Natur
Technik
Kultur
Gesellschaft

UNIKASSEL
VERSITÄT

# Electrophysiological analysis of the olfactory signal transduction cascade in the hawkmoth *Manduca sexta*

**Dissertation**

zur Erlangung des akademischen Grades eines
Doktors der Naturwissenschaften (Dr. rer. nat.)

vorgelegt von

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Kassel, 22.10.2014

Vom Fachbereich 10, Mathematik und Naturwissenschaften, der Universität Kassel als Dissertation am 22.10.2014 angenommen.

## Prüfungskommission

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Tag der mündlichen Prüfung: 14.12.2015

*„Wissenschaft: Es ist nicht ihr Ziel, der unendlichen Weisheit eine Tür zu öffnen, sondern eine Grenze zu setzen dem unendlichen Irrtum.“*

Bertolt Brecht (1898-1956)

## Eidesstattliche Erklärung

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Andreas Nolte

# Content

# I Contribution statements

My contributions for each chapter will be stated clearly according to the "Allgemeine Bestimmungen für Promotionen an der Universität Kassel (AB-PromO) vom 13. Juni 2012".

**In situ tip recordings found no evidence for an Orco-based ionotropic mechanism of pheromone-transduction in *Manduca sexta***

- Development and implementation of long-term and short-term tip recording experiments (55 of 58)
- Development and implementation of all spontaneous activity experiments
- Evaluation and analysis of all tip recording experiments
- Wrote the manuscript in cooperation with Prof. Dr. Monika Stengl
- Data are published in Nolte et al. (2013) in PLoS ONE 8(5): e62648

**Effects of prospective Orco antagonists on pheromone responses in the hawkmoth *Manduca sexta***

- Development and implementation of long-term and short-term tip recording experiments (79 of 79)
- Development and implementation of competitive blockage experiments
- Current injection experiments (Fig. 21) were performed to the same extent by Sarah Körte
- Evaluation and analysis of all tip recording experiments
- Wrote the manuscript in cooperation with Prof. Dr. Monika Stengl

**Blocking the metabotropic pathway by G-protein blocker GDP-β-S reduced pheromone responses in the hawkmoth *Manduca sexta***

- Planning and construction of a new setup
- Development and implementation of all long-term tip recording experiments (32 of 32)
- Evaluation and analysis of all experiments
- Wrote the manuscript in cooperation with Prof. Dr. Monika Stengl

# II Zusammenfassung

Insekten spielen im Ökosystem der Erde eine wichtige Rolle und sind auch für den Menschen von großer ökologischer und ökonomischer Bedeutung. Die Insekten als artenreichste Klasse der Tiere sind von enormer Wichtigkeit bei der Bestäubung von Pflanzen und somit verantwortlich für einen beträchtlichen Teil des landwirtschaftlichen Ertrags. Besonders relevant für Menschen sind auch Schadinsekten, welche jedes Jahr enorme agrarwirtschaftliche Schäden verursachen und somit an Missernten und Hungersnöten beteiligt sind. Zudem sind eine Vielzahl von Insekten Überträger von gefährlichen Krankheiten, wie z. B. der Malaria. Allein dieser von verschiedenen Stechmückenarten der Gattung *Anopheles* übertragenen Krankheit fallen jährlich mehr als 1 Millionen Menschen zum Opfer (Malaria-Report 2013, WHO). Eine Vielzahl von Verhaltensweisen von Insekten, wie z. B. die Futtersuche oder das Paarungsverhalten, werden hauptsächlich durch den Geruchssinn gesteuert. Der Tabakschwärmer *Manduca sexta* ist bei der Erforschung des Geruchssinns zu einem wichtigen Modelorganismus geworden. Er ist wie viele flugfähige Insekten in der Lage, selbst kleinste Duftstoffkonzentrationen über große Entfernungen wahrzunehmen (Kaissling and Priesner 1970; Kaissling 1987). Dazu befähigt werden sie von speziellen Duftstoffrezeptoren, welche sich auf den Dendriten von olfaktorischen Rezeptorneuronen (ORNs) befinden (Clyne et al. 1999; Vosshall et al. 1999). Die dendritischen Ausläufer ziehen in Sensillen unterschiedlichster Morphologie, welche über die ganze Antenne in enormer Anzahl verteilt sind (Lee and Strausfeld 1990). Die Pheromon-sensitiven Trichoidsensillen werden von zwei oder mehr ORNs innerviert (Sanes and Hildebrand 1976; Keil 1989; Lee and Strausfeld 1990), wobei jedes ORN einen oder wenige olfaktorische Rezeptortypen exprimiert (Hildebrand and Shepherd 1997; Couto et al. 2005). Neben diesem duftstoffbindenden Rezeptor konnte zudem bei den meisten ORNs in Insekten ein Korezeptor gefunden werden (Orco, *olfactory receptor co-receptor*) (Vosshall et al. 1999). Dieser spielt eine essentielle Rolle bei Einbau und Lokalisation des olfaktorischen Rezeptors in die dendritische Membran (Larsson et al. 2004). Fehlt der Korezeptor Orco, z. B. durch *Knock-Out*-Mutationen, so wird deshalb auch die olfaktorische Antwort stark reduziert (Larsson et al. 2004; Neuhaus et al. 2005). An der Bindung von Duftstoffen scheint er

jedoch nicht beteiligt zu sein. Der Korezeptor Orco ist hochgradig konserviert und somit zwischen unterschiedlichen Insektenspezies austauschbar (Krieger et al. 2003). Welche Rolle Orco allerdings im Einzelnen bei der olfaktorischen Signaltransduktionskaskade spielt, ist bislang nicht bekannt. Viele Forschungsgruppen gehen der Frage der olfaktorischen Reizweiterleitung nach, kamen dabei allerdings zu teilweise widersprüchlichen Ergebnissen. Während einige Ergebnisse für eine ausschließlich ionotrope Transduktion sprechen (Sato et al. 2008), konnte in anderen Veröffentlichungen zumindest die Beteiligung von metabotropen Prozessen bei der Bildung einer Duftstoff-abhängigen Reizantwort gezeigt werden (Wicher et al. 2008). Somit ist der Mechanismus der Signalweiterleitung bis heute nicht vollständig geklärt. Zudem stellt sich auch die Frage, welche Rolle der Korezeptors hierbei einnimmt. Durch die Entwicklung von speziellen Orco-Agonisten und Antagonisten (Jones et al. 2011; Chen and Luetje 2012) innerhalb der letzen Jahre konnte ein neuer Ansatz bei der Erforschung dieser Fragestellung geschaffen werden. In dieser Arbeit soll daher die Rolle von Orco *in-vivo* anhand von extrazellulären Einzelsensillenableitungen beim olfaktorischen Modelorganismus *Manduca sexta* ergründet werden. Zusätzlich wurde ein G-Protein-Blocker verwendet, mit dem weitere Details über die Beteiligung einer möglichen metabotropen Signalkasdade ermittelt werden sollten. Für alle Experimente wurden zudem tageszeitabhängige Unterschiede ergründet. Die Versuche erfolgten in zwei Zeitfenstern, zum einen am Ende der Aktivitätsphase der Tiere zwischen Zeitgeberzeit (*Zeitgebertime*, ZT) 1 - 3 und zum anderen in der Ruhephase der nachtaktiven Schwärmer zwischen ZT 9 - 11.

**Auswirkungen einer Orco-Aktivierung auf Spontanaktivität und Duftstoffantwort**

Durch die Forschergruppe Jones konnte im Jahre 2011 in einer breit angelegten Suche der spezifische Orco-Agonist *N-(4-ethylphenyl)-2((4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl) thio)acetamide* (VUAA1) ermitteln werden. Calcium-Imaging-Versuche von Nico Funk und Latha Mukunda an HEK-Zellen, in die der *Manduca* Orco MsexOrco transfiziert wurde, konnten die Spezifität von VUAA1 auf den Korezeptor nachweisen. Im Rahmen dieser Arbeit wurden daraufhin extrazelluläre Einzelsensillenableitungen durchgeführt. Neben dem Einfluss von VUAA1 auf die Spontanaktivität der ORNs

wurden zudem die Effekte auf die pheromonabhängige Antwort der Neurone untersucht. Die Sensillenpotentialamplitude (SPA), welche zum Teil das nach Pheromonbindung entstehende Rezeptorpotential der olfaktorischen Nervenzellen repräsentiert, wurde durch VUAA1 nicht beeinflusst. Eine Beteiligung von Orco an der Ausbildung der SPA erscheint daher unwahrscheinlich. Während die Aktionspotentialfrequenz der ersten sechs stimulationsbedingten Aktionspotentiale innerhalb der ersten 20 Minuten der Ableitungen keine deutliche Veränderung unter dem Einfluss von VUAA1 aufwies, nahm die AP-Frequenz im Verlauf der zweistündigen Langzeitableitungen signifikant ab. Die Kinetiken der Ableitungen wurden innerhalb der ersten 20 Minuten, in denen das abgeleitete Neuron mehrfach mit Pheromon stimuliert wurde, weder in der Aktivitätsphase noch in der Ruhephase stark beeinflusst. Zum Ende der Ableitungen zeigte sich allerdings eine deutliche Abnahme der phasischen Komponente in der Pheromonantwort. Weiterhin erhöhte VUAA1 die Latenzzeit des ersten APs nach Pheromonstimululation zum Ende der Ableitungen in beiden Zeitfenstern. Da Duftstoffe ausschließlich an den olfaktorischen Rezeptoren binden, VUAA1 hingegen spezifisch an Orco, wäre bei einer ionotropen Signalweiterleitung ein synergistischer Effekt zu erwarten. Dieser sollte sich in einer Steigerung der Sensitivität und somit in einer schnelleren und stärkeren Pheromonantwort manifestieren. Diese konnte allerdings in keinem der durchgeführten Experimente gezeigt werden. Vielmehr zeigte sich häufig eine Reduktion der Sensitivität. Daher konnte kein Hinweis für eine ionotrope Signalweiterleitung gefunden werden. Im Gegensatz zu den dargestellten Parametern der schnellen Pheromonantwort wurde die langanhaltende Pheromonantwort, die noch Minuten nach der Stimulation erkennbar ist, durch die Applikation von VUAA1 deutlich erhöht. Dieses zeigte sich ebenfalls bei der Spontanaktivität. Während in Kontrollexperimenten ohne vorherige Pheromonstimulation nahezu keine Spontanaktivität zu verzeichnen war, wurde diese durch VUAA1 Dosis-abhängig gesteigert. Beides ist mit der Eigenschaft von Orco als kontinuierlich offenem Ionenkanal zu erklären, dessen Offenwahrscheinlichkeit durch die Wirkung von VUAA1 verstärkt wird. Orco scheint daher eher modulierend auf den generellen Erregungszustand der Zelle einzuwirken.

**Auswirkungen potentieller Orco-Antagonisten auf die Pheromonantwort**

Nachdem die spezifische Aktivierung des olfaktorischen Korezeptors in *M. sexta* gezeigt wurde, stellt sich zudem die Frage, ob Orco ebenso gezielt gehemmt werden kann. Die Entwicklung eines Orco-selektiven Blockers wäre aufgrund der hohen Konservierung innerhalb der Insekten ein breit nutzbares Instrument bei der Bekämpfung von Schadinsekten. Neben eher unspezifisch wirksamen Substanzen wie Ruthenium-Rot oder Diethyltoluamid (DEET) konnte gezeigt werden, dass Amiloride Duftstoffantworten blockieren können (Frings and Lindemann 1988; Frings et al. 1992). Hierzu zählen unter anderem die Substanzen 5-(N,N-hexamethylene)amiloride (HMA) und 5-(N-methyl-N-isobutyl)amiloride (MIA), dessen Wirkung bereits in Zellsystemexperimenten nachgewiesen wurde (Pask et al. 2013). Ob diese allerdings spezifisch auf Orco wirken, ist bislang nicht geklärt und wurde im Rahmen dieser Arbeit untersucht. Eine weitere vielversprechende Substanz, die für eine Blockierung von Orco in Frage kommt ist OLC15 (Chen and Luetje 2012). Diese ist strukturell verwandt mit dem entwickelten Orco-Agonisten VUAA1. Eine spezifische Bindung an Orco ist daher denkbar. Neben der Auswertung der Pheromonantworten erfolgte in einigen Experimenten die Stimulation mittels Strominjektion. Ziel war es, den Einfluss von OLC15 auf die auf diese Weise depolarisierten Zellen zu ergründen. Des Weiteren wurden ORNs durch VUAA1 stimuliert, wobei die zusätzliche Gabe von OLC15 eine mögliche kompetitive Hemmung aufklären sollte. Bei letzerem stellte sich heraus, dass die Applikation von OLC15 in unterschiedlichen Konzentrationen eine Dosis-abhängige Blockierung der durch VUAA1 erzielten neuronalen Aktivität bewirkte, welches auf eine kompetitive Blockierung und somit Orco-Spezifität von OLC15 hinweist. Die SPA blieb bei Kontrollversuchen im Laufe der Aufzeichnungen unverändert. Gleiches konnte bei Versuchen unter dem Einfluss von OLC15 gezeigt werden. Deshalb wurde kein Hinweis dafür gefunden, dass Orco an der Pheromon-abhängigen SPA beteiligt ist. Hingegen zeigten die Amiloride HMA und MIA bereits zu Beginn der Ableitungen eine Reduktion der SPA, welche sich bis zum Ende der Ableitungen fortsetzte. Die Kinetik der Pheromon-abhängigen Aktionspotentialfrequenz innerhalb der ersten 20 Minuten war nicht beeinflusst durch OLC15, wohingegen beide Amiloride zu einer Verminderung der phasischen Pheromon-abhängigen Aktionspotentialfrequenz

führten. Beide Amiloride scheinen somit an weitere unbekannte Orco-unspezifische Ziele zu binden, welche Teil der phasischen Pheromonantworten sind. Die Aktionspotentialfrequenz der ersten sechs APs wurde von allen getesteten Substanzen gemindert. Im Falle von HMA zeigte sich eine stärkere Wirksamkeit während der Ruhephase. Die Latenzzeit des ersten APs nach Pheromonstimulation wurde von allen Agentien erhöht. Den stärksten Effekt zeigte hierbei HMA. Ein Effekt von OLC15 auf bestimmte Parameter der direkten Pheromonantwort konnte somit gefunden werden. Dieser war allerdings im Vergleich zu den Effekten der getesteten Amiloriden nicht vorherrschend. Die späte, langanhaltende Pheromonantwort (late, *long-lasting pheromone response;* LLPR) wurde durch die Amiloride und OLC15 während der Aktivitätsphase geblockt. Zur Ruhephase konnte ein Effekt hauptsächlich unter dem Einfluss von HMA verzeichnet werden. Der vermutliche Orco-Antagonist war somit während der Aktivitätsphase besonders wirksam. In Strominjektionsexperimenten ohne Pheromonstimulation zeigte sich eine erhöhte Aktivität der ORNs nach Gabe von 300 pA. Diese konnte unter dem Einfluss von OLC15 besonders während der Aktivitätsphase deutlich gemindert werden. Die Aktivität von Orco scheint somit spannungsabhängig zu sein. OLC15 konnte jedoch die Spontanaktivität zu keiner Zeit vollständig blocken. Dies spricht für eine Beteiligung von mindestens einem weiteren Ionenkanal bei der Generierung der Spontanaktivität.

**Auswirkungen des G-Protein-Blockers GDP-β-S auf die Pheromonantwort**

Die Versuche mit dem Orco-Agonisten VUAA1 konnten keine überzeugenden Hinweise für eine ionotrope Orco-OR-getragene Pheromontransduktion bei *M. sexta* offenbaren. Dieses legt die Beteiligung einer metabotropen Kaskade nahe. Um diese Hypothese zu testen, wurden Einzelsensillenableitungen unter dem Einfluss des G-Protein-Blockers GDP-β-S am Tabakschwärmer durchgeführt. Die SPA wurde durch GDP-β-S besonders während der Aktivitätsphase langsam aber signifikant reduziert. Auch die Pheromon-abhängige Aktionspotentialfrequenz der ersten sechs APs wurde im Verlauf der Ableitungen durch GDP-β-S zur Aktivitätsphase stärker als zur Ruhephase reduziert. Die Reduktion trat auch hier langsam auf, war aber in beiden Zeitfenstern signifikant. Die deutliche Erhöhung der Latenzzeit des ersten APs nach

Pheromonstimulation durch GDP-β-S zeigte sich ebenfalls erst zum Ende der Ableitungen. Im Vergleich zur Kontrolle konnte somit im Verlauf der Ableitungen beobachtet werden, dass die Sensitivität der ORNs durch den Einfluss von GDP-β-S sukzessive nachließ und die Kinetik der Pheromonantwort erst zum Ende der Ableitungen zu beiden Zeitfenstern signifikant beeinflusst wurde. Zusammenfassend ist zu sagen, dass alle ausgewerteten Parameter der phasischen Pheromonantwort durch GDP-β-S im Laufe der Ableitungen reduziert wurden, dass aber GDP-β-S offensichtlich sehr lange brauchte um seine Wirkung zu entfalten und deutlich langsamer wirkte, als Amiloride und Orco-Agonisten/-Antagonisten. Die wirksame Reduktion der phasischen Pheromonantwort deutet darauf, dass eine metabotrope Signalstransduktion in den ersten Millisekunden nach Stimulation eine wichtige Rolle spielt. Im Gegensatz dazu konnte gezeigt werden, das GDP-β-S keinen Einfluss auf die späte, langanhaltende Pheromonantwort im Bereich von Sekunden bis Minuten hat.

Die Ergebnisse meiner Arbeit zeigen, dass Orco in erster Linie die Spontanaktivität und die späte, langanhaltende Pheromonantwort beeinflusst und die Kinetik und Sensitivität der phasischen Pheromonantwort nur zu einem geringeren Teil moduliert. Orco wird offensichtlich spannungsabhängig und sehr wahrscheinlich auch $Ca^{2+}$-abhängig aber nicht direkt G-Protein-abhängig durch die Pheromon-Signaltransduktionskaskade gesteuert. Es ist anzunehmen, dass Orco in der Lage ist das Ruhepotential und die intrazelluläre $Ca^{2+}$-Basislinie der ORNs zu steuern und auf diese Weise die Schwelle und Kinetik der Pheromonantwort beeinflusst. Die phasische Pheromonantwort innerhalb der ersten Millisekunden wird offensichtlich in erster Linie durch einen G-Protein-gekoppelten Pheromonrezeptor gesteuert. Dies ist konsistent mit früheren Patch-Clamp-Experimenten an ORNs des Tabakschwärmers aus primärer Zellkultur, aus denen gefolgert wurde, dass Pheromone einen G-Protein-gekoppelten Pheromonrezeptor aktivieren, der zu einer Aktivierung der Phospholipase Cβ führt. Es konnten im Rahmen dieser Arbeit keine Hinweise für eine ionotrope Bombykal-abhängige Signaltransduktionskaskade in ORNs des Tabakschwärmers gefunden werden. Dieses und die Hinweise auf eine Beteiligung von G-Proteinen an der phasischen Pheromonantwort unterstützen die Hypothese einer metabotropen Signalweiterleitung der Pheromonrezeptoren des Tabakschwärmers.

# 1 Summary

Since insects are responsible for large agricultural damage and are involved in spreading serious diseases killing millions of people it is necessary to evolve broadly usable insect repellents. Insect behavior is mostly, either directly or indirectly, controlled by the sense of olfaction. Therefore, understanding olfactory signal transduction is a crucial prerequisite for solving this task. The ligand-binding olfactory receptors (ORs) and a conserved co-receptor (Orco) play a major role for odor perception. However, it is not yet clarified whether and how ionotropic or metabotropic mechanisms are involved.

In single sensillum recordings (tip-recordings) from male *M. sexta* pheromone-transduction was explored in the intact hawkmoth. Orco agonists and prospective antagonists were employed for analysis of the co-receptor's functions. Stimulation of Orco by the Orco agonist VUAA1 and simultaneous stimulation of the ligand-binding OR by the main pheromone component bombykal was expected to synergistically increase pheromone responses in an ionotropic bombykal transduction pathway. Since VUAA1 did not increase phasic pheromone responses in the first 20 minutes of the tip-recordings a sole ionotropic pheromone transduction is unlikely. This hypothesis is supported by the Orco antagonist OLC15 which only moderately affected phasic bombykal responses during the activity phase of *M. sexta*. However, VUAA1 and OLC15 significantly decreased the late, long-lasting pheromone response, suggesting that Orco is not part of an ionotropic pheromone receptor complex but rather is a voltage- and possibly $Ca^{2+}$-dependent ion channel which controls sensitivity and kinetics of pheromone responses via modulation of membrane potential and intracellular $Ca^{2+}$ concentrations. Since in contrast to VUAA1 and OLC15 the amilorides HMA and MIA strongly affected the phasic pheromone responses they appear to target also other pheromone-dependent ion channels than Orco. The antagonist of G-protein-dependent metabotropic cascades GDP-β-S strongly diminished the phasic pheromone responses. Thus it is very likely that the bombykal receptor is G-protein-coupled. Since GDP-β-S in contrast to Orco-agonists and antagonists did not affect the late long-lasting pheromone response, Orco appears not directly gated via G-proteins.

# 2 Introduction

## 2.1 The hawkmoth *Manduca sexta*

Under natural conditions *Manduca sexta* (Carl Nilsson Linnæus, 1763) (Fig. 1), belonging to the moth family Sphingidae, can be mainly found in Central America. Its common name, tobacco hornworm, indicates its significance as pest insect. Even if the human benefit of this plant can be called into question, *M. sexta* larvae also feed on tomato plants, potatoes, pepper crops and *Datura*, and therefore is responsible for economic damage (Madden 1945). Eggs of the moth are laid by the females on the underside of the plants' leaves (Alwood 1898). After hatching larvae begin to feed. In the next three weeks they pass five larval stages, whereby an enormous increase in body mass can be observed. They can protect themselves against predators with internal storage of nicotine from the tobacco plant which renders the larvae very poisonous.

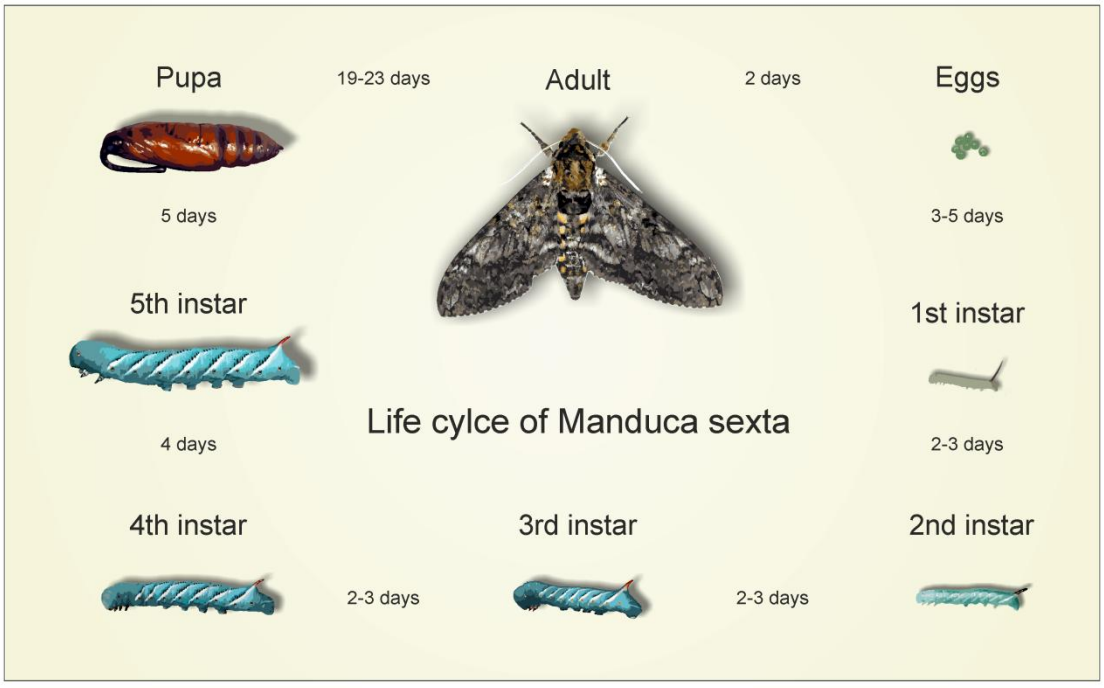


**Fig. 1 - Life cycle of *Manduca sexta*.** After oviposition larvae hedge after 2 days and pass 5 instars. During pupation the animal undergoes a complete metamorphism within approximately 20 days until the adult stage. Adult animals have a life expectancy of about 50 days.

At the end of the fifth instar larvae can reach a length of up to 70 mm when pupation is initiated. The larvae start wandering away from the host plant and bury into the ground. Since *M. sexta* is a holometabolous insect, it undergoes a complete metamorphism which ends in eclosion of the adult animal approximately around 20 days after pupation (Garman and Jewett 1920; Gilmore 1938). The wings of the adult animals can reach a span up to 12 cm which provides them with impressive flying skills. Adults are nectarivorous and mainly feed on the Datura plant while hovering in front of the flower. They are nocturnal, so their activity phase occurs between dusk and dawn (Madden 1945). Female adults release sex pheromones for attracting a mating partner. Whereas females usually mate only once, males are able to mate several times. After mating the fertilized eggs are laid on the plants' leaves and the life cycle is closed. The lifespan of an adult animal is up to 50 days.

## 2.2 Antennal structure and the peripheral olfactory system

The antenna is a very important sensory organ in insects. In pterygote insects antennae are subdivided into scape, pedicel and flagellum. Scape and pedicel possess muscles, and therefore are responsible for movement of the antennae (Eaton 1971). The pedicel also houses Johnston's organ, which can detect vibrations of the flagellum. These can occur during transmission of sounds (Caldwell and Eberl 2002; Kernan 2007), but also may be triggered by wind exposure (Yorozu et al. 2009).

It was shown that the antenna is an important mediator of flight control in moths (Sane et al. 2007). The flagellum, which represents the major part of the antennae, can only be moved passively. It can reach a length of about 2 cm and is segmented into a different number of annuli, in *M. sexta* in up to 80 (Sanes and Hildebrand 1976). Two nerves, a blood vessel and a trachea are running through the whole antenna (Sanes and Hildebrand 1976) (Fig. 2). Through the entire length of the flagellum different types of sensilla are distributed, which represent structural units in the peripheral sensing system of the insects. They serve for olfactory sensing, but also for taste, mechano-, hygro- and thermoreception and are innervated by one to several receptor cells (Schneider 1964).

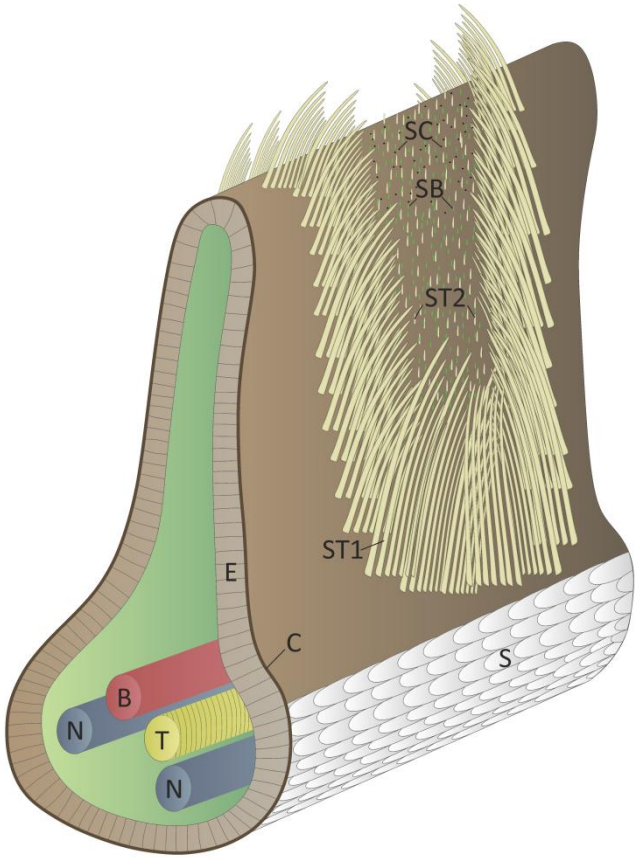


**Fig. 2 - Scheme of a flagellar annulus from male *M. sexta* antennae.** The male antenna is keyhole-shaped. Two nerve trunks run through the antenna housing the axons of nerve cells. In addition a blood vessel and a trachea can be found. Different sensilla types serving for olfactory perception are located on the leading edge of the antenna. Sensilla with additional functions are not shown. The trailing edge is covered by scales. *B* blood vessel, *C* cuticle, *E* epidermis, *N* nerve trunk, *S* scales, *SB* sensilla basiconica, *SC* sensilla coeloconica, *ST1* sensilla trichoidea type 1, *ST2* sensilla trichoidea type 2, *T* trachea.

In many moth species a dimorphism between male and female flagella can be observed (Boeckh et al. 1960). This is mainly due to the fact that males have to detect pheromones released by females. While with 100.000 – 125.000 the hair-like pheromone-sensitive sensilla trichoidea (type 1 and 2) are the most common type of sensilla on the antenna of male *Manduca*, these kinds of sensilla are absent in females (Sanes and Hildebrand 1976). Olfactory cone-like basiconic sensilla with a number of less than 40.000 are the second most frequent type of sensilla on the male's antennal surface. Compared to the total number of 250.000 sensory sensilla on a male's antenna (Sanes and Hildebrand 1976) this clearly shows that the sense of smell in *M. sexta* is the most important sense housed on the antenna and it can be assumed to be the most important sense for the crepuscular animal since it is a necessary prerequisite to reproduction. Three additional types of sensilla were found on the male's antenna: Firstly, bristle-like sensilla chaetica, which are gustatory and/or mechanosensory. Secondly, sensilla coelonica, which form a peg in a pit, with

olfactory/thermosensory or thermosensory/hygrosensory functions, respectively. The latter is also assumed for the third type, the peg-like sensilla styliform complex. However, these three types of sensilla are represented at significant lower abundance (hundreds to a few thousands) on the male's antenna (Lee and Strausfeld 1990). The functionality of the sensilla types often was assumed due to ultrastructural characteristics as proposed by Altner (1977) without electrophysiological verification, e.g. sensilla having wall-pores are assumed to be olfactory sensilla, which was gradually proven in electrophysiological studies. A specific spatial distribution of all types of sensilla can be observed on each annulus (Lee and Strausfeld 1990) (Fig. 2).

Axons of the sensory cells are bundled in two nerves per larval or adult antenna projecting to the deutocerebrum of the insects' brain (Schoonhoven 1966). In the pupal stage the antennal nerves elongate with the growing antenna and are employed as guiding posts for developing axons (Sanes and Hildebrand 1975). Evidence suggests that in vertebrates and invertebrates alike each olfactory neuron expresses one type of ligand-gated olfactory receptor (OR) (Hildebrand and Shepherd 1997; Couto et al. 2005). All axons of the sensory cells expressing the same type of OR converge into one specific glomerulus within the antennal lobe (Gao et al. 2000; Vosshall et al. 2000; Bhalerao et al. 2003). However, also the co-expression of two functional ORs was observed in one neuron (Goldman et al. 2005) and the one receptor – one neuron rule was questioned (Mombaerts 2004).

The number of glomeruli receiving information about general odors is more or less equal in male and female moths. Male moths additionally possess sex-specifically enlarged glomeruli. Together they form the macroglomerular complex which collects solely input from pheromone-sensitive neurons (Rospars and Hildebrand 1992). In glomeruli the olfactory information is processed by interneurons and projected to higher brain centers (Tolbert and Hildebrand 1981; Distler and Boeckh 1996), e.g. the mushroom body, which serves for olfactory learning and memory (McGuire et al. 2001), or the lateral horn, which appears to be involved in the extraction of stimulus features such as odor intensity (Gupta and Stopfer 2012). Many ORs can respond to multiple odors (Hallem and Carlson 2006), thus stimulating glomeruli in a specific pattern. Therefore, a combinatorial receptor coding scheme is assumed to play the key role in perception of a specific odor (Malnic et al. 1999).

