003).

In a following study again a significantly higher percentage of males showed behavioural response to the pheromone in a walking olfactometer after pre-exposure in two time windows: a short-term effect was observed 15 minutes after pre-exposure, indicating a rise in selective attention for the sex pheromone. A long-term effect was shown after 27 hours and 51 hours respectively, indicating a form of long-term sensitization for the odour.

In parallel, at the central nervous level the sensitivity of AL neurons was tested between 22 and 28 hours after the short pheromone experience. The brief pre-exposure lowered the response thresholds of AL neurons for the pheromone significantly, whereas EAG

(electroantennogram) recordings of the peripheral olfactory system did not reveal any difference in sensitivity between naïve and pre-exposed males (Fig. 4) (Anderson et al. 2007).

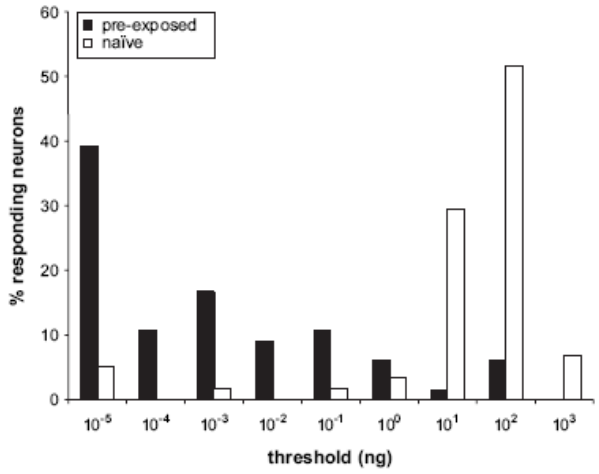

Figure 4: The response threshold for the main compound of the sex pheromone in antennal lobe neurons of *Spodoptera littoralis* males is much lower in pheromone pre-exposed than in naïve animals. (From: Anderson et al. 2007)

1.4.2. Intra-modal effects of short pre-exposure in taste

Pre-exposure effects within the gustatory system have been studied in humans already: in a study examining glucose sensitivity, participants who were treated with a 43 mM fructose solution during 10 days for 10 seconds daily were able to discriminate glucose as sugar at significantly lower levels than non-treated participants on day 11 and 12 as well as on day 22 or 23. They had returned back to their pre-treatment glucose discrimination thresholds by day 33 or 34 (Gonzalez et al. 2008). It is not known if the underlying neuronal changes take place within the peripheral or the central nervous system.

Other vertebrate data suggest changes in chemosensory sensitivity to take place at either level: in hamsters, the magnitudes of chorda tympani responses increased after repeated stimulation with novel taste stimuli, whereas in humans psychophysical and fMRI responses showed "familiarization" when repeatedly exposed to novel taste stimuli. At the same time those CNS changes could have resulted from changes in the periphery (for more detailed information see Berteretche et al. 2005; Faurion et al. 2002; Wang et al. 2003).

Experiments on *Drosophila* showed that firing rates of taste receptor cells increased after repeated stimulation with sucrose, assuming the locus of plasticity to lie in the taste receptor cell (Kennedy and Halpern 1980).

All these data are based on repeated presentation of a stimulus which leads to sensitization. The central question in this study, however, will be, if a one-time brief exposure to a certain stimulus can also lead to long-term sensitization at the level of the gustatory sense and across

modalities respectively, since in behavioural experiments sensitivity-increasing effects of taste pre-exposure onto the gustatory as well as the olfactory system have been observed.

Behavioural experiments examining the role of experience in the gustatory system of *Spodoptera littoralis* males showed an effect of a unique brief exposure to appetitive and aversive taste stimuli onto a gustatory test with sucrose 24 h later (S. Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data): When tested to low concentrations of sucrose, a significantly higher percentage of males pre-exposed to 1 M sucrose as well as to 0.1 M quinine extended their proboscis (proboscis extension reflex, PER), compared to the control group pre-exposed to H_2O (Fig. 5).

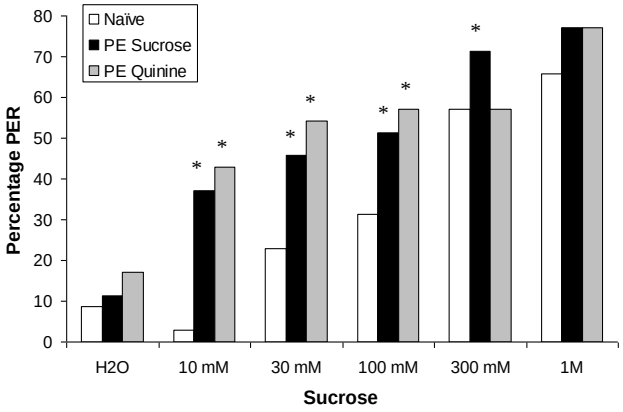

Figure 5: *Spodoptera littoralis* males show a higher behavioural sensitivity for sucrose 24 h after gustatory pre-exposure to sucrose as well as to quinine than naïve males. N for each bar = 35 (S. Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data).

These results already show that the above-described pre-exposure effect is rather not specific for the sex pheromone, nor limited to the olfactory system, but can obviously be revealed using other behaviourally important sensory stimuli such as attractive and repellent tastants for pre-exposure and test as well.

However, it was thus far not known if pre-exposure effects occur only within one modality or also across modalities, meaning that either sex pheromone exposure might affect the sensitivity of other sensory systems or if incoming information of a different modality might have a similar effect on sex pheromone responses.

1.4.3. Cross-modal effects of brief pre-exposure

Behavioural experiments on *S. littoralis* males using female pheromone as olfactory pre-exposure stimulus showed a positive cross-modal effect on the response to gustatory stimuli (S. Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data): A significantly higher percentage of males pre-exposed to 1 female equivalent (FE) gland extract extended their proboscis as a response to 0.03 M sucrose solution 24 h later.

In addition, pre-exposure to other sensory stimuli enhanced the pheromone sensitivity of male moths: Experiments in a flight tunnel showed a significantly higher percentage of orientation towards a 0.25 FE gland extract stimulus in males pre-exposed to the plant compound geraniol 24 h before, compared to naïve males (Pingard 2009).

Experiments using pulsed high-frequency sound mimicking bat sound as a predator signal revealed that males pre-exposed to the bat-like ultrasound 24 hours before, were more sensitive for low doses of sex pheromone compared to naïve males as well as males pre-exposed to a non-pulsed tone of the same frequency at both the behavioural and central nervous level in the antennal lobe (S. Anton, K. Evengaard, J. Blomqvist, N. Skals, P. Anderson, unpublished data).

Behavioural data on *S. littoralis* males collected prior to the present study showed that brief experience with sucrose (attractant) as well as quinine (repellent) enhanced activation as well as orientation towards the stimulus source on a locomotion compensator when tested to 0.25 FE of the female pheromone 24 h later (Fig.6). The same type of effect was obtained in the opposite way: males pre-exposed to the pheromone presented higher PER frequency at low sucrose concentrations than naïve males (S. Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data).

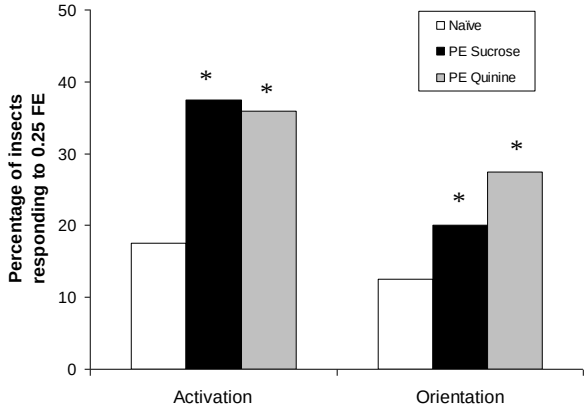

Figure 6: Gustatory pre-exposure to sucrose enhanced the activation as well as the orientation towards the female pheromone 24 h later in *Spodoptera littoralis* males compared to naïve males. N = 120, n for each bar = 40 (S. Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data).

The fact that long-term effects of pre-exposure appeared across sensory modalities indicates that it might cause a form of long-term sensitization leading to maturation processes that increase general sensitivity for behaviourally relevant sensory input rather than evoke selective attention for a certain kind of stimulus.

1.5. Aim of the present study

A behavioural intra-modal long-term effect of taste experience onto the sensitivity for sugar as well as a cross-modal long-term effect of taste experience on the response to the female pheromone has already been shown in *S. littoralis* males (see 1.4.2. and 1.4.3.).

However, nothing is known so far about the neuronal processes underlying the observed increase in behavioural sensitivity.

For this reason it should be examined in the present study if the presumed neuronal maturation processes and long-lasting structural changes caused by experience-dependent plasticity are housed in the central nervous system or rather in the periphery.

Two different electrophysiological approaches are thus used: extracellular tip-recording and intracellular single cell recording, examining intra- as well as inter-modal effects of gustatory pre-exposure in the peripheral and central nervous system. For examining the responses of gustatory receptor neurons on the antennae, the tip-recording technique was used. Extracellular recording of peripheral structures was chosen because of easy accessibility and feasibility within the given time. The sensitivity of AL neurons in the CNS on the other hand was investigated by intracellular recording techniques because we considered unlikely to find an effect of taste pre-exposure in the periphery (in ORNs).

1.5.1. Intra-modality: monosensillar recordings of GRNs after taste pre-exposure

In this part of the study the response characteristics of GRNs within taste sensilla (sensilla chaetica) on the peripheral part of the gustatory system of *S. littoralis* males, the antennae, will be investigated. A possible effect of gustatory pre-exposure with an attractant (sucrose) and a repellent (quinine) at the level of sensory input, influencing the response of GRNs for sucrose stimuli between 22 and 28 h after pre-exposure will be investigated using the tip-recording technique.

It should be shown if the response characteristics of GRNs in gustatory pre-exposed males are different from those in naïve males and if differences in sucrose sensitivity between animals pre-exposed to the attractant and animals pre-exposed to the repellent can be observed.

1.5.2. Cross-modality: single cell recordings of AL neurons after taste pre-exposure

As mentioned above, cross-modality effects of auditory pre-exposure with bat sound on pheromone responses in the antennal lobe have been shown in *S. littoralis* males. Effects of

gustatory pre-exposure on responses to female sex pheromone have also been shown, but so far only at the level of behaviour.

This part of the study is focused on the central nervous level, where the response thresholds of pheromone-sensitive AL neurons within the MGC of naïve and gustatory pre-exposed males will be compared. A possible long-term effect of gustatory pre-exposure with an attractant (sucrose) on the sensitivity for sex pheromone, as observed in behaviour will be investigated using intracellular recording techniques between 22 and 28 h later. The central question arising is, if pre-exposure to an attractive, behaviourally relevant taste stimulus will across sensory modalities raise pheromone-sensitivity within AL neurons significantly in an analogous manner to the experiments conducted with bat-sound serving as pre-exposure stimulus.

2. MATERIAL AND METHODS

2.1. Insects

Spodoptera littoralis larvae were reared on a semi-artificial diet according to Poitout and Bues (1974). Pupae were sexed and males and females were kept separately in groups of 20-30 individuals at 22 - 24°C and 65 – 75 % relative humidity under a 16:8 h light:dark cycle (photophase from 7pm to 11am, scotophase from 11am to 7pm). Every morning (before 11am) emerged adults were separated from pupae. They were considered to be one day old at this time.

2.2. Pre-exposure procedure

Newly emerged males used for the experiments were separated from pupae and kept without feeding. During their first photophase (day 1) they were prepared for the pre-exposure procedure by fixing their thorax and abdomen in cut Eppendorf-tubes with their head protruding, so that they could not move and the antennae were accessible at the same time.

The pre-exposure took place during darkness, 2h into the scotophase (i.e. 1pm on day 1), which is the natural time of the animals' peak activity, with red light as light source.

2.2.1. Gustatory pre-exposure for taste experiments

Three groups of animals were either pre-exposed to sucrose (1 M, $C_{12}H_{22}O_{11,}$ Sigma-Aldrich), quinine (0.1 M, $C_{20}H_{24}N_2O_{2,}$ Sigma-Aldrich) or ultra pure water (control group). To apply the pre-exposure stimulus onto the antenna, a wooden toothpick was wetted with the particular solution, which was applied onto the left antenna by touching it with the toothpick and moving the toothpick along the flagellum for 10 seconds in such a way, that a maximum of gustatory sensilla had been in contact with the solution (Fig. 7). Animals extending their proboscis (usually as a response to the Sucrose-stimulus) were not allowed to feed on the solution, to avoid a reward situation and exclude associative learning.