## 2.3 Pheromone-sensitive trichoid sensilla

Pheromone-sensitive trichoid sensilla (type 1) can reach a length of up to 400 µM (Sanes and Hildebrand 1976) and are arranged in a horseshoe-form (Fig. 2). Each sensillum houses two olfactory receptor neurons (ORNs) (Sanes and Hildebrand 1976; Keil 1989; Lee and Strausfeld 1990) (Fig. 3). The ORNs are forming an unbranched dendritic cilium, which can be separated into an inner and outer dendritic segment (Keil 1989). The outer dendritic segment houses the olfactory receptors, thus representing the origin of the signal transduction (Clyne et al. 1999; Vosshall et al. 1999). The outer dendrites are cilia which contain microtubules. They can be moved actively, possibly resulting in an improved detection of odors (Keil 1993).

In *M. sexta* one ORN per sensillum can almost always be stimulated by bombykal (BAL; (E,Z)- 10,12-hexadecadienal), the other one mainly by (E,E,Z)-10,12,14-hexadecatrienal or its isomer (E,E,E)-10,12,14-hexadecatrienal (Kalinova et al. 2001). However, the specific pheromone blend of female *M. sexta* consists of at least 12 components

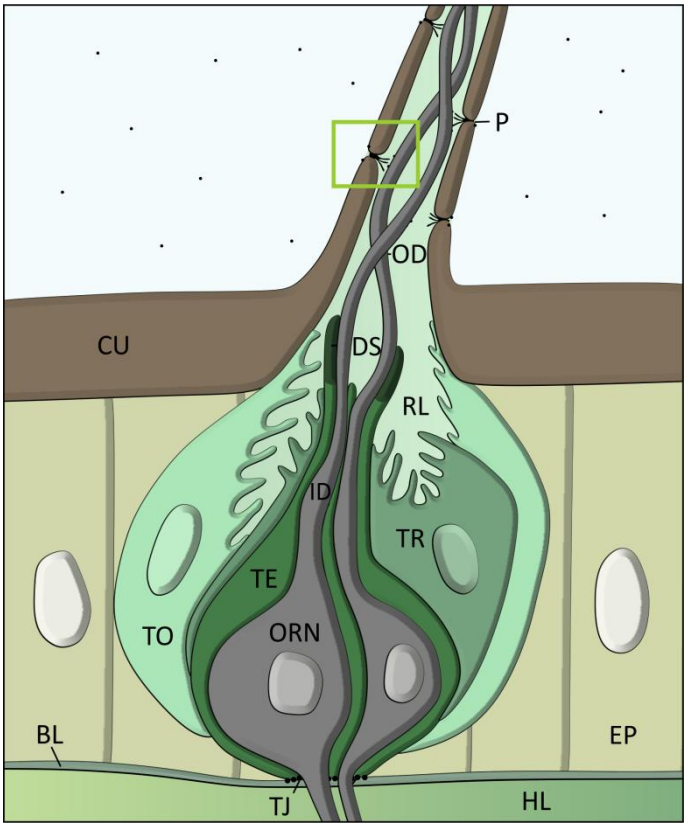


**Fig. 3 - Schematic trichoid sensillum.** Olfactory receptor cells are surrounded by three accessory cells, which isolate the ORNs and produce the receptor lymph. Odors pass through pores into the receptor lymph cavity and reach the outer dendritic segment (= the cilium) of the ORN where receptors are located. *BL* basal lamina, *CU* cuticle, *DS* dendritic shaft, *EP* epidermis, *HL* hemolymph, *ID* inner dendritic segment, *OD* outer dendritic segment, *ORN* olfactory receptor neuron, *P* pore, *RL* receptor lymph, *TE* thecogen cell, *TJ* tight junctions, *TO* tormogen cell, *TR* trichogen cell. *Area within green rectangle enlarged in Fig. 4.*

(Tumlinson 1989), with eight playing a major role in attracting the male moth (Starrat et al. 1979; Tumlinson 1989). ORNs responding to pheromone are extremely sensitive. They are assumed to detect even single molecules. In addition, it was shown that ORNs can discriminate pheromone concentrations over at least 4 log units (Kaissling and Priesner 1970; Kaissling 1987; Dolzer et al. 2003).

The ORNs are surrounded by three accessory cells (Fig. 3). The inner thecogen cell functions as glia cell, isolating the ORNs from axon to the outer dendritic segment. Due to tight cover of the ORN by its sister thecogen cell, only the external part of the outer dendritic segment is in contact with the sensillum lymph (Keil and Steinbrecht 1987). The trichogen cell as well as the tormogen cell produce the cuticular sensillum hair shaft and form the receptor lymph cavity. Both cells turned out to have high concentrations of V-ATPases in the membranes, which face the receptor lymph (Klein and Zimmermann 1991; Wieczorek et al. 1991; Wieczorek 1992). V-ATPases cause $K^+$-transport into the lymph cavity (Küppers and Bunse 1996), leading to enrichment of potassium within the receptor lymph. Hence, a transepithelial potential gradient is formed, which can be used for generating a receptor potential (Thurm and Wessel 1979; Thurm and Küppers 1980). Furthermore, sensillum lymph cavity and hemolymph are strictly separated by septate junctions (Keil and Steinbrecht 1987) allowing for different ion compositions in the sensillum lymph as compared to the hemolymph.

After the hydrophobic odors enter through sensilla pores they have to pass the aqueous sensillum lymph (Fig. 4). This can be achieved by specific odorant binding proteins (Leal et al. 2005; Leal 2013). General odorant-binding proteins (OBP) (Breer et al. 1990; Vogt et al. 1991; Vogt 2003) and pheromone-binding proteins (PBPs) (Vogt and Riddiford 1981; Vogt 2003) were isolated from the moth olfactory sensillum lymph. They were assumed to complex with odor molecules (Vogt et al. 1985; Van den Berg and Ziegelberger 1991). They are concentrated in the sensillum lymph of moths in a millimolar range (Vogt and Riddiford 1981; Klein 1987) indicating their importance in olfactory sensing. Electrophysiological experiments also confirmed this suggestion since simultaneous application of PBPs increased pheromone responses (Van den Berg and Ziegelberger 1991). The PBPs are specific for their pheromone ligands, thereby

increasing the specificity and sensitivity of an ORN (Laue et al. 1994; Steinbrecht et al. 1995; Steinbrecht 1996).

Besides binding proteins odor degrading enzymes are essential in olfactory sensing (Ishida and Leal 2005; Ishida and Leal 2008; Durand et al. 2011). The first pheromone-degrading enzyme in a moth was found in 1981 (Vogt and Riddiford) and its degradation kinetics were analyzed (Vogt et al. 1985; Vogt and Riddiford 1986; Ishida and Leal 2005). A half-life time of the pheromone of about 15 ms was estimated. The degrading process was only delayed when the pheromone was bound to binding proteins (Vogt and Riddiford 1986; Vogt 1987). However, a fast degradation is strictly necessary for detecting subsequent stimuli.

For bombykol and its pheromone-binding protein it was indicated that the protein changes its conformation when bound to bombykol (Maida et al. 2000; Lautenschlager et al. 2005). This was also shown in a pH-dependent manner (Wojtasek and Leal 1999; Horst et al. 2001; Leal 2004). In this way it might be possible to release the odor around the surface of the outer dendrite due to pH-differences compared to the lymph, thus being able to activate the odorant receptor. However, it is still unknown if the odor alone or the complex of odor and binding protein is necessary for receptor

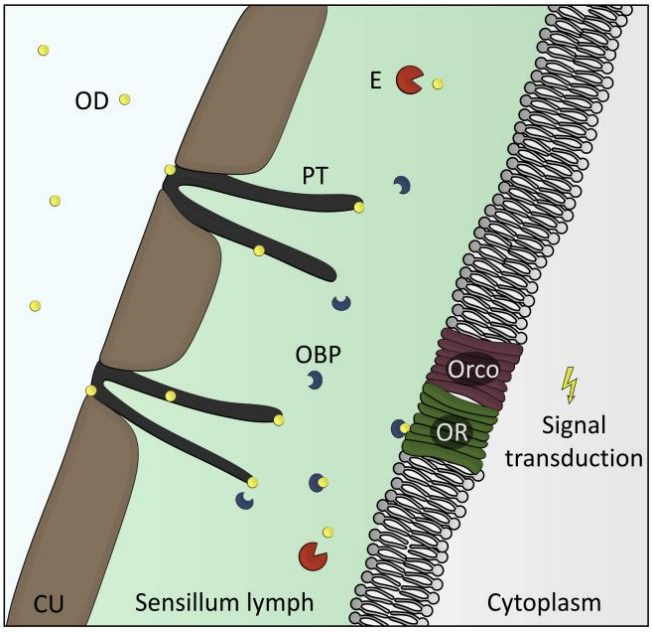


**Fig. 4 - Perireceptor events.** Odors pass through the pore and are transported along pore tubuli into the sensillum lymph. There, odors bind to odorant binding proteins, which protect the odor against enzymatic degradation and carry the molecule to the dendrites of the olfactory receptor neurons where the olfactory receptors (OR) are located together with the ion channel/ olfactory coreceptor Orco. Binding of the odor to the OR initiates signal transduction. Finally odors are released and degraded. *CU* cuticle, *E* enzyme, *OBP* odor binding protein, *OD* odor, *OR* olfactory receptor, *Orco* olfactory co-receptor, *PT* pore tubuli.

activation.

Besides ORs and the olfactory co-receptor Orco, described in detail in the next chapter, another family of membrane proteins seems to be involved in perireceptor processes. They were found in the dendritic membranes of moths (Rogers et al. 1997; Rogers et al. 2001; Forstner et al. 2008) and were called sensory neuron membrane proteins (SNMPs), belonging to the family of CD36 receptors (Rogers et al. 1997). They are possibly co-receptors for the PBP-pheromone complex (Rogers et al. 2001) and were suggested to be necessary for pheromone detection in *Drosophila* (Benton et al. 2007; Jin et al. 2008). However, the functions of the SNMPs are not clarified yet.

## 2.4 Chemosensory receptors

***Olfactory receptors (ORs) and suggested co-receptors***

After being transported through the sensillum lymph general odors or pheromones activate dendrite-located ORs (Clyne et al. 1999; Vosshall et al. 1999), which belong to the family of seven transmembrane (7TM) domain proteins (Clyne et al. 1999; Gao and Chess 1999; Vosshall et al. 1999; Stortkuhl and Kettler 2001; Hallem and Carlson 2006). While ORs detecting food odors seem to be generalists and can be activated by different ligands, pheromone receptors are suggested to be more specific (Hallem and Carlson 2006). In some vertebrate species more than 1000 OR-genes are expressed (Mombaerts 2004). On the contrary, most insect species analyzed so far express considerably fewer ORs. For example, 62 OR genes were found in the genome of *D. melanogaster* (Robertson et al. 2003), 79 ORs in *A. gambiae* (Hill et al. 2002) and 66 ORs in *Bombyx mori* (The International Silkworm Genome 2008). As described above, mostly the one neuron – one receptor hypothesis can be assumed (see chapter "2.2 Antennal structure and the peripheral olfactory system").

Unlike conventional G-protein-coupled 7TM receptors observed in vertebrates, the C-terminus of insect ORs is exposed to the extracellular space and comparison of insect and vertebrate ORs reveal little sequence similarity (Benton et al. 2006; Wistrand et al. 2006; Lundin et al. 2007; Smart et al. 2008; Tsitoura et al. 2010). The ligand binding

insect ORs were found to be highly divergent between insect species (Clyne et al. 1999; Mombaerts 1999; Krieger et al. 2002). On the contrary, another larger 7TM protein was found (Vosshall et al. 1999), which is highly conserved across insect species (Hill et al. 2002; Krieger et al. 2003; Pitts et al. 2004; Jones et al. 2005; Patch et al. 2009; Olafson 2013). Due to its colocalization with ligand-binding ORs in antennae of many insect orders it was generally termed Orco (olfactory receptor co-receptor) (Vosshall and Hansson 2011). Orco was found in OR-expressing ORNs in various insect species (Vosshall et al. 1999; Krieger et al. 2003; Larsson et al. 2004; Pitts et al. 2004; Jones et al. 2005; Nakagawa et al. 2005), indicating its importance for OR-dependent insect olfaction. However, Orco was found in legs and proboscis, too, but it remained to be examined whether it colocalizes also with gustatory receptors (GRs) and participates in gustatory perception (Krieger et al. 2002; Larsson et al. 2004; Melo et al. 2004; Pitts et al. 2004). Odor ligands are suggested to exclusively bind to ORs but not to Orco, thus triggering an olfactory response (Dobritsa et al. 2003; Elmore et al. 2003; Hallem et al. 2004). Indeed, OR expression alone is sufficient for ligand-depending responses (Wetzel et al. 2001; Sakurai et al. 2004; Nakagawa et al. 2005; Neuhaus et al. 2005; Grosse-Wilde et al. 2006; Smart et al. 2008; Deng et al. 2011), although higher odor concentrations are needed, as compared to ORs coexpressed with Orco. The expression of different OR subunits co-expressed with the same Orco-protein changes the odor-sensitivity, the type of the response (excitatory or inhibitory), the response termination rate, and also the spontaneous activity (Hallem and Carlson 2006). Furthermore, Orco alone was not shown to elicit a response to natural odors (Nichols et al. 2011).

This raises the question about the functionality of the Orco protein. Orco mutants of *D. melanogaster* showed an impaired integration of the ORs into the dendritic membrane of the ORNs and localization stability of the ORs is likely depending on Orco (Larsson et al. 2004). Therefore, it is not surprising that loss of Orco leads to reduced odor responses (Larsson et al. 2004; Neuhaus et al. 2005). It was suggested that ORs as well as Orco can form homo- or heteromultimeric complexes of unknown stoichiometry (Neuhaus et al. 2005; German et al. 2013). Not only ORs but also the Orco protein controls the generation of spontaneous activity. In contrast to ORs the heterologous expression of Orco alone already allows for ion influx. Thus, the Orco protein forms an

ion channel without the need for heteromerization with ORs (see chapter "2.5 Spontaneous activity").

***Gustatory receptors and ionotropic receptors***

Next to the OR/Orco-complex two additional chemical receptor families were found in insects. One family, belonging to the 7TM receptors (Jones et al. 2007; Kwon et al. 2007), was mainly found in gustatory tissues (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001), and therefore termed gustatory receptors (GRs). Due to its similarity to ORs and since GRs appeared earlier during evolution it is assumed that ORs were evolutionarily derived from GRs (Robertson et al. 2003; Nordstrom et al. 2011).
The second family is the family of ionotropic receptors (IRs) belonging to 3TM proteins (Benton et al. 2009; Croset et al. 2010; Abuin et al. 2011). They were found in coeloconic sensilla (Benton et al. 2009; Croset et al. 2010) and they mainly respond to amines and acids (Yao et al. 2005; Ai et al. 2010). The IRs are highly conserved and possibly represent the evolutionary oldest olfactory receptor family in insects (Croset et al. 2010). Comparably to ORs in *D. melanogaster* IR64a and IR8a built a functional complex (Ai et al. 2013). Furthermore, there is evidence for broadly expressed co-receptors as well, which seems to be involved in the trafficking of IRs into the cilia (Abuin et al. 2011).

## 2.5 Spontaneous activity

The spontaneous generation of action potentials without previous stimulation is termed "spontaneous activity". In insect antennae spontaneous activity was observed in ORNs (Boeckh et al. 1965; Kaissling 1986). It is still unclear which ion channels are involved in the generation of spontaneous activity in ORNs. Hyperpolarization-activated cyclic nucleotide-gated channels (HCN-channels) play an important role in the generation of the resting potential and of pacemaker activity in different cell types (Robinson and Siegelbaum 2003) and were found also in *M. sexta* ORNs (Krannich and

Stengl 2008). Blockage of hyperpolarization-activated $I_h$-currents in neurons of the vomeronasal organ in mice caused stronger hyperpolarization of the cells and, therefore, lower spontaneous activity (Dibattista et al. 2008).

Besides HCN channels ORs in vertebrates and insects mediate spontaneous activity. In mammals the metabotropic ORs mediate spontaneous activity of the ORNs (Reisert 2010). Heterologous expression of the *Drosophila* OR Or22a alone was sufficient for generation of spontaneous activity (Wicher et al. 2008) and activity levels were influenced by loss of the OR protein (Dobritsa et al. 2003; Elmore et al. 2003; Hallem et al. 2004). In addition, Orco-specific agonists and antagonists confirmed a participation of Orco in generating spontaneous activity in ORNs of different insects (Jones et al. 2011; Su et al. 2012). Orco alone is able to form a leaky, non-specific ion channel (Sato et al. 2008; Wicher et al. 2008; Jones et al. 2011; Sargsyan et al. 2011). Thus, caused by a steady ion influx, Orco can drive spontaneous activity (Larsson et al. 2004; Benton et al. 2007; Deng et al. 2011) and might influence sensitivity and kinetics to olfactory stimulation (Stengl 2010). In general, spontaneous activity of ORNs seems to be more related to the olfactory co-receptor since non-functional Orco mutants of *D. melanogaster* did not show any spontaneous activity, whereas Orco expression alone elicited spontaneous activity (Dobritsa et al. 2003; Elmore et al. 2003; Larsson et al. 2004).

Finally, also SNMPs influence spontaneous activity of ORNs. SNMP mutants increased the spontaneous activity in *D. melanogaster* (Benton et al. 2007), indicating an interaction with the OR/Orco complex.

It is suggested that spontaneous activity is supported by subthreshold membrane potential oscillations (Stengl 2010). This is consistent with the fact that interspike intervals of spontaneously active ORNs of the hawk moth did not show a random distribution (Dolzer et al. 2001). However, spontaneous activity of ORNs is not yet fully understood.

## 2.6 Signal transduction in ORNs

***Ionotropic and metabotropic pathways***

The simplest way for transmitting signals in a receptor neuron is an ionotropic mechanism. It has the advantage of being very fast in the µs range. However, it also has the decisive disadvantage of insensitivity since there are no components for amplification. From an evolutionary point of view a highly sensitive olfaction system seems to be very useful. In vertebrates' olfactory neurons G-proteins were found and cAMP-rises after odor stimulation were observed (Pace et al. 1985; Pace and Lancet 1986). The pathway was further specified in the following years. Activation of the olfaction-specific G-protein $G_{olf}$ increases the activity of an adenylyl cylcase, thus increasing cAMP concentrations (Breer et al. 1990). cAMP-gated CNG channels open (Nakamura and Gold 1987) and cause $Ca^{2+}$-influx (Frings et al. 1995; Leinders-Zufall et al. 1997). Higher $Ca^{2+}$-concentrations lead to the opening of $Ca^{2+}$-dependent chloride-channels, which cause chloride efflux and thereby further depolarization (Kleene and Gesteland 1991; Kurahashi and Yau 1993; Lowe and Gold 1993).

However, the insect olfactory system appears to work differently as compared to vertebrate olfaction. Nevertheless, particularly in insects a sensitive system should be an evolutionary advantage, since smallest amounts of odors have to be detected for successful mating and food search. Especially detection of pheromones is an enormous task for flying insects. Measured pheromone release by the female oriental fruit moth showed an emitted amount of pheromone components in a range of nanograms per hour (Lacey and Sanders 1992), making it necessary for the olfactory system to be sensitized for detection of a few molecules. Therefore, it is a reasonable hypothesis that an amplifying metabotropic cascade has to be involved.

However, unexpectedly in some studies it was shown that the OR/Orco complex elicited a solely ionotropic ion current after odor stimulation (Sato et al. 2008; Smart et al. 2008; Yao and Carlson 2010; Nakagawa et al. 2012) (Fig. 5-A) and interruption of metabotropic pathways caused little changes in the response of some ORNs to odors (Yao and Carlson 2010).

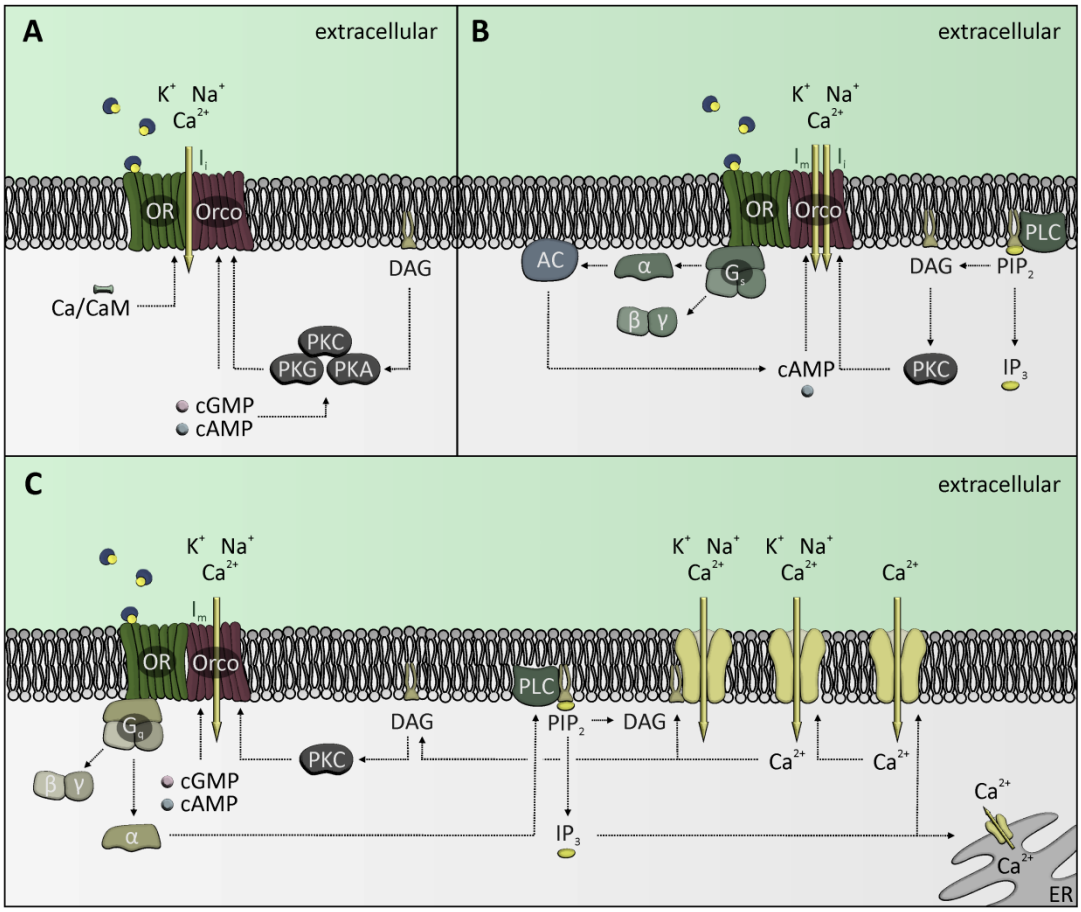


**Fig. 5 - Hypotheses in insect olfactory signal transduction. A.** Sato et al. (2008) proposed a solely ionotropic odor transduction cascade for insect olfaction. After ligand binding to the OR an ionotropic current is triggered by OR and Orco. This current might be metabotropically modulated by protein kinases (PKA, PKC, PKG) or cyclic nucleotides (cAMP, cGMP) as suggested by Nakagawa and Vosshall (2009). **B** Wicher et al. (2008) found an ionotropic and metabotropic cascade provided by Orco. After the fast ionotropic cation influx the more sensitive metabotropic response occurs. In this model the activation of the OR leads to a G-protein-dependent increase of cAMP by an adenylyl cyclase (AC), which elicits the metabotropic current by activating the Orco protein. However, a prior phosphorylation of Orco by PKC is necessary. **C** Indications for a solely metabotropic cascade were found for *M. sexta* (Stengl, 2010). Ligand binding to ORs results in a G-protein-dependent increase in phospholipase Cβ (PLCβ) activity. Phosphatidylinositol 4,5-bisphosphate ($PIP_2$) is hydrolyzed to inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). $IP_3$ affects $Ca^{2+}$-release from the endoplasmatic reticulum (ER) and $IP_3$-dependent opening of $Ca^{2+}$-permeable ion channels. Increased calcium levels lead to opening of $Ca^{2+}$-dependent cation channels. PKC activation by $Ca^{2+}$ and DAG results in a phosphorylation of ion channels and Orco. Orco changes its sensitivity to cyclic nucleotides. Thus, sensitivity and response kinetics are modulated. Ca/CaM Calcium calmodulin.

Other studies suggest an ionotropic as well as a metabotropic component in insect olfaction (Kain et al. 2008; Wicher et al. 2008; Chatterjee et al. 2009; Deng et al. 2011; Sargsyan et al. 2011; Getahun et al. 2013). E.g. Wicher et al. (2008) proposed a model of a fast ionotropic component followed by a slower $G_s$-protein mediated metabotropic pathway (Fig. 5-B). Such a system would benefit from both advantages since it would be fast at high odor concentrations, but sensitive enough to detect also lower concentrations. Nevertheless, the relevance of G-proteins in insect olfaction has to be assumed since different G-proteins, e.g. $G_{o/i}$, $G_s$ and $G_q$, were found to be expressed in insect ORNs (Miura et al. 2005; Boto et al. 2010). Therefore, it is conceivable that also parallel signaling pathways might be used in ORNs (Hermans 2003).

Deng et al. (2011) found in *in vivo* electrophysiology on *Drosophila* antennae and single sensilla that the $G_s$-pathway plays a crucial role in signal transduction. Similar to this, also a disruption of odor induced currents by the G-protein blocker GDP-β-S was observed which were assigned to be mediated by a $G_s$-protein (Wicher et al. 2008). However, most evidence was found for a $G_q$-coupled pathway (Fig. 5-C). The $G_q$-protein, which can activate a phospholipase Cβ (PLCβ), was found using immunocytochemistry or in cloning studies in ORN dendrites of different lepidopteran species (Jacquin-Joly et al. 2002). Furthermore, the $G_\alpha$-subunits of $G_o$- and $G_q$-proteins were found in the antennae of moth, fly and cockroach (Breer et al. 1988; Boekhoff et al. 1990; Raming et al. 1990; Talluri et al. 1995). Additionally, $G_q$-like proteins were shown in the dendrites of *B. mori* and *Antheraea pernyi* (Laue et al. 1997). It was observed that $G_{\alpha q}$-deletion in *Drosophila* reduced odorant responses (Kain et al. 2008). An RNAi assay, suppressing the expression of $G_q$, led to impaired performance in odorant-depending behavioral tests (Kalidas and Smith 2002). Besides the $G_q$-protein also a PLC was found in dendritic homogenates of ORNs from *A. pernyi* (Maida et al. 2000), indirectly evidencing the involvement of a $G_q$-pathway. Furthermore, mutation of a PLC in *Drosophila* influenced the ability of odor detection (Riesgo-Escovar et al. 1995).

Next to the $G_\alpha$-subunit pathway the mode of action of the $G_{\beta\gamma}$-subunit remains largely unknown in insects. In vertebrates it was shown to regulate the GTPase activity of the $G_\alpha$-subunit, thus, regulating the activity of the $G_\alpha$-subunit (Witherow and Slepak 2003).

Moreover, in vertebrates the $G_{\beta\gamma}$-subunit was shown to modulate PLC activity and thus $IP_3$ concentrations (Akgoz et al. 2002). Therefore, it must be assumed that also in insects the $G_{\beta\gamma}$-subunit is involved in the olfactory signal transduction cascade, as reported recently by Ignatio Raja et al. (2014).

More evidence for an involvement of G-proteins in insect olfaction was found in various studies by using G-protein blockers (see chapter "2.7 Pharmacological interference with the olfactory system").

***Role of $IP_3$ and DAG***

In a $G_q$-pathway an activated PLCβ induces the cleavage of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into inositol 1,4,5-trisphosphate ($IP_3$) and diacylglycerol (DAG). Since after stimulation with general odors or pheromones $IP_3$ levels were increased in different insects (Boekhoff et al. 1990; Boekhoff et al. 1990; Breer et al. 1990; Kaissling and Boekhoff 1993) this supports the possible participation of the $G_q$-pathway in olfactory signal transduction. Furthermore, in ORNs of *M. sexta* a $IP_3$-dependent $Ca^{2+}$-channel was observed *in vitro*, which causes a subsequent serial opening of further ion channels (Stengl 1994) (Fig. 5-C), thus, leading to a depolarization of the cell. $IP_3$-dependent receptors were localized in the outer dendritic membrane of male moth sensilla (Laue and Steinbrecht 1997), possibly affecting an influx of calcium ions. Together with the increased DAG level $Ca^{2+}$ influx leads to an activation of protein kinase C (PKC) (Breer et al. 1990; Stengl et al. 1992; Boekhoff et al. 1993; Stengl 1993; Stengl 1994). It was stated that $IP_3$ levels are negatively regulated by PKC (Schleicher et al. 1994), thus delimiting the response of the $G_q$-pathway. Furthermore, intracellular $Ca^{2+}$-levels were decreased by PKC (Dolzer et al. 2008). After strong, adapting stimulation of antennal homogenates of *Antherea polyphemus* PKC activity was increased (Maida et al. 2000). Therefore, PKC is assumed to play a role as modulator of the sensitivity during overstimulation and as possible component in short term adaptation.

DAG was found to stimulate ORNs in different moth species, including *M. sexta* (Maida et al. 2000; Pophof and Van der Goes van Naters 2002; Krannich and Stengl 2008). This

is in agreement with the finding of sensitized pheromone responses in tip recordings on *M. sexta* under influence of a DAG analog (Petra Gawalek, Department of Animal Physiology, University of Kassel, unpublished data). Furthermore, DAG is able to activate DAG-sensitive transient receptor potential channels (TRP-channels) (Lucas et al. 2003; Trebak et al. 2003; Venkatachalam et al. 2003; Krannich 2008). Thus, these findings strongly suggest an involvement of a $G_q$-protein based olfactory signal transduction cascade in the respective insects.

***Role of cAMP and cGMP***

After adapting pheromone stimulation delayed and long-lasting rises in cGMP concentration were observed in different moth species (Ziegelberger et al. 1990; Boekhoff et al. 1993). The transient rise of $IP_3$ levels upon pheromone stimulation was strongly reduced under the influence of cGMP (Boekhoff et al. 1993). Levels of cGMP can be modulated by guanylyl cyclases (GC) of which various types were found to be located in ORNs (Simpson et al. 1999; Nighorn et al. 2001; Stengl et al. 2001; Morton and Nighorn 2003; Morton 2004). Furthermore, different ion channels in ORNs were modulated by cGMP. Rises in cGMP blocked $Ca^{2+}$-dependent cation channels in *A. polyphemus,* while the open probability of PKC-dependent cation channels was increased (Zufall and Hatt 1991; Zufall et al. 1991). Additionally, in *M. sexta* delayed rectifier channels were blocked (Zufall et al. 1991). Tip recordings from *M. sexta* trichoid sensilla revealed a more tonic response and a decreased AP frequency under the influence of cGMP (Flecke et al. 2006). The same was previously found for pheromone responses in *B. mori* (Redkozubov 2000). Thus, cGMP allows for pheromone responses under conditions of adaptation.

In contrast to cGMP little is known about the role of cAMP in olfactory signal transduction in insects. In vertebrates it plays a major role since odors activate the G-protein $G_{Olf}$ which activates adenylyl cyclase, thus increasing cAMP levels. In electroantennogramms from *A. pernyi* cAMP application increased pheromone responses (Villet 1978). However, after stimulation with odors or pheromones cAMP levels were not elevated in insect species (Ziegelberger et al. 1990; Boekhoff et al.

1993). Nevertheless, cyclic nucleotid-gated CNG-channels were found in insect ORNs (Baumann et al. 1994; Krieger et al. 1997). Reminiscent of its action in crustacea cAMP was suggested to hyperpolarize ORNs via activation of $K^+$ outward currents (Krieger et al. 1997). Alternatively, it was suggested to play an important role for hormone-dependent circadian modulation of pheromone responses in the hawkmoth (Stengl 2010).

This is supported by the fact that octopamine (OA) receptors which trigger adenylyl cyclases (Evans and Maqueira 2005; Farooqui 2007) were found in the antennae of different moth species, including *M. sexta* (Von Nickisch-Rosenegk et al. 1996; Dacks et al. 2006). Different kinds of circadian physiological stages were modulated by OA (see chapter "2.9 Circadian rhythms in moth olfaction"). In tip recordings octopamine application and, thus, a possible increase of cAMP levels disadapted ORNs of *M. sexta* and *B. mori* (Pophof 2000; Pophof and Van der Goes van Naters 2002; Flecke and Stengl 2009), suggesting a sensitizing effect of cAMP depending on the time of day. However, it has to be mentioned that OA receptors also employ other second messenger systems such as the PLCβ cascade (Blenau and Baumann 2001; Blenau and Baumann 2003).

Next to CNG-type cation channels and cyclic nucleotide-dependent $K^+$ channels also Orco is modulated via cGMP and cAMP (Wicher et al. 2008). Thus, Orco could be a target for the regulation of sensitivity. However, previous phosphorylation of Orco by PKC seems is a prerequisite for its nucleotide sensitivity (Sargsyan et al. 2011; Getahun et al. 2012).

***Role of arrestins***

Additionally, a role of arrestins in olfactory transduction is suggested. It was shown that arrestins can cause desensitization of cells by decoupling the G-protein-coupled receptor (GPCR) from the G-protein (Inglese et al. 1993; Freedman and Lefkowitz 1996). For *D. melanogaster* and *A. gambiae* it was found out that arrestin mutants exhibited impaired EAG-responses to olfactory stimulation (Merrill et al. 2002). Thus,

arrestin is another modulator of olfactory sensitivity which also hints an involvement of G-protein-coupled pathways in insect olfaction.

## 2.7 Pharmacological interference with the olfactory system

A variety of insect-borne human diseases can be observed worldwide. Malaria is considered to be one of the worst since it causes hundreds of thousands of people to lose their lives from this disease every year as stated in the World Malaria Reports of the World Health Organization[1]. Beside this, insects compete with humans for food and can cause massive agricultural damage, e.g. the loss of 14 percent of the worldwide crop resulting in an economical damage of 2 trillion dollar each year (Pimentel 2009). Pesticides were used around the world to reduce the number of harmful insects. However, the massive usage brought forward widespread resistances against those chemicals. Furthermore, also beneficial insects were affected. Thus, more specific repellents have to be developed.

One approach is to investigate agents which are capable of influencing the olfactory system of insects directly since loss would also impact mating and feeding behavior. Main potential targets are ion channels, which are involved in the generation of the receptor potential or APs in the ORNs. Other candidates are the ligand binding ORs and the co-receptor Orco. Targeting specific ORs might allow the control of restricted pest species. On the other hand since Orco is conserved across insect species, impairment should influence the olfactory sense for multiple species, and thus agents targeting Orco could be used as broadly usable insect repellents. Furthermore, also G-proteins can be targeted, since a kind of involvement of G-proteins in the olfactory signal transduction has to be assumed as described above. Various agents were tested for their effects on olfactory responses upon stimulation in different studies. However, only a few can already be used for the control of insects since first of all studies have to be performed according to environmental safety concerns. Beside this, the synthesis of specific agents is expensive, and therefore not affordable, especially for third world

[1] http://www.who.int/malaria/publications/world_malaria_report_2013/report/en/

countries. Nevertheless, agents specifically binding to one of the mentioned targets can help to understand the specific functions of those components due to their effects on the olfactory transduction in insects and thus bringing forward the development of further repellents.

***Ruthenium red***

A widely used agent is ruthenium red. This agent was shown to be an unspecific cation channel blocker, which inhibits insect OR-dependent currents (Nakagawa et al. 2005; Sato et al. 2008; Jones et al. 2011; Nichols et al. 2011; Pask et al. 2011). The sensitivity to this agent was shown to depend on the composition of OR and Orco, since exchanged OR subunits showed different levels of blockage (Nichols et al. 2011). However, due to its various targets on e.g. ryanodine receptors, ion channels or uniporter (Tapia and Velasco 1997; Xu et al. 1998; Hajnoczky et al. 2006; Ma et al. 2012) its role as specific blocker of a pore built by the OR/Orco complex can be called into question.

***DEET, IR3535 and Picaridin***

One of the most used insect repellents is the agent N,N-Diethyl-meta-toluamide, known as DEET. It reduces the risk of bite from blood feeding insects (Debboun et al. 2007) since it seems to influence the olfactory system of insects. However, its mode of action is still not fully understood. There are two mechanisms how DEET might act. On the one hand it could act as a repellent. On the other hand it could change the responses of ORNs to specific ligands. The latter was supported by the fact that electrophysiological responses to host odors were impaired in *A. gambiae* and *D. melanogaster* (Ditzen et al. 2008). Furthermore, responses to L-lactic acid, an attractant of the mosquito *Aedes aegypti*, were inhibited by DEET (Davis and Sokolove 1976; Dogan et al. 1999). However, there is also evidence for direct binding of DEET to olfactory receptors. It was shown that DEET selectively activates an OR of *A. aegypti*

(Bohbot and Dickens 2010). In addition, DEET appears to directly interact with the *Drosophila* OR Or59b *in vivo* and an amino acid polymorphism of the OR was able to inhibit the effect of DEET (Pellegrino et al. 2011). In the mosquito *Culex quinquefasciatus* DEET alone was able to elicit an excitatory response of ORNs and it was suggested, that DEET was actively avoided by the animals (Syed and Leal 2008). In addition, various gustatory receptors were found to be also activated by DEET in *Drosophila* (Lee et al. 2010). The same was discovered for the ionotropic receptor Ir40a (Kain et al. 2013). Again, an activation of the neuronal activity by DEET seems to result in an avoidance of the chemical. Additionally, in both studies loss or mutation of the receptor led to a reduced avoidance level. Nevertheless, further functionalities of DEET have to be assumed since it was also shown to affect acetylcholinesterases (Corbel et al. 2009). One possible target might be the olfactory co-receptor Orco. Orco mutant mosquitoes were shown to be not repelled by DEET anymore (Degennaro et al. 2013). However, this also might be due to chaperon and additional functions of the Orco protein. Additionally, resistances of insect species to DEET were shown (Reeder et al. 2001; Klun et al. 2004; Stanczyk et al. 2010), probably due to the massive worldwide application of this repellent. Therefore, it is questionable how long DEET can be used effectively in the future. Besides DEET two more agents, IR3535 and Picaridin, having a prospective effect on ORs have to be mentioned. In the maxilliary palps of *Drosophila* the activity of an ORN was enhanced by all three agents (Syed et al. 2011). Moreover, it was found that the receptor DmOr42a is sensitive for the agents thus attributing to active avoidance of those chemicals. However, since it was shown that the effects of IR3535 were OR-independent, binding to the Orco protein is possible, too (Bohbot and Dickens 2012).

***Amilorides***

Also members of the amiloride group were found to influence olfactory sensing. In vertebrates amilorides blocked bursting of isolated ORNs (Frings and Lindemann 1988). Furthermore, amilorides reduced cyclic-nucleotide-mediated odor responses (Frings and Lindemann 1988; Frings et al. 1992). In lobster amilorides reversibly inhibited

odor-dependent neuronal activity of ORNs (Bobkov and Ache 2007). They furthermore were also able to block insect olfactory receptor currents (Pask et al. 2013; Rollecke et al. 2013). Furthermore, currents evoked by the Orco agonist VUAA1 (Jones et al. 2011) were blocked by the amiloride HMA in a cell expression system as well (Pask et al. 2013). Thus amilorides might directly bind to the Orco protein. However, additional targets of amilorides were reported (Kleyman and Cragoe 1988). Thus the specific blockage of components in odor transduction has to be investigated.

***Orco agonists and antagonists***

With the characterization of the specific Orco agonist VUAA1 a new tool for exploring insect olfactory signal transduction was found. The agonist function of VUAA1 was analyzed in a cell expression system, where it was able to elicit responses of cells co-expressing the *Anopheles gambiae* olfactory receptor AgOr10 together with the specific Orco protein (Jones et al. 2011). Since in the same study this was also true for expression of the Orco protein alone an agonist function of VUAA1 is very likely. Additionally, VUAA1 was shown to activate the Orco protein of various insect species, thus showing VUAA1 to be a broadly usable Orco agonist (Jones et al. 2011).

New Orco ligand candidates (OLC) structurally related to VUAA1 were found to be prospective agonists as well as antagonists of the Orco protein (Chen and Luetje 2012; Jones et al. 2012). The most effective antagonist was OLC15 (Chen and Luetje 2012). Application of OLC15 inhibited responses to hexanol in a cell expression system when expressing *the D. melanogaster* Orco and the olfactory receptor OR35a (Chen and Luetje 2012). In addition to these results the expression of the OR/Orco complex from other insect species reduced responses when stimulating with their key ligands, thus suggesting the block of the conserved Orco protein. Furthermore, the inhibition of Orco seems to be non-competitive to OR ligands, since the IC50 value was not changed at different odor concentrations upon stimulation (Chen and Luetje 2012). Since the Orco protein is highly conserved as described above, this agent might be an excellent candidate in developing a broadly usable insect repellent.

***G-protein blocker***

The involvement of G-proteins in the insect olfaction system was analyzed by different agents which target metabotropic pathways. One agent is pertussis toxin which inhibits $G_0$-activity by affecting the ADP-ribosylation of the α-subunit (West et al. 1985; Moss and Vaughan 1988). It was able to inhibit the PLC activity which was increased by pheromone stimulation (Boekhoff et al. 1990). Furthermore, it was shown that the block of the $G_0$-pathway in olfactory signal transduction by pertussis toxin led to behavioral deficits in the fruit fly (Ignatious Raja et al. 2014). The G-protein blocker GDP-β-S, which is a non-hydrolyzable analog of GDP, prevents the activation of G-proteins and was also employed in the examination of insect olfactory transduction. In a heterologous cell system, expressing the *B. mori* OR BmOr1 with its co-receptor no effect on the current response to its ligand bombykol was observed under influence of the general G-protein blocker GDP-β-S (Sato et al. 2008). In agreement to this GDP-β-S was neither able to inhibit the maximal response of the *Drosophila* OR43b to its ligand (Smart et al. 2008). On the contrary, a slower G-protein mediated pathway was mentioned in the same study since a modulation of $Ca^{2+}$-levels under influence of GDP-β-S was reported. Additionally, Wicher et al. (2008) found in *Drosophila*, that GDP-β-S reduced the sensitivity of OR22a and Orco to its key ligand, indicating a metabotropic pathway.

## 2.8 Temporal resolution of pheromone pulses

Pulsed release of pheromones from female pheromone glands (Itagaki and Conner 1985; Crnjar et al. 1988; Itagaki and Conner 1988; Valeur et al. 1999), turbulent wind and finally the flapping of the moths' wings cause a generation of small pheromone filaments and, thus, a nonlinear pheromone gradient within the air. The capability of detecting pulsed pheromone signals of ORNs, therefore, is very important and was examined for *M. sexta* (Marion-Poll and Tobin 1992) and other moths (Almaas et al. 1991; Kodadova 1996). Electrophysiological experiments on whole antennae revealed maximal frequency resolution of around 30 Hz (Bau et al. 2002; Bau et al. 2005) which

fulfill the qualification of sampling the airflow generated by wing beat frequencies of around 30 Hz (Tripathy et al. 2010). However, single receptor cells of *A. polyphemus* were able to resolve only odor pulses up to a frequency of 5 Hz (Rumbo and Kaissling 1989; Kodadova 1996). Details of sampling are not fully understood yet. Nevertheless, intermittent signals are a prerequisite for generating behavioral responses. Upwind flight is rather triggered by intermitted than continuous pheromone stimulation, as shown for *Heliothis virescens* (Vickers and Baker 1992) and *Grapholita molesta* (Baker et al. 1985). Furthermore, it is also needed for generating the moth's typical zigzag upwind search for females (Kennedy et al. 1981; Murlis and Jones 1981; Baker et al. 1985; Vickers and Baker 1992; Vickers 2000; Koehl 2006; Lei et al. 2009).

## 2.9 Circadian rhythms in moth olfaction

Many biochemical and physiological processes are dependent on endogenous, circadian mechanisms which are changing in the course of the day. Therefore, it is not surprising that as a result also the animal's behavior expresses circadian rhythms. Circadian pacemakers influence production and release of pheromones (Martinez and Camps 1988; Rafaeli and Soroker 1989; Rafaeli et al. 1993; Rafaeli and Klein 1994; Alstein et al. 1995; Zhu et al. 1995; Choi et al. 1998; Rosen 2002) as well as the calling behavior in female moths (Baker and Carde 1979). The pheromone responsiveness of males (Linn et al. 1992; Linn et al. 1996; Rosen et al. 2003) is also modulated in a daytime-dependent manner, leading to synchronized behavior of both sexes (Sasaki and Riddiford 1984; Itagaki and Conner 1988; Rosen 2002; Rosen et al. 2003; Silvegren et al. 2005). Female hawk moths show increased pheromone release and calling behavior at the end of the scotophase under control of a circadian pacemaker (Itagaki and Conner 1988). At the same time also mating behavior and flight activity of males occur more frequently (Lingren et al. 1977; Sasaki and Riddiford 1984). Daytime-dependent changes in sensitivity and temporal dynamics were found for pheromone sensitive ORNs of *M. sexta* (Flecke et al. 2006). Furthermore, during photophase the AP frequency after pheromone stimulation was reduced, compared to scotophase (Flecke and Stengl 2009).

Levels of the biogenic amine octopamine (OA) were found to correlate with the flight activity of *M. sexta* in a daytime-dependent manner (Lehman 1990) (Fig. 6). Changes in OA concentration in the hemolymph during the course of day occurred in the moth *Trichoplusia ni* (Linn et al. 1994) under influence of an endogenous oscillator (Linn et al. 1996). In accordance with this, for *M. sexta* higher OA concentrations were found during activity phase of the animals (Thomas Schendzielorz, University of Kassel, unpuplished data). Since it was shown that the stress hormone OA possesses neuromodulatory, neurohormonal and neuromediating properties (Roeder 2005; Farooqui 2007), it seems to be a good candidate for linking circadian rhythms in physiology with behavioral rhythms. In behavioral assays pheromone responses of several moth species were increased by OA-application into the hemolymph (Linn and Roelofs 1986; Linn et al. 1992; Linn and Roelofs 1992). Furthermore, electrophysiological experiments revealed a sensitization of ORNs due to OA-treatment (Pophof 2000; Grosmaitre et al. 2001; Pophof and Van der Goes van Naters 2002; Flecke and Stengl 2009) and an OA-dependent disadaptation of the ORNs in a daytime-dependent manner (Flecke and Stengl 2009). OA-receptors were found also in *M. sexta* (Dacks et al. 2006). They are G-protein-coupled receptors and suggested to increase cAMP concentration due to activation of an adenyly cylcase (Farooqui 2007) resulting in changed levels of sensitivity.

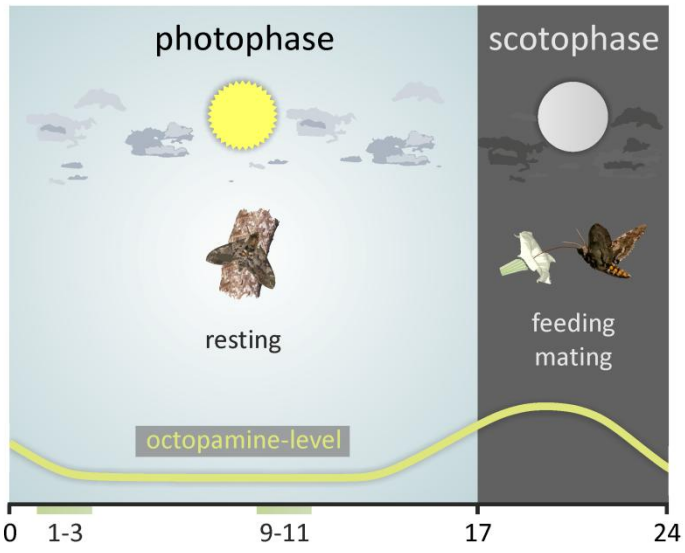


**Fig. 6 - Circadian behavior of *M. sexta*.** Animals used in the experiments were kept in an artificial light-dark cycle serving as subjective Zeitgeber (ZT). Light phase lasted from ZT 0 to ZT 17 followed by 7 hours of darkness. Feeding and mating behavior mainly occurs during scotophase when the animal is active. During this time the level of the stress hormone octopamine is elevated. Recordings were performed at the end of the activity phase at ZT 1 - 3 and during rest at ZT 9 - 11.

The circadian pacemaker which controls circadian locomotor activity rhythms was located to the optic lobes of the brain (Nishiitsutsuji-Uwo and Pittendrigh 1968). Later, lesion and transplantation experiments showed that the accessory medulla at the ventral medulla with associated pigment-dispersing hormone-releasing neurons controls circadian locomotor activity rhythms (Stengl and Homberg 1994; Reischig and Stengl 2003). Accordingly, rhythmic mating behavior of cockroaches was abolished when extracting the optic lobes of Madeira cockroaches (Rymer et al. 2007). However, rhythmicity in ORNs persisted, indicating that peripheral circadian clocks in the antenna might control the olfactory response in a daytime-dependent manner (Saifullah and Page 2009). Indeed, several autonomous peripheral oscillators were found in different tissues (Plautz et al. 1997), also in the antennae of *D. melanogaster* (Tanoue et al. 2004).

The endogenous rhythmicity of these cells in turn is controlled by so-called circadian clock genes, e.g. the gene *period*. These genes are expressed in a circadian rhythm and control their own expression via negative feedback loops (Hardin et al. 1990; Glossop et al. 1999). Thus, they oscillate independently of external Zeitgeber and serve as circadian pacemakers. Clock-gene mutants in *D. melanogaster* also lost circadian modulation of olfactory responses (Zhou et al. 2005), indicating the importance of circadian gene-based pacemakers for circadian behavior. Additionally, fruit fly per-null mutants, which do not express the per-gene in antennae, showed an abolished circadian rhythmicity of olfactory responses (Krishnan et al. 1999). Furthermore, the expression of clock genes in olfactory sensory neurons of moths such as *M. sexta* (Schuckel et al. 2007) and *Mamestra brassicae* was indicated (Merlin et al. 2006) and different clock genes were identified in the antenna of *Spodoptera littoralis* (Merlin et al. 2007).

Moreover, it was shown that the localization of ORs and the open probability of OR-dependent ion channels seems to be regulated by circadian expression of G-protein-coupled receptor kinases, thus influencing the amplitudes of spontaneously generated APs (Krishnan et al. 2008).

# 3 Aims of this study

To determine whether MsexOrco is involved in an ionotropic or metabotropic pheromone signal transduction mechanism the specific role of Orco in *M. sexta* bombykal transduction was examined with *in vivo* tip recordings under influence of the Orco agonist VUAA1. Additionally, further insights were gained by employing not only the agonist VUAA1, but also the prospective Orco antagonist OLC15 and the amilorides HMA and MIA. Furthermore, the involvement of a metabotropic pathway in the signal transduction cascade of *Manduca* was tested by using the general G-protein blocker GDP-β-S.

Since Orco was shown to be a spontaneously opening cation channel its role as a pacemaker channel controlling the resting potential and thereby the spontaneous activity of olfactory receptor neurons was explored by examining the effects of VUAA1 on spontaneous activity. Additionally, current injection experiments without pheromone stimulation were performed to show whether Orco is gated voltage-dependently.

Experiments were performed during the activity and resting phase of the animals, to analyze daytime-dependent changes in spontaneous and pheromone-dependent activity of the ORNs. Thereby, changes in the modulation or expression rates of the Orco protein over the course of the day were analyzed.

# 4 Material and methods

## 4.1 Animal rearing

*M. sexta* is a frequently examined model organism due to the fact of easy mass rearing (Yamamoto 1969). Animals were raised in the in-house breading facilities of the Department of Animal Physiology at the University of Kassel. As host tobacco plants were used which belong to the family of nightshades (Solanaceae). Eggs are attached on the leaves by female moths and afterwards were collected by the animal keepers. After hatching larva were fed on artificial diet (Tab.1) (modified after: Bell and Joachim 1976). In the fifth larva stage *Manduca* caterpillar were placed into wooden tubes for pupation when entering the wandering phase. After metamorphosis pupae were separated by sex since only male moths were used in experiments. Male pupae were cleaned and afterwards kept isolated to avoid contact to pheromones after eclosion. All adult animals were fed on sugar solution. This was served by tubes within flower shaped paper. An artificial scent of the host plant *Datura wrightii* or bergamotte oil was put on the paper to support the foraging of the animals. At any time animals were held under a long-day photoperiod of 17 h light followed by 7 h darkness. Time of

| Flour-mix | | Vitamin-mix | | Feed-mix | |
|---|---|---|---|---|---|
| Ingredients | Amount (g) | Ingredients | Amount (mg) | Ingredients | Amount |
| Soy flour | 312.5 | Nicotine acid | 100.3 | Tap water | 1800 ml |
| Rye flour | 288.8 | Riboflavin | 50.2 | Flour-mix | 500 g |
| Wheat flour | 288.8 | Thiamine | 23.4 | Vitamin-mix | 32 ml |
| Casein | 77.0 | Pyridoxin | 23.4 | Agar | 85 g |
| Ascorbic acid | 29.0 | Folic acid | 23.4 | Vegetable oil | 4.5 ml |
| Methylparaben | 9.6 | Biotin | 2.0 | | |
| Sorbic acid | 9.6 | | | | |
| Calcium carbonate | 2.9 | | | | |
| Sodium chloride | 2.9 | | | | |

**Tab. 1 - Ingredients of *M. sexta* larval diet. Left** Ingredients for 1000 g flour-mix. **Middle** Chemicals for vitamin-mix were diluted in 100 ml tab water. **Right** Components for final feed-mixture.

| Chemical | Formula | HLR | SLR |
|---|---|---|---|
| Potassium chloride | KCl | 6.4 | 171.9 |
| Magnesium chloride | $MgCl_2*6H_20$ | 12.0 | 3.0 |
| Calcium chloride | $CaCl_2*2H_20$ | 1.0 | 1.0 |
| Sodium chloride | NaCl | 12.0 | 25.0 |
| HEPES | $C_8H_{18}N_2O_4S$ | 10.0 | 10.0 |
| Glucose-monohydrate | $C_6H_{12}O_{6*H2O}$ | 354.0 | 22.5 |

**Tab. 2 - Ringer solution composition.** Solutions for hemolymph ringer (HLR) and sensillum lymph ringer (SLR). Concentrations of agents shown in mmol/l.

turning lights on is defined as Zeitgebertime (ZT) 0. Accordingly, the lights are turned off at ZT 17. Humidity was kept between 40 - 60 % at an air temperature from 24 - 27 °C. Animals were employed at the earliest 2 days after eclosion.

## 4.2 Solutions and agents

In case of no further statements chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Co., Munich, Germany). The compositions of hemolymph (HLR) and sensillum lymph ringer solution (SLR) (Tab. 2) were mainly applied as described by Kaissling (1995). Hemolymph and sensillum lymph cavity are separated by septate junctions (Keil and Steinbrecht 1987). Thus, varieties in their composition can be observed. A main difference is the high concentrations of potassium since this is enriched in the sensillum lymph cavity (see chapter “2.3 Pheromone-sensitive trichoid sensilla”).

Ringer solutions were adjusted to a pH of 6.5. The osmolarity was adjusted by mannitol for sensillum lymph ringer solution to 475 mOsmol/l and for hemolymph ringer solution to 450 mOsmol/l respectively. All tested agents were diluted in dimethyl sulfoxide (DMSO) and stored in stock solutions. When required, those were added to the SLR. The resulting working solutions always had a final concentration of 0.1 % DMSO, whereas the concentration of the respective agent was adjusted to 10 µM unless otherwise specified.

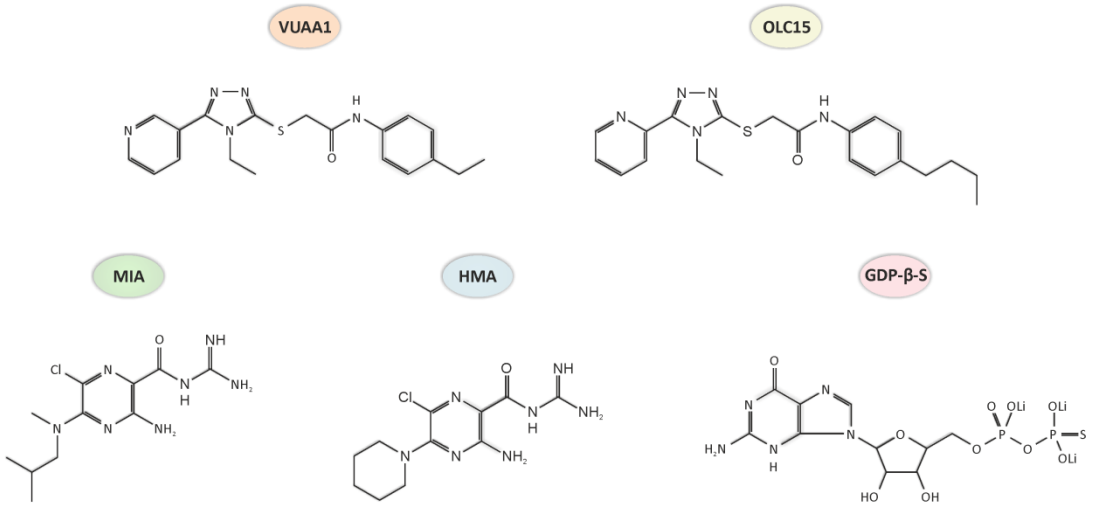


**Fig. 7 - Structural formula of tested agents.** Putative Orco agonist VUAA1 (orange) and the structurally related antagonist OLC15 (yellow). Amilorides MIA (green) and HMA (blue) as further prospective antagonists. G-protein blocker GDP-β-S (pink). Color code was used throughout the whole manuscript.

Used agents (Fig. 7) were the prospective Orco agonist *N-(4-ethylphenyl)-2((4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl)-thio)-acetamide* (VUAA1) [2], the prospective Orco antagonist *N-(4-butylphenyl)-2-((4-et-5-(2-pyridinyl)-4H-1,2,4-triazol-3-yl)thio) acetamide* (OLC15) [2], the amilorides *5-(N-methyl-N-isobutyl)-amiloride* (MIA) and *5-(N,N-hexamethylene)-amiloride* (HMA) as well as antagonist and the G-protein blocker *Guanosine 5'-[β-thio]diphosphate trilithium salt* (GDP-β-S).

## 4.3 Tip recording

Electrophysiological research on insect olfaction was initiated by finding voltage fluctuations between tip and basis of an silkmoth antenna after pheromone stimulation (Schneider 1957), also known as electroantennography (EAG). At about the same time (1959) the first pheromone was extracted by Adolf Butenandt and purified from pheromone glands of 500.000 female *B. mori*. Subsequently EAG was developed and first single sensillum recordings were performed (Boeckh 1962; Schneider et al.

[2] generously provided by the Max Planck Institute for Chemical Ecology, Jena, Germany

1964). In 1979 the major pheromone component of *Manduca*, bombykal (BAL), was detected (Starrat et al. 1979). Beside this, eleven additional components were found in the blend of the female pheromone glands (Tumlinson 1989). It was shown that trichoid sensilla of *M. sexta* always house two ORNs and one of them always responds to BAL (Sanes and Hildebrand 1976; Keil 1989) (Fig. 3). In tip recordings it was observed that the two ORNs of a trichoid sensillum can be easily distinguished from each other. They have different odor specificity and one of the two ORNs, the BAL-sensitive ORN has a distinctly larger action potential amplitude than the other (Dolzer et al. 2001). Therefore, tip recordings, as a minimal invasive procedure, combined with BAL stimulations is ideally suited for the detailed investigation of the olfactory system of *Manduca*.

### 4.3.1 Preparation

The preparation always started by taking an isolated male moth which was fixed into a Teflon holder with adhesive tape. The antenna was kept still by using dental wax (Boxing wax, Sybron/Kerr, Romulus, MI, USA) at the basis of the antenna. Around 15 apical annuli were clipped off by a micro scissor. Two glass capillaries were produced by an electrode-puller (DMZ-Universal Puller, Zeitz Instruments, Martinsried, Germany). One capillary was filled with HLR and was slipped over an Ag/AgCl-electrode, serving as reference electrode, the other one was filled with SLR and was connected to the recording electrode (Fig. 8), sending their signal to the amplifier (see chapter “4.4 Data acquisition and analysis”). Both electrodes were attached to micormanipulators for precise movement. The capillary containing the reference electrode was inserted by a micromanipulator into the cut antenna in every experiment up to the level of the second outermost annulus. Afterwards the antenna was protected against drying out by electrode gel (GE Medical Systems Information Technologies, Freiburg, Germany). Next trichoid sensilla of the examined annulus were shortened with a sharpened forceps by around one third of the sensillum length. The glass capillary containing the recording electrode was pulled over one cut trichoid sensillum by a micromanipulator.

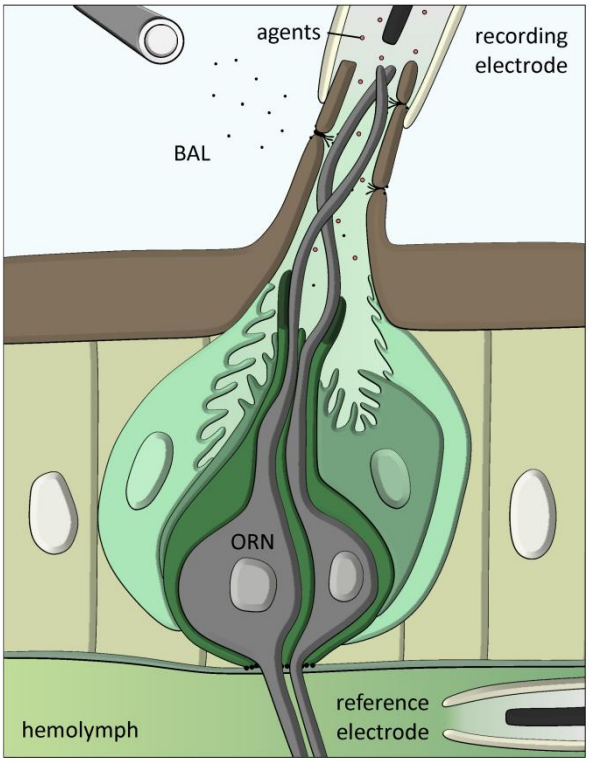


**Fig. 8 - Schematic representation of tip recording and drug application.** After one sensillum is cut by a forceps the recording electrode is slipped over. The reference electrode is put into the hemolymph of the antenna. Afterwards the transepithelial potential between both electrodes was measured. Pheromone stimulation with the main pheromone component bombykal (*BAL*) was applied via an air pressure system. Agents were diluted in the sensillum lymph ringer and diffused passively into the sensillum. *ORN* olfactory receptor neuron.

In order to detect daytime-dependent effects experiments were performed at different ZTs. One aspect of particular importance is the difference between activity and resting phase of the animals. Therefore, experiments were performed at ZT 1 - 3 representing the end of the moths' active phase and ZT 9 - 11 which lies in the middle of the resting phase.