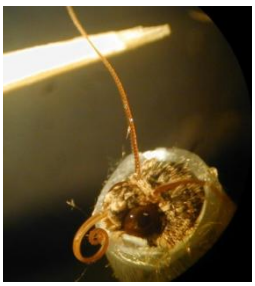

Figure 7: Gustatory pre-exposure 2 h into the scotophase. A toothpick is wetted with the pre-exposure stimulus (sucrose, quinine or H_2O) and moved along the antenna, touching it for 10 seconds. (Photo: S. Minoli)

2.2.2. Gustatory pre-exposure for olfactory experiments

The pre-exposure for olfactory testing was conducted in an analogous way as for the taste-experiments. One group of animals was pre-exposed to 1 M sucrose, the other one to ultra pure water (control group). Quinine was not used for pre-exposure. The stimuli were applied as described in 2.2.1. onto one antenna, usually the right one for reasons of better accessibility in the electrophysiology set-up.

After the treatment the insects were removed from the tubes and kept separately in groups according to their treatment under the same conditions as mentioned above (22 – 24°C, 65 – 75 % RH, 16:8 h l:d), without feeding for approximately 24 hours.

Since the pre-exposure procedure took place in a different chamber than the rearing, it was important not to disturb the light:dark cycle of the animals. For this reason, they were transported in a non-transparent box.

2.3. Test procedure

To reveal a possible long-term effect of gustatory pre-exposure with appetitive and aversive stimuli on the peripheral response to sucrose stimuli (intra-modality, within gustation) as well as a possible effect of pre-exposure with an attractive tastant on the central nervous responses to sex pheromone (cross-modality, across gustation and olfaction), experiments were carried out 22-28 h after pre-exposure, i.e. during the scotophase of day 2, at room temperature. Electrophysiological experiments were, however, conducted under daylight conditions to allow the preparation and dissection of the animals.

The Experiments were carried out between March and July 2009 at the laboratories of the PISC (Physiologie de l'Insecte: Signalisation et Communication) unit at the INRA (Institut National de la Recherche Agronomique) Centre of Versailles, France.

2.3.1. Intra-modality: test to gustatory stimuli

2.3.1.1. Animal preparation

Insects were again fixed in cut Eppendorf-tubes with their head and antennae protruding. The tube was then attached to a piece of polystyrene covered with a dental-wax layer, with the left antenna situated at the same level as the wax. The antenna was attached to the wax by use of two tiny metal hooks fixing its base and tip (Fig. 8A). The animal was positioned in the

electrophysiology set-up under optical control (Microscope Leica MZ12) in such a way that the gustatory sensilla of the left antenna were easily accessible by the glass capillary of the recording electrode, i.e. they were orientated in the same plane (Fig. 8B).

To ground the animal, the antenna was connected to a silver wire serving as grounding electrode by means of conductive gel (Fig 8A).

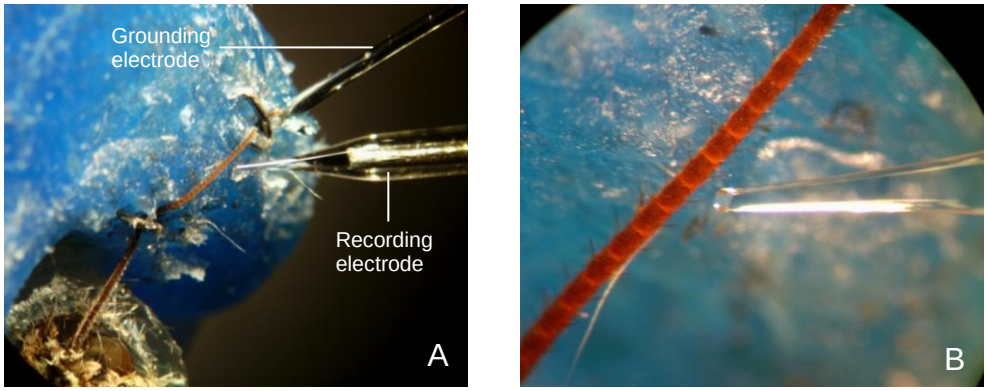

Figure 8 A-B: Stimulation and recording of gustatory sensilla on the left antenna of a *S. littoralis* male. **A**: The antenna is fixed onto a dental wax layer by means of two metal hooks. The recording electrode containing the stimulus solution together with the electrolyte is put over a single sensillum to trigger recording. **B**: Detailed view of the tip of the glass microelectrode approaching a single sensillum on the antennal flagellum, situated in the same plane. (Photos: I. Kauer)

2.3.1.2. Sucrose stimulation

Four different concentrations of sucrose served as test stimuli, ultra pure water as control stimulus. A 1M sucrose solution was diluted in ultra pure water to obtain test concentrations of 0.1 mM, 1 mM, 10 mM and 100 mM. To ensure conductivity, 10^{-3} M KCl was added to both the stimulating and control solutions. They were prepared once and stored in the refrigerator at 4°C.

Four taste sensilla standing in line laterally on the distal half of the left antenna were tested per animal (Fig.9). The tip of each sensillum was covered with the recording electrode containing the stimulus solution for two seconds.

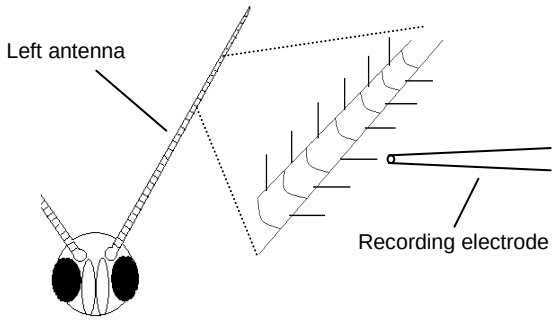

Figure 9: Four lateral sensilla standing in line on the distal half of the flagellum of the left antenna are approached by the recording electrode and one after the other tested with the stimulus for two seconds.

Each sensillum was first tested with the control solution, and then stimuli were presented always in the same increasing order from 0.1 mM to 100 mM in decadic steps, once per sensillum for two seconds and with an interstimulus interval of at least 1 minute to avoid adaptation.

2.3.1.3. Peripheral recording

Glass microelectrodes with a tip diameter of 40 – 60 µm were pulled in two steps out of borosilicate glass capillaries (GC 100T-10, Harvard Apparatus), using a vertical puller (PC-10, Narishige). Loaded with the stimulus or control solution respectively, the electrode was fitted onto a silver wire connected to the probe of a preamplifier (amplification 10-fold, TasteProbe DTP-02, Syntech). It could be positioned by means of a manual (Leitz) and a motorised micromanipulator (MS314, Markhäuser) (Fig.10). Electrical signals were further amplified (amplification 50-fold, CyberAmp 320, Axon Instruments) and filtered by a band-pass filter (10-2800 Hz) (Fig. 10).

Recording was triggered by voltage signals as soon as contact of the pore at the tip of a taste sensillum with the stimulus solution was established.

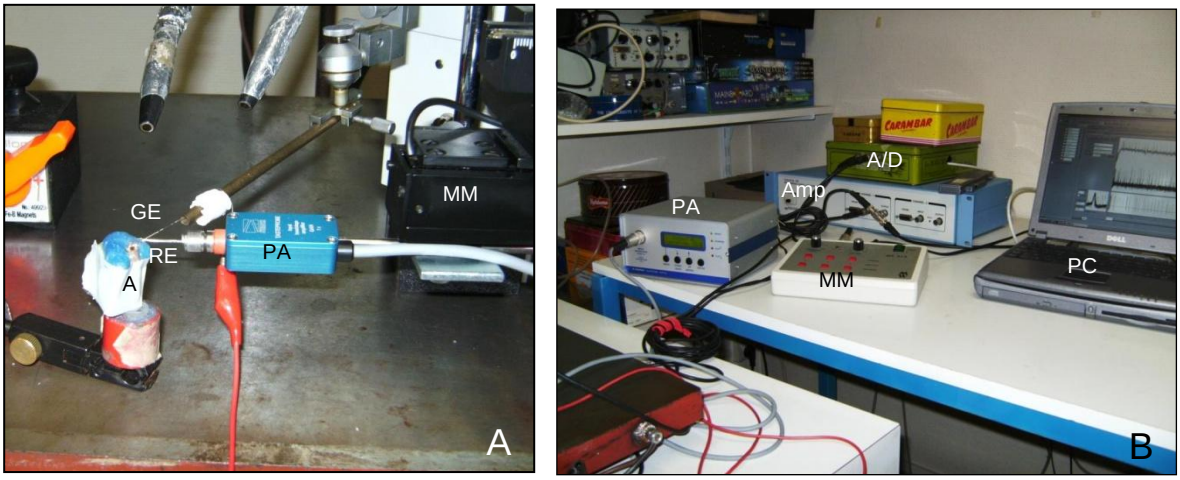

Figure 10: **A**: Tip-recording set-up. A animal, GE grounding electrode, MM micromanipulator (manual), PA preamplifier headstage, RE recording electrode. **B**: Data acquisition unit. A/D Analogue-digital converter, Amp amplifier, MM micromanipulator (motorised), PA preamplifier, PC computer. (Photos: I. Kauer)

2.3.1.4. Data analysis

Spikes were detected and counted during two seconds after the recording trigger using the programme dbWave. For each recording a horizontal threshold to detect the total number of spikes and separate them from background noise was determined.

Further analysis was done with the aid of the programme MS Excel 2003. The mean number of spikes over all recordings during the first second was calculated for each stimulus concentration and separately for naïve, sucrose- and quinine-treated males. Post stimulus time histograms (PSTH) for each treatment group were done as well, using a bin size of 100 ms.

2.3.2. Cross-modality: test to olfactory stimuli

2.3.2.1. Animal preparation

Insects were mounted into cut plastic pipette tips with their head protruding, and dental wax was attached around the neck to fix their head in an immobile position. To allow access to the cuticle from the frontal part of the head, scales and labial palps were removed under optical control (Stemi 2000, Zeiss). A window between the bases of the antennae and the two eyes was cut into the cuticle of the head capsule and the cuticle was removed together with the proboscis, the oesophagus and associated muscles (Fig 11B). For stabilization and to allow better access to the antennal lobes, the dissection was held open by micro needles inserted close to the eyes. As soon as the head capsule was opened, the tissue was constantly rinsed with physiological solution (Tucson Ringer, see appendix) to avoid drying of the brain tissue.

Antennal muscles as well as tracheal tubes were removed using fine forceps. To allow penetration of the antennal lobe with the tip of the glass capillary of the recording electrode, the fine membrane covering the tissue called neurolemma was removed carefully by avoiding damage of the lobe and the antennal nerve. The antenna which was going to be stimulated was supported by a small hook, to allow its positioning within the centre of the stimulating air stream (Fig.11A).

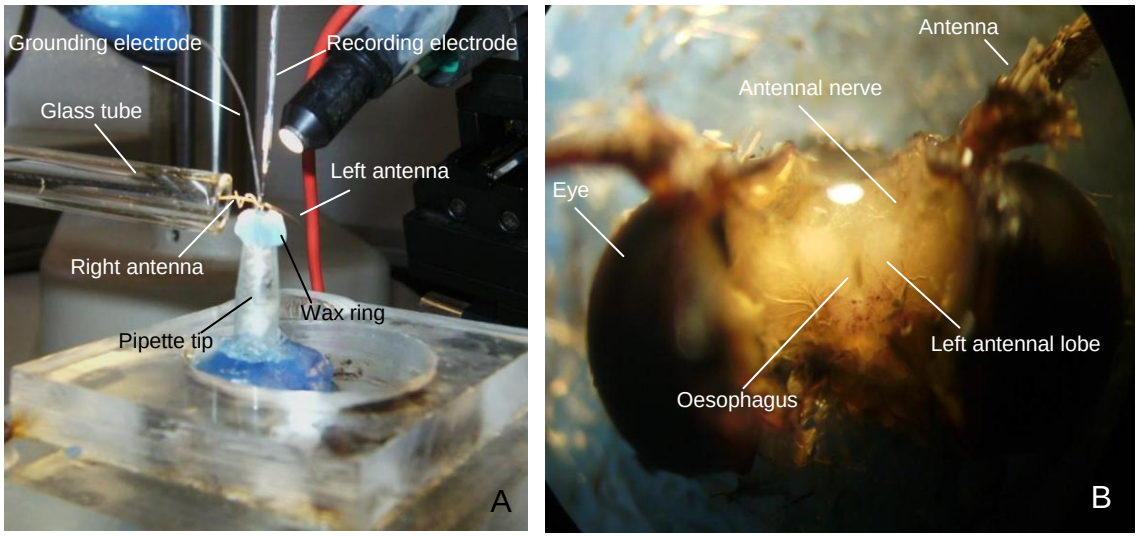

Figure 11 A-B: Preparation of the insect and its positioning inside the electrophysiology setup. **A**: The animal is fixed in a plastic pipette tip with its head protruding, surrounded by a wax ring. The right antenna is supported by a small metal hook and positioned in the centre of the glass tube transporting the stimulus through an air stream. Voltage signals are recorded from the ipsilateral antennal lobe with a glass microelectrode. The grounding electrode is placed within the tissue. **B**: Frontal view of the opened head capsule showing the two antennal lobes and the antennal nerves. (Photos: I. Kauer)

2.3.2.2. Pheromone stimulation

For better reproducibility, the olfactory stimuli did not consist of female gland extracts but of the synthetic main component of the female-emitted *S. littoralis* sex pheromone, which is (Z,E)-9-11-tetradecadienylacetate (Z9,E11-14:OAc). One female equivalent gland extract (1 FE) has been shown to contain approximately 15 ng of Z,E-9,11-14:OAc (Hartlieb and Hansson 1999).