### 4.3.2 Pheromone stimulation and drug application

Two air pressure systems were installed in the setup. Air streaming into both systems was previously cleaned by an active charcoal filter. Afterwards air was moistened by passing a gas washing bottle. The first air pressure system provided a steady air stream on the recording site of the antenna. There are two main reasons for this: Firstly, pheromones released during stimulation are quickly blown away from the recording site, preventing adaptation of the pheromone sensitive neurons. Secondly, antennal

mechanical receptors around the recording site adapt throughout constant stimulation. The second air pressure system (Fig. 8) was used to provide pheromone stimulation. Each stimulation persisted for 50 ms, timed by a computer-controlled valve (JFMH-5-PK-3, Festo, Esslingen, Germany). The pheromone component BAL [3], was diluted in n-hexan in a concentration of 0.1 µg / µl. From this stock solution 10 µl were applied on a filter paper. In a fume hood n-hexan was evaporated completely. Thus, the mass of pheromones remaining on the filter paper was 1 µg BAL, corresponding to an amount of substance of 4,23 pM BAL. Afterwards, the filter paper was stored within a cartridge. In case of stimulation the air stream was redirected and passed the cartridge. A second valve (PA 202-004 P, Staiger, Erligheim, Germany) prevented an uncontrolled efflux of pheromones between actual stimuli. It was directly attached to a glass tube which was directed to the recording side of the antenna. All valves were controlled by self-made protocols for pClamp software (Clampex 8, Molecular Devices, Sunnyvale, CA, USA). Stimulations occurred every five minutes, since this has been shown not to have an adaptive effect (Dolzer et al. 2003). Recordings with pheromone stimulation lasted 30 or 120 minutes. Accordingly there were 6 or 24 subsequent stimulations for each recording.

Drugs were added to the sensillum lymph ringer solution as described (see chapter "4.2 Solutions and agents"). Thus, all tested agents passively diffused into the sensillum lymph when slipping the electrode over the sensillum.

### 4.3.3 Current injections

Current injection was used as a possible approach of stimulating ORNs without pheromone stimulation. Here the neuronal activity after or during current application was compared to that observed before stimulation. The amplifiers stimulation, which allowed the adjustment of the current strength, was connected to the PC via TTL link. The signal was controlled with the PCs' parallel printer port by software (Diyk74, Freeware) which also enabled the adjustment of stimulus duration. Currents were

[3] generously provided by the Institute of Physiology, University of Hohenheim, Germany

given for 10 s or 60 s, respectively. If not further specified the current strength was 300 pA.

## 4.4 Data acquisition and analysis

In the first instance voltage changes measured by the electrodes within one trichoid sensillum were sent to the headstage of the used extracellular amplifier (BA-03x, NPI Electronic GmbH, Tamm, Germany). Output signals were converted by the analog-to-digital converter (DigiData 1200 Series Interface, Molecular Devices, Sunnyvale, CA, USA) and forwarded to the amplifier. Here the signal was amplified 200-fold with a cutoff frequency of 1.3 kHz and transmitted to the PC, where it was recorded by Clampex 8 software (Molecular Devices, Sunnyvale, CA, USA). Data acquisition was implemented in two ways. During pheromone stimulation the signal was sampled gap-free with a sampling rate of 20 kHz for five seconds and each stimulation was stored as individual file, which were used after recordings to obtain all parameters of the pheromone response (see chapter “4.5 Analyzed parameters”). In the following 295 seconds the signal of the long lasting pheromone response was recorded as fixed length events. The length of each detected event was 12.75 ms and it was sampled with a rate of 19.6 kHz. Only if a set threshold was passed by the signal an event was recorded. This type of acquisition was also performed for analyzing spontaneous or current-induced neuronal activity. For analysis of parameters concerning the properties of APs a high-pass filter was applied (cutoff frequency: 150 Hz) (Fig. 9-B). Thus, slow potential changes, e.g. from the arising receptor potential, were filtered out. Secondly, for the analysis of the sensillum potential, the signal was low-pass filtered (cutoff frequency: 50 Hz; with median filter: time constant = 0.05 ms; smooth process: time constant= 0.01) (Fig. 9-A). Afterwards data analysis was performed using self-written scripts for Spike2 (Version 7; Cambridge Electronig Design Limited, Cambridge, England) and Matlab software (Version R2007b; The MathWorks Inc., Natrick, Massachusetts, USA). For calculation of parameters concerning the sensillum potential the time point of the beginning and maximal amplitude of the potential deflection were selected manually by cursors (Fig. 9-A).

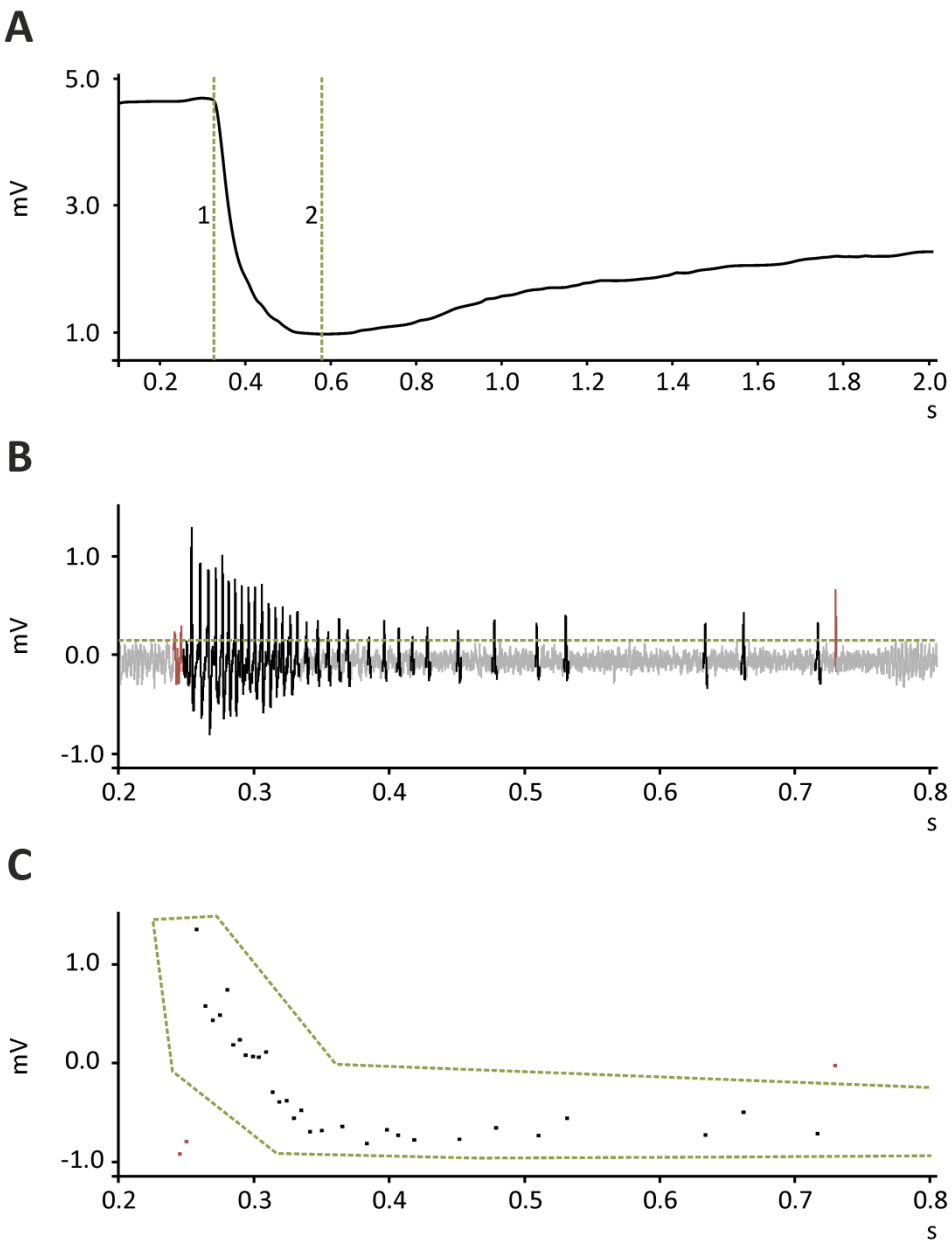


**Fig. 9 - Evaluation of parameters. A** Low-pass-filtered electrophysiological signal (cutoff frequency: 50 Hz) for analysis of the pheromone-dependent sensillum potential amplitude. Cursors marking start and maximum of the sensillum potential were set manually. **B** High-pass-filtered response (cutoff frequency: 150 Hz) after pheromone application for the analysis of occurring APs. For peak search a threshold level (green line) was set. Some artifacts (red) also exceeded this threshold and had to be excluded. **C** Analyzed peak values taken from B. Evident APs had to be framed. Only values inside the frame were used for further analysis.

## 4.5 Analyzed parameters

### 4.5.1 Direct pheromone response

A few parameters can be analyzed within the first five seconds after pheromone stimulation (Fig. 10-A). After stimulation of ORs with their specific ligand the cell responds with opening of ion channels. In case of BAL this leads to a negative deflection of the transepithelial potential measured between the two electrodes. Although it can be assumed that this deflection is mainly due to the receptor potential of the ORN, also the accessory cells possibly contribute to changes of the potential. Therefore, it is termed sensillum potential. Its main parameter is its maximal amplitude (sensillum potential amplitude, SPA). Since the absolute values between recordings strongly differed even due to small changes in preparation this parameter is always normalized to the value of the first SPA during the time course of each recording.

Superimposed on the slow changes of the transepithelial potential APs can be found. They were differently evaluated. The AP frequency of the very first six APs is of particular importance since it changes in a dose-dependent manner (Dolzer et al. 2003), thus being an indicator for sensitivity levels of the ORN. Also the temporal distribution of APs within the first 1000 ms after pheromone stimulation was analyzed in a binwidth of 10 ms. As a result post-stimulus time histograms (PSTHs) were obtained (Fig. 11). Additionally, the number of APs within the first 150 APs was calculated and compared to the number of APs within the first 1000 ms. Thus, changes in the temporal distribution of APs were shown and different response kinetics were observed. The last evaluated parameter of the direct pheromone stimulus response is the latency of the first AP after the onset of the SPA. This parameter relates to the threshold level for generating APs and thus to the sensitivity of the ORN.

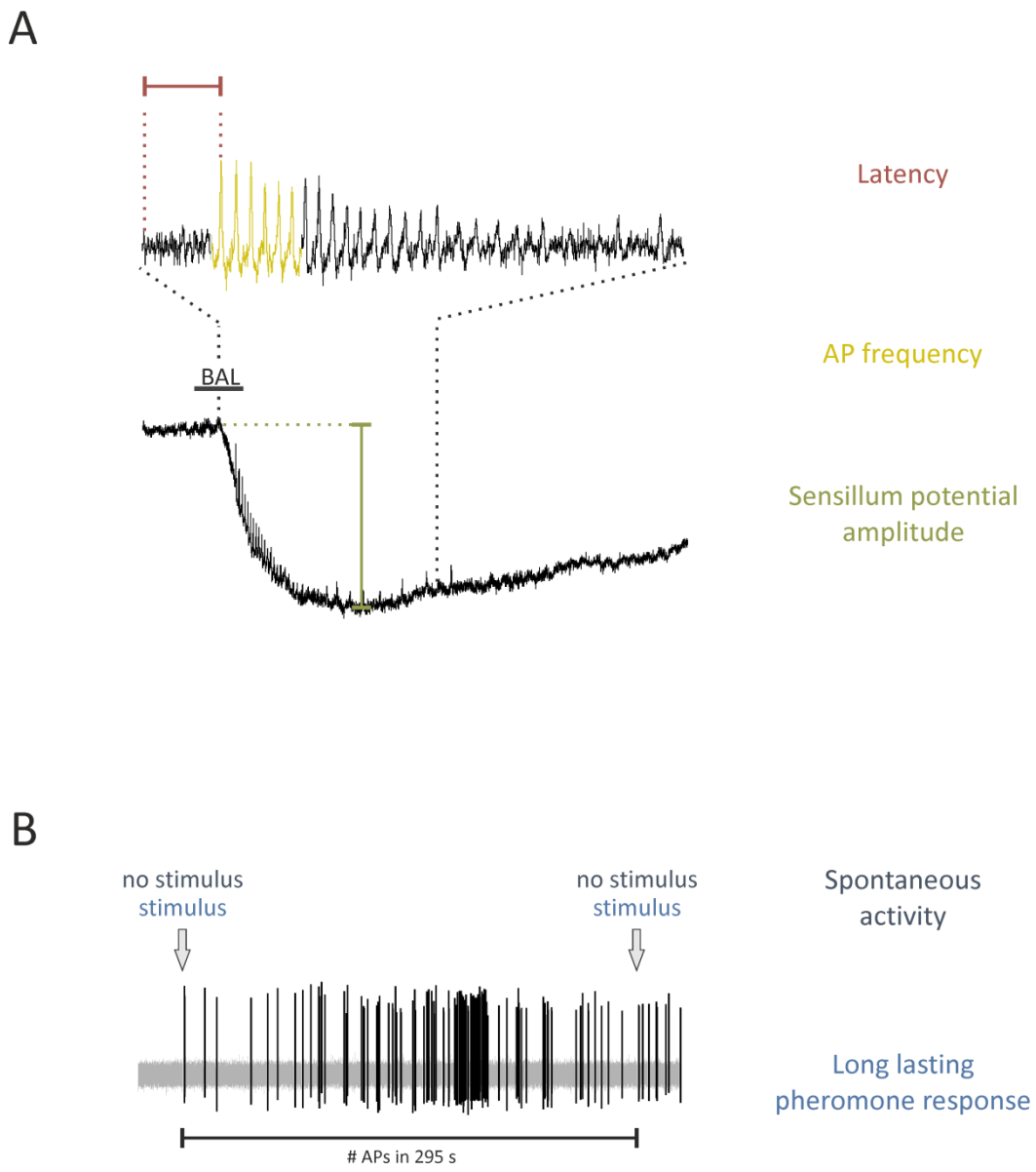


**Fig. 10 - Analyzed parameters: A** Pheromone response parameters within the first 5 seconds after stimulation. The maximal deflection of the transepithelial potential is defined as sensillum potential amplitude (green). Latency describes the interval between onset of pheromone stimulation upon first AP (red). The AP frequency was calculated for the first six APs after pheromone stimulation (yellow). **B** Long lasting pheromone response was measured between pheromone stimulations, whereas spontaneous activity occurs pheromone-independently. Both parameters were analyzed for 295 seconds.

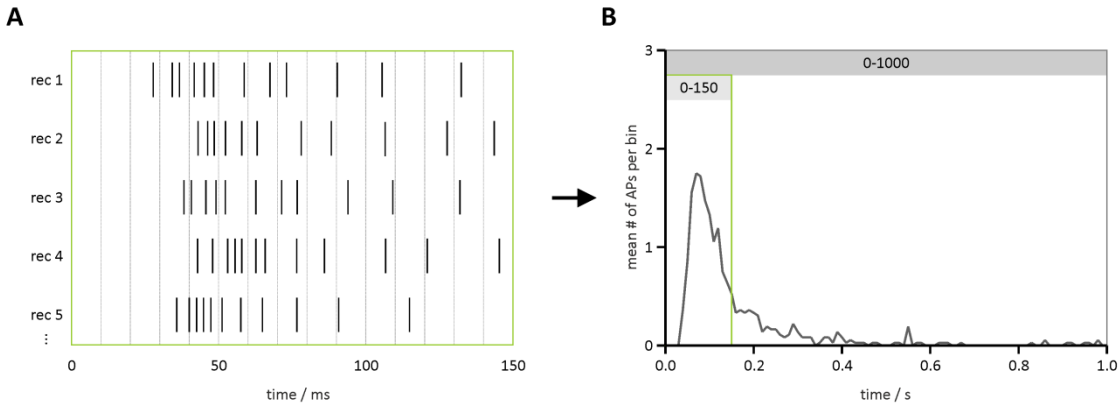


**Fig. 11 - Preparing post stimulus time histograms: A** APs (indicated by strokes) after pheromone stimulation of all analyzed recordings were summed for each bin (binsize: 10 ms). **B** Afterwards the mean number of APs was plotted for each bin afterwards in histograms for the first 1000 ms after pheromone stimulation. Finally the numbers occurring within the first 150 ms and 1000 ms were compared between different recordings for analysis of the pheromone response kinetics.

### 4.5.2 Long lasting pheromone response and spontaneous activity

Furthermore, long lasting effects of pheromone stimulation were observed (Fig. 10-B). Therefore, only APs occurring between 5 and 300 seconds after pheromone stimulation were analyzed. The number of APs within this time interval was defined as long-lasting pheromone response (LLPR). Accordingly, also the spontaneous activity was measured for 295 s each (Fig. 10-B) for better comparison of LLPR and spontaneous activity. In contrast to the LLPR analysis in recordings of spontaneous activity the cells were never exposed to pheromone stimuli.

### 4.5.3 Current injections

Current injections were performed as described in chapter “4.3.3 Current injections”. They were performed for 10 or 60 s. In both cases the spontaneous activity before current stimulation was measured for 60 s and the number of APs during this time interval was analyzed. In case of recordings where current injections lasted 10 s the neuronal activity was measured directly after stimulation for a period of 60 s. If

stimulations lasted 60 s the measurement of the neuronal activity was performed during stimulation. In both cases the number of APs occurring in 60 s was analyzed.
Afterwards, for analysis of the occurring APs, high frequent signals were determined by peak search and the amplitudes were plotted in a scatter chart. Afterwards evident APs had to be framed for further analysis in accordance to the analysis shown in figures 9-B and 9-C.

## 4.6 Graphs and statistics

All statistics were raised using GraphPad Prism software. First, data were tested for Gaussian distribution using Shapiro-Wilk normality test which most data did not reveal. Therefore, data concerning two groups were statistically tested by Mann-Whitney test. In case of comparison of three or more groups Kruskal-Wallis test with a Dunn's post-hoc analysis was used. For both tests a significance level of $\alpha=0.05$ was applied. Significances in figures were shown by asterisk (*** ≙ $p<0.001$; ** ≙ $p<0.01$; * ≙ $p<0.05$; n.s. ≙ not significant,). If not presented in the related figure, all statistics are available in the appendix. Significant differences were marked with asterisks (for Kruskal-Wallis test) and p-values (for Mann-Whitney test). They were summed in tables of the appendix. Same was true for all median values.
The mean values of all parameters were plotted in graphs over recording time. Error bars always indicate the standard error of the mean (SEM). In long-term recordings comparisons between begin (0 - 20 min) and end of recordings (100 - 120 min) were made to detect long-term effects of the tested agents. Some data are presented in box-plots. All box plots were drawn with whiskers from 10 to 90 percentile. All figures were created by means of GraphPad Prism (Version 5.01; GraphPad Software Inc, La Jolla, CA, USA) and Corel Draw software (Version X3; Corel Corporation, Ottawa, Ontario, Canada).

# 5 Results

## 5.1 In situ tip recordings found no evidence for an Orco-based ionotropic mechanism of pheromone transduction in *Manduca sexta*

The role of the coreceptor Orco in insect olfactory transduction is not clarified yet. With the identification of VUAA1 as Orco agonist in *D. melanogaster* (Jones et al. 2011) an important tool for researchers was provided which will help to further examine the functions of the Orco protein in different insect species. In particular the possible involvement of Orco in an ionotropic signal transduction process can now be examined with the prospective Orco agonist and antagonists in tip recordings of pheromone-sensitive trichoid sensilla on the antenna of intact male *Manduca sexta* during simultaneous stimulation of bombykal (BAL), the main pheromone component of *M. sexta* females.

In calcium imaging experiments it was previously shown by Nico Funk and Latha Mukunda that VUAA1 is a specific agonist for the Orco protein also in the hawkmoth *M. sexta* (Nolte et al. 2013). In tip recordings VUAA1 was infused into pheromone-sensitive trichoid sensilla on antennae of *M. sexta in vivo*. Since only the spontaneous activity as well as the late pheromone response parameters increased Orco is very likely not part of an ionotropic pheromone transduction cascade underlying phasic pheromone responses in *M. sexta*.

### 5.1.1 Effects of the Orco agonist VUAA1 on pheromone response parameters

In tip recordings of pheromone-sensitive trichoid sensilla the function of the Orco agonist VUAA1 was examined. Recordings were performed at two different Zeitgeber times, at ZT 1-3 and ZT 9-11, representing the end of the activity phase and the resting phase of *Manduca* respectively. In this time windows long-term recordings lasting 2 h were employed with VUAA1 concentration of 100 µM. Additionally, at the same ZTs short-term recordings were performed for 30 minutes with 1 µM VUAA1.

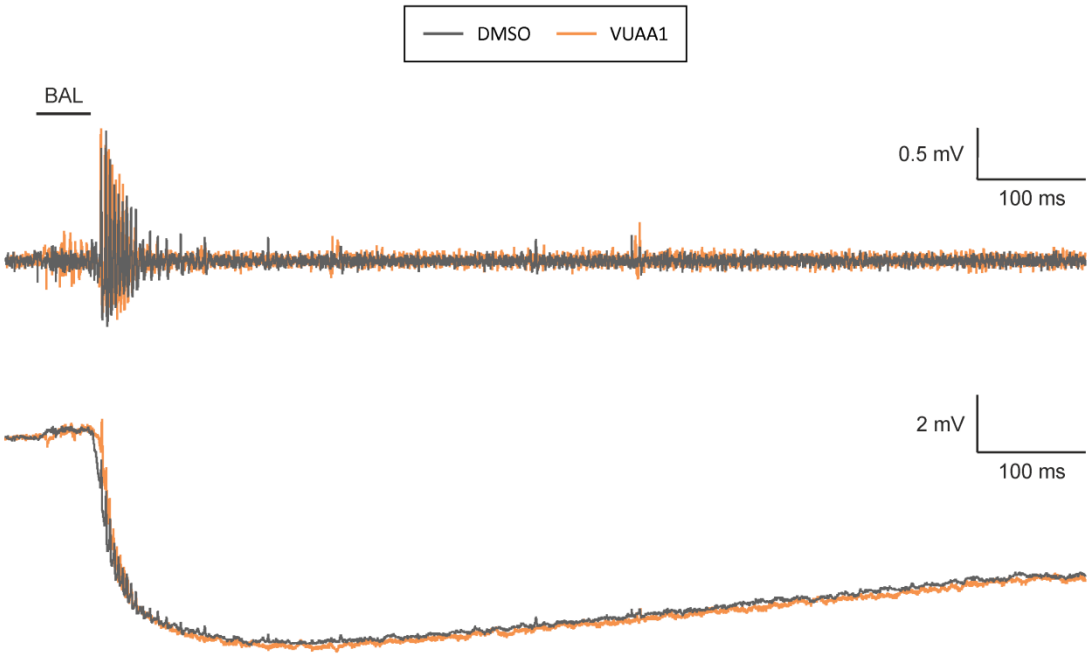


**Fig. 12 - Original traces of VUAA1 recordings at the beginning of recordings during activity phase. Above** High-pass filtered signal with APs after bombykal (BAL) stimulation. **Below** Unfiltered signal with the slow deflection of the sensillum potential amplitude and superimposed APs. The sensillum potential did not change in VUAA1 recordings (orange) compared to control recordings (grey). Same was found for the response kinetics to pheromone stimulation.

### *Orco agonist VUAA1 does not affect bombykal-dependent sensillum potential amplitude*

Control recordings did not reveal any differences in the sensillum potential amplitude (SPA) during the time course of long-term tip recordings when comparing beginning and end (Fig. 12, 13). This was true for activity ($p=0.796$, Mann-Whitney-test with $\alpha=0.05$ unless otherwise specified) as well as for resting phase ($p=0.904$). Furthermore, no ZT-dependent differences were found in control recordings for the beginning ($p=0.629$) as well as the end ($p=0.393$) of the recordings. Application of the Orco agonist VUAA1 did not affect the SPA (Fig. 13). No differences in the SPA at the beginning (activity: $p=0.340$; end: $p=0.381$) as well as the end (activity: $p=0.979$; end: $p=0.657$) were found in comparison to control recordings. Furthermore, no changes

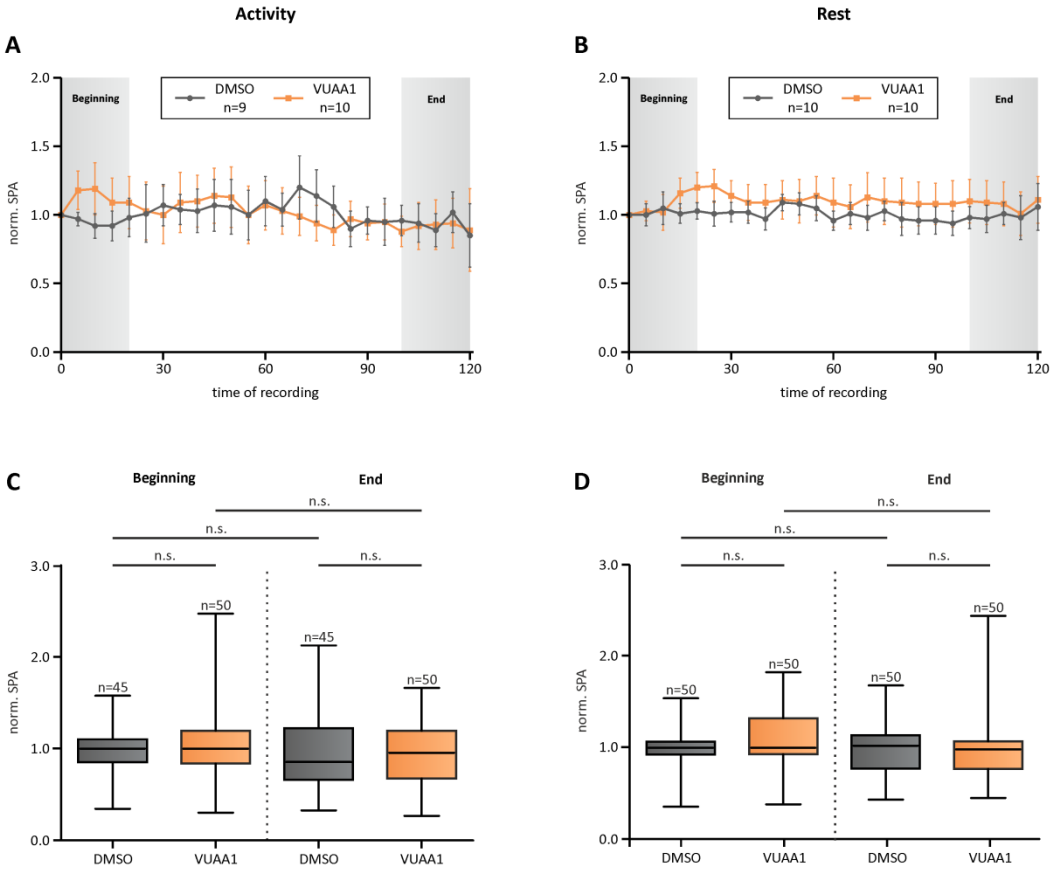


**Fig. 13 - Sensillum potential amplitudes was not affected by Orco agonist VUAA1. A-B** The sensillum potential amplitude (SPA) was measured every 5 minutes after bombykal (BAL; 4.23 pM on filter paper) stimulation in long-term tip recordings of pheromone-sensitive trichoid sensilla. Control recording as well as VUAA1 (100 µM) recordings did not show any changes in the normalized BAL-dependent SPA over the course of the recordings. Error bars show SEM. **C-D** Furthermore, no changes were found when comparing beginning (first 20 minutes) and end (last 20 minutes) of long-term recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant; Mann-Whitney-test with α=0.05).

within the time course of long-term VUAA1 (100 µM) recordings (activity: p=0.194; rest: p=0.145) nor ZT-dependent differences (beginning: p=0.893; end: p=0.459) in recordings at both ZTs were detected.

In short-term recordings under influence of 1 µM VUAA1 in the first 20 minutes at both ZTs no effects were observed in comparison to control recordings (activity: p=0.855; rest: p=0.779) or recordings with the higher VUAA1 concentration (activity:

p=0.263; rest: p=0.474). Furthermore, also no ZT-dependent changes were found for those recordings (beginning: p=0.383).

Since the sensillum potential amplitude is mainly based upon the receptor potential of the BAL-sensitive ORNs, caused by BAL-dependent opening of ion channels in the dendrites of ORNs, it is unlikely that Orco is an ion channel which contributes to the pheromone-dependent receptor potential. Consequently, no evidence for BAL-dependent OR-Orco-based ionotropic pheromone-receptors in *M. sexta in vivo* was found.

***VUAA1 did not increase the BAL-dependent AP frequency***

Next, it was examined whether Orco affects the phasic action potential (AP) response of the first six BAL-dependent APs, generated by the rise of the depolarizing receptor potential. Control recordings at both ZTs showed a significant decline of the AP frequency when comparing the first and the last 20 minutes of 2h-lasting long-term tip recordings (Fig. 14; activity: p=0.039; rest: p<0.001; Mann-Whitney-test with α=0.05 unless otherwise specified). The BAL-dependent AP frequency in control recordings was lower during resting phase. Whereas this was significant for the end of recordings (p<0.001) it was only found by trend for the beginning (p=0.133). Infusion of 100 µM VUAA1 via the tip recording capillary caused a significant decline of BAL-dependent phasic AP frequencies during the course of the tip recordings at both ZTs (Fig. 14; activity: p<0.001; rest: p<0.001). Furthermore, also for long-term recordings with 100 µM VUAA1 ZT-dependent differences were found as already observed in control recordings. Both beginning (p=0.002) and end (p=0.055) of VUAA1 recordings showed a lower AP frequency during rest. Short-term recordings with 1 µM VUAA1 (activity: p=0.572; rest: p=0.710) as well as recordings with 100 µM VUAA1 during activity phase (p=0.507) did not affect the AP frequency in the first 20 minutes of recordings. However, infusion of 100 µM VUAA1 during rest caused a significant reduction of the AP frequency (p=0.002). Comparison of the end of long-term recordings showed that the observed reduction of AP frequencies during the time courses of control as well as

in VUAA1 recordings (100 µM) was stronger during application of VUAA1 (activity: p<0.001; rest: p<0.001).

Since the BAL-dependent phasic AP frequency did not increase at any time under influence of VUAA1 the open probability of Orco was not increased by the Orco agonist VUAA1 in the first milliseconds after pheromone stimulation.

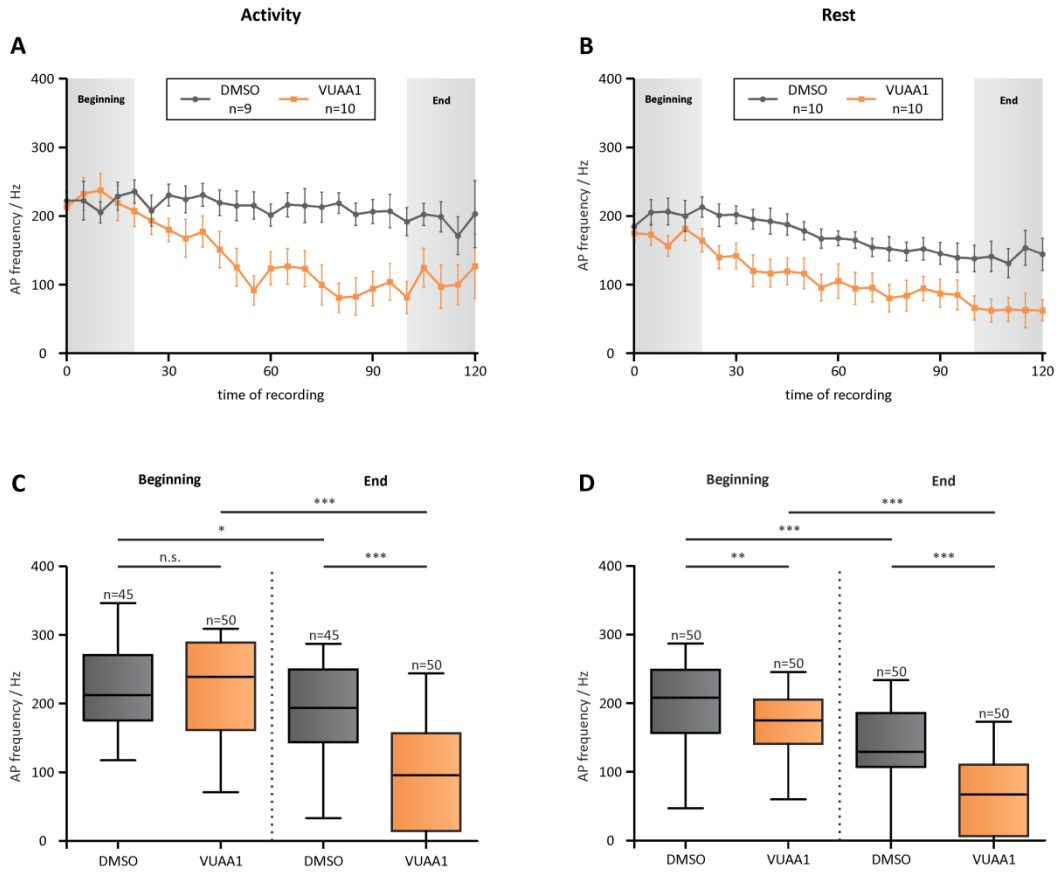


**Fig. 14 - Orco agonist VUAA1 did not increase the bombykal-dependent phasic pheromone responses.** **A-B** The AP frequency after bombykal (BAL; 4.23 pM on filter paper) stimulation was measured every 5 minutes in long-term tip recordings. VUAA1 did not affect the AP frequency at the beginning of activity phase. During activity phase recordings revealed higher AP frequencies in comparison to resting phase. For the later part of recordings at both ZTs VUAA1 decreased the AP frequency compared to control recordings. Error bars show SEM. **C-D** Comparison of beginning and end of the recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, *P<0.05, **P<0.01, ***P<0.001; Mann-Whitney-test with α=0.05).