Based on a concentration of 1 µg/µl, the pheromone was diluted with hexane in decadic steps, resulting in eight different concentrations used, reaching from 0.01 pg/10 µl to 100 ng/10 µl.

The stimulus solutions were applied in quantities of 10 µl each onto small filter papers (2 x 0.5 cm) and inserted into labelled Pasteur pipettes. Pure hexane as well as an empty filter paper (blank stimulus) was used as control stimuli to measure the response of antennal lobe neurons to the solvent itself and to the air stream respectively.

Filter papers were used during two experimentation days, reloaded with the stimuli at the beginning of the second day, before being disposed.

A constant charcoal-filtered and humidified airflow was blown over the antenna through a glass tube with an inner diameter of 8 mm, whose outlet surrounded the tip of the antennal

flagellum (Fig. 11A). The continuous airflow velocity was 17 ml/s. Stimuli were presented by inserting the Pasteur pipette into the glass tube about 20 cm from the antenna. An air pulse (7 ml/s) with 500 ms duration was then sent through the pipette which was loaded with the synthetic pheromone component or with the control stimulus by means of a stimulation device (Stimulus Controller CS-55, Syntech). In order to keep mechanical stimulation of the antenna to a minimum, an airflow of the same velocity as the stimulus was permanently added to the continuous airflow and removed during stimulation.

As soon as contact with neurons of the ipsilateral antennal lobe was established, naïve animals were first of all tested with 10 ng, pre-exposed ones with 1 ng of Z,E-9,11-14:OAc, to check if the neuron was responding (i.e. if it was part of the MGC). If so, the following stimulus was hexane, serving as control. After this, stimuli were presented randomly, starting with lower pheromone concentrations in order to detect the response threshold. The threshold was defined as the lowest pheromone concentration eliciting an excitatory neuronal response.

Once the threshold was obvious to the "naked eye", concentrations around it were tested several times followed by hexane and the blank stimulus. Stimuli were presented with a minimal interstimulus time of 10 s to allow neurons to fully recover after responding. Up to five responding neurons per animal were taken into account for subsequent statistical analysis.

Sucrose-pre-exposed males were treated in two different ways: in one group the odour stimuli were applied onto the pre-exposed antenna, in the second group stimuli were applied onto the non-treated antenna. Responses were always recorded from the ipsilateral lobe as to the stimulated antenna.

2.3.2.3. Central electrophysiology

Intracellular recordings of AL neurons were performed according to standard methods (Christensen and Hildebrand 1987). Glass microelectrodes were produced using a horizontal puller (P-97 Flaming/Brown Micropipette Puller, Sutter Instruments). Filled with 3 M KCl, resistances lay between 10 and 25 MΩ, measured in the extracellular medium (Tucson Ringer).

The microelectrode was connected to the preamplifier headstage (HS-2A Headstage, Axon Instruments) and located near or inside the MGC of the ipsilateral AL (Fig. 12) by means of a manual micromanipulator (Leitz). A buzzing device was used to facilitate the penetration of the tissue.

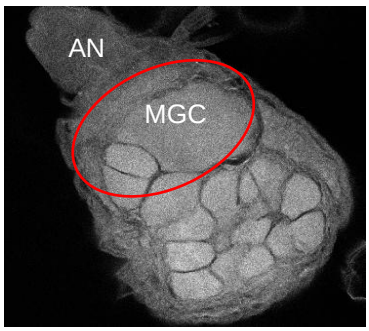

Figure 12: Confocal microscope image of the antennal lobe of a male moth. The red circle indicates the MGC-area, where neurons responding to the pheromone were recorded. It covers approximately a third of the lobe and is located near the entrance of the antennal nerve. (Image: S. Anton)

The activity of the penetrated neurons was constantly monitored and registered 1.5 s before, 500ms during and 3 s after odour stimulation of the antenna, using Autospike software (Syntech). Recorded signals were amplified (Axoclamp-2B amplifier, Axon Instruments), subsequently digitalized and analyzed with Autospike.

During the entire recording period the tissue was rinsed with Tucson Ringer to avoid desiccation. A Faraday cage protected the recording from electrical noise and an extractor placed above the electrophysiology setup removed odorants.

2.3.2.4. Neuronal staining

Several neurons were stained. When neuronal staining was attempted, the tip of the recording electrode was filled with 4% Lucifer Yellow and the electrode was then backfilled with 2 M LiCl, leading to tip resistances of 120 – 170 MΩ measured in the extracellular medium.

When stable intracellular contact with a neuron responding to the pheromone was established and in case of the spike amplitude exceeding 8 mV, a hyperpolarizing negative current of 1 nA was passed through the recording electrode into the cell for up to 10 minutes to fill it with the fluorescent dye. The brain was then dissected out of the head capsule and fixed in Lucifer Fix solution (see appendix) for at least 12 h. After washing it in Millonig's Buffer (see appendix) it was cleared in Vectashield mounting medium (Vector laboratories) for at least 24 hours. The cleared brain was transferred onto a microscopy glass slide, surrounded by the mounting medium and analysed as wholemount by means of a confocal microscope (Leica DMIRE2, equipped with an argon/neon laser and a 10x dry objective). Brains were scanned frontally and partial maximum projections of optical sections were done to visualize AL neuron branches within the antennal lobe.

2.3.2.5. Data analysis

Spikes were counted in Autospike to detect the response threshold of pheromone-sensitive AL neurons. To reveal excitatory responses to the different pheromone doses, the spike number was counted during the duration of the response and compared to an equal duration before the response onset (see Fig.18). The total number of action potentials before the response (monitoring spontaneous activity of a neuron) was then subtracted from the number of action potentials during the response. Spiking rates exceeding spontaneous activity by more than 20 percent were defined as response.

Since a very high percentage of cells responded to the hexane and the blank stimulus as well as to the pheromone (indicating a mechanical stimulation), responses to pheromone stimuli were compared to responses to the control stimuli and defined as pheromone responses when they exceeded the hexane response by more than 10 percent (Jarriault et al. 2009).

3. RESULTS

3.1. Peripheral electrophysiology

A total number of 1849 recordings of 290 sensilla on 72 animals were performed, 25 of which (31.6%) pre-exposed to sucrose, 23 (29.1%) pre-exposed to quinine and 24 (30.3%) pre-exposed to H_2O as control group. The majority of the tested sensilla were situated laterally in the distal half of the left antenna for reasons of accessibility (see Fig. 8).

3.1.1. Response characteristics of gustatory receptor neurons

The majority of approached sensilla chaetica yielded an electrical contact, confirming the terminal pore to be open and the sensillum to be intact. The amplitude of the recorded action potentials varied between 0.2 and 0.8 mV on a baseline noise of 0.1 mV peak to peak. In most cases the spikes could easily be distinguished from the noise. After stimulus onset the GRN response was recorded during two seconds (Fig. 13).

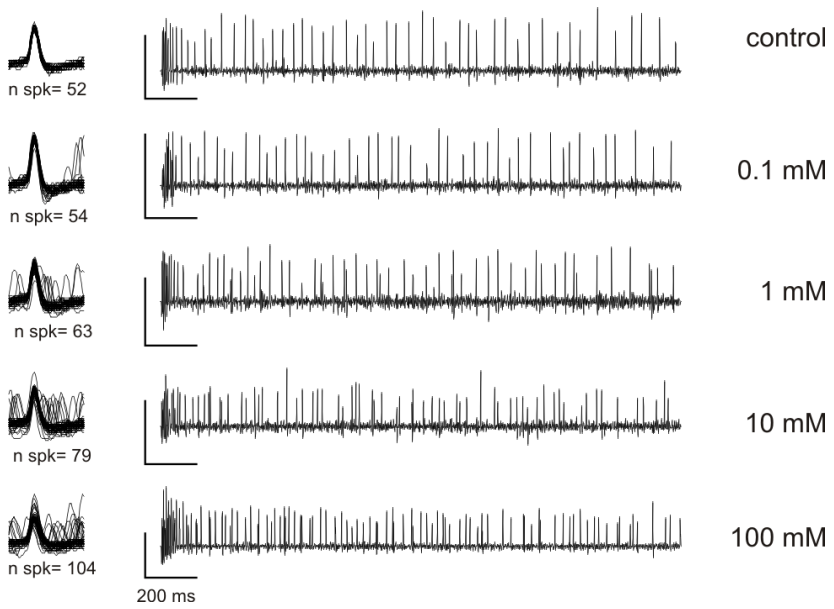

Figure 13: Original recordings of the GRNs in one sensillum of a quinine pre-exposed male *Spodoptera littoralis*, showing an increasing spike number as a response to increasing concentrations of sucrose. At least two neurons with different spike amplitudes can be distinguished in each recording. Vertical scale bars: 1 mV, total recording duration: 2s.

The results show a distinct dose-response correlation between stimulus intensity and the mean number of spikes during the two recorded seconds in all three tested groups (Fig. 13, 14, 16). The spiking rate was monitored in 20 time windows of 100 ms bin size each, showing phasic-tonic response characteristics of the GRNs with a distinct burst of action potentials at

the stimulus onset, lasting between 50 and 100 ms, and a stable frequency after a sharp decrease (Fig. 14 B-D). During the first bin of 100ms duration the total number of spikes is high in all three groups and increases only slightly with the stimulus intensity (Fig.14A). This might be due to mechanical stimulation of the sensillum and therefore excitement of the mechanosensory cell.

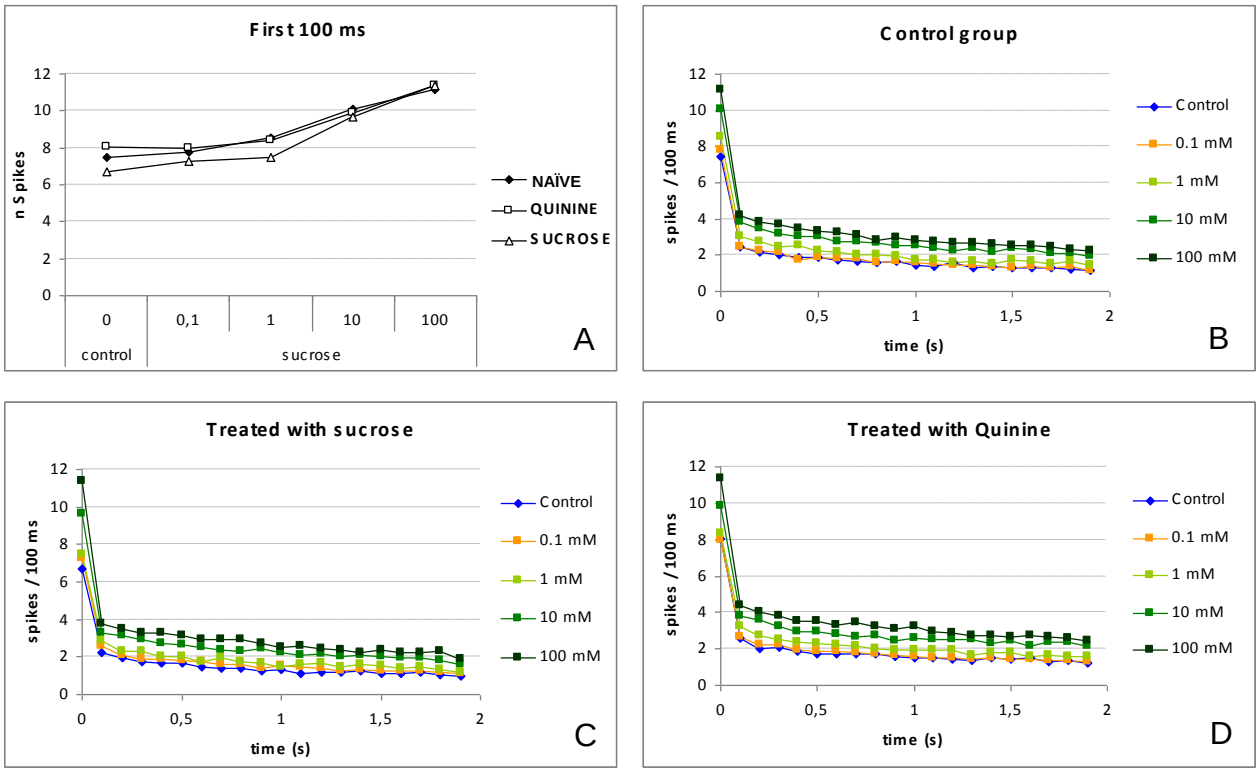

Figure 14 A-D: Post-stimulus time histograms (PSTH) of GRNs, showing the phasic-tonic character of the neuronal responses in the three groups tested. **A**: The spiking rate monitored during the first 100 ms is similarly high in naïve, sucrose- and quinine-pre-exposed animals and increases only slightly with the stimulus intensity. **B**, **C**, **D**: Similar phasic-tonic patterns of spiking activity during two seconds in the three groups showing a sharp decrease following an initial burst and a rather constant frequency afterwards. Bin size: 100ms.