### *VUAA1 prolonged the latencies of the first BAL-dependent AP*

The latency of the first AP after BAL stimulation significantly increased at the end of long-term control recordings compared to the beginning at both ZTs tested (Fig. 15, activity: p=0.002; rest: p<0.001; Mann-Whitney-test with α=0.05 unless otherwise specified). This increase in control recordings was stronger at the end of recordings during rest as compared to the activity phase (p<0.001), whereas no ZT-dependent differences could be found at the beginning (p=0.905). During the activity phase 1 µM (p=0.191) as well as 100 µM VUAA1 (p=0.052) did not significantly change the latency at the beginning of recordings compared to control recordings (Tab. A1-A).

However, during the resting phase a significant increase was observed at the beginning for 1 µM (p=0.008) and 100 µM VUAA1 (p<0.001). Furthermore, at the end of recordings the latency was prolonged under influence of 100 µM VUAA1 at both ZTs tested (activity: p<0.001; rest=p<0.001).

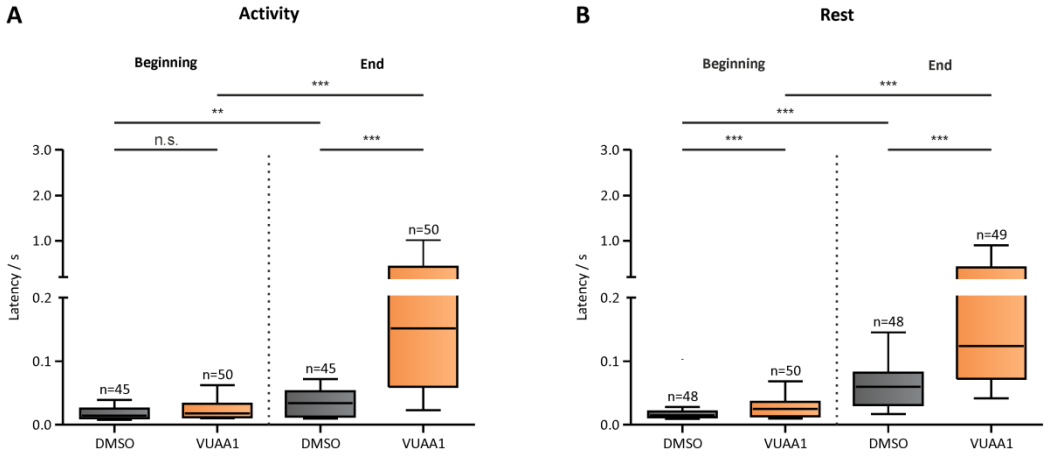


**Fig. 15 - Latency of bombykal-dependent first action potential was prolonged by VUAA1. A-B** For control and VUAA1 (100 µM) tip-recordings the bombykal-dependent (4.23 pM on filter paper) latency was longer at the end of recordings in comparison to the beginning. VUAA1 infusion via the recording electrode increased the latency compared to control recordings, except for the first 20 min of tip recordings during the activity phase. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, ***P*<0.01, ****P*<0.001; Mann-Whitney-test with α=0.05).

***VUAA1 slowed the response kinetics at the end of the recordings***

Next, AP distributions within the first 1000 ms after BAL-stimulation were analyzed. The numbers of APs within the first 150 ms and 1000 ms were determined. Post stimulus time histograms (PSTHs) were performed as described above (Fig. 11) for better comparison of the measured kinetics.

During activity a change in response kinetics during long-term control recordings did not occur (Fig. 12, 16). Neither the numbers of APs observed within the first 150 ms ($p=0.134$; Mann-Whitney-test with $\alpha=0.05$ unless otherwise specified) nor within 1000 ms ($p=0.693$) changed during the course of recordings. During resting phases the numbers of APs within 150 ms ($p<0.001$) and 1000 ms ($p=0.012$) were reduced significantly at the end of control recordings. Significant ZT-dependent differences were only observed at the end of control recordings where the number of APs within the first 150 ms was lower during rest ($p<0.001$). Therefore, response kinetics of control recordings were less phasic towards the end of the recordings compared to the activity phase (Fig. 16). Application of VUAA1 at concentrations of 1 µM and 100 µM did not significantly changed the kinetics at the beginning of recordings at both ZTs (Tab. A1-A), except for 1 µM VUAA1 which caused a significant reduction of the number of APs in the first 1000 ms ($p=0.002$). During the course of VUAA1 (100 µM) recordings a decrease of the AP frequency within 150 ms was observed for both ZTs tested (activity: $p<0.001$; rest: $p<0.001$), whereas the number of APs in 1000 ms was not significant affected (activity: $p=0.078$; rest $p=0.103$). Compared to control recordings the number of BAP-dependent phasic APs within 150 ms were significantly reduced under influence of 100 µM VUAA1 at the end of the recordings (activity: $p<0.001$; rest: $p<0.001$). Same was found for the numbers of APs within 1000 ms during activity phase ($p=0.008$), whereas the reduction during rest was not significant ($p=0.373$).

Therefore, application of VUAA1 during the time course of recordings changed kinetics from a phasic to a more tonic response pattern. This was also seen during rest in control recordings, even though not as strongly as observed for VUAA1 recordings.

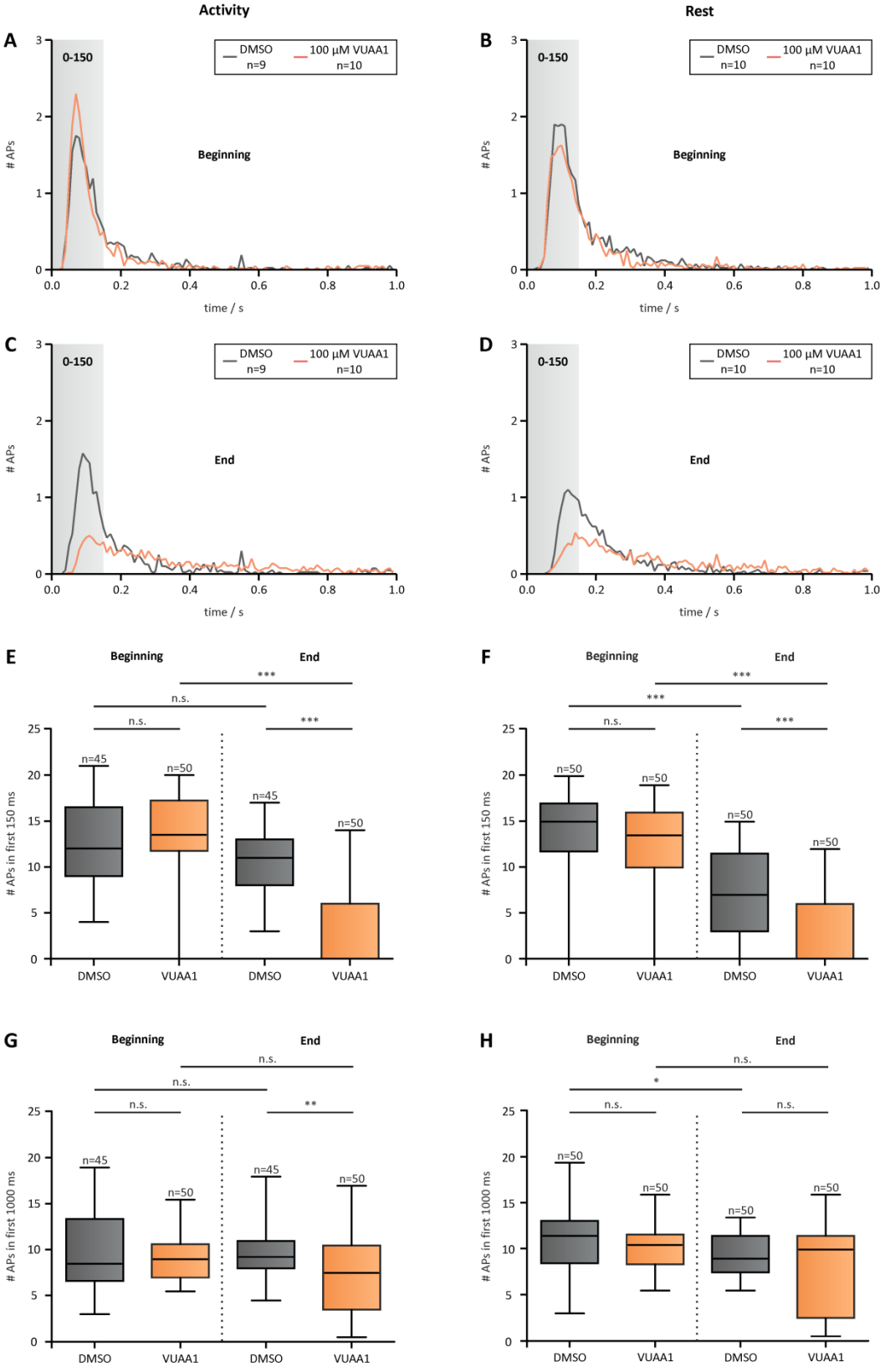


**Fig. 16 - VUAA1 slowed the response kinetics. A-D** Post-stimulus time histograms of response kinetics. In control recordings response kinetics during activity did not change, whereas kinetics became more tonic at the end of the 2 hr tip recordings during rest. VUAA1 (100 µM) had no effect on response kinetics at the beginning of the recordings while it decreased the phasic pheromone response at the end of the long-term tip recordings. Grey areas indicate the first 150 ms after pheromone stimulation. **E-H** Comparison of the numbers of APs within 150 or 1000 ms at beginning and end of the recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, *$P$<0.05, **$P$<0.01, ***$P$<0.001; Mann-Whitney-test with α=0.05).

### 5.1.2 Spontaneous activity was dose-dependently increased by VUAA1

Since Orco was previously shown to affect the spontaneous activity in other insect species (Larsson et al. 2004; Jones et al. 2011) Orco-effects on AP frequency in the absence of BAL stimulation were examined. In tip recordings of trichoid sensilla VUAA1 increased the spontaneous activity dose-dependently (Fig. 17). APs occurring without

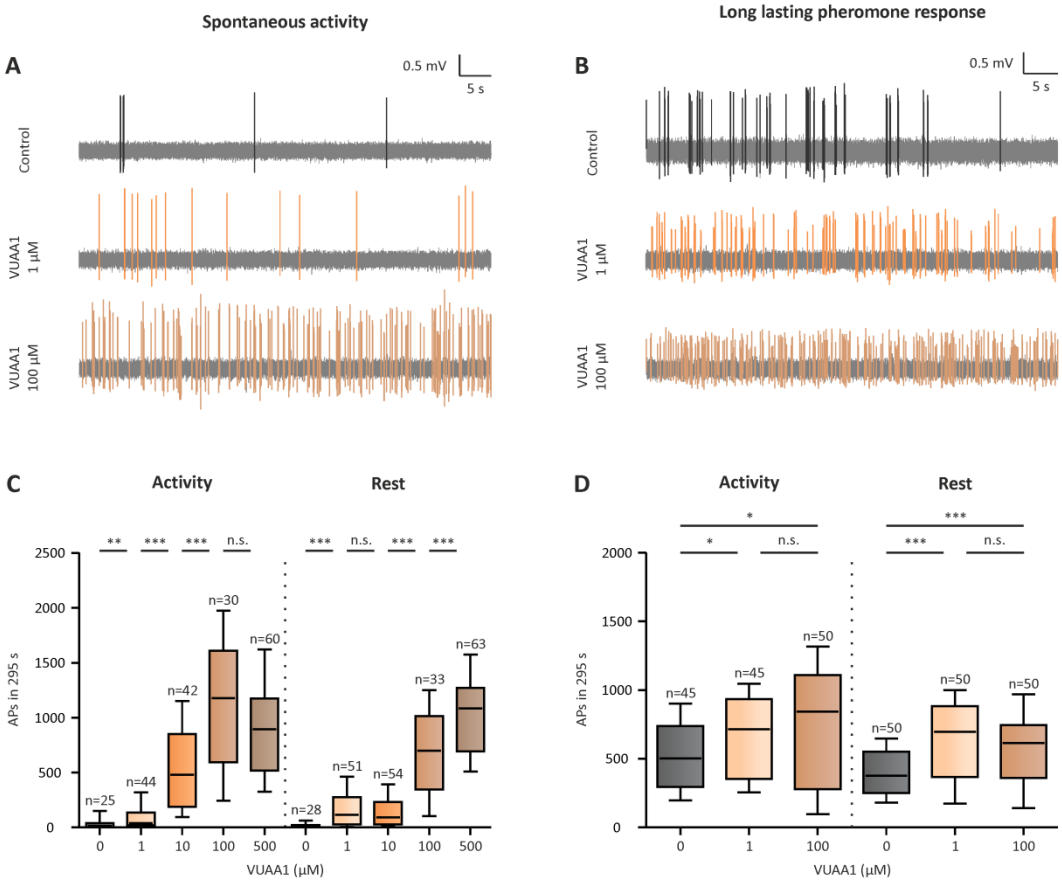


**Fig. 17 - VUAA1-dependent activation of MsexOrco increased spontaneous activity dose-dependently.** **A-B** Original traces of the neuronal activity in recordings with (4.23 pM BAL on filter paper) or without pheromone stimulation. ORNs showed little spontaneous activity. VUAA1 significantly increased the spontaneous activity in a dose-dependent manner. A lasting increase in neuronal activity was observed after pheromone stimulation. **C-D** VUAA1 increased the spontaneous activity more potent during activity compared to resting phase. The bombykal-dependent later, long-lasting pheromone-response (5-300 s after pheromone stimulus) was increased during VUAA1 infusion (1, 100 µM). Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant; *$P$<0.05; **$P$<0.01; ***$P$<0.001; Mann-Whitney-test with α=0.05).

previous pheromone stimulation were already strongly enhanced after application of 1 µM VUAA1 at both ZTs tested (Fig. 17: activity: $p<0.01$; rest: $p<0.001$; Mann-Whitney-test with $\alpha=0.05$ unless otherwise specified). With higher VUAA1 concentration the numbers of APs further increased (Fig. 17).

VUAA1 was more effective in increasing the spontaneous activity during activity as compared to resting phase for concentrations of 10 µM ($p<0.001$) and 100 µM ($p=0.003$), whereas no significant differences were found for control recordings ($p=0.879$), recordings with infusion of 1 µM VUAA1 ($p=0.054$) or 500 µM (0.091). A saturation of the VUAA1 effect was seen at a concentration of 100 µM VUAA1 during activity, whereas the same concentration was not able to elicit a maximal AP response during rest, indicating higher threshold levels during resting phase (Fig. 17).

### 5.1.3 VUAA1 increased the late, long-lasting pheromone response

Beside spontaneous activity also the late, long-lasting pheromone response (LLPR) after BAL stimulation was analyzed (Fig. 18). In control recordings a decline of the LLPR was observed during the time course at both ZTs. At activity ($p<0.001$; Mann-Whitney-test with $\alpha=0.05$ unless otherwise specified) and rest phase ($p<0.001$) the LLPR was lower at the end of long-term control recordings in comparison to the beginning. The LLPR of control recordings was stronger during activity as compared to rest at the beginning ($p=0.017$) as well as the end of long-term recordings ($p<0.001$). VUAA1 increased the LLPR at both ZTs (Fig. 17, 18). The BAL-dependent LLPR during beginning was significantly increased by VUAA1 (100 µM) compared to control recordings already at the beginning of the recordings at activity ($p=0.012$) and rest phase (Fig. 18; $p=0.001$). Same effect was also found for the end of the recordings (activity: $p<0.001$; rest: $p<0.001$). ZT-dependent differences in the effectiveness of 100 µM VUAA1 were discovered, since VUAA1 recordings during activity phase showed significantly higher values at the beginning ($p=0.032$) and at the end ($p=0.004$) of the recordings in contrast to recordings during rest (Fig. 18).

When comparing spontaneous activity (no VUAA1 application, no BAL-stimulation) and the BAL-dependent LLPR (no VUAA1 application) during the beginning of the

recordings a considerably higher amount of APs was seen for the LLPR (Fig. 17; p<0.001). Same was found for the spontaneous activity (no BAL-stimulation) in recordings under influence of 1 µM VUAA1 compared to the BAL-dependent LLPR (1 µM VUAA1; p<0.001). This changed during application of 100 µM VUAA1. Under these conditions no difference between spontaneous activity (no BAL-stimulation) and BAL-dependent LLPR at the beginning of recordings was observed during rest (p=0.357), whereas the spontaneous activity was even higher than the LLPR during the activity phase (p=0.006).

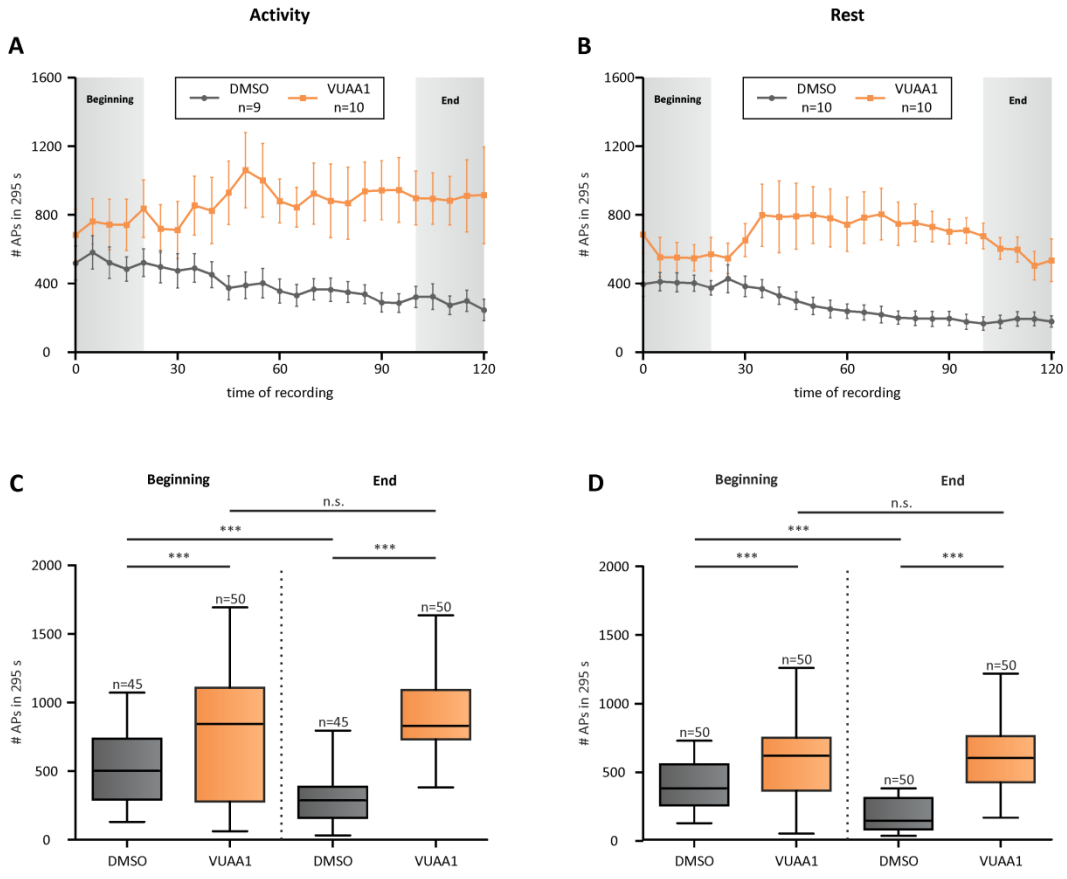


**Fig. 18 - Late, long-lasting pheromone response was strongly increased by VUAA1.** **A-B** Control recordings showed a reduction of the late, long-lasting pheromone response (LLPR) after bombykal stimulation (4.23 pM on filter paper) during the course of long-term tip recordings. Furthermore, a ZT-dependent decrease was observed during rest (ZT 9-11). Error bars show SEM. **C-D** Already within the first 20 minutes VUAA1 (100 µM) strongly increased the LLPR up to the end of the recordings. This VUAA1 effect was stronger during activity. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant; *$P$<0.05; **$P$<0.01; ***$P$<0.001; Mann-Whitney-test with α=0.05).

## 5.2 Effects of prospective Orco antagonists on pheromone responses in the hawkmoth *Manduca sexta*

Since long time the impact of amilorides on insect olfaction is known (Frings and Lindemann 1988), amilorides might be prospective blocker of the Orco protein. This was strengthened by the fact that insect olfactory receptor currents were blocked by amilorides (Rollecke et al. 2013) and that the neuronal activity generated by the Orco agonist VUAA1 was reduced by the amiloride HMA (Pask et al. 2013). However, additional targets of amilorides are known (Kleyman and Cragoe 1988), e.g. ion channels such as $Ca^{2+}$-dependent cation channels as observed for the silkmoth Antheraea polyphemus (Zufall and Hatt 1991). Thus, the specific binding of the amilorides HMA and MIA were determined in vivo in tip recordings on ORNs of *M. sexta*.

To examine the role of Orco in insect pheromone transduction prospective agonists and antagonists were developed recently by producing chemical components structurally related to the Orco agonist VUAA1 (Chen and Luetje 2012; Jones et al. 2012). One of these components is OLC15 which was shown to be a specific Orco antagonist in a cell expression system (Chen and Luetje 2012). If this is also true for MsexOrco was revealed in vivo in *Manduca*. Since this became obvious an involvement of Orco in an ionotropic signal transduction and its participation as a pacemaker channel was analyzed.

### 5.2.1 Block of VUAA1-induced activity by OLC15

First, it was tested whether OLC15 is an antagonist of MsexOrco. Since VUAA1 was shown to be a specific MsexOrco agonist (Jones et al. 2011) it was tested whether OLC15 interferes with VUAA1-dependent increases of spontaneous activity without previous pheromone stimulation in olfactory receptor neurons of *M. sexta*. As shown previously infusion of VUAA1 in tip recordings of pheromone-sensitive trichoid sensilla strongly increased spontaneous activity of BAL-sensitive ORNs (Fig. 17, 19) (Nolte et al. 2013). This activity was dose-dependently blocked by co-application of OLC15 (1, 10,

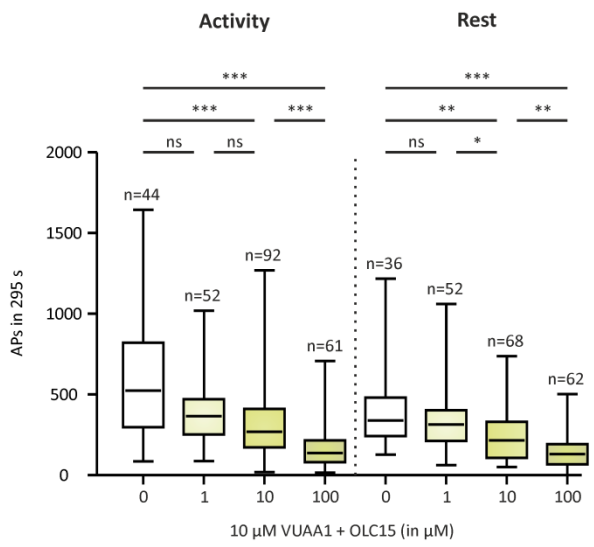


**Fig. 19 - OLC15 blocked VUAA1-dependent spontaneous activity competitively and dose-dependently.** Without previous pheromone stimulation the neuronal activity was strongly increased by application of VUAA1 (10 µM). Additional presence of OLC15 (1, 10, 100 µM) dose-dependently decreased VUAA1-dependent neuronal activity. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, **P*<0.05, ***P*<0.01, ****P*<0.001; Kruskal-Wallis-Test with α=0.05).

100 µM) at both ZTs (Fig. 19). ZT-dependent differences between activity and resting phase were found in control recordings (10 µM VUAA1, no OLC15) with higher activity levels during activity phase (Fig. 19; p=0.011; Mann-Whitney-test with α=0.05 unless otherwise specified).

Significant ZT-dependent differences during co-application of VUAA1 (10 µM) and OLC15 (1, 10, 100 µM) were only found for OLC15 at a concentration of 10 µM with lower activity levels during rest (Fig. 19, p=0.032). Lower spontaneous activity during rest was also observed for co-application of OLC15 in concentrations of 1 µM (p=0.105) and 100 µM (p=0.409). However, these changes were not found to be significant.

### 5.2.2 Injections of depolarizing current increased spontaneous activity of bombykal-sensitive ORNs

To examine whether Orco is voltage-dependently activated, current injection experiments were performed. Depolarizing currents were injected into trichoid sensilla in the absence of pheromone stimulations. To ensure sufficient depolarization of the ORNs by current injections different current strengths initially were tested (Fig. 20). After previous current stimulation (200, 300, 400 pA) with duration of 10 s the

neuronal activity was measured for 60 s and the elicited spontaneous activity was compared to controls without current injection. Previous injection of 200 pA did not significantly affect the neuronal activity during both activity and resting phase in comparison to the spontaneous activity in control recordings (Fig. 20).

Recordings with current injection of 400 pA quickly became unstable and could not be analyzed. On the contrary, current injection with 300 pA stably increase neuronal activity at both ZTs (Fig. 20; activity: $p<0.001$, rest: $p<0.001$; Mann-Whitney-test with $\alpha=0.05$ unless otherwise specified), and therefore was used for further analysis. Again, ZT-dependent effects were observed. Control recordings without current injections showed higher spontaneous activity during activity phase compared to rest (Fig. 20, 20-A, $p=0.011$). This could also be observed for the activity after a 10 s long 300 pA current injection in control recordings (Fig. 21-A, $p=0.001$). Application of OLC15 without current injection showed no effect compared to control recordings during activity phase (Fig. 21-A, $p=0.265$). However, depolarization-dependent increases of neuronal activity after previous 10 s stimulation with 300 pA were significantly abolished by OLC15 during activity phase (Fig. 21-A, $p<0.001$), whereas due to high variability no significant changes were observed during rest (Fig. 21-A, $p=0.234$).

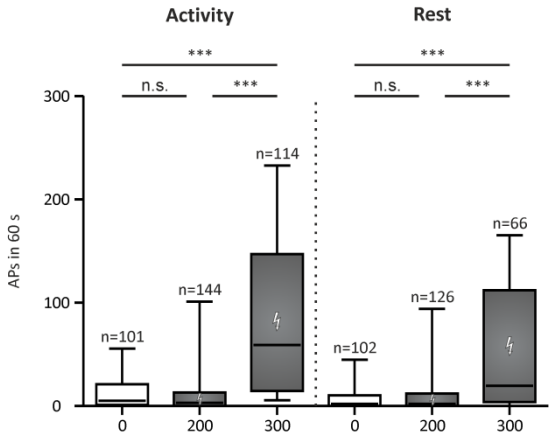


**Fig. 20 - Injections of depolarizing current increased spontaneous activity of bombykal-sensitive ORNs.** After a previous 10 s long injection of depolarizing current the neuronal activity of ORNs not stimulated by bombykal was measured. While injections of 200 pA were ineffective, stimulations with 300 pA significantly increased spontaneous activity of ORNs. Higher current values could not be analyzed due to high interferences, and therefore were excluded for further analysis. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, ****P*<0.001; Mann-Whitney-Test with $\alpha=0.05$).

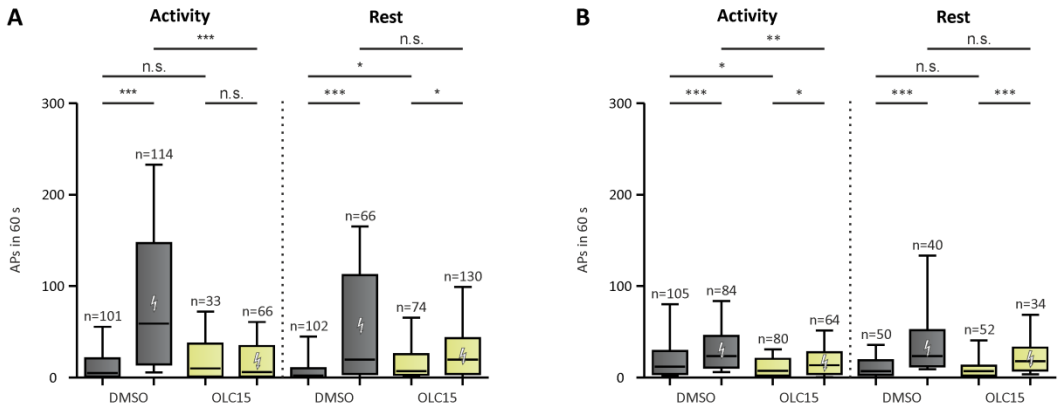


**Fig. 21 - Orco is a voltage-dependent cation channel since OLC15 blocked depolarization-dependent spontaneous activity. A** Recordings with (flash symbol) and without previous 10 s lasting current injection of 300 pA. Control recordings without current stimulation showed low spontaneous activity, which was higher during the activity in comparison to resting phase. Current injections strongly increased the spontaneous, not pheromone-dependent neuronal activity in a ZT-dependent manner. Simultaneous application of OLC15 (10 µM) counteracted this increase at both ZTs with higher effectiveness during activity phase. **B** Recordings with (flash symbol) and without simultaneous 60 s lasting stimulation with 300 pA. During current stimulation the neuronal activity was higher in control recordings at both ZTs. This could also be observed for OLC15 (10 µM) recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, **P*<0.05, ***P*<0.001, ****P*<0.001; Mann-Whitney-Test with α=0.05).

To test for possible long-term effects of current stimulations additional injection experiments were performed. Here, the current injections (300 pA) lasted for 60 s and the neuronal activity was measured simultaneously. Afterwards it was compared to the previous spontaneous activity without current application. Neuronal activity in control recordings was strongly increased during current injections at both ZTs tested in comparison to spontaneous activity (Fig. 21-B, activity: p<0.001, rest: p<0.001). In contrast to current injections with durations of 10 s an increase was also observed for OLC15 recordings at both ZTs (Fig. 21-B, activity: p=0.038, rest: p=0.003). Comparison of the current-induced (60 s, 300 pA) activity of control and OLC15 recordings showed higher activity levels for the control recordings at both ZTs (Fig. 21-B). Whereas this was significant at activity phase (p=0.002) it was only seen by trend at rest (p=0.134). In control recordings with 60-s-lasting current injections no ZT-dependent changes on the neuronal activity were revealed, which were found in recordings with previous 10 s current stimulation (Fig. 21-A, p=0.667). Furthermore, during activity phase the activity

generated by previous 10 s current stimulation was higher compared to activity measured during 60 s current stimulation (Fig. 21-A, 21-B, $p<0.0001$). However, this was not the case during resting phase, where no significant difference was seen.