During the first second, which was taken into account for the frequency analysis, spike frequency varied between 0 and 101 Hz, depending mostly on the intensity of the stimulus but also on an obviously high natural variability of GRN activity in different sensilla.

In many sensilla at least two firing neurons could be determined (Fig. 13, 15), however, they were not separated into individual classes but all together taken into account to calculate the mean spike frequency, since they were very difficult to distinguish in most cases.

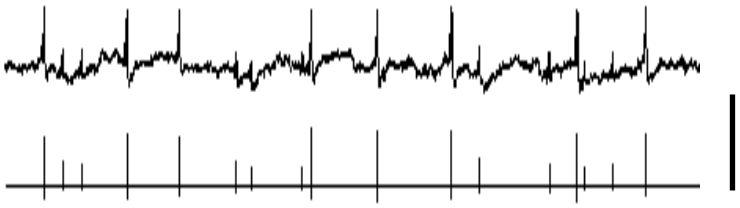

Figure 15: Detail (0.4ms) of an original recording showing two easily distinguishable GRNs in the same sensillum, responding to 1 mM sucrose.
The lower trace shows the discriminated spikes. Vertical scale bar indicates 1 mV.

3.1.2. Sucrose sensitivity as a function of pre-exposure

No long-term effect of pre-exposure to taste stimuli onto the sucrose-response of GRNs on the antennae of *S. littoralis* males could so far be observed. Strikingly similar for all three tested groups, mean spiking rates of GRNs reached from 20.6 to 24.5 spikes/ s for the control stimulus to 40.9 to 43.5 spikes/ s for the highest (100 mM) sucrose solution, showing positive correlation between dose and response. Standard errors lie between 0.99 and 1.19 (Fig. 16).

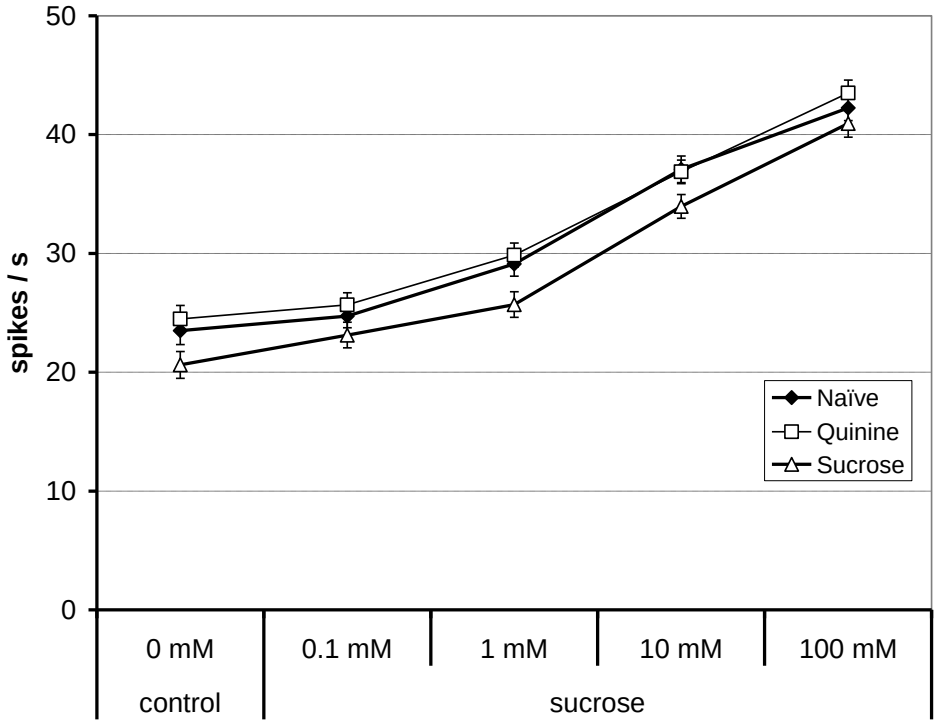

Figure 16: Dose response curves from taste sensilla of male *Spodoptera littoralis* in response to sucrose diluted in water and 10^{-3} M KCl after different pre-exposure treatments. The spike frequency of GRNs rises with the stimulus intensity. n (for each stimulus) naïve = 128, n quinine PE = 125, n sucrose PE = 133. Error bars indicate the standard error. The spike frequency rises with the stimulus intensity.

It is noticeable that the response in naïve as well as in quinine treated animals is stronger than in sucrose pre-exposed ones for each of the tested concentrations as well as for the control stimulus (Fig. 16). This could be due to a lower activation of either the salt- or the water-sensitive neurons in sucrose-pre-exposed animals compared to naïve and quinine pre-exposed ones.

To filter the response to sucrose exclusively out of the collected data, the mean number of spikes as a response to the control solution was subtracted from the mean number of spikes in the responses to the four sucrose solutions. The discrepancy between sucrose- and quinine-treated animals disappears like this and the curves for all three tested groups overlap (graphs not shown). This indicates that there is no effect of pre-exposure neither to sucrose nor to quinine onto the response characteristics of GRNs when tested to sucrose, which can be measured at the peripheral level of the gustatory pathway.

3.2. Central electrophysiology

The threshold for the synthetic main component of the sex pheromone to elicit a neuronal response could be determined in a total number of 84 neurons in the antennal lobe of male *Spodoptera littoralis*, 31 (36.9%) of which in 16 naïve animals and 53 (63.1%) in 25 animals pre-exposed with sucrose 22-28 hours before. The group of pre-exposed males was separated into two groups according to the antenna on which the test-stimuli were applied: the sensitivity threshold was determined in 32 neurons housed in the lobe of the pre-exposed (ipsilateral) antenna of 16 males and in 21 neurons housed in the lobe of the non-treated (contralateral) antenna of 9 other males (see table 1).

Many more neurons were encountered but a large part did not respond to the pheromone (see discussion) or responded to the pheromone as well as to the control stimuli with the same intensity (Fig. 17). In addition, intracellular contact often could not be kept as long as needed to apply the whole range of test stimuli. Only neurons whose response to at least one of the tested pheromone concentrations exceeded the response to the control stimuli (blank and hexane) by more than 10 percent were classified as responding to the pheromone and thus taken into account for data analysis (see 2.3.2.5.).

3.2.1. Response characteristics of antennal lobe neurons

Not only response patterns but also action potential amplitude and spontaneous activity differed widely among the recorded neurons. However, there was no obvious difference

between naive and pre-exposed animals. Spontaneous activity between 0 and 130 Hz could be found, and spike amplitude varied between 2 and 40 mV, depending on the quality of the cell contact. Durations of the excitatory period of pheromone responses between 80 and 800 ms were observed.

Neurons that were responding to mechanical stimulation were discarded. Mechanically elicited responses were often characterised by inhibition rather than by excitation. Excitatory responses to mechanical stimulation lasted longer than excitatory responses to sex pheromone on average and no initial burst of APs was noticeable compared to the pheromone responses (Fig. 17).

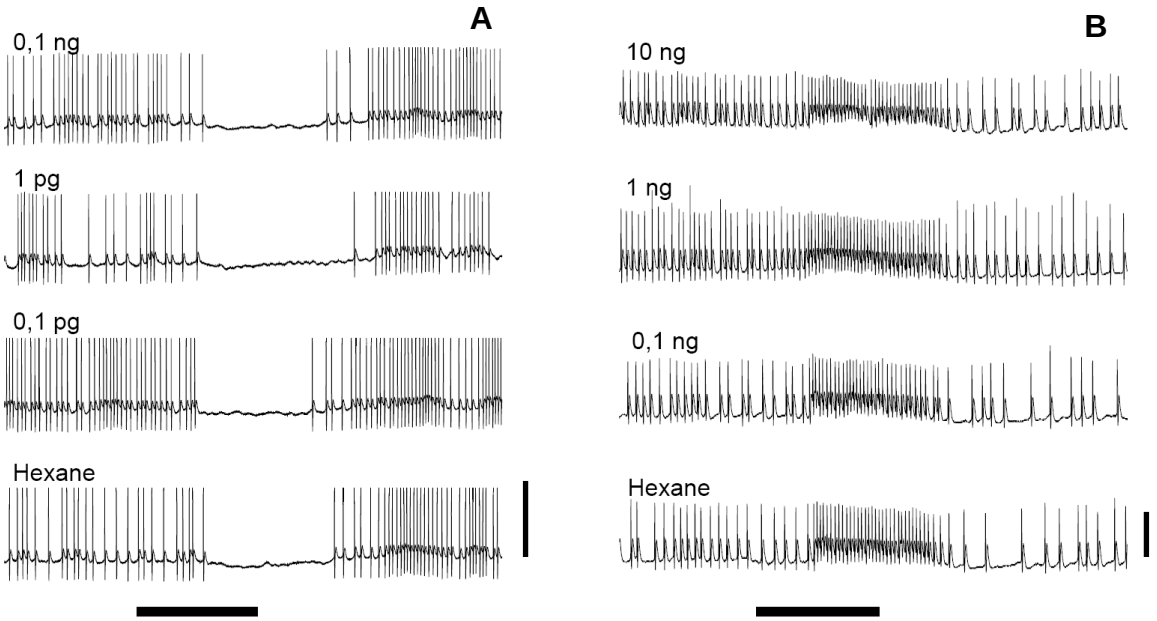

Figure 17 A-B: Typical neuronal responses to mechanical stimulation of AL neurons caused by the air stream. **A**: inhibitory responses to different pheromone doses as well as to the control stimulus (hexane), **B**: excitatory responses to similar stimuli in another neuron. Note the long response duration and immediately recovering spontaneous activity. The horizontal scale bar marks the stimulus duration (500 ms), the vertical scale bars indicate 20 mV.

A characteristic excitatory response of an AL neuron to the main component of the sex pheromone is shown in figure 18: a burst of action potentials is initialising the excitatory phase followed by a decline in AP frequency and an inhibitory phase before the spontaneous activity recovers again.

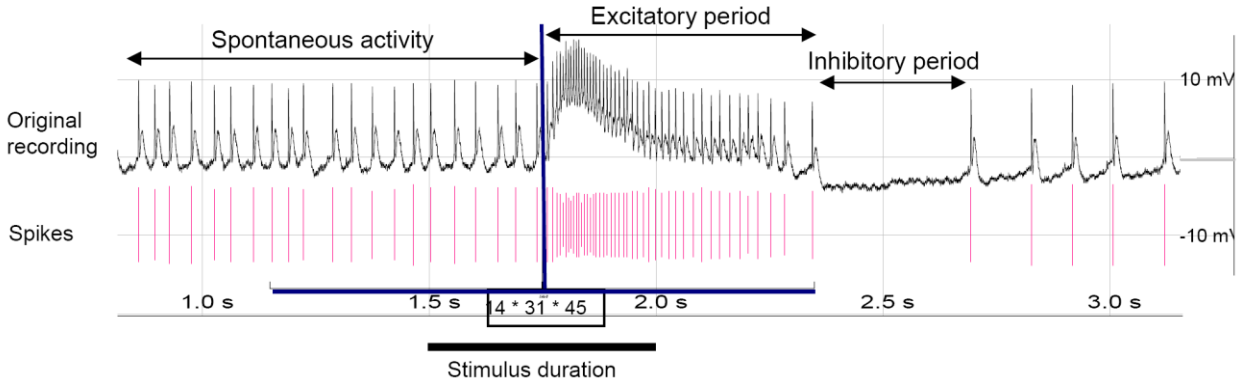

Figure 18: Response of an AL neuron inside the MGC to 10 ng of the synthetic main component of the *S. littoralis* sex pheromone. At 1.5 s a stimulating air stream of 500 ms duration is applied onto the antenna. With a slight latency that indicates the time the odour information needs to reach the AL, the neuron responds, characterised by a burst-like pattern. During this excitatory period the AP frequency rises markedly and is followed by an inhibitory period during which APs are absent. After this the cell comes back to spontaneous activity and is ready to respond again. The blue mark indicates the spike-counting mark. The total spike number is counted before and after the response onset during two identical time windows. In this case the time windows measure 600 ms and spike numbers rise from 14 before to 45 during the response (a gain of 31 spikes or 221% respectively).

The majority of the recorded neurons showed no visible inhibitory period after the excitatory response, and if so, it was observed only at high pheromone doses (10 ng and 100 ng) (Fig. 19 A, B). Usually the frequency of action potentials during the response increased slightly with an increase of the pheromone dose (Fig.19). Figure 19 illustrates the variability of spontaneous neuronal activity and response patterns encountered across the recorded neurons in naïve and pre-exposed males.