Particularly remarkable was the fact that OLC15 was not able to block the spontaneous activity without current injections at any time (Fig. 21), indicating further ion channels, which are involved in the generation of the spontaneous activity.

### 5.2.3 Effects on pheromone response parameters

***In contrast to amilorides the Orco antagonist OLC15 did not affect the bombykal-dependent sensillum potential amplitude***

In 2-h-lasting long-term control tip recordings the bombykal (BAL)-dependent sensillum potential amplitude (SPA) remained stable at both ZTs tested (Fig. 24). In recordings under the influence of the Orco antagonist OLC15 no effects on the SPA were found within the first 20 minutes in short-term recordings at both ZTs in comparison to control recording (Fig. 22, 24; n.s. each; Kruskal-Wallis-Test with $\alpha$=0.05 unless otherwise specified). In contrast to control and OLC15 recordings, the tested agents MIA and HMA already induced a reduction of the SPA at the beginning of the recordings at activity (Fig. 23, 24; $p<0.05$ each) and rest (MIA: $p<0.01$, HMA: $p<0.001$). This decline of the SPA further increased up to the end of the recordings when compared to the control recordings ($p<0.001$ each). HMA seems to be more effective in the beginning of the resting phase compared to MIA. However, these differences were significant.

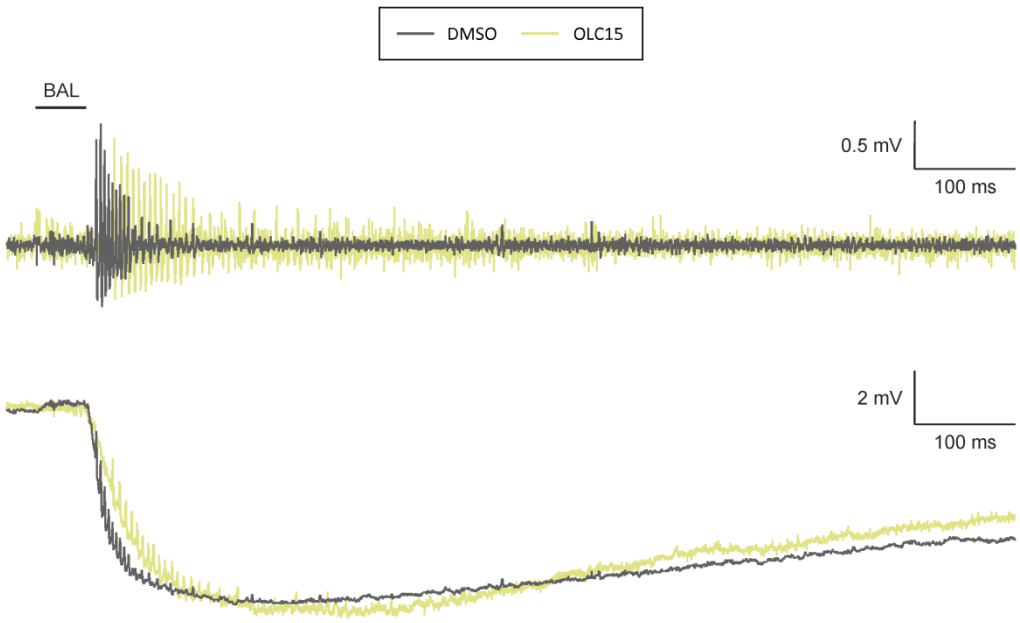


**Fig. 22 - Original traces of OLC15 recordings at the beginning of recordings during activity phase.** ***Above*** High-pass filtered signal with APs after bombykal (BAL) stimulation. ***Below*** Unfiltered signal with the slow deflection of the sensillum potential amplitude and superimposed APs. The sensillum potential was not changed by VUAA1 recordings (yellow) compared to control recordings (grey) (Fig. 24). A reduction of the AP frequency of the first six APs (Fig. 25) and a prolonged latency of the first AP (Fig. 26) was observed under influence of VUAA1. However, no significant changes in the number of APs within the first 150 or 1000 ms were found at the beginning of recordings (Fig. 27).

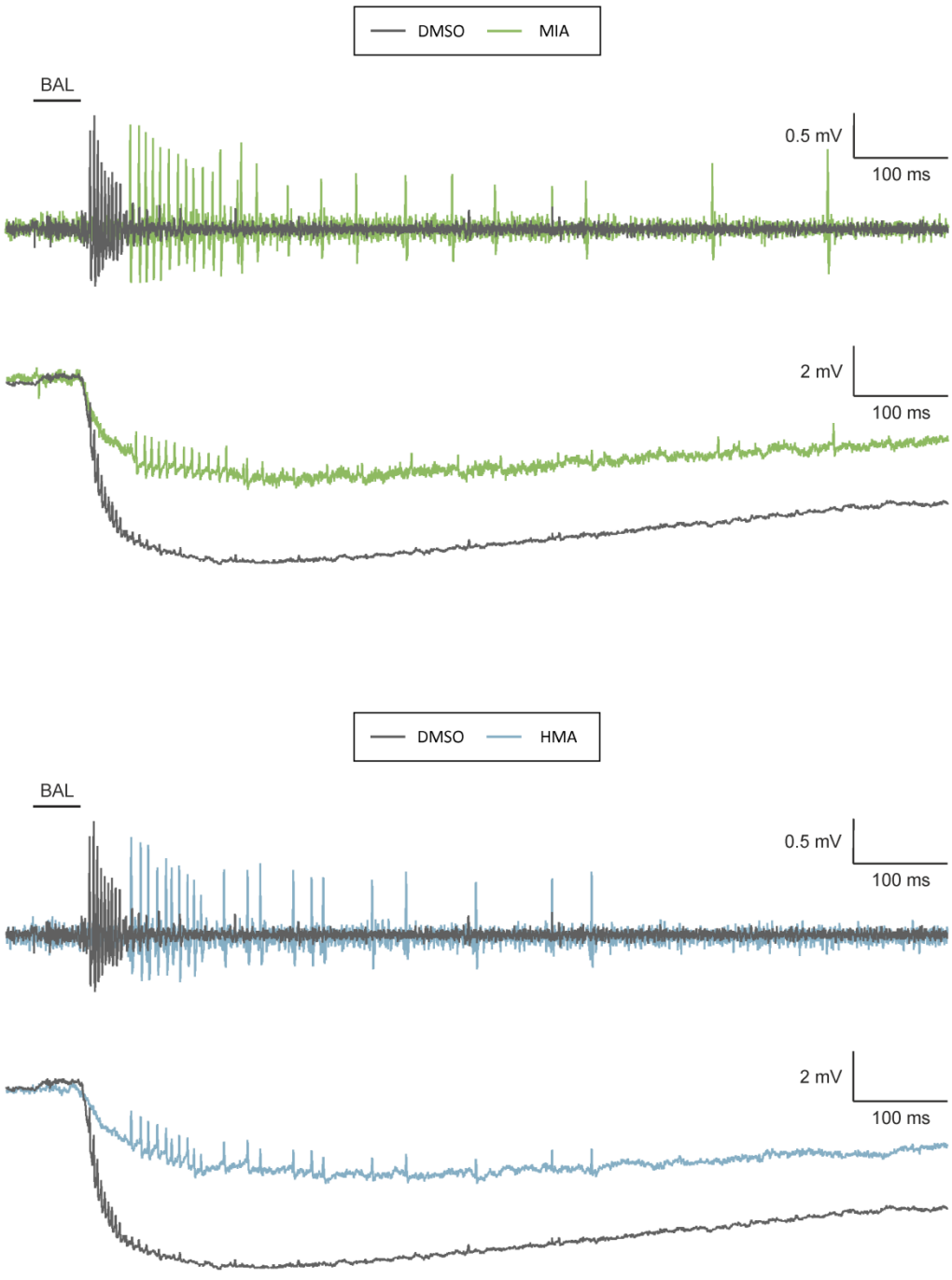


**Fig. 23 - Original traces of amiloride recordings at the beginning of recordings during activity phase.** High-pass filtered signals (upper traces) with APs after bombykal (BAL) stimulation and unfiltered signals (lower traces) with the slow deflection of the sensillum potential amplitude and superimposed APs. The sensillum potential was decreased by the amilorides MIA (green) as well as HMA (blue) compared to control recordings (grey) (Fig. 24). Furthermore, also the AP frequency of the first six APs was reduced (Fig. 25). The latency of the first pheromone-dependent AP increased under influence of both amilorides (Fig. 26).

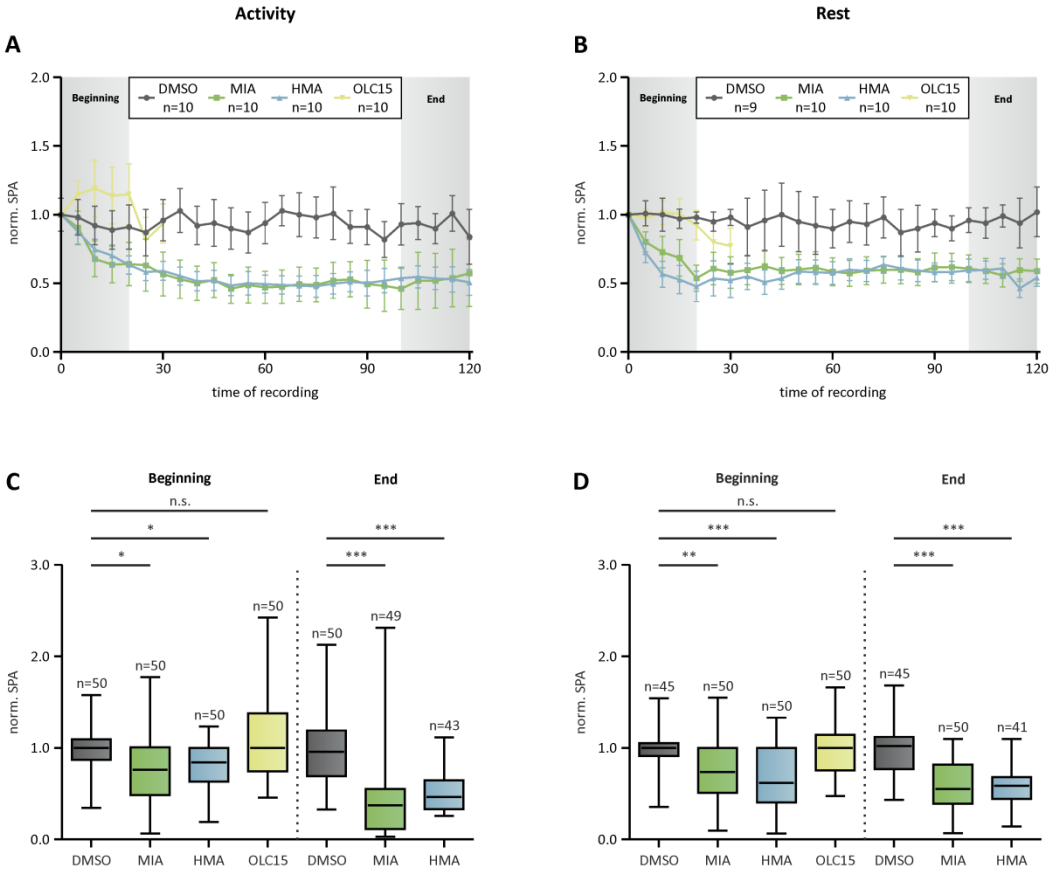


**Fig. 24 - Contrary to amilorides OLC15 did not affect the sensillum potential amplitude. A-B** The sensillum potential amplitude (SPA) in control recordings remained stable during long-term tip recordings at both ZTs tested. OLC15 (10 µM) had no significant effect on the bombykal-dependent (4.23 pM on filter paper) SPA during the first 20 minutes of tip recordings. Amilorides HMA and MIA (10 µM) reduced the SPA. Error bars show SEM. **C-D** Comparison of beginning and end of recordings. Amilorides already reduced the SPA during the beginning of recordings. This further increased at the end of recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, *$P$<0.05, **$P$<0.001, ***$P$<0.001; Mann-Whitney-Test with α=0.05).

### *The phasic bombykal-dependent AP frequency was decreased most strongly by amilorides*

Control recordings at both ZTs showed a reduction of the phasic BAL-dependent AP frequency during the time course of long-term tip recordings. At the end of control recordings of activity (p=0.003; Mann-Whitney-test with α=0.05 unless otherwise specified) and rest (p<0.001) the AP frequency was significantly lower compared to the

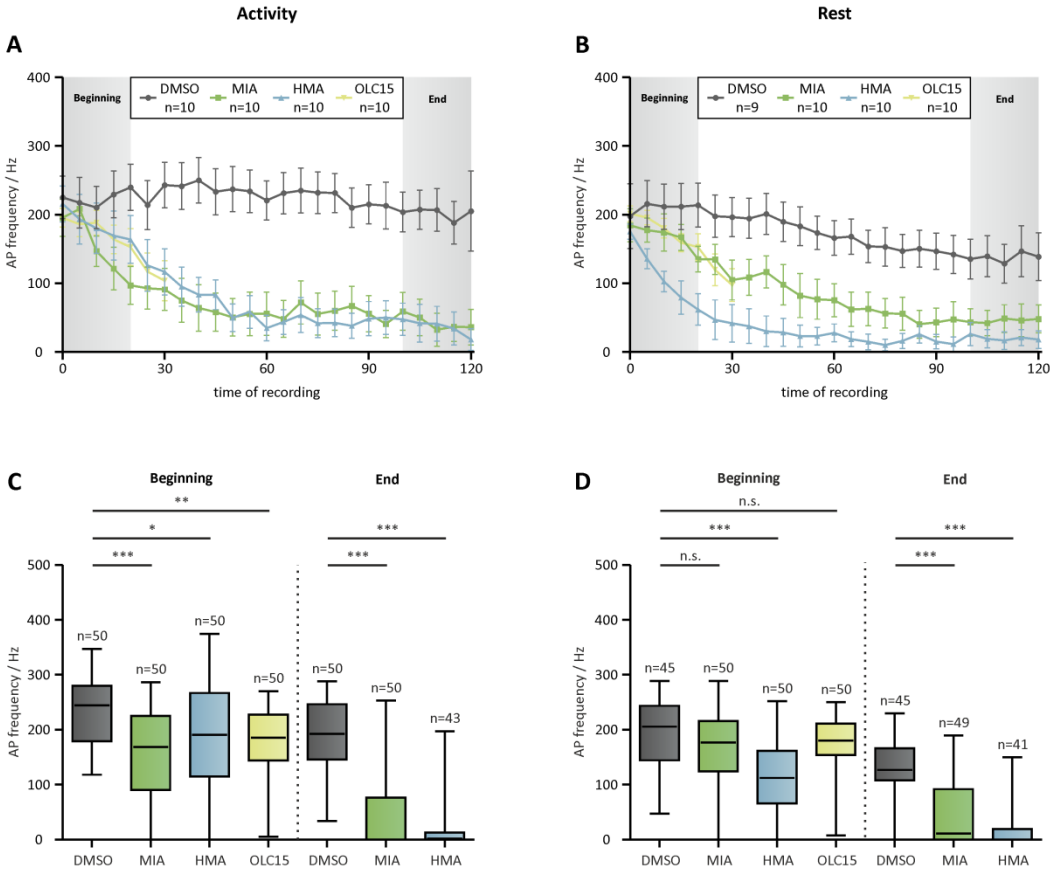


**Fig. 25 - Blockers reduced the bombykal-dependent phasic AP frequency to different extends at the two Zeitgebertimes tested. A-B** The phasic bombykal-dependent (4.23 pM on filter paper) AP frequency of the first six APs in control recordings slightly decreased during long-term tip recordings. Furthermore, in control recordings the AP frequency was in general higher during the activity phase. Amilorides strongly decreased the AP frequency within the time course of recordings. Error bars show SEM. **C-D** Comparison of beginning and end of recordings. All blockers (MIA, HMA, OLC15; 10 µM) reduced the AP frequency already at the beginning of recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, *P<0.05, **P<0.001, ***P<0.001; Mann-Whitney-Test with α=0.05).

beginning (Fig. 25). Furthermore, a ZT-dependent change of the phasic BAL-dependent AP frequency in control recordings was observed for the beginning (p=0.008) and end (p<0.001) with lower rates during rest. During activity phase amilorides (Fig. 23) and OLC15 (Fig. 22) decreased the phasic BAL response compared to controls in the first 20 minutes with the highest effect for the amiloride MIA (Fig. 25, MIA: p<0.001, HMA: p<0.05, OLC15: p<0.01; Kruskal-Wallis-Test with α=0.05). In the further course of the

recordings both amilorides similarly affected the phasic BAL-dependent AP frequency, and both amilorides strongly reduced the phasic pheromone responses at the end of long-term recordings during activity phase compared to control recordings ($p<0.001$ each; Kruskal-Wallis-Test with $\alpha=0.05$). During resting phase only HMA significantly decreased the phasic BAL responses during the first 20 minutes of the tip recordings even if a reduction was also observed for MIA and OLC15 (Fig. 25, MIA: n.s., HMA: $p<0.001$, OLC15: n.s.; Kruskal-Wallis-Test with $\alpha=0.05$). At the end of long-term tip recordings both amilorides reduced the phasic BAL-dependent AP frequency significantly compared to control (Fig. 25; $p<0.001$ each; Kruskal-Wallis-Test with $\alpha=0.05$). During resting phase HMA in general was more potent than MIA, although differences at the end of the recordings could only be seen by trend (n.s.; Kruskal-Wallis-Test with $\alpha=0.05$).

***Both amilorides strongly increased the latency of the first bombykal-dependent action potential***

During long-term control recordings at both ZTs an increase of the latency of the first AP after BAL stimulation was observed when comparing beginning and end of recordings (Fig. 26; activity: $p<0.001$, rest: $p<0.001$; Mann-Whitney-test with $\alpha=0.05$ unless otherwise specified). Whereas no ZT-dependent differences in the latency were seen at the beginning of the recordings ($p=0.535$), at the end of recordings the latency was longer during rest than during activity phase ($p<0.001$). Application of the amilorides MIA (activity: $p<0.01$, rest: $p<0.01$; Kruskal-Wallis-Test with $\alpha=0.05$) and HMA (activity: $p<0.001$, rest: $p<0.001$; Kruskal-Wallis-Test with $\alpha=0.05$) as well as OLC 15 (activity: $p<0.01$, rest: $p<0.05$; Kruskal-Wallis-Test with $\alpha=0.05$) were able to already increase the latency within the first 20 minutes of 2h-lasting tip recordings compared to control recordings at both ZTs, with highest under influence of HMA (Fig. 22, 23, 26). At the end of the recordings at both ZTs the latency was further increased to the same extent by both amilorides compared to control recordings ($p<0.001$ for all; Kruskal-Wallis-Test with $\alpha=0.05$).

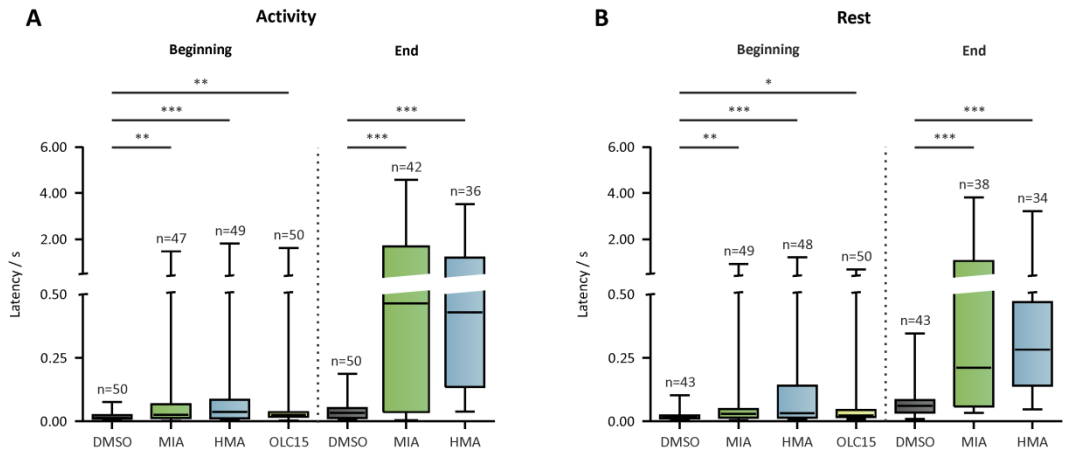


**Fig. 26 - All blockers prolonged the latency of the first bombykal-dependent AP. A-B** In control tip recordings the latency of the first bombykal-dependent (4.23 pM on filter paper) AP was higher at the end of recordings compared to the beginning. All blockers (MIA, HMA, OLC15; 10 µM) significantly increased the latency at the beginning of recordings at both ZTs. The latency was strongly enhanced by amilorides in the last 20 minutes of the long-term tip recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (**P*<0.05; ***P*<0.01; ****P*<0.001; Mann-Whitney-Test with α=0.05).

### *OLC15 did not change the response kinetics*

The phasic and tonic BAL responses occurring in the first 1000 ms after pheromone stimulation were analyzed in post stimulus time histograms. The number of APs within the first 150 (p=0.147; Mann-Whitney-Test with α=0.05 unless otherwise specified) and 1000 ms (p=0.689) were not affected in control recordings during activity phase when comparing beginning and end (Fig. 27). During rest the phasic response from the beginning shifted to a more tonic response and the number of APs within the first 150 (p<0.001) and 1000 ms (p=0.018) significantly declined (Fig. 27).

During activity phase both the number of APs in the first 150 as well as in 1000 ms were not significantly affected in the first 20 minutes under the influence of MIA, HMA or OLC15 compared to control recordings (Fig. 27; n.s. for all; Kruskal-Wallis-Test with α=0.05), although a trend of a reduction of the phasic component by amilorides was observed. This was strengthened during resting phase, where the number of APs in the first 150 ms after BAL stimulation was decreased by MIA (p<0.05; Kruskal-Wallis-Test with α=0.05) and even more strongly by HMA (p<0.001; Kruskal-Wallis-Test with

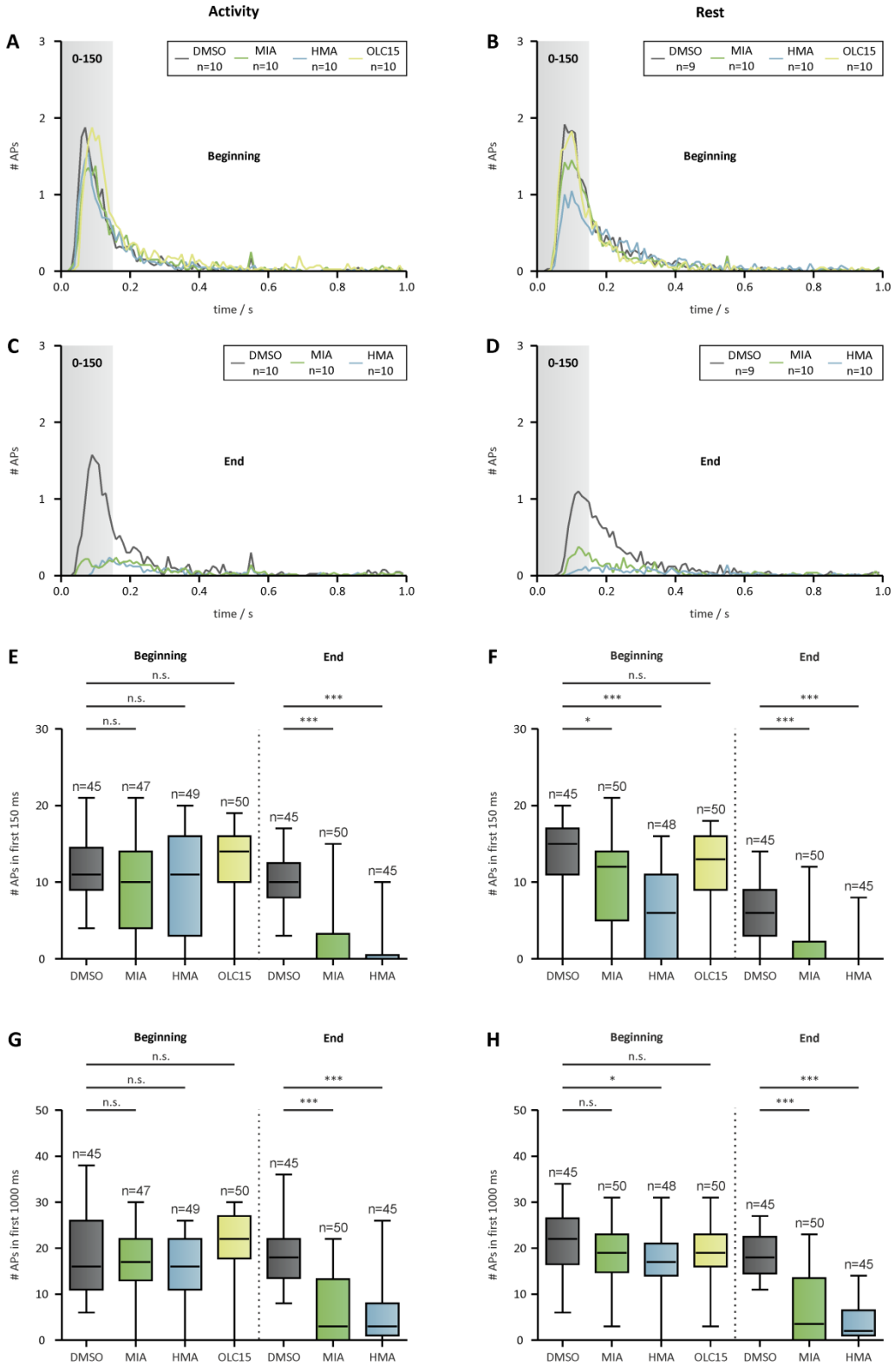


**Fig. 27 - Orco antagonist OLC15 did not significantly affect the phasic pheromone response during the first 20 min of tip recordings. A-D** Post-stimulus time histograms of pheromone responses in control and blocker recordings. During activity the kinetics of control recordings remained stable throughout the long-term tip recordings, whereas at rest a lower phasic response component was observed at the end of control recordings. Continues on next page.

↑ **Fig. 27 -** OLC15 (10 µM) did not significantly affect the kinetics in the first 20 minutes at both ZTs tested, whereas amilorides HMA and MIA (10 µM) decreased the phasic pheromone response kinetics, in particular at the end of the recordings. Grey areas indicate first 150 ms after pheromone stimulation (4.23 pM on filter paper). **E-H** Comparison of the numbers of APs within 150 or 1000 ms at beginning and end of the recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, **P*<0.05, ***P*<0.01, ****P*<0.001; Mann-Whitney-test with α=0.05).

α=0.05) during the beginning of the recordings (Fig. 27). However, OLC15 had no significant effect (Kruskal-Wallis-Test with α=0.05). During the beginning of recordings at rest the number of BAL-dependent APs in the first 1000 ms was only significantly influenced by HMA ($p<0.05$; Kruskal-Wallis-Test with α=0.05). In long-term amiloride recordings MIA as well as HMA reduced the number of APs in 150 ms as well as in 1000 ms at both ZTs compared to control recordings ($p<0.001$ for all; Kruskal-Wallis-Test with α=0.05).

### 5.2.4 Orco antagonist OLC15 reduced the late, long-lasting pheromone response during activity phase

Control recordings at activity ($p<0.001$; Mann-Whitney-Test with $\alpha=0.05$ unless otherwise specified) and resting phase ($p<0.001$) showed a strong reduction of the late, long-lasting pheromone response (LLPR) during the time courses when comparing

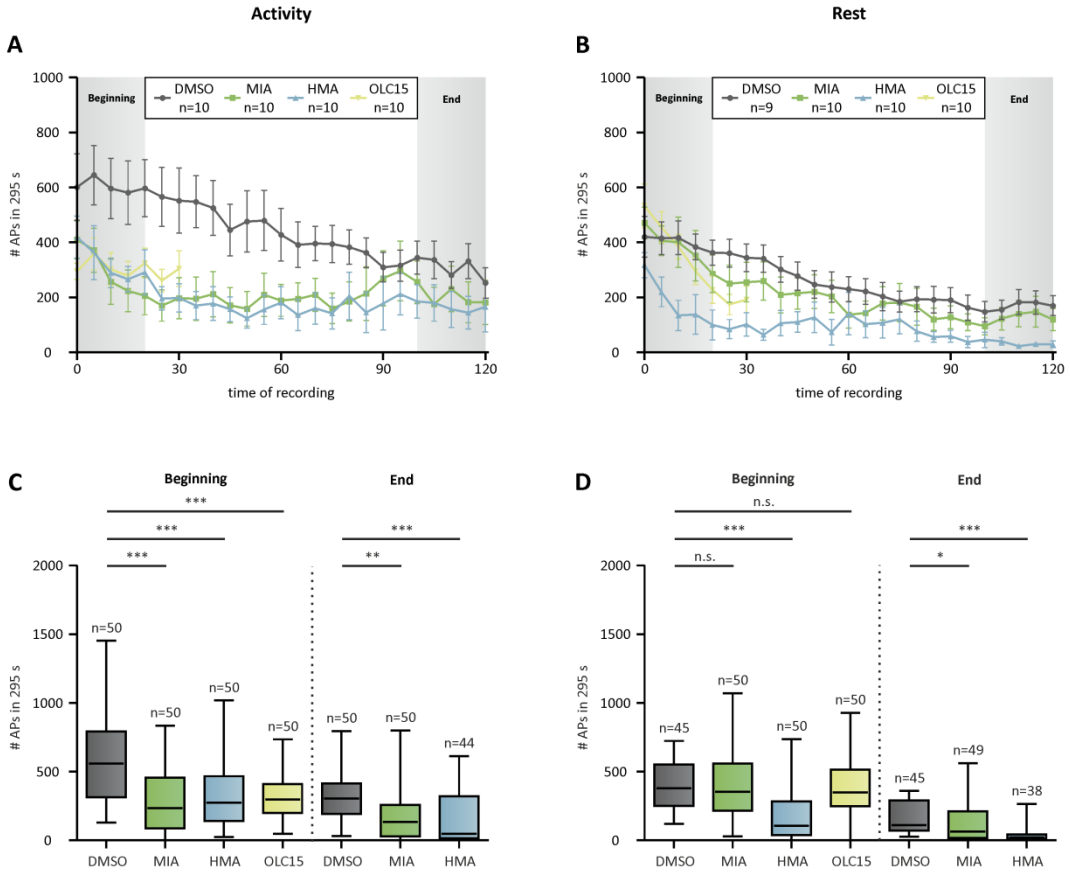


**Fig. 28 - Blockers decreased late, long-lasting pheromone response. A-B** The bombykal-dependent (4.23 pM on filter paper) late, long-lasting pheromone response (LLPR) diminished in control recordings over the course of time at both ZTs tested. In control recordings it was in general higher during activity compared to resting phase. Error bars show SEM. **C-D** Whereas all blockers (MIA, HMA, OLC15; 10 µM) significantly decreased the LLPR within the first 20 minutes of the activity phase, only HMA significantly reduced the LLPR during the first 20 min of the recording during rest. In general HMA was the most potent antagonist during resting phase. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, *$P$<0.05, **$P$<0.01, ***$P$<0.001; Mann-Whitney-test with $\alpha=0.05$).

beginning and end of the recordings (Fig. 28). In control recordings the LLPR was more present during the activity phase compared to resting phase at beginning ($p=0.003$) as well as end of the recordings ($p<0.001$). In the activity phase both amilorides and OLC15 decreased the LLPR right at the beginning of the recordings (Fig. 28; $p<0.001$ for all; Kruskal-Wallis-Test with $\alpha=0.05$). Moreover, this effect was already observed within the first 295 s after first pheromone stimulation for all agents (Fig. 28), lasting up to the end of the recordings (MIA: $p<0.01$; HMA: $p<0.001$; Kruskal-Wallis-Test with $\alpha=0.05$). During rest a significant effect in the first 20 minutes was only observed under the influence of HMA ($p<0.001$; Kruskal-Wallis-Test with $\alpha=0.05$), causing a decreased LLPR. At the end of recordings both amilorides significantly reduced the LLPR during resting phase, with higher effectiveness for HMA (MIA: $p<0.05$; HMA: $p<0.001$; Kruskal-Wallis-Test with $\alpha=0.05$).