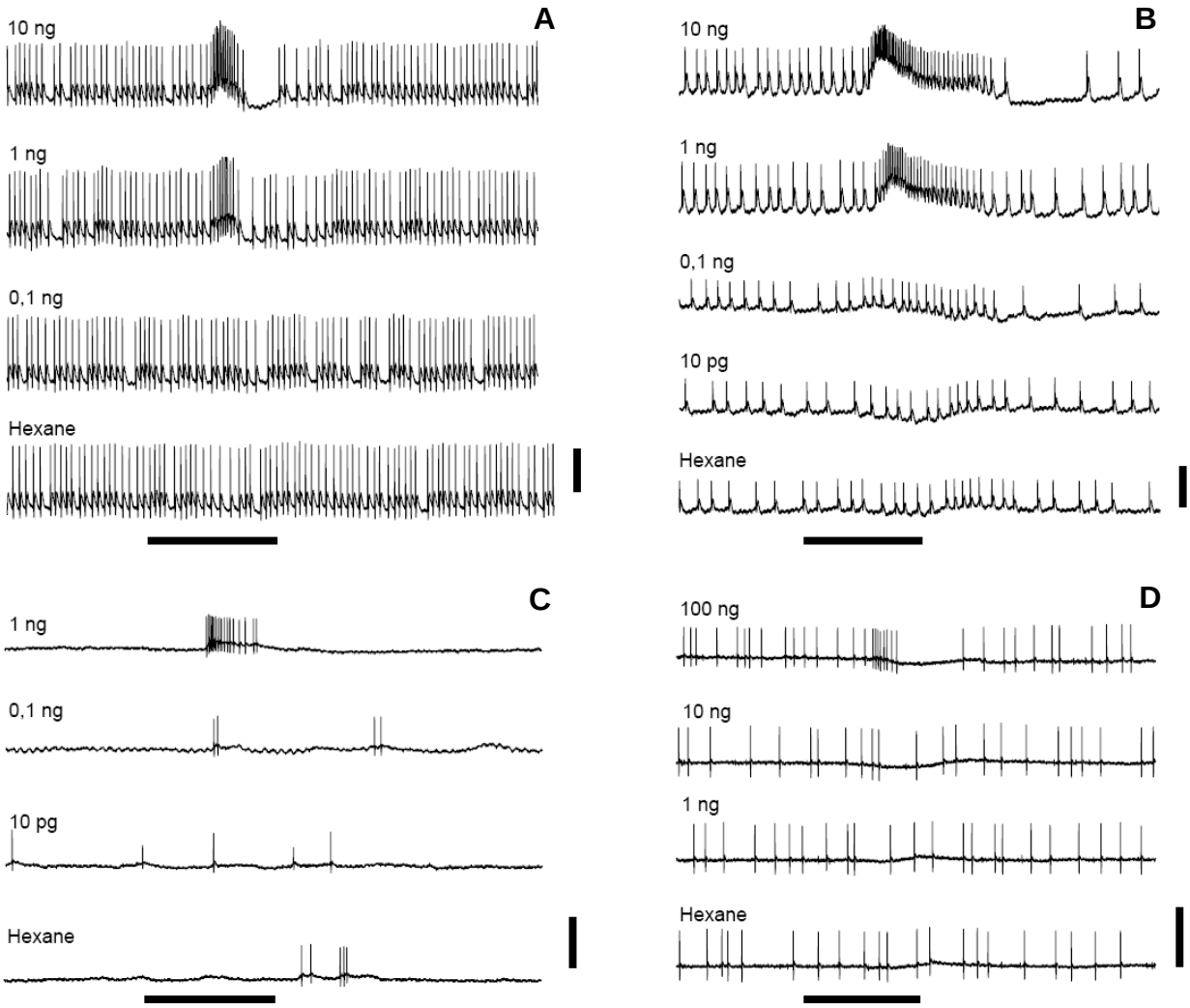

Figure 19 A-D: Different types of responses to the female pheromone in *Spodoptera littoralis* males. **A**: a neuron with a high spontaneous activity and a clearly distinguishable threshold at a concentration of 1 ng/ 10 µl of the sex pheromone. **B**: a cell with its visible threshold at 1 ng, the calculated threshold however lies at 0.1 ng (spike frequency rises more than 20% and exceeds the response to hexane). A and B show an increase of AP frequency with higher pheromone doses and inhibition after excitation. **C**: a cell with very low spontaneous activity and a clear threshold at 1 ng. **D** shows a rather insensitive neuron with a high threshold and a feeble response. Note the strong variation in response duration. Hexane stimuli are shown as control. The horizontal scale bar marks the stimulus duration (500 ms), the vertical scale bars indicate 10 mV.

3.2.2. Response thresholds as a function of pre-exposure

Concerning the response characteristics as well as the response thresholds, antennal lobe neurons in pre-treated and in naïve males did not differ significantly. The threshold was defined as the lowest pheromone dose that still elicited a neuronal response which exceeded the response to control stimuli.

Test doses reached from 0.01 pg to 100 ng in decadic steps. Table 1 shows the thresholds found in naïve and pre-exposed males, tested on the pre-treated (ipsilateral) and the non-treated (contralateral) antenna.

Table 1: Response thresholds of AL neurons in male *Spodoptera littoralis* in the naïve control group and two pre-treated (PE) test groups. Absolute numbers represent numbers of neurons with their response threshold at a certain pheromone dose; numbers in brackets are percentages of the total number of responding neurons.

	Responding neurons	Sex pheromone dose indicating the response threshold							
		0,01pg	**0,1pg**	**1pg**	**10pg**	**0,1ng**	**1ng**	**10ng**	**100ng**
Naïve	31 (100%)	2 (6.45%)	0	0	2 (6.45%)	8 (25.81%)	13 (41.94%)	5 (16.13%)	1 (3.22%)
PE: Test ipsilateral	32 (100%)	2 (6.25%)	0	0	2 (6.25%)	7 (21.88%)	12 (37.49%)	7 (21.88%)	2 (6.25%)
PE: Test contralateral	21 (100%)	1 (4.76%)	0	0	0	4 (19.05%)	13 (61.91%	3 (14.28%)	0

The percentage of AL neurons starting to respond at certain pheromone thresholds was calculated. Statistical analysis was performed using an R X C test of independence using a G-test and applying the Williams's correction (Sokal and Rohlf 1995).

The test revealed no significant difference between pre-exposed males tested on the pre-treated (ipsilateral) and on the non-treated antenna (contalateral) (G_{adj} = 3.472, d.f. = 3, n.s.) (Fig.20A), thus the collected data from all pre-exposed males was pooled (n=53 neurons in N= 25 males). Between pre-exposed and naïve males no significant difference in AL neuron sensitivity was found either (G_{adj} = 0.712, d.f. = 5, n.s.) (Fig. 20B).

The threshold for pheromone detection was situated at a dose of 1 ng in the majority of neurons in naïve (41.9%) and pre-exposed (47.2%) males equally. 20.8% of the neurons in pre-exposed and 25.8% in naïve animals were sensitive to lower doses starting with 0.1 ng. 18.9% of the neurons in pre-exposed and 16.1% in naïve males seemed to be less sensitive, starting to respond to the sex pheromone only at 10 ng. Still more sensitive (up to 0.01 pg) as well as less sensitive (threshold only at 100 ng) neurons were recorded in both groups, but only at percentages of less than 10% (Table 1, Fig. 20).

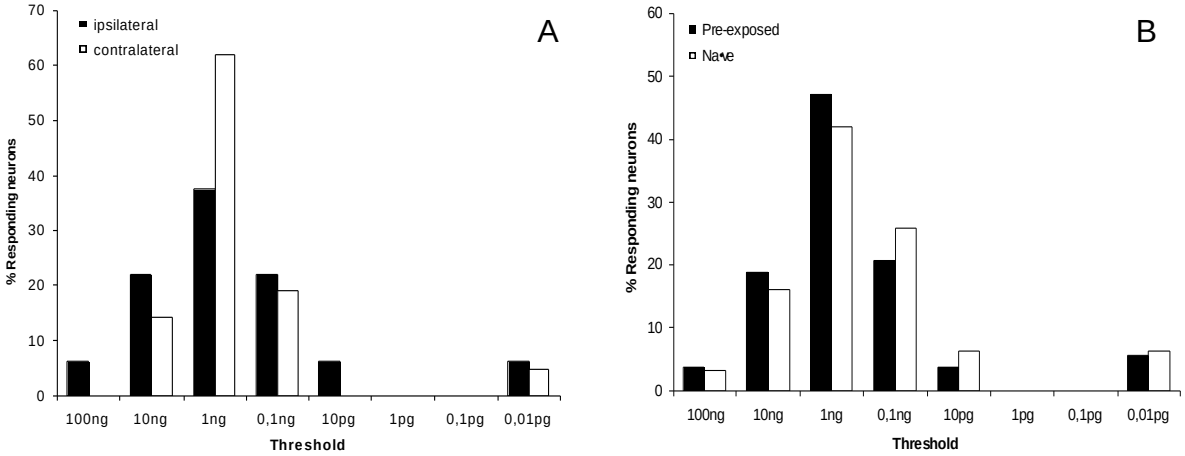

Figure 20 A-B: Sensitivity of AL neurons in *Spodoptera littoralis* males for the main pheromone component 24 h after pre-exposure. The histogram in **A** shows the threshold distribution among sucrose pre-exposed males: No significant difference was found between animals tested on the ipsilateral (n=32) and contralateral (n=21) antenna (G_{adj} = 3.472, d.f. = 3, n.s.). **B**: Threshold distribution in naïve (n=31) and pre-exposed (n=53) males. The pattern is similar for the two groups, indicating the threshold to lie between 0.1 ng and 10 ng in most neurons. Statistical analysis showed no significant difference among the threshold distribution between naïve and pre-exposed animals (G_{adj} = 0.712, d.f. = 5, n.s).

The results indicate that there is no effect of gustatory pre-exposure at the level of the primary olfactory centre, the antennal lobe, that can be measured 22-28 h later. The threshold distribution pattern for sex pheromone detection examined in naïve and gustatory pre-exposed males of *S. littoralis* is strikingly similar (Fig.20B).

As figure 20 shows, there seem to be differently tuned neurons: "very sensitive" and "less sensitive" ones. In less sensitive neurons pheromone doses from 10 pg, 0.1 ng, 1 ng or even higher elicit excitatory responses, whereas very sensitive neurons were observed responding to doses as feeble as 0.01 pg, and the actual threshold might still be lower (Fig. 21). Not a single neuron with its response threshold at 0.1 pg or 1 pg respectively was recorded.

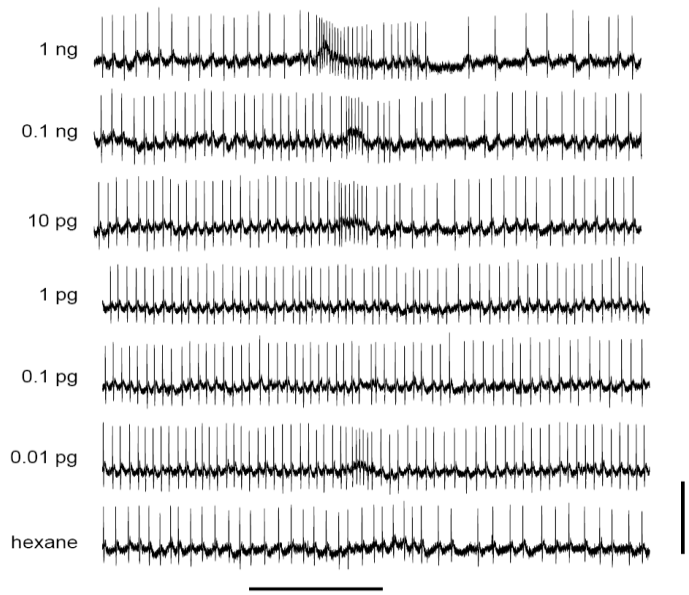

Figure 21: Original recording of an AL neuron in a sucrose pre-exposed male. The response threshold for the female pheromone seems to be located at 0.01pg or lower, but however, responses are short and feeble and no responses to 0.1 and 1 pg could be observed.
Horizontal scale bar: 500ms stimulus duration, vertical scale bar: 10 mV spike amplitude

3.2.3. Neuronal staining

One neuron could successfully be stained using Lucifer Yellow as fluorescent dye and be identified as a projection neuron (Fig. 22A). Its maximal response to the female pheromone was recorded at 0.1 ng, which suggests that the neuron is kind of tuned to this dose and thus not responding like a 'common' MGC neuron (Fig.22B). Indeed, the innervated glomerulus does not seem to be part of the MGC (Fig. 22A) but a response to the sex pheromone did nevertheless occur.

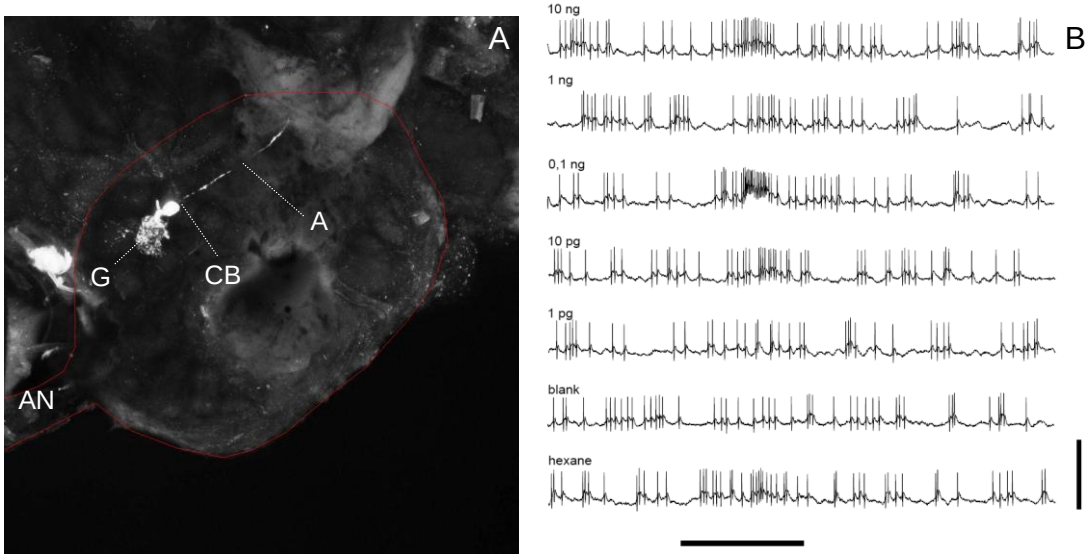

Figure 22: **A**: Staining of a projection neuron within the AL of a naïve male *Spodoptera littoralis*, using Lucifer Yellow as fluorescent dye. The right antennal lobe is outlined, with the antennal nerve (AN) coming from bottom left. The innervated glomerulus (G) is situated close to the cell body (CB), which seems to be part of the medial cluster. It sends its axon (A) to higher brain centres
B: Original recordings of the stained neuron. The threshold to elicit a response exceeding the hexane-response lies at 10 pg of the female pheromone, however, no response to 1 ng could be observed and the response to 0.1 ng exceeds the one at 10 ng. Horizontal scale bar: 500ms stimulus duration, vertical scale bar: 40 mV.

4. DISCUSSION

4.1. Intra-modality: gustatory experiments

The results of this part of the study show that the behaviourally observed long-term effect of gustatory pre-exposure onto behavioural taste sensitivity is not due to changes at the level of sensory input, the taste sensilla, but might rather take place at a higher brain level.

We assumed neuronal plasticity and maturation processes to occur at some level of sensory processing in the gustatory system, between the peripheral sense organs (the gustatory receptor neurons in the antennae) and the central nervous taste areas (the deutocerebrum and the SOG/ Tritocerebrum) (Jørgensen et al. 2006; Popescu 2008). Since the antennal GRNs now can be ruled out as loci of neuronal gustatory plasticity, further research could focus on the central nervous system like the primary gustatory projection centres within the deutocerebrum and the SOG.

4.1.1. Behavioural output

Prior behavioural experiments had shown increased sensitivity for sugar discrimination in male individuals of *Spodoptera littoralis* when they had briefly experienced either an appetitive tastant (sucrose, indicating a food reward) or a repellent tastant (quinine, indicating toxicity of a plant) 24 hours before (S. Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data).

Sucrose as well as quinine used as pre-exposure stimuli could be shown to increase the sensitivity for sucrose concentrations between 0.01 and 0.1 M measured by the percentage of PER, significantly, leading to the assumption that this long-term effect is based on an increase in sensitivity through neuronal maturation processes within the gustatory system, caused by neuronal plasticity depending on experience.

A second series of experiments had also shown a behavioural long-term effect of quinine- and sucrose-pre-exposure onto a test with the repellent quinine 24 h later: *S. littoralis* males pre-exposed to quinine responded with a higher percentage of PER to a mixture of quinine and sucrose than naïve males, whereas pre-exposure with sucrose led to less PER than in naïve males (S. Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data). Different mixtures containing increasing concentrations of quinine (10^{-7} to 10^{-1} M) and a fixed concentration of sucrose (1 M) have been used and effects have been observed at intermediate concentrations of quinine.

These findings suggest that the animals might "compare" the quality of a test stimulus with the pre-exposure stimulus and therefore react to the quality of a tastant (in this case a mixture of sucrose and quinine) according to their prior experience: the mixture tastes "better" on the one hand after pre-exposure to pure bitter quinine and "worse" on the other hand after pre-exposure to pure sweet sucrose.

So where do underlying neuronal effects of the observed behaviour take place? At the sensory or rather at the central nervous level?

4.1.2. Localisation of the neuronal basis of gustatory plasticity

Experiments on olfaction conducted by Anderson et al. in 2007 showed a rise in sensitivity for the female sex pheromone in *S. littoralis* males at the behavioural as well as the central nervous level in AL neurons, when tested 22-28 hours after brief exposure with the same stimulus. Electroantennogram (EAG) recordings, however, showed no changes at the level of the ORNs, indicating that the pre-exposure effect in this case did not occur at the level of sensory input but rather at the level of perception, assuming neuronal plasticity to take place within the CNS (Anderson et al. 2007).

For this reason the working hypothesis of the present study somehow already predicted the negative results that were actually obtained: we assumed that pre-exposure of taste sensilla on the antennae of male moths would not change the sensitivity of the gustatory receptor neurons either but rather lead to neuronal changes within the CNS.

However, since data on *Drosophila* and vertebrate chemosensory sensitivity showed changes to take place at the level of sensory input also (Berteretche et al. 2005; Faurion et al. 2002; Kennedy and Halpern 1980), we still wanted to test this possibility.

The obtained results show a clearly positive correlation between the dose of the sucrose stimulus and the frequency of action potentials in GRNs. Action potential frequency, however, was obviously not affected by pre-exposure and therefore very similar in naïve and pre-exposed animals, leading to the conclusion that experience with neither an attractive nor a repellent tastant affects the sucrose sensitive neurons of the peripheral taste organs within the tested time interval (22-28 h).

The AP frequency of GRNs in sucrose-pre-exposed males was slightly lower for each test stimulus than in naïve and quinine-pre-exposed males (Fig.16). Since this discrepancy was also observed for the control solution, which did not contain sucrose but water and 10^{-3} KCl,

the pre-exposure with 1M sucrose might not affect the responses of sugar-sensitive GRNs but possibly the response characteristics of the salt and/or the water-sensitive GRNs.

To prove this hypothesis, future experiments could involve sucrose- and quinine-pre-exposure and tests to different concentrations of NaCl or KCl using the tip-recording technique to characterise the corresponding GRNs. If sucrose pre-exposure actually influences the response characteristics of the salt-sensitive neurons in an analogous way as shown here, they should be less sensitive to salt than in naïve or quinine pre-exposed animals.

In order to determine at which level within the CNS changes might occur after pre-exposure with gustatory stimuli, more knowledge on central gustatory pathways is necessary.

In the moth *Heliothis virescens* GRNs from the proboscis and the antennae project to two closely located but distinct areas in the SOG/tritocerebrum. Projections from antennal GRNs (SOG) are located posterior-laterally to those of proboscis GRNs (SOG/tritocerebrum) (Jørgensen et al. 2006). In *Spodoptera littoralis* even four different target areas (A1-A4) of GRNs of sensilla chaetica on the antennae could be identified in the deutocerebrum and the tritocerebrum/SOG (Popescu 2008). Mass staining as well as staining of individual sensilla showed one group of neurons projecting to the AMMC of the deutocerebrum, another one to a deutocerebral projection area located posterior to the AL, and two distinct areas in the SOG/tritocerebrum complex. All axons from one individual taste sensillum run together and bypass the ipsilateral AL. One axon does not enter the AMMC but projects to another part of the deutocerebrum (A1), the others all project to the AMMC. One of them terminates within the AMMC (A2), presumably the mechanosensory cell. A third type of axon arborises within the AMMC and projects to the SOG (A3), and a fourth type passes through the AMMC and projects with widespread arborisations into the SOG/tritocerebrum (A4) (Popescu 2008).

4.1.3. Conclusions and outlook

The results of the present study allow ruling out the behaviourally observed long-term effect of taste experience with sucrose and quinine to take place at the level of sensory input, since GRNs of sensilla chaetica on the antennae of *Spodoptera littoralis* males did not change their sensitivity for sucrose within the tested time interval.

The existence of 3 gustatory neurons with different response characteristics in taste sensilla of *Spodoptera littoralis* had been confirmed in a prior study (Popescu 2008, see 4.1.2.): in addition to the mechanoreceptive cell they contain one sugar-sensitive neuron, one

sugar and salt sensitive neuron and a water-sensitive cell. The separate projection areas of the GRNs of one individual sensillum lead to the hypothesis, that the projection areas each gather neurons with the same response characteristics.

Therefore, the next step to reveal the neuronal basis of the sensitivity changes would be, to conduct either extra- or intracellular recordings within these central nervous projection areas while the sensilla chaetica on the ipsilateral antenna are stimulated by sucrose solutions, to see if modulation already occurs within the primary integration area of gustatory information or rather at higher brain levels.

4.2. Cross-modality: olfactory experiments after taste pre-exposure

4.2.1. Response characteristics of antennal lobe neurons

The sensitivity of AL neurons after brief taste experience did not differ in naïve and pre-exposed males. Most animals started to respond to the main pheromone component at a dose of 1 ng. One female equivalent gland extract contains approximately 15 ng of the main pheromone component Z,E-9,11-14:OAc (Hartlieb and Hansson 1999). If we take into account that the pheromone doses were applied onto filter paper and reached the antenna through the stimulating air stream, we can conclude that the amount of pheromone reaching the antenna was lower than the one applied onto the paper, but however we do not know the actually perceived stimulus intensity.

The amounts of pheromones emitted by female moths in their natural environment are highly distributed in the air and reach males over high distances in very low doses (Hansson 1995), so it does not astonish that most males started to respond to pheromone doses far lower than 1 FE.

However, compared to the naïve control group in the studies of Anderson et al. (2007) (Fig.4) and Anton et al. (unpublished, data not shown), where the same electrophysiology set-up with the same stimulating device as well as the same basic pheromone dilution (1µg of the main component diluted in 1 µl hexane) was used, naïve as well as pre-exposed males were more sensitive for the sex pheromone in the present study. Whereas in both preceding studies most animals started to respond only at doses of 10 or even 100 ng of Z,E-9,11-14:OAc, the animals in the present one showed their sensitivity threshold at 0.1 and 1 ng mainly.

This could be due to genetic variations between generations, to variations in activity and sensitivity according to the period of the year in which experiments were conducted, or

simply to a contamination of the basic dilution caused by hexane evaporation and therefore stronger pheromone concentration.

Excitatory responses were in general rather short (often between 200 and 300 ms), feeble and therefore not easily distinguishable from spontaneous neuronal activity (Fig.21).

Responses within the MGC were not only recorded when pheromone was applied onto the antenna but also with hexane as stimulus. The hexane response was in some cases stronger than the reaction to the blank stimulus, indicating that there are neurons which are sensitive for the solvent itself. Many neurons were encountered which responded to hexane as well as to the pheromone stimulus, so obviously not all mechanosensitive neurons project into the AMMC but some either branch directly or via feedback connections into the AL, as already described by Han et al. (2005).

One of only four attempted neuronal stainings was successful. It shows a projection neuron which is probably not part of the MGC. The lack of neuronal staining leaves the proportion of local interneurons and projection neurons within the recorded neurons unknown. Recordings of ORNs can largely be excluded, since their cell bodies are situated within the antennal flagellum and their axon diameters are too small to successfully place the recording electrode within.

4.2.2. Physiological plasticity within the CNS

In order to locate the level at which the pre-exposure effect on pheromone responses takes place in case of brief gustatory experience, I examined the sensitivity of antennal lobe neurons in the MGC for different doses of the synthetic main component of the sex pheromone, after prior brief exposure to sucrose.

The present results are not consistent with prior findings obtained by auditory pre-exposure and allow ruling out the effect to take place within the primary olfactory centre of the CNS in this case, as AL neurons in gustatory pre-exposed males did not change their sensitivity within the tested time interval.

Hence, the behaviourally observed cross-modal long-term effect between taste and olfaction is not correlated with neuronal changes within the antennal lobe.

Behavioural experiments had shown higher sensitivity for female sex pheromone to occur in males when they had briefly experienced an attractive tastant (sucrose) 24 hours before (S.

Minoli, V. Colson, F. Marion-Poll, S. Anton, unpublished data). The same results were observed when a repellent, the bitter tasting alkaloid quinine, was used as pre-exposure stimulus.