## 5.3 Blocking the metabotropic pathway by G-protein blocker GDP-β-S reduced pheromone responses in the hawkmoth *Manduca sexta*

Tip recordings under influence of the Orco agonist VUAA1 found no evidence for an Orco-based ionotropic pheromone transduction pathway in *M. sexta* (see Chapter 5.1). Thus, it was tested whether a metabotropic pathway is involved in the signal transduction of pheromones in *Manduca*. For this, the non-hydrolyzable GDP analog GDP-β-S which interferes with the GDP binding site of G-proteins was employed in long-term tip recordings of pheromone-sensitive trichoid sensilla *in vivo* and the effects on the pheromone responses were analyzed.

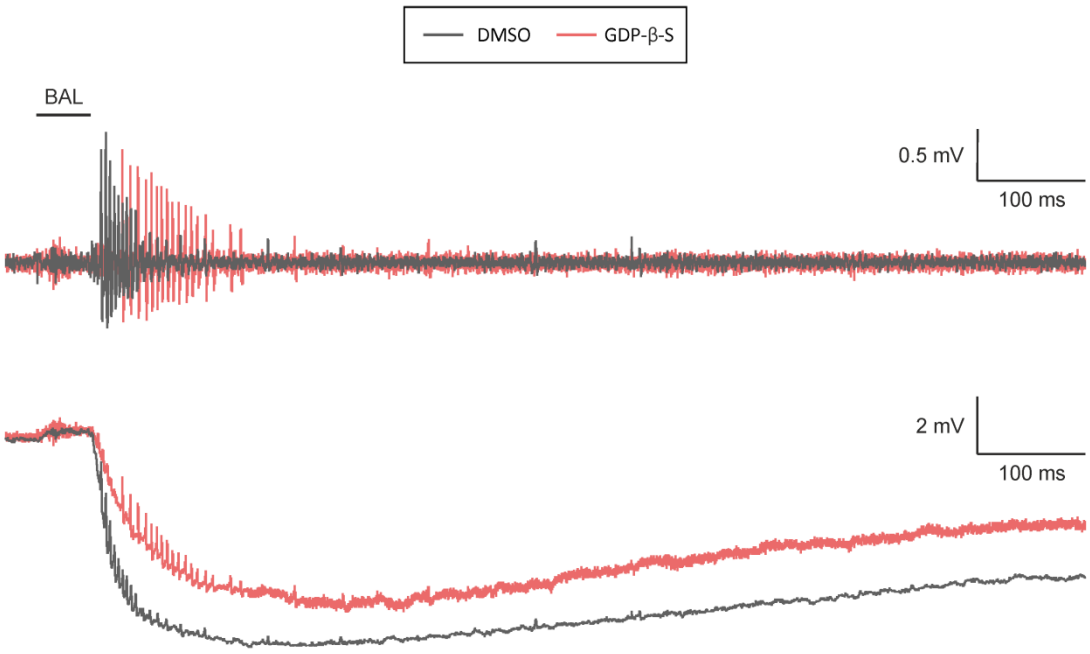


**Fig. 29 - Original traces of GDP-β-S recordings at the beginning of recordings during activity phase. Above** High-pass filtered signals with APs after bombykal (BAL) stimulation. **Below** Unfiltered signals with the slow deflection of the sensillum potential amplitude and superimposed APs. In general the BAL-dependent sensillum potential amplitude (Fig. 30) decreased under the influence of GDP-β-S (red) in comparison to control recordings (grey). Furthermore, the AP frequency of the first six APs was decreased (Fig. 31) and the latency prolonged (Fig. 32) under influence of GDP-β-S. However, these changes were not found to be significant at the beginning of the recordings, whereas they were prominent at the end of long-term recordings.

### 5.3.1 Effects on pheromone response parameters

***Sensillum potential amplitude was decreased by G-protein blocker***

While control recordings were stable over the course of long-term recordings the normalized BAL-dependent SPA decreased in the presence of GDP-β-S (Fig. 29, 30). During activity (p=0.004; Mann-Whitney-Test with α=0.05 unless otherwise specified)

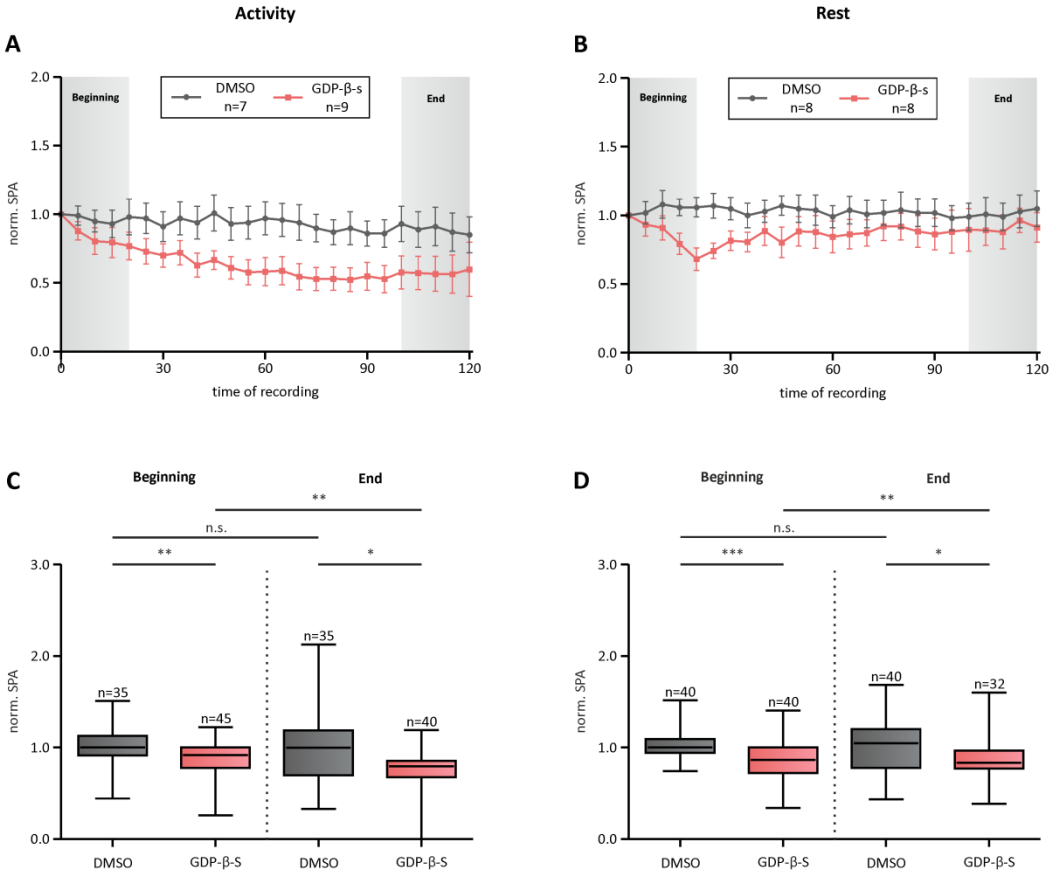


**Fig. 30 - GDP-β-S reduced the bombykal-dependent sensillum potential amplitude mostly during the activity phase. A-B** The sensillum potential amplitude (SPA) was measured every 5 minutes after bombykal (BAL; 4.23 pM on filter paper) stimulation in long-term tip recordings of pheromone-sensitive trichoid sensilla. In control recordings no changes in the normalized bombykal-dependent SPA were found in the time course of the recordings and between both ZTs tested. GDP-β-S (10 µM) significantly reduced the SPA at both ZTs within the first 20 minutes up to the end of the recordings. Grey areas indicate beginning and end of tip recordings. Error bars show SEM. **C-D** Comparing of beginning (first 20 minutes) and end (last 20 minutes) of long-term recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant; Mann-Whitney-test with α=0.05).

as well as resting phase ($p<0.001$) the G-protein blocker GDP-β-S reduced the SPA significantly already within the first 20 minutes. Same was found for the last 20 minutes of long-term recordings (activity: $p=0.016$; rest: $p=0.043$). In addition, in contrast to controls which did not show any daytime-dependent changes, the GDP-β-S-dependent decline of SPA occurred more prominent during the activity phase (Fig. 30). However, neither at the beginning ($p=0.642$) nor at the end of the recordings ($p=0.069$) this was shown to be significant.

***G-protein block led to reduced BAL-dependent AP frequency***

In the beginning of control recordings the AP frequency was significantly higher compared to the end of the long-term recordings (Fig. 31). This was true for activity ($p=0.014$) as well as resting phase ($p<0.001$; Mann-Whitney-Test with $\alpha=0.05$ unless otherwise specified). Furthermore, ZT-dependent differences in control recordings were found. Both, beginning ($p=0.082$) and end ($p=0.003$) of control recordings showed higher AP frequencies during activity phase as compared to recordings during rest.

Recordings with infusion of GDP-β-S increasingly declined during the course of the recordings, with a steeper decline at the activity phase (Fig. 29, 31). At both ZTs the AP frequency was reduced at the end of the recordings, compared to the beginning ($p<0.001$ each). ZT-dependent differences were only found for the beginning of GDP-β-S recordings, when the AP frequency during activity phase was higher as compared to resting phase ($p=0.022$). This effect changed at the end of the recordings, where the AP frequency during activity phase was by trend lower ($p=0.051$). Compared to control recordings GDP-β-S reduced the BAL-dependent AP frequencies significantly during rest phase at the beginning ($p=0.002$) as well as the end ($p<0.001$) of the recordings. For the activity phase significant differences to control recordings were only found for the end of the recordings ($p<0.001$), but not for the beginning ($p=0.304$).

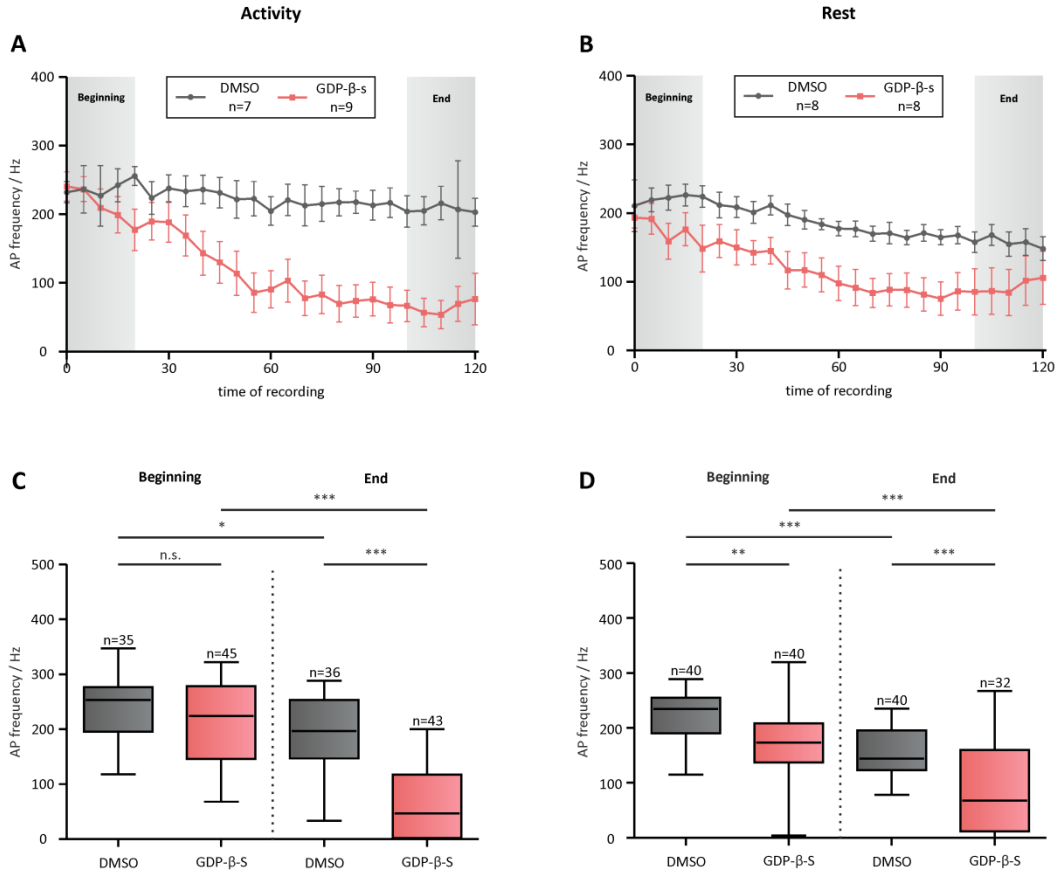


**Fig. 31 - The bombykal-dependent phasic AP frequency was decreased by GDP-β-S infusion at both ZTs. A-B** Control as well as GDP-β-S (10 µM) recordings had lower bombykal-dependent (4.23 pM on filter paper) phasic AP frequencies at the end of the recordings compared to the beginning. Infusion of GDP-β-S reduced the AP frequencies at both ZTs compared to control recordings. During the course of tip recordings it almost completely blocked any pheromone responses during the activity phase. Grey areas indicate beginning and end of recordings. Error bars show SEM. **C-D** Comparing of beginning (first 20 minutes) and end (last 20 minutes) of long-term recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant; Mann-Whitney-test with α=0.05).

### *Prolonged latency under influence of G-protein blocker*

At both ZTs the latency of the first AP after BAL stimulation at the beginning of control recordings was lower than observed at the end of the recordings (Fig. 32; $p<0.001$ each; Mann-Whitney-Test with α=0.05 unless otherwise specified). Whereas in

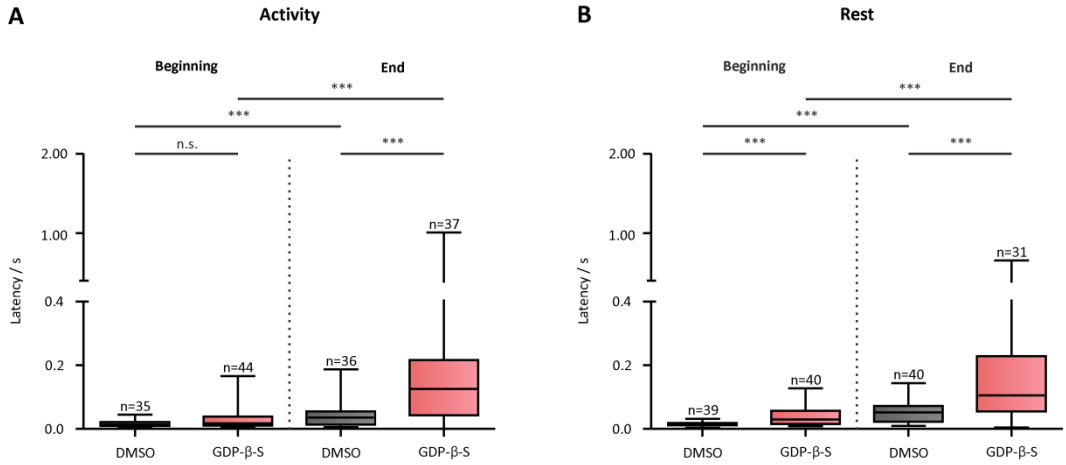


**Fig. 32** - **Latency of the first bombykal-dependent action potential increased under the influence of GDP-β-S. A-B** The latency of the first AP upon bombykal stimulation (4.23 pM on filter paper) was higher at the end of the control recordings in comparison to the beginning. This was also observed in GDP-β-S (10 µM) recordings. Furthermore, GDP-β-S led to an increase of the latency, particularly at the end of the recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant; ***P<0.001).

controls no ZT-dependent differences were found at the beginning (p=0.871), the latency was higher at the end of recordings during resting phase (p=0.014).

Recordings with GDP-β-S also showed an increase of the latency when comparing beginning and end of the recordings at both ZTs (p<0.001 each). When compared to control recordings the latency was prolonged by GDP-β-S at the beginning of recordings during rest (p<0.001), whereas for activity phase this was only seen by trend (p=0.087). However, at the end of GDP-β-S recordings the latency was significantly prolonged at both ZTs in comparison to control recordings (p<0.001 each).

### *Response kinetics were decreased by GDP-β-S*

Control recordings during activity phase did not show any differences in the numbers of APs during the first 150 ms (p=0.494; Mann-Whitney-Test with α=0.05 unless otherwise specified) as well as 1000 ms (p=0.724) after pheromone stimulation when comparing beginning and end of the recordings (Fig. 33). During rest the phasic

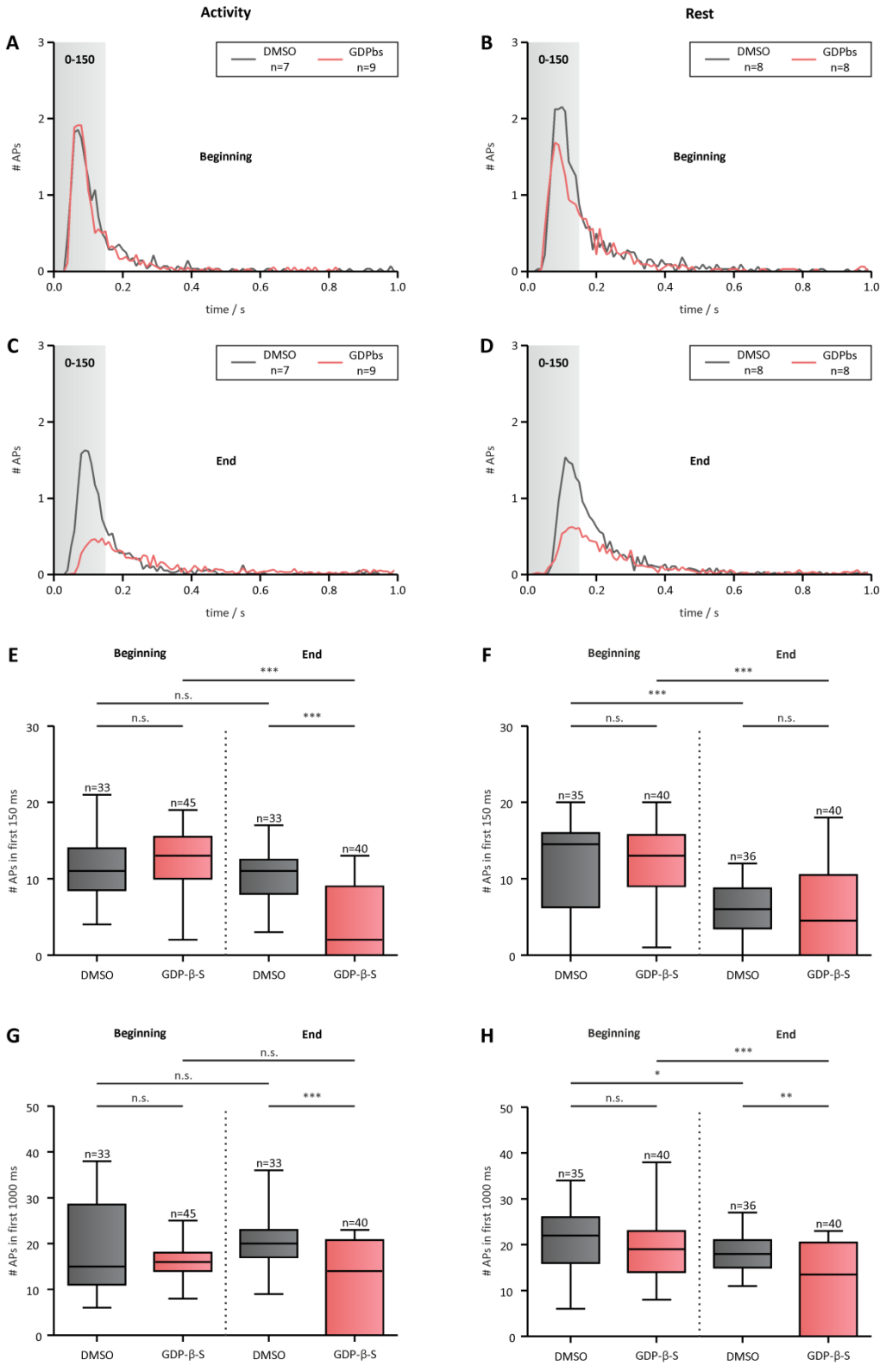


**Fig. 33 - GDP-β-S decreased phasic pheromone responses at both ZTs tested. A-D** Post-stimulus time histograms of pheromone responses in control and GDP-β-S recordings. Control recordings did not show a significant change in bombykal-dependent (4.23 pM on filter paper) response kinetics during the time course of 2h-lasting tip recordings during the activity phase. At rest there was a small reduction of the phasic component at the last 20 min (end) of the control recordings. Continues on next page.

↑ **Fig. 33 -** GDP-β-S (10 µM) significantly decreased the phasic bombykal response only at the end of the tip recordings as compared to controls. Grey areas indicate the first 150 ms after pheromone stimulation. **E-H** Comparison of the numbers of APs within 150 or 1000 ms at beginning and end of the recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant, *$P$<0.05, **$P$<0.01, ***$P$<0.001; Mann-Whitney-test with α=0.05).

response within the first 150 ms declined significantly at the end of the recordings ($p<0.001$), whereas the number of APs within the first 1000 ms did not change ($p=0.217$). For control recordings no ZT-dependent differences in the numbers of APs within the first 150 ms ($p=0.301$) as well as 1000 ms ($p=0.658$) were observed at the beginning of the recordings. At the end of the recordings a significant reduction of the number of APs within 150 ms was observed during rest compared to activity phase ($p<0.001$). By trend this was also true for the number of APs within 1000 ms ($p=0.087$).

### 5.3.2 No effects on the late, long-lasting pheromone response by GDP-β-S

The late, long-lasting pheromone response (LLPR) in control recordings declined in the course of the recordings at both ZTs, where higher levels were found at the beginning of the recordings (Fig. 34; $p<0.001$ each; Mann-Whitney-Test with α=0.05 unless otherwise specified). At the beginning of control recordings the LLPR was higher during activity phase compared to rest ($p=0.041$), whereas no significant ZT-dependent differences were found at the end of the recordings ($p=0.137$). In contrast to the phasic BAL responses the late, long-lasting pheromone response was not affected by GDP-β-S at the beginning (activity: $p=0.617$; rest: $p=0.949$) as well as the end (activity: $p=0.395$; rest: $p=0.408$) of recordings at both ZTs when compared to control recordings (Fig. 34). Thus, the blockage of G-proteins by GDP-β-S obviously had no influence on the LLPR.

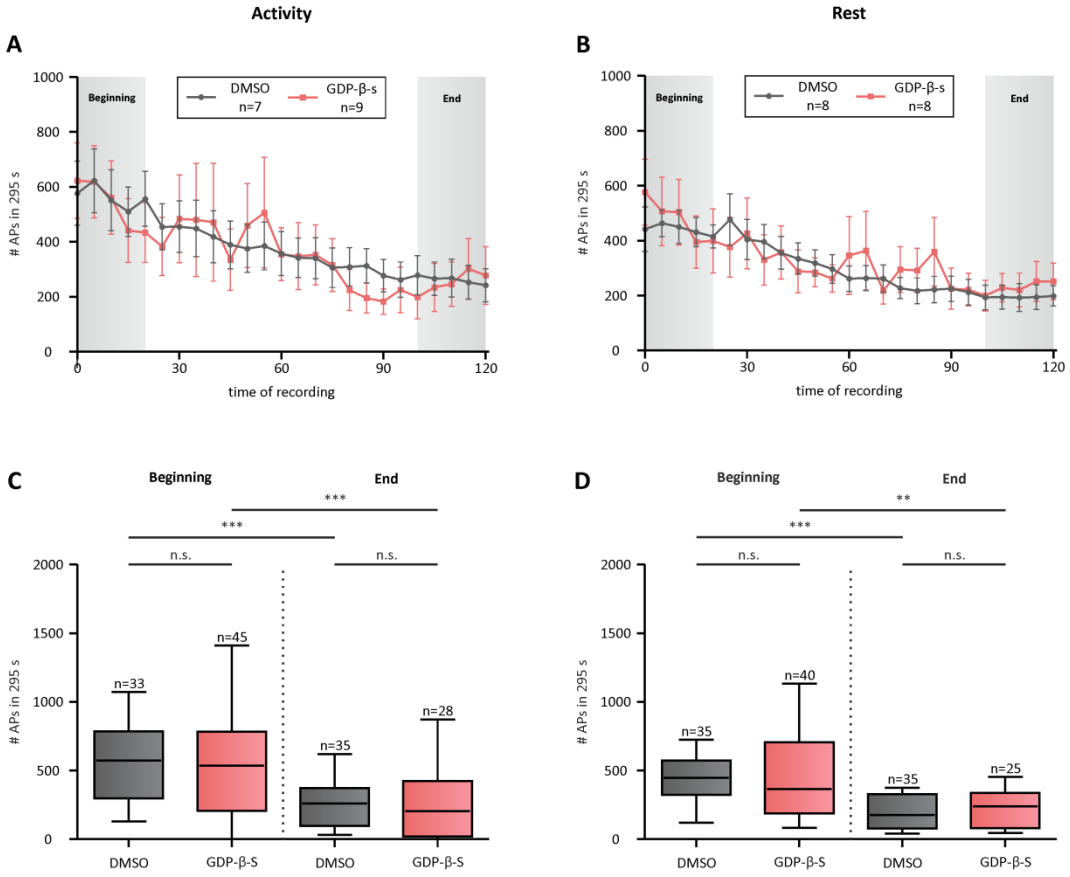


**Fig. 34 - Infusion of GDP-β-S did not affect the late, long-lasting pheromone response. A-B** Control recordings revealed a reduction of the late, long-lasting pheromone response (LLPR) in the time course of recordings at both ZTs. A ZT-dependent difference was observed with higher LLPR during the beginning of the activity phase. Remarkably, application of GDP-β-S (10 µM) never significantly affected the LLPR at both ZTs. Just like control recordings GDP-β-S recordings showed higher LLPR at the beginning of the recordings at both ZTs. **C-D** Comparison of begin (first 20 minutes) and end (last 20 minutes) of long-term recordings. Box plots with whiskers from 10 to 90 percentiles. Significant differences are indicated by asterisks (n.s. = not significant; Mann-Whitney-test with $\alpha$=0.05).

# 6 Discussion

## 6.1 Specificity of prospective agonists and antagonists of the Orco protein in *M. sexta*

Orco function in insect olfaction is still not fully understood. Since Orco is highly conserved in insects (Krieger et al. 2003; Larsson et al. 2004; Pitts et al. 2004; Jones et al. 2005; Vosshall and Hansson 2011) and functional loss of Orco reduced responses to odors (Larsson et al. 2004; Neuhaus et al. 2005) this demonstrates the importance of Orco in odor detection. Therefore, Orco modulators could be used across insect species as a broadly applicable insect repellent and for the further elucidation of the processes involved in olfactory signal transduction. For this, the specificity of prospective agonists and antagonists of the Orco protein had to be tested.

***VUAA1 is a specific agonist of MsexOrco***

In the last years several prospective Orco agonists and antagonists were characterized which helped to address the importance of Orco in insect signal transduction (Jones et al. 2011; Bohbot and Dickens 2012; Chen and Luetje 2012; Jones et al. 2012). One of them is the Orco agonist VUAA1 (Jones et al. 2011). It was shown that VUAA1 application in cells expressing Orco alone elicited calcium ($Ca^{2+}$) currents (Jones et al. 2011). The same study showed that the activation of currents by VUAA1 was more effective during co-expression of Orco and odor ligand binding olfactory receptors (ORs) of different insect species. Thus, conformational changes due to binding of VUAA1 to the Orco protein affected current flow in OR-Orco heteromeric complexes.

We successfully showed in our *in vitro* and *in vivo* experiments that VUAA1 is also a specific Orco agonist in *M. sexta* (Nolte et al. 2013). The $Ca^{2+}$ imaging experiments performed by Nico Funk and Latha Mukunda in HEK293 cells transiently transfected with the *Manduca* Orco MsexOrco showed VUAA1-dependent rises in intracellular $Ca^{2+}$ levels (Nolte et al. 2013). Furthermore, MsexOrco transfected HEK293 cells revealed higher $Ca^{2+}$ levels in general, either with or without simultaneous application of

VUAA1. Thus, VUAA1 is also an agonist of MsexOrco and Orco itself is a spontaneously opening cation channel, whose activity can be enhanced by VUAA1. Additionally, co-transfection of MsexOrco, the sensory neuron membrane protein MsexSNMP1 and the pheromone receptor binding candidates MsexOR-1 or MsexOR4 (Grosse-Wilde et al. 2010; Grosse-Wilde et al. 2011) increased $Ca^{2+}$ levels after VUAA1 stimulation indicating an interaction of these proteins. This is consistent with the finding of associated complexes of ligand-binding ORs and the coreceptor Orco (Neuhaus et al. 2005; Benton et al. 2006; German et al. 2013). However, the *in vitro* studies in vertebrate-derived HEK-cells could not determine whether VUAA1 also activates MsexOrco in the intact hawkmoth in the pheromone-sensitive trichoid sensilla. Therefore, *in vivo* tip recordings were performed under influence of VUAA1. Changes in the spontaneous activity as well as late, long-lasting pheromone responses were able to show the activation of Orco in the intact animal (Fig. 17; see chapter "6.3 Orco as a pacemaker channel"), also indicating the specificity of VUAA1 to MsexOrco.

***OLC15 specifically blocks MsexOrco***

As a result of the finding of the first specific Orco agonist VUAA1 (Jones et al. 2011) structurally related agonists and antagonists could be determined more easily (Chen and Luetje 2012; Jones et al. 2012; Taylor et al. 2012). One of them was the highly effective Orco antagonist OLC15 (Chen and Luetje 2012). As part of this thesis it was determined whether OLC15 also is a potent antagonist of MsexOrco in intact ORNs of *M. sexta*. Thus, it was tested how effectively OLC15 can inhibit Orco-dependent spontaneous activity in pheromone-dependent ORNs of the hawkmoth. Since the spontaneous activity of ORNs is very low the spontaneous activity was increased initially with the Orco agonist VUAA1 (Fig. 17). In case of a competitive binding of VUAA1 and OLC15 the co-application of VUAA1 and OLC15 should decrease the VUAA1-dependent "spontaneous" activity. In fact it was found that increasing concentrations of OLC15 counteract the VUAA1-generated spontaneous activity (Fig. 19). Thus, as already observed for other insect Orco proteins (Chen and Luetje 2012), also in *M. sexta* OLC15 is a potent Orco antagonist. However, it cannot be determined

with these experiments whether OLC15 is specific for the Orco protein or whether it also affects other targets involved in the generation of spontaneous activity.

***Unspecific effects on pheromone responses by amilorides***

In recent times it was suggested that also the amilorides MIA and HMA are potent, specific antagonists of Orco. However, amilorides reversibly inhibited odor-dependent neuronal activity of ORNs in lobsters which do not express any Orco proteins and which employ IR but not OR gene families for odor detection (Bobkov and Ache 2007). In addition, amilorides blocked a $Ca^{2+}$-dependent cation channel expressed in dendrites of moth ORNs (Zufall and Hatt 1991). In favor for amiloride-dependent block of Orco it was shown that amilorides are able to block odor-dependent currents of the OR/Orco complex in *A. gambiae*., *D. melanogaster,* and *B. mori* (Pask et al. 2013; Rollecke et al. 2013). Highest effectiveness was found for the amilorides HMA and MIA. However, the Orco specificity of both amilorides has not been clarified yet in these expression systems. Thus, the effects of VUAA1 and OLC15 in comparison to those observed with amilorides HMA or MIA on bombykal (BAL) transduction in the intact hawkmoth were examined.