The findings of this study indicate neuronal plasticity not to take place at the AL level but probably at an interface between the gustatory and the olfactory system, where input of different sensory modalities is brought together, or upstream, at a higher common processing level. The mushroom bodies as well as the lateral protocerebrum are possible locations for interactions between sensory modalities and could therefore be responsible for the observed cross-modal behavioural effects of pre-exposure.

Experiments on *Spodoptera littoralis* males, where auditory stimuli like a predator signal (pulsed high frequency sound) and a tone (same frequency without pulses) were used for pre-exposure, had revealed an astonishing effect of this brief experience onto the sensitivity of AL neurons as well as onto behaviour, when tested 24 hours later (S. Anton, K. Evengaard, J. Blomqvist, N. Skals, P. Anderson, unpublished data): Experience with the predator sound lowered the threshold for pheromone detection significantly, whereas pre-exposure to a simple tone did not. In addition, pre-exposure with 1 FE of the sex pheromone did have no effect onto the behavioural sensitivity for the predator sound, neither did pre-exposure with the predator sound affect sensitivity for it when tested 24 h later. A possible interpretation for this phenomenon could be that the system to detect a predator is already at maximum sensitivity, due to its indispensable function, and can thus not further mature.

However, these findings indicated that an auditory stimulus which reaches the CNS not even via the antenna but via two sensory neurons ending in the thoracic ganglia and an unknown area within the brain, respectively, can lead to neuronal plasticity which causes the sensitivity for the sex pheromone to rise within the antennal lobe. This requires integration of information reaching the brain via different input channels. Multimodal integration is expected to take place in the protocerebrum (De Belle and Kanzaki and references therein 1999), but nevertheless auditory information must somehow reach the deutocerebrum to modulate AL neurons. Most probably this connection is provided by feedback neurons that project from the protocerebrum back into the MGC of the AL.

Predator sound experience might either cause general maturation within all sensory systems (like an alarm signal telling the moth to be more attentive), or lead to an increase in general or pheromone sensitivity, limited to the olfactory system. The fact that pre-exposure

with a simple tone did not change the sensitivity for the pheromone could be due to a selection at some higher brain level, which lets sensory input only pass when its pattern is behaviourally relevant.

In comparison with the reported results on auditory pre-exposure, we examined in the present study, if this cross-modal effect onto the olfactory system also occurs at the antennal lobe level when pre-exposure stimuli of other modalities, like taste, are used.

Gustatory stimuli are not as crucial as the predator signal but nevertheless relevant for survival. Anyway, in this case no feedback from higher brain centres into the AL seems to take place. Gustatory pre-exposure does not change the sensitivity of AL neurons, therefore neuronal interactions and modulations must take place at a different level. Probably this happens within the protocerebrum, at the level of the taste/smell interface which is likely to be located within the MBs (De Belle and Kanzaki and references therein 1999), or further upstream, where gustatory and olfactory input is processed together to co-ordinate appropriate output.

In contrast to the sensitivity for a predator sound, the behavioural sensitivity for sucrose could be increased by brief olfactory experience with the female sex pheromone. A possible interpretation would be that the detection of food is not as crucial as the detection of a predator, thus maturation of the food detecting system caused by brief olfactory experience of an evolutionary relevant stimulus like the female pheromone is still possible.

Since the olfactory information about the pheromone is directly fed into the AL it is very likely that in case of brief pheromone experience neuronal plasticity already takes place at the AL level. This assumption is further strengthened by anatomical findings that show greater AL volume in males pre-exposed to the sex pheromone than in naïve males (F. Guerreri, unpublished data). PNs from the AL further transfer the odour information to the protocerebrum, from where in this case probably central gustatory areas are affected via feedback neurons after smell/taste integration, which causes gustatory sensitivity to change.

4.2.3. Structural plasticity within the CNS

Neuronal activity is known to influence neuronal architecture, both during development and as a direct function of activity itself (De Belle and Kanzaki and references therein 1999).

In regard to experience-dependent plasticity, the mushroom bodies seem to be especially plastic: In honeybees olfactory experience-dependent structural plasticity occurs within the

MBs of nurses, in foraging workers multimodal sensory input correlates with neuronal plasticity (Durst et al. 1994).

Experiments on *Drosophila* showed similar results: Adult flies that are maintained in cultures enriched with plants and odours of different fruits developed more axons in their peduncles than those deprived of an interesting chemical environment (Technau 1984). *Drospohila* larvae grown in dense populations show a significantly higher number of Kenyon cell processes in their adulthood than individuals reared in sparse populations. As dense populations leave more traces of presence in the food, this stimulates additional fibre re-growth and may consolidate more KC-PNs connections (Heisenberg et al. 1995).

It is not known whether in the case of brief pre-exposure anatomical changes occur too, but regarding the described cases, we can expect structural changes most likely to occur within the mushroom bodies.

4.2.4. Conclusions and outlook

Since no difference in sensitivity between naïve and gustatory pre-exposed males were found at the antennal lobe level, neuronal plasticity must take place elsewhere: either in higher olfactory brain centres, within the gustatory system or at an interface between the two. To test this, extra- or intracellular recordings could be attempted within the protocerebrum, while the ipsilateral antenna is stimulated by the female pheromone.

4.3. General conclusions

Responses to environmental stimuli in *Spodoptera littoralis* are not hard-wired but can be influenced by experience. Behavioural studies have shown that the long-term effect of pre-exposure is induced by general neuronal maturation processes rather than by selective attention, since it could be evoked by cross-modal stimulation with behaviourally relevant stimuli, which male moths likely encounter during their search for available females.

According to the present findings, the neuronal basis for this cross-modal effect is not located within the antennal lobe, but presumably within the protocerebrum, where a synthesis of gustatory and olfactory input takes place. Neuronal plasticity within the gustatory pathway could not be observed at the peripheral sensory level, thus it must be located within the CNS, maybe in the primary integration centre (SOG/tritocerebrum, deutocerebrum) or at a higher processing level within the protocerebrum.

4.4. Problems encountered

The main objective of this study would have been the characterization of the effect of pre-exposure of different sensory modalities onto the processing of sex pheromone within the central olfactory system. For this reason it was planned not only to do gustatory pre-exposure with sucrose, but also pre-exposure with plant odours and test to the female pheromone to complete the results found in behavioural experiments in the wind tunnel (Pingard 2009). In addition it was planned to stain recorded neurons that respond to the sex pheromone to be able to characterize their branching pattern and to determine their projections.

The experiments just did not work. Until the end of May I had collected a fistful of recordings of neurons responding to the sex pheromone, which was of course far too few to do statistical analysis and see any difference – if present – between naïve and pre-exposed animals. The dissection of an insect brain proved to be everything else than simple. Especially in the beginning the time needed for the dissection was the limiting factor for the amount of animals that could be tested during one experimentation day (which means between 11h and 17h on day 2 following pre-exposure).

Another problem within the intracellular experimental design was the quality of the recording electrodes. They were pulled out of borosilicate glass using a horizontal puller, which was seemingly very sensitive to variations in temperature, air pressure and humidity, resulting in variations of electrode shape and resistance. However, to guarantee an electrophysiological recording to be intracellular, these parameters are of crucial importance.

Troubles with the electrode parameters often resulted in too little resistance, which led to extracellular instead of intracellular contact, very low spike size and troubles keeping the cell contact. Small spikes on a constant background noise made it sometimes hard to detect responses. Furthermore these problems made the staining of neurons, which requires intracellular contact established for several minutes as well as the neuron's tolerance of about 1 nA negative current injection nearly impossible.

To gain some results nevertheless, I started to examine in parallel the peripheral gustatory system of male *Spodoptera littoralis* males. For these experiments pre-exposure was done in the same way as for the single cell recordings, but the test procedure was far easier to do. As for the tip recording technique no brain dissection needs to be done, the number of tested animals per experimentation day was much higher and data sampling could be finished in less than one month.

References

Anderson P, Hansson BS, Nilsson U, Han Q, Sjöholm M, Skals N, Anton S (2007) Increased Behavioral and Neuronal Sensitivity to Sex Pheromone after Brief Odor Experience in a Moth. Chemical Senses 32: 483-491

Anderson P, Sadek MM, Hansson BS (2003) Pre-exposure Modulates Attraction to Sex Pheromone in a Moth. Chemical Senses 28: 285-291

Anton S, Dufour MC, Gadenne C (2007) Plasticity of olfactory-guided behaviour and its neurobiological basis: lessons from moths and locusts. Entomologia Experimentalis et Applicata 123: 1-11

Anton S, Gadenne C (1999) Effect of juvenile hormone on the central nervous processing of sex pheromone in an insect. Proceedings of the National Academy of Sciences USA 96: 5764-5767

Anton S, Homberg U (1999) Antennal Lobe Structure. In: Hansson BS (editor) Insect Olfaction. Springer Verlag Berlin (Germany): p 98-125

Baker TC (1989) Sex pheromone communication in the Lepidoptera: New research progress. Experientia 45: 248-262

Berteretche MV, Boireau-Ducept N, Pillias AM, Faurion A (2005) Stimulus-increase of taste responses in the hamster chorda tympani by repeated exposure to 'novel' tastants. Appetite 45: 324-333

Blaney WM, Simmonds MSJ (1990) A behavioural and electrophysiological study of the role of tarsal chemoreceptors in feeding by adults of *Spodoptera*, *Heliothis virescens* and *Helicoverpa armigera*. Journal of Insect Physiology 36: 743-756

Christensen TA, Hildebrand JG (1987) Male-specific, sex pheromone-selective projection neurons in the antennal lobes of the moth *Manduca sexta*. Journal of Comparative Physiology A 160: 553-569

Carlsson MA, Galizia CG, Hansson BS (2002) Spatial representations of odours in the antennal lobe of the moth *Spodoptera littoralis* (Lepidoptera: Noctuidae). Chemical Senses 27 (3): 231-244

Dahanukar A, Hallem EA, Carlson JR (2005) Insect Chemoreception. Current Opinion in Neurobiology 15: 423-430

De Belle JS, Kanzaki R (1999) Protocerebrum Olfactory Processing. In: Hansson BS (editor) Insect Olfaction. Springer Verlag Berlin (Germany): p 243-281

Dethier VG (1976) The Hungry Fly: a Physiological Study of the Behavior Associated with Feeding. Harvard University Press, Cambridge

Dufour MC, Gadenne C (2006) Adult neurogenesis in a moth brain. Journal of Comparative Neurology 495: 635-643

Durst C, Eichmüller S, Menzel R (1994) Development and experience lead to increased volume of subcompartments of the honeybee mushroom body. Behavioural and Neural Biology 62: 259-263

EPPO (European and Mediterranean Plant Protection Organisation) A2 List of pests recommended for regulation as quarantine pests (version 2008-09), Data sheets on *Spodoptera littoralis* and *Spodoptera littura*, Internet download

Fan RJ, Anderson P, Hansson BS (1997) Behavioural analysis of olfactory conditioning in the moth *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Journal of Experimental Biology 200: 2969-2976

Faurion A, Cerf B, Pillias AM, Boireau N (2002) Increased taste sensitivity by familiarization to novel stimuli: psychophysics, fMRI, and electrophysiological techniques suggest modulation at peripheral and central levels. In: Ruby C (ed.) Chapter in Olfaction, Taste and Cognition. Cambridge University Press

Gadenne C, Dufour MC, Anton S (2001) Transient post-mating inhibition of behavioural and central nervous responses to sex pheromone in an insect. Proceedings of the Royal Society B 268: 1631-1635

Gadenne C, Renou M, Streng L (1993) Hormonal control of sex pheromone responsiveness in the male black cutworm, *Agrotis ipsilon*. Experientia 49: 721-724

Gonzalez KM, Peo C, Livdahl T, Kennedy LM (2008) Experience-Induced Changes in Sugar Taste Discrimination. Chemical Senses 33: 173-179

Hallem EA, Dahanukar A, Carlson JR (2006) Insect Odor and Taste Receptors.
Annual Review of Entomology 51: 113-135

Han Q, Hansson BS, Anton S (2005) Interactions of mechanical stimuli and sex pheromone information in antennal lobe neurons of a male moth, *Spodoptera littoralis*. Journal of Comparative Physiology A 191: 521-528

Hansson BS (1995) Olfaction in Lepidoptera. Experientia 51: 1003-1027

Hartlieb E, Anderson P, Hansson BS (1999) Appetitive Learning of Odours with Different Behavioural Meaning in Moths. Physiology and Behavior 67: 671-677

Hartlieb E, Hansson BS (1999) Sex or Food? Appetitive Learning of Sex Odors in a Male Moth. Naturwissenschaften 86: 396-399

Heisenberg M, Heusipp M, Wanke C (1995) Structural plasticity in the *Drosophila* brain. Journal of Neuroscience 15: 1951-1960

Hildebrand JG (1996) Olfactory control of behaviour in moths: central processing of odor information and the functional significance of olfactory glomeruli. Journal of Comparative Physiology A 178: 5-19

Hiroi M, Meunier N, Marion-Poll F, Tanimura T (2004) Two antagonistic gustatory receptor neurons responding to sweet-salty and bitter taste in *Drosophila*. Journal of Neurobiology 61: 333-342

Hodgson ES, Lettvin JY, Roeder KD (1955) Physiology of a primary chemoreceptor unit. Science 122: 417-418

Inoshita T, Tanimura T (2006) Cellular identification of water gustatory receptor neurons and their central projection pattern in *Drosophila*. Proceedings of the National Academy of Sciences of the United States of America 103: 1094-1099

Jarriault D, Barrozo RB, Carvalho Pinto CJ, Greiner B, Dufour MC, Masante-Roca I, Gramsbergen JB, Anton S, Gadenne C (2009) Age-dependent plasticity of sex pheromone response in the moth, *Agrotis ipsilon*: Combined effects of octopamine and juvenile hormone. Hormones and Behavior 56: 185-191

Jørgensen K, Kvello P, Almaas TJ, Mustaparta H (2006) Two Closely Located Areas in the Suboesophageal Ganglion and the Tritocerebrum Receive Projections of Gustatory receptor Neurons Located on the Antenna and the Proboscis in the Moth *Heliothis virescens*. Journal of Comparative Neurology 496: 121-134

Kehat M, Dunkelblum E (1993) Sex pheromones: Achievements in monitoring and mating disruption of cotton pests in Israel. Insect Biochemistry and Physiology 22: 425-431

Kennedy LM, Halpern BP (1980) Fly chemoreceptors: a model system for the taste modifier Ziziphin. Physiology and Behaviour 24: 135-143

Klun JA, and cooperators (1975) Insect sex pheromones: intraspecific variability of *Ostrinia nubilalis* in North America and Europe. Environmental Entomology 4: 891-894

Krieger J, Gänßle H, Raming K, Breer H (1993) Odorant binding proteins of *Heliothis virescens*. Insect Biochemistry and molecular Biology 23: 449-456

Kvello P, Almaas TJ, Mustaparta H (2006) A confined taste area in the lepidopteran brain. Arthropod Structure and Development 35: 35-45

Ljungberg H, Anderson P, Hansson BS (1993) Physiology and Morphology of Pheromone-specific Sensilla on the Antennae of Male and Female *Spodoptera littoralis* (Lepidoptera: Noctuidae). Journal of Insect Physiology 39: 253-260

Masante-Roca I, Anton S, Delbac L, Dufour MC, Gadenne C (2007) Attraction of the grapevine moth to host and non-host plant parts in a wind tunnel: effects of plant phenology, sex, and mating status. Entomologia Experimentalis et Applicata 122: 239-245

Mitchell BK, Itagaki H, Rivet MP (1999) Peripheral and Central Structures Involved in Insect Gustation. Microscopy Research and Technique 47: 401-415

Ochieng SA, Anderson P, Hansson BS (1995) Antennal lobe projection patterns of olfactory receptor neurons involved in sex pheromone detection in *Spodoptera Litoralis* (Lepidoptera: Noctuidae). Tissue and Cell 27 (2) 221-232

Ozaki M, Tominaga Y (1999) Contact Chemoreceptors. In: Eguchi E and Tominaga Y (eds.) Atlas of arthropod sensory receptors, dynamic morphology in relation to function. Springer Verlag (Tokyo) pp. 143-154

Pingard C (2009) Effet de la pré-exposition olfactive sur la réponse comportementale aux odeurs chez le mâle de la noctuelle du coton, *Spodoptera littoralis*. Rapport de Stage, Université Tours

Poitout S, Bues R (1974) Rearing Larvae of 28 species of Noctuidae and 2 species of Arctiidae (Lepidoptera) on a simple artificial diet breeding peculiarities according to the different species. Annales de Zoologie Ecologie Animale 6: 431-441

Popescu A (2008) Mechanisms of gustatory coding in *Spodoptera littoralis.* Doctoral thesis, Agro Paris Tech

Sato K, Pellegrino M, Nakagawa T, Vosshall LB, Touhara K (2008) Insect olfactory receptors are heteromeric ligand-gated ion channels. Nature 452: 1002-1009

Sokal RR, Rohlf FJ (1995) Biometry. The Principle and Practise of Statistics in Biological Research. Third edition, W.H. Freeman, New York.

Steinbrecht RA (1987) Functional morphology of pheromone-sensitive sensilla, in: Prestwich GD and Blomquist GJ (eds.) Pheromone Biochemistry. Academic Press (New York): pp. 353-383

Steinbrecht RA, Gnatzy W (1984) Pheromone receptors in *Bombyx mori* and *Antherea pemyi*. I. Reconstruction of the cellular organisation of sensilla trichodea. Cell and Tissue Research 235: 25-34

Stengl M, Ziegelberger G, Boekhoff I, Krieger J (1999) Perireceptor Events and Transduction Mechanisms in Insect Olfaction. In: Hansson BS (ed.) Insect Olfaction. Springer Verlag Berlin (Germany): p 49-66

Technau GM (1984) Fibre number in the mushroom bodies of adult *Drosophila melanogaster* depends on age, sex and experience. Journal of Neurogenetics 1: 113-126

Thorne N, Chromey C, Bray S, Amrein H (2004) Taste perception and coding in *Drosophila*. Current Biology 14: 1065-1079

Tolbert LP, Hildebrand JG (1981) Organization and synaptic ultrastructure of glomeruli in the in the antennal lobes of the moth *Manduca sexta*: a study using thin sections and freeze-structure. Phil. Trans. Royal Society London B213: 297-301

Vogt RG, Riddiford LM (1981) Pheromone binding and inactivation by moth antennae. Nature 293: 161-163

Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96: 725-736

Wang L, Chen L, Jacob T (2003) Evidence for peripheral plasticity in human odour response. Journal of Physiology 554: 236-244

Wicher D, Schafer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS (2008) *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. Nature 452: 1007-1010

Zacharuk RY (1980) Ultrastructure and function of insect chemosensilla. Annual Review of Entomology 25: 27-47

Image sources

Figure 1A: Internet, EPPO homepage http://photos.eppo.org
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Figure 1B: Internet, INRA homepage http://www-physiologie-insecte.versailles.inra.fr
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Figure 2: Hansson BS ed. (1999) Insect Olfaction. Springer Verlag Berlin (Germany)

Figure 3: Popescu A (2008) Mechanisms of gustatory coding in *Spodoptera littoralis.* Doctoral thesis, Agro Paris Tech

Figure 4: Anderson P, Hansson BS, Nilsson U, Han Q, Sjöholm M, Skals N, Anton S (2007) Increased Behavioral and Neuronal Sensitivity to Sex Pheromone after Brief Odor Experience in a Moth. Chemical Senses 32: 483-491

Figure 7: photographed by Sebastian Minoli

Figures 8, 10, 11: photographed by Isabella Kauer

APPENDIX

Tucson Ringer:

8.76 g NaCl
0.333 g $CaCl_2$
0.224 g KCl

2.29g TES (buffer)

Dilute ingredients in 1 litre of ultra pure H_2O
Add NaOH for pH (6.9)
Add 8.55 g sucrose and store refrigerated

Lucifer Yellow Fix:

I
5% formaldehyde
(67.5 ml 37% formaldehyde + 432.5 ml H_2O)

II
Solution A: 27.8 g NaH_2PO_4 / l
Solution B: 28.4 g Na_2HPO_4 / l

Mix 1 part of A and 4 parts of B (5 ml of A + 20 ml of B)

Mix 50 ml of I and 50 ml of II
Add 3 g sucrose and store refrigerated

Millonig's Buffer

Solution A: 2.26% $NaH_2PO_4 \times H_2O$
Solution B: 2.52% NaOH as pellets

For pH 7.2 mix 83.9 ml A and 16.1 ml B
Add 1.3 g sucrose and store refrigerated

ZUSAMMENFASSUNG

Männliche Nachtfalter besitzen eine bereits angeborene hohe Empfindlichkeit für die Sexualpheromone arteigener Weibchen. Verhaltensreaktionen auf das Weibchenpheromon können bei Männchen der Spezies *Spodoptera littoralis* dennoch durch eine sehr kurze, einmalige Vorexponierung mit dem Pheromon weiter verstärkt werden (Anderson et al. 2003, 2007). Diese Empfindlichkeitssteigerung wurde in zwei Zeitfenstern nach der Vorexponierung beobachtet: es kam zu einem nach 15 Minuten gemessenen Kurzzeiteffekt und einem Langzeiteffekt, der bis zu 51 Stunden anhielt. Parallel dazu erhöhte sich die Pheromon-Empfindlichkeit der Neurone in den primären olfaktorischen Zentren, den Antennalloben (AL), 27 Stunden nach der Vorexponierung mit dem Pheromon.

In der vorliegenden Arbeit wurden Geschmacksreize für die Vorexponierung und den darauffolgenden Test verwendet: die Empfindlichkeit für Zucker in den gustatorischen Rezeptorneuronen auf den Antennen von *Spodoptera littoralis* Männchen wurde 24 Stunden nach einer kurzen Vorbehandlung mit Zucker bzw. Chinin mithilfe von extrazellulären elektrophysiologischen Ableittechniken gemessen, um die neurobiologischen Vorgänge zu studieren, die der in einer vorhergehenden Studie beobachteten erfahrungsabhängigen Plastizität im Verhalten zugrunde liegen.

In einer zweiten Serie von Experimenten wurde die Empfindlichkeit für das Weibchenpheromon nach kurzer Vorexponierung mit Geschmacksreizen getestet: die Empfindlichkeitsschwelle zentraler olfaktorischer Neurone im Antennallobus für das Sexualpheromon zwischen Zucker-exponierten und naiven Tieren wurde mithilfe von intrazellulären Ableitmethoden verglichen. Ergebnisse einer parallel durchgeführten Verhaltensstudie hatten darauf hingewiesen, dass Geschmackserfahrung auch die Empfindlichkeit für das Pheromon steigert.

Die Ergebnisse der vorliegenden Arbeit deuten darauf hin, dass neuronale Plastizität innerhalb des Geschmackssystems nicht auf der peripheren sensorischen Ebene stattfindet, da keine Unterschiede in der Empfindlichkeit gustatorischer Rezeptorneurone für Saccharose zwischen naiven und vorexponierten Männchen gefunden wurde. Die neuronale Basis der intra-modalen Plastizität befindet sich demnach vermutlich im zentralen Nervensystem, es ist bisher jedoch unbekannt, ob zentrale Geschmacksneurone ihre Antworteigenschaften überhaupt erfahrungsabhängig ändern, oder ob die im Verhalten beobachtete Effekte anders verursacht wurden.

Außerdem scheint sich die neuronale Grundlage des auf Verhaltensebene beobachteten, Sinnesmodalitäten überkreuzenden Effekts der gustatorischen Vorbehandlung nicht auf Ebene des Antennallobus zu befinden, da sich die Empfindlichkeit der AL-Neurone für das weibliche Sexualpheromon in naiven und mit Zucker vorbehandelten Männchen nicht unterschied. Die nicht-assoziativen Lernprozesse, die durch die Präexponierung verursacht werden laufen demnach vermutlich auf höheren Verarbeitungsebenen im Protocerebrum, wie den Pilzkörpern und dem lateralen Protocerebrum, ab, wo multimodale Sinneseindrücke verschaltet und gemeinsam verarbeitet werden.

Curriculum vitae

Name: Isabella Kauer
Geboren: 21.06.1985, Linz (Oberösterreich)
Eltern: Ulrike Kauer, HS-Lehrerin
Ing. Robert Kauer, pensionierter Innenarchitekt
Staatsbürgerschaft: Österreich
Familienstand: ledig

Schulbildung

09/1991 - 07/1995: Volksschule Puchenau (Oberösterreich)
09/1995 - 06/2003: Georg von Peuerbach Gymnasium, Linz
AHS-Matura mit ausgezeichnetem Erfolg

Studienverlauf

09/2003 - 07/2004: Außerordentliches Studium im Fach Violine bei Josef Sabaini
Anton Bruckner Privatuniversität, Linz
10/2004 - 03/2006: Grundstudium Biologie (A437), Universität Wien
Abschluss mit der 1. Diplomprüfung
03/2006 - 11/2009: Diplomstudium Zoologie (A439), Universität Wien
Forschungsschwerpunkt Neurobiologie
Abschluss mit der 2. Diplomprüfung
02/2009 – 08/2009: Forschungsaufenthalt am Institut National de la Recherche Agronomique (INRA) in Versailles (Frankreich) bei Dr. Sylvia Anton zum Anfertigen der Diplomarbeit unter Mitarbeit am staatlich geförderten Projekt „Pre-Exposure“; Präsentation der Ergebnisse am 19. Kongress der European Chemoreception Research Organization (ECRO) in Villasimius (Sardinien)

Fremdsprachenkenntnisse: Englisch fließend, Französisch fließend