Since distinct differences in the effects of these different agents were found it has to be assumed that they do not share identical targets in hawkmoth pheromone-sensilla. As shown above, the SPA was not influenced by OLC15 at all (Fig. 22, 24), which was also observed for the Orco agonist VUAA1 (Fig. 12; see chapter “6.2 Involvement of Orco in ionotropic and metabotropic signal transduction”). On the contrary the amilorides HMA and MIA both induced a reduction of the SPA (Fig. 23, 24). Thus, amilorides affected BAL-transduction but Orco agonists and antagonists did not. Furthermore, also latency times (Fig. 23, 26) and kinetics of BAL-dependent phasic action potential response (Fig. 27) were stronger affected by at least one of both amilorides in comparison to OLC15. Therefore, also factors determining the transformation of the BAL-dependent receptor potential into the action potential response such as the membrane potential are more strongly affected by the amilorides than by Orco.

Although a binding of amilorides to the Orco protein is possible, at least one additional target for amilorides exists beside the Orco protein. This hypothesis is supported by the finding that amilorides affect a broad range of targets like $Na^+$-channels, $Na^+/H^+$-antiporter, $Na^{2+}/Ca^{2+}$-exchanger or $Na^+/K^+$-ATPases (reviewed in: Kleyman and Cragoe 1988). Thus, it is unlikely that amilorides are specific for the MsexOrco protein.

Whereas during activity phase for the LLPR similar effects were found for both amilorides compared to OLC15, during rest HMA was the only agent which affected the LLPR (Fig. 28). Thus different targets for both amilorides have to be assumed. This is consistent with the fact, that HMA in general seems to be more potent during resting phase compared to MIA recordings. Additionally, MIA showed higher similarity to the effects observed under influence of OLC15. Thus in general it might have a higher affinity to the Orco protein than HMA.

## 6.2 Involvement of Orco in ionotropic and metabotropic signal transduction

Signal transduction in insect olfaction is still under discussion. Especially studies examining Orco-based ionotropic transduction revealed controversial data and thus various hypotheses resulted (Sato et al. 2008; Wicher et al. 2008; Nakagawa and Vosshall 2009; Stengl 2010) (Fig. 5). The role of the co-receptor protein Orco appears to be of particular importance since Orco was shown to be highly conserved across insect species (Krieger et al. 2003; Larsson et al. 2004; Pitts et al. 2004; Jones et al. 2005; Vosshall and Hansson 2011). As reported before, Orco is a spontaneously opening “leaky” cation channel described in different insects (Sato et al. 2008; Wicher et al. 2008; Jones et al. 2011; Sargsyan et al. 2011). Nevertheless, it was suggested that Orco plays a crucial role for ionotropic odor transduction (Fig. 5).

As discussed above, it is very likely that VUAA1 and OLC15 specifically bind to MsexOrco *in vivo*. Since it was shown that Orco proteins from *D. melanogaster*, *A. gambiae* and *C. quinquefasciatus* were not affected by stimulation with odorants (Elmore et al. 2003; Neuhaus et al. 2005; Sato et al. 2008; Wicher et al. 2008; Jones et al. 2011; Pask et al. 2011; Chen and Luetje 2012), odorants bind only to ORs.

Consequently, pheromone-dependent activation of ORs and simultaneous modulation of Orco by OLC15 or VUAA1 should act independently of each other without competitive effects. Thus, all effects observed in tip recordings under influence of OLC15 and VUAA1 are likely to be restricted to the Orco protein.

***MsexOrco activation did not increase pheromone response parameters***

If an ionotropic odor transduction pathway dominates, the activation of the OR by pheromone and the simultaneously allosteric activation of Orco by VUAA1 should lead to an additive pheromone response since both components should non-competitively contribute to a resulting ion influx. This additive response should mimic pheromone responses observed at higher BAL concentrations. As a result of an ionotropic cascade the co-stimulation with VUAA1 and BAL should particularly increase pheromone parameters occurring within the first few milliseconds after pheromone stimulation. Thus, the SPA, AP frequency of the first APs, the latency of the first AP as well as the general kinetics should exhibit sensitization.

Surprisingly, for none of these parameters this was the case during VUAA1-dependent Orco activation (Fig. 13-16). Actually, pheromone response parameters occurring within the first 1000 ms after pheromone stimulation were almost unaffected within the beginning of recordings under influence of VUAA1. The sensillum potential amplitude was never affected by VUAA1. Both different ZTs and VUAA1 concentrations did not have an impact on the SPA in long-term tip recordings (Fig. 13). This suggests that MsexOrco does not contribute to the generation of the SPA and the forming of the ORN's receptor potential upon pheromone stimulation. Furthermore, the AP frequency of the first six APs was also unaffected by VUAA1 in the first 20 minutes of the recordings (Fig. 14). VUAA1 application extended latency times of the first AP after BAL stimulation in the time course of recordings (Fig. 15). In addition, the phasic component within the post stimulus kinetics was strongly reduced in long-term tip recordings, indicating increased threshold levels, possibly due to Orco-dependent increases in $Ca^{2+}$ baseline levels in the ORNs (Fig. 16). Additionally, the AP frequency

showed reduced values in the further time course of control as well as VUAA1 recordings.

All of this might be seen as an indication for cGMP-dependent adaptation, which was observed in *M. sexta* by Flecke et al. (2006). Possibly, a $Ca^{2+}$-dependent increase in cGMP concentrations reduced pheromone responses. However, adaptation due to saturation of BAL responses can be excluded since higher concentrations of BAL were still able to increase the pheromone responses in *M. sexta* (Dolzer et al. 2003; Flecke et al. 2006; Flecke and Stengl 2009; Flecke et al. 2010). Furthermore, VUAA1 concentrations employed were not saturating and a lower VUAA1 concentration of 1 µM did not affect the AP frequency (Tab. A1). Accordingly, VUAA1 was still able to change the spontaneous activity in a dose-dependent manner (Fig. 17).

Since these findings contradicts the hypothesis of a solely ionotropic pathway it encourages the suggestion of an involvement of a metabotropic cascade influencing the open-probability of an MsexOrco-participating ion channel.

***Block of Orco reduced the pheromone responses***

The SPA was not influenced by OLC15 at all (Fig. 24). This was already found for the Orco agonist VUAA1 (Fig. 13). Thus, an involvement of MsexOrco in the generation of the SPA and the forming of the ORN's receptor potential upon pheromone stimulation becomes increasingly unlikely. By OLC15 the AP frequency of the first six APs was reduced (Fig. 25) and the latency prolonged (Fig. 26). Thus, a contribution of the Orco protein in the phasic BAL-response was observed. However, at least one amiloride showed a higher effectiveness in the desensitization of pheromone parameters than observed for OLC15 at any time (Fig. 24-27). This and the fact that OLC15 like VUAA1 did not change the SPA suggest that Orco only partially contribute to the phasic BAL-response, but does not predominately control it. This hypothesis is confirmed by the finding that in contrast to amilorides the kinetics were not influenced in the beginning of recordings by OLC15 (Fig. 27), similar as already observed for VUAA1. Therefore, it is unlikely that Orco is involved in a fast ionotropic pathway in *M. sexta*.

***Block of metabotropic pathways reduced pheromone responses***

Since an ionotropic pathway is unlikely in *M. sexta* a metabotropic cascade has to be assumed. Blockage of G-protein pathways should lead to strong desensitization of the pheromone response. Unlike to the Orco blocker OLC15 as well as the Orco agonist VUAA1 recordings under influence of the G-protein blocker GDP-β-S had an impact on the sensillum potential amplitude (Fig. 29, 30). Thus, a G-protein pathway seems to be involved in the generation of the receptor potential. Since the cAMP pathway seems to be more modulating (Flecke et al. 2010) the involvement of a PLC-depending pathway has to be assumed. GDP-β-S also influenced the AP frequency (Fig. 31). As observed for the SPA the AP frequency was reduced during the time course of recordings. Also, the latency of the first AP after pheromone stimulation increased (Fig. 32) and kinetics during the time course of recordings showed a less prominent phasic component (Fig. 33). However, in the first 20 minutes of recordings during activity phase no significant changes were found.

However, in general the effects observed under influence of GDP-β-S were relatively slow. Furthermore, a complete block of the pheromone responses did not occur as expected for a solely metabotropic signal transduction. However, contrary to all other agents tested in this thesis, the targets of GDP-β-S can be assumed to be intracellular. Therefore, GDP-β-S has to pass the dendritic membrane of the ORNs, which might explain the delayed and slightly rising effect of GDP-β-S. Additional GDP-β-S has to competitively block the G-protein and GDP produced by the cell has to be displaced. Thus, the used GDP-β-S concentration of 10 µM might be insufficient to fully block the pheromone responses.

Nevertheless, all parameters of the direct pheromone response were impaired by GDP-β-S which strongly suggests an involvement of at least one G-protein pathway, and thus, a metabotropic signal transduction in the olfactory system of *M. sexta*. However, since GDP-β-S unspecifically blocks G-proteins finally no statement can be made which pathway is involved, even if other studies pointed out that this is likely a $G_q$-dependent pathway (Laue et al. 1997; Kain et al. 2008).

## 6.3 Orco as a pacemaker channel

***Orco is a current-dependent ion channel***

Orco activation by VUAA1 was shown to increase the spontaneous activity as well as the late, long-lasting pheromone response (LLPR) (Fig. 17). This might be explained by the property of Orco in forming a leaky cation channel. Thereby, Orco increases the membrane potential. Thus, the ORN lies closer to the spike threshold which results in an increased sensitivity of the ORN. To test whether Orco is voltage-dependent and whether membrane depolarization via current injection can replace Orco action, ORNs were depolarized by current application with or without coapplication of OLC15. It was hypothesized that block of Orco by OLC15 should decrease the neuronal activity initiated by current injections.

To initially determine sufficient current strength for depolarization of the ORNs 3 different currents were tested while recording neuronal activity. ORNs injected with currents of 200 pA did not change the neuronal activity in comparison to control recordings (Fig. 20). Therefore, this stimulation was not sufficient to significantly depolarize the membrane potential of the ORNs. Experiments with injection of 400 pA could not be analyzed since this caused impairment of the cells, probably due to damage of the cells membrane. Recordings of ORNs stimulated with 300 pA were stable and increased neuronal activity which was blocked by OLC15 (Fig. 20, 21). Thus, Orco, which controls spontaneous activity (Dobritsa et al. 2003; Elmore et al. 2003; Larsson et al. 2004), appears to be opened via depolarization and is a voltage-dependent cation channel.

The neuronal activity was lower in control recordings during 60-s-lasting current stimulations than those observed after previous 10-s-lasting stimulation (Fig. 21). Therefore, it has to be assumed that due to long current stimulations adapting mechanisms are initiated, such as $Ca^{2+}$-calmodulin-dependent block of Orco. In addition, the concentrations of cGMP might also be increased via sustained depolarizations which increase intracellular $Ca^{2+}$ baseline levels which in turn desensitize the ORNs in *M. sexta* (Flecke et al. 2006).

Remarkably, OLC15 was not able to completely prevent the generation of spontaneous activity (Fig. 21). Thus, at least one additional ion channel, which is Orco-independent, seems to be involved in the control of spontaneous activity. Since HCN-channels were shown to be involved in the generation of the resting potential (Robinson and Siegelbaum 2003), these might be an possible candidate. This is supported by the fact that HCN-channels were also found in *M. sexta* (Krannich and Stengl 2008).

### *Involvement of MsexOrco in spontaneous activity and late, long-lasting pheromone responses*

Accumulating evidence was provided for a participation of Orco in generation of spontaneous activity in different insect species (Larsson et al. 2004; Benton et al. 2007; Jones et al. 2011; Su et al. 2012). Thus, Orco appears to depolarize ORNs. This assumption is supported by the observation that Orco forms a leaky cation channel which depolarizes ORNs via influx of $Ca^{2+}$ and other cations, thereby increasing spontaneous activity (Sato et al. 2008; Wicher et al. 2008; Jones et al. 2011; Sargsyan et al. 2011). In $Ca^{2+}$ imaging experiments (performed by Nico Funk and Latha Mukunda as mentioned above) it was confirmed that this is also true for *M. sexta* (Nolte et al. 2013).

Since MsexOrco activation by VUAA1 in tip recordings without pheromone stimulations strongly increased spontaneous activity of the ORNs also MsexOrco depolarizes ORNs (Fig. 17). This supports the hypothesis of Orco being a pacemaker channel which controls the spontaneous activity and the LLPR acting as a leaky cation channel such as other pacemaker channels.

Since stimulation with BAL increased AP frequencies in ORNs over several minutes (Fig. 17; LLPR) and since pheromones are degraded within milliseconds (Vogt and Riddiford 1986; Vogt 1987) brief BAL stimuli must elicit long-lasting changes in ORNs such as sustained depolarizations or sustained changes in intracellular second messenger levels. This LLPR cannot be due to sustained rises in $IP_3$ levels since pheromone-dependent $IP_3$ levels declined within milliseconds correlating with time

courses of phasic pheromone responses (Boekhoff et al. 1990; Boekhoff et al. 1990; Breer et al. 1990; Kaissling and Boekhoff 1993).

Since previous evidence suggested the involvement of metabotropic BAL transduction cascades (review: Stengl 2010) it is not surprising that the spontaneous activity after VUAA1 application differed from VUAA1 effects observed in the LLPR (Fig. 17). It was shown that the BAL-dependent LLPR during VUAA1 application (100 µM) was lower than the observed spontaneous activity under influence of VUAA1 during activity phase (Fig. 17). This might be explainable by changed cGMP concentrations after pheromone stimulation (Boekhoff et al. 1990; Ziegelberger et al. 1990). It was previously revealed that cGMP is able to regulate the Orco function in *D. melanogaster* (Wicher et al. 2008), possibly resulting in changed calcium baseline levels, which itself might influence the neuronal activity.

As shown spontaneous activity and long-lasting pheromone response was strongly increased by the Orco agonist VUAA1 in *M. sexta* (Fig. 17, 18), structurally related to OLC15. This indicates lower threshold levels due to Orco activation. Orco blockage thus should lead to an opposite effect. Indeed, OLC15 strongly reduced the LLPR already within the first 295 seconds in the activity phase (Fig. 28). However, this was not seen during resting phase (for discussion see chapter “6.4 Circadian modification in pheromone responses and sensitivity”).

***G-proteins are not involved in late, long-lasting pheromone responses***

Interestingly GDP-β-S showed no significant differences during the time course of recordings in the LLPR at both ZTs (Fig. 34). Thus the ligand-binding OR and potential affiliated G-proteins seems not being involved in the generation of a longer lasting pheromone response when the G-protein pathways are blocked. However, higher GDP-β-S might be necessary to influence the LLPR. Nevertheless, since all other pheromone parameter were impaired this explanation is unlikely.

## 6.4 Circadian modification in pheromone responses and sensitivity

Since daytime-dependent changes in sensitivity and temporal dynamics were found in pheromone sensitive ORNs of *M. sexta* (Flecke et al. 2006), tip recordings were performed at activity phase (ZT 1-3) and resting phase (ZT 9-11) of the animal. By analysis of daytime-dependent effects of the used drugs further insides of the olfactory signal transduction in the hawkmoth were gained.

***Higher sensitivity levels of ORNs during activity phase***

In control recordings in general the AP frequency of the first six APs after BAL stimulation was lower and the latency was prolonged during rest (e.g. Fig. 14, 15). Additional, response kinetics became increasingly less phasic during the time course of recordings (e.g. Fig. 16). Thus, threshold levels seem to be increased during resting phase and in general the cells are less sensitive. This could be caused by a more depolarized resting potential which results in increased intracellular $Ca^{2+}$ baseline levels during rest, possibly due to circadian control of respective ion channels. Calcium thus could be a possible modulator of MsexOrco and might influence its open-probability (Stengl 2010). Evidence is increasing, that intracellular $Ca^{2+}$-levels are controlled by circadian clocks within the ORNs (Schuckel et al. 2007; Stengl 2010; Schendzielorz et al. 2012). Thereby, Orco might modulate threshold levels of the ORNs in *M. sexta* in a ZT-dependent manner. In addition, it was shown that the stress hormone octopamine is involved in the sensitization of the ORNs in Manduca in a daytime-dependent manner (Flecke and Stengl 2009). Possibly, octopamine levels are controlled in a circadian manner resulting in circadian changes of cAMP concentrations (Farooqui 2007; Stengl 2010). Since the Orco protein is modulated by cyclic nucleotides (Wicher et al. 2008) it might play the key role in the daytime-dependent sensitization.

***ZT-dependent changes in Orco expression***

VUAA1 recordings showed also a lower AP frequency lower during rest when compared to activity phase (Fig. 14). Furthermore, VUAA1 stronger affected the spontaneous activity during activity phase, which was also found for the LLPR (Fig. 17). Both might be an indication for ZT-dependent changes in Orco expression rates with lower expression during rest. This assumption is supported by the finding, that OLC15 did not show any changes in the LLPR during resting phase, whereas a strong decline was observed during activity phase (Fig. 28). Current injection experiments under influence of OLC15 further provided evidence for this hypothesis, since it was shown, that the reduction of the current-induced activity by OLC15 was more prominent during activity phase (Fig. 21).

***ZT-dependent effectiveness of amilorides***

The effectiveness of the amilorides HMA and MIA also differed in a ZT-dependent manner. During resting phase HMA diminished the pheromone response and its kinetics to a greater extent than observed for MIA (Fig. 24-27). During activity phase for most parameters no differences were observed between both amilorides (Fig. 24, 26, 27). However, the AP frequency was reduced more potently by MIA during activity phase (Fig. 25). Thus, this again supports the assumption that both amilorides may bind to different targets. Both amilorides were able to decrease the LLPR during activity phase, whereas this only was true for HMA during rest (Fig. 28), supporting the hypothesis of a higher affinity to Orco by MIA as compared to HMA, as mentioned above.

# 7 Conclusion and outlook

Tip recordings of pheromone sensitive long trichoid sensilla on antennae of *M. sexta* demonstrated the specificity of the agonist VUAA1 and the antagonist OLC15 for MsexOrco. The amilorides MIA and HMA in contrast are not specific for MsexOrco and additional targets in ORNs have to be assumed, with the $Ca^{2+}$-dependent cation channel being the most likely target. With MsexOrco agonist VUAA1 no evidence was found for an ionotropic pathway, since the Orco agonist did not increase the phasic pheromone response as would have been expected. Furthermore, also the sensillum potential amplitude as well as the kinetics of the pheromone response remained unchanged. On the other hand there is strong evidence for an involvement of a G-protein-coupled pathway, which control the pheromone response and its kinetics within the first milliseconds after pheromone stimulation, since all parameters were affected by the G-protein blocker GDP-β-S. Since the pheromone response was not fully blocked, higher GDP-β-S concentrations have to be tested in future experiments to further provide evidence for an involvement of G-protein coupled signal transduction cascades.

Since VUAA1 increased spontaneous activity as well as LLPR in the time scale of seconds to minutes after the pheromone application, this effect cannot only be due to pheromone-dependent membrane potential activation but could result from rises in intracellular second messengers. Thus, also based upon current injection studies MsexOrco seems to be a spontaneously opening pacemaker channel which can control the sensitivity of the ORNs in *M. sexta* membrane-potential and second messenger-dependently. Complying with this hypothesis, Orco block by OLC15 strongly impaired the LLPR during activity phase, whereas GDP-β-S showed no effect on the LLPR. However, it still needs to be confirmed whether MsexOrco is indeed regulated via second messenger levels as shown for DmelOrco.

Both, VUAA1 as well as OLC15 were more potent during activity phase. This might be an indication for higher expression levels of the Orco protein during this time of day. However, it remains to be studied whether circadian mechanisms control Orco expression and modification.

# 8 Acknowledgments

First of all I would like to thank Prof. Dr Monika Stengl for giving me the opportunity for performing my doctor thesis in the department of animal physiology. I would like to thank her for the support and for introducing me into the field of insect olfaction.

I would like to thank PD Dr. Dieter Wicher for reviewing my doctor thesis and his collaboration. Furthermore, I thank Prof. Dr. Hellmuth Zöltzer and Prof. Dr. Jörg Helmut Kleinschmidt for participating in the examination committee.

Also I like to thank Dr. Christian Flecke for teaching me making real good tip recordings and his special support. I hope I was able to justice to this task.

Thanks to all the members of the department of animal physiology. I really enjoyed working with all of you. Particular I would like to thank Gisela Kaschlaw and Chistin Sender for raising certainly the best Manduca in the world. Special thanks I would like to give to my “fellow-sufferer” Petra Gawalek, for solving one or another problem and her support in any matter.

I would like to thank Hanzey and Sarah for having a lot of fun and nice discussions and their support. I also thank Fonso for being Fonso. Good luck for you in the sunny Spain. I would like to thank Achim for introducing me into the Matlab world. A special thanks goes to Nico, for his knowledge and support concerning all this insect olfaction stuff and his cooperation.

I would like to thank my parents, grandparents and friends for any backing. Especially I like to thank my “little” sister.

# 9 Appendix

## Statistics for Orco agonist VUAA1

| A | Begin | | SPA | AP frequency | Latency | # APs 0-150 ms | # APs 0-1000 ms | LLPR | Spont. activity |
|---|---|---|---|---|---|---|---|---|---|
| Activity | DMSO | 1 µM VUAA1 | 0.855 | 0.572 | 0.191 | 0.898 | 0.124 | **0.04** | **0.001** |
| | DMSO | 100 µM VUAA1 | 0.340 | 0.507 | 0.052 | 0.146 | 0.484 | **0.012** | **< 0.001** |
| | 1 µM VUAA1 | 100 µM VUAA1 | 0.263 | 0.985 | 0.617 | 0.068 | 0.333 | 0.217 | **< 0.001** |
| Rest | DMSO | 1 µM VUAA1 | 0.779 | 0.710 | **0.008** | 0.620 | **0.002** | **< 0.001** | **< 0.001** |
| | DMSO | 100 µM VUAA1 | 0.381 | **0.002** | **< 0.001** | 0.097 | 0.113 | **0.001** | **< 0.001** |
| | 1 µM VUAA1 | 100 µM VUAA1 | 0.474 | **< 0.001** | 0.136 | 0.124 | 0.059 | 0.331 | **< 0.001** |
| Activity vs. Rest | DMSO | DMSO | 0.629 | 0.133 | 0.905 | 0.132 | 0.172 | **0.017** | 0.879 |
| | 1 µM VUAA1 | 1 µM VUAA1 | 0.383 | **0.013** | 0.342 | **0.035** | 0.173 | 0.704 | **0.044** |
| | 100 µM VUAA1 | 100 µM VUAA1 | 0.893 | **0.002** | 0.143 | 0.179 | **0.013** | **0.032** | **0.003** |
| **B** | **Begin vs. End** | | **SPA** | **AP frequency** | **Latency** | **# APs 0-150 ms** | **# APs 0-1000 ms** | **LLPR** | **Spont. activity** |
| Activity | DMSO | DMSO | 0.796 | **0.039** | **0.002** | 0.134 | 0.693 | **< 0.001** | - |
| | 100 µM VUAA1 | 100 µM VUAA1 | 0.194 | **< 0.001** | **< 0.001** | **< 0.001** | 0.078 | 0.273 | - |
| Rest | DMSO | DMSO | 0.904 | **< 0.001** | **< 0.001** | **< 0.001** | **0.012** | **< 0.001** | - |
| | 100 µM VUAA1 | 100 µM VUAA1 | 0.145 | **< 0.001** | **< 0.001** | **< 0.001** | 0.103 | 0.918 | - |
| **C** | **End** | | **SPA** | **AP frequency** | **Latency** | **# APs 0-150 ms** | **# APs 0-1000 ms** | **LLPR** | **Spont. activity** |
| Activity | DMSO | 100 µM VUAA1 | 0.979 | **< 0.001** | **< 0.001** | **< 0.001** | **0.008** | **< 0.001** | - |
| Rest | DMSO | 100 µM VUAA1 | 0.657 | **< 0.001** | **< 0.001** | **< 0.001** | 0.373 | **< 0.001** | - |
| Activity vs. Rest | DMSO | DMSO | 0.393 | **< 0.001** | **< 0.001** | **< 0.001** | 0.726 | **0.004** | - |
| | 100 µM VUAA1 | 100 µM VUAA1 | 0.459 | 0.055 | 0.953 | 0.964 | 0.418 | **< 0.001** | - |

**Tab. A1 - Significant differences within the time course of recordings or between different ZTs for G-protein blocker experiments. A-C** Data groups were compared using Mann-Whitney-test (α=0.05). Corresponding p-values are shown (significant differences in fat letters).

| A | Begin | norm. SPA | APF | Latency | PSTH 0-150 ms | PSTHs 0-1000 ms | LLPR | Spont. activity |
|---|---|---|---|---|---|---|---|---|
| Activity | DMSO | 1.00 | 213.0 | 0.014 | 12.0 | 17.0 | 503.0 | 11.0 |
| | | n=45 | n=45 | n=45 | n=45 | n=45 | n=45 | n=25 |
| | 1 µM VUAA1 | 1.00 | 241.4 | 0.016 | 13.0 | 16.0 | 715.0 | 38.0 |
| | | n=45 | n=45 | n=44 | n=45 | n=45 | n=45 | n=44 |
| | 100 µM VUAA1 | 1.00 | 239.9 | 0.018 | 13.5 | 18.0 | 844.5 | 1178.0 |
| | | n=50 | n=50 | n=50 | n=50 | n=50 | n=50 | n=30 |
| Rest | DMSO | 1.00 | 209.7 | 0.015 | 15.0 | 23.0 | 375.0 | 11.0 |
| | | n=50 | n=50 | n=48 | n=50 | n=50 | n=50 | n=28 |
| | 1 µM VUAA1 | 1.00 | 206.8 | 0.020 | 15.0 | 18.5 | 696.0 | 113.0 |
| | | n=50 | n=50 | n=50 | n=50 | n=50 | n=50 | n=51 |
| | 100 µM VUAA1 | 1.00 | 176.6 | 0.025 | 13.5 | 21.0 | 614.0 | 698.0 |
| | | n=50 | n=50 | n=50 | n=50 | n=50 | n=50 | n=33 |
| **B** | **End** | **norm. SPA** | **APF** | **Latency** | **PSTH 0-150 ms** | **PSTHs 0-1000 ms** | **LLPR** | **Spont. activity** |
| Activity | DMSO | 0.86 | 194.3 | 0.034 | 11.0 | 18.5 | 288.0 | - |
| | | n=45 | n=45 | n=45 | n=45 | n=45 | n=45 | - |
| | 100 µM VUAA1 | 0.96 | 95.7 | 0.152 | 0.0 | 15.0 | 831.5 | - |
| | | n=50 | n=50 | n=50 | n=50 | n=50 | n=50 | - |
| Rest | DMSO | 1.02 | 130.4 | 0.060 | 7.0 | 18.0 | 139.5 | - |
| | | n=50 | n=50 | n=48 | n=50 | n=50 | n=50 | - |
| | 100 µM VUAA1 | 0.98 | 67.5 | 0.124 | 0.0 | 20.0 | 598.0 | - |
| | | n=50 | n=50 | n=49 | n=50 | n=50 | n=50 | - |

**Tab. A2 - Medians and numbers for VUAA1 recordings. A-B** Median values and numbers for analyzed parameters in VUAA1 recordings and associated control recordings are shown.

## Statistics for Orco antagonists OLC15 and amilorides HMA and MIA

| A | Begin | | SPA | AP frequency | Latency | # APs 0-150 ms | # APs 0-1000 ms | LLPR |
|---|---|---|---|---|---|---|---|---|
| Activity | DMSO | MIA | * | *** | ** | ns | ns | *** |
| | DMSO | HMA | * | * | *** | ns | ns | *** |
| | DMSO | OLC15 | ns | ** | ** | ns | ns | *** |
| | MIA | HMA | ns | ns | ns | ns | ns | ns |
| | MIA | OLC15 | ** | ns | ns | ns | ** | ns |
| | HMA | OLC15 | ** | ns | ns | ns | ** | ns |
| Rest | DMSO | MIA | ** | ns | ** | * | ns | ns |
| | DMSO | HMA | *** | *** | *** | *** | * | *** |
| | DMSO | OLC15 | ns | ns | * | ns | ns | ns |
| | MIA | HMA | ns | *** | ns | * | ns | *** |
| | MIA | OLC15 | *** | ns | ns | ns | ns | ns |
| | HMA | OLC15 | *** | *** | ns | *** | ns | *** |
| **B** | **Begin** | | **SPA** | **AP frequency** | **Latency** | **# APs 0-150 ms** | **# APs 0-1000 ms** | **LLPR** |
| Activity vs. Rest | DMSO | DMSO | 0,8722 | **0,0077** | 0,5351 | 0,1085 | 0,0704 | **0,003** |
| | MIA | MIA | 0,6805 | 0,7303 | 0,9825 | 0,5218 | 0,2624 | **0,0367** |
| | HMA | HMA | **0,0214** | **< 0.0001** | 0,5859 | **0,0047** | 0,7642 | **0,0002** |
| | OLC15 | OLC15 | | | | | | |
| **C** | **Begin vs. End** | | **SPA** | **AP frequency** | **Latency** | **# APs 0-150 ms** | **# APs 0-1000 ms** | **LLPR** |
| Activity | DMSO | DMSO | 0,5882 | **0,0031** | **0,0002** | 0,1469 | 0,6892 | **< 0.0001** |
| | MIA | MIA | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **0,0315** |
| | HMA | HMA | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** |
| Rest | DMSO | DMSO | 0,9421 | **< 0.0001** | **< 0.0001** | **< 0.0001** | **0,0183** | **< 0.0001** |
| | MIA | MIA | **0,0094** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** |
| | HMA | HMA | 0,1919 | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **< 0.0001** |

**Tab. A3 - Significant differences within the time course of recordings or between different ZTs for Orco blocker experiments.** Continued on next page.

| D | End | | SPA | AP frequency | Latency | # APs 0-150 ms | # APs 0-1000 ms | LLPR |
|---|---|---|---|---|---|---|---|---|
| Activity | DMSO | MIA | *** | *** | *** | *** | *** | ** |
| | DMSO | HMA | *** | *** | *** | *** | *** | *** |
| | MIA | HMA | ns | ns | ns | ns | ns | ns |
| Rest | DMSO | MIA | *** | *** | *** | *** | *** | * |
| | DMSO | HMA | *** | *** | *** | *** | *** | *** |
| | MIA | HMA | ns | ns | ns | ns | ns | *** |
| **E** | **End** | | **SPA** | **AP frequency** | **Latency** | **# APs 0-150 ms** | **# APs 0-1000 ms** | **LLPR** |
| Activity vs. Rest | DMSO | DMSO | 0,5047 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0,9742 | **0,0002** |
| | MIA | MIA | **0,0017** | 0,2999 | 0,8585 | 0,6367 | 0,9638 | 0,169 |
| | HMA | HMA | 0,1216 | 0,4701 | 0,3207 | 0,5271 | 0,8901 | **0,0045** |

**Tab. A3 - Significant differences within the time course of recordings or between different ZTs for Orco blocker experiments. A,D** Data groups were compared using Kruskal-Wallis-Test (α=0.05). P***<0.001; P**<0.01; P*<0.05, n.s = not significant. **B,C,E** Data groups were compared using Mann-Whitney-test (α=0.05). Corresponding p-values are shown (significant differences in fat letters).

| A | Begin | norm. SPA | APF | Latency | PSTH 0-150 ms | PSTHs 0-1000 ms | LLPR |
|---|---|---|---|---|---|---|---|
| Activity | DMSO | 1 | 244,4 | 0,01324 | 11 | 16 | 558,5 |
| | | n=50 | n=50 | n=50 | n=45 | n=45 | n=50 |
| | MIA | 0,7611 | 168,6 | 0,02526 | 10 | 17 | 234,5 |
| | | n=50 | n=50 | n=47 | n=47 | n=47 | n=50 |
| | HMA | 0,8435 | 190,8 | 0,03728 | 11 | 16 | 274 |
| | | n=50 | n=50 | n=49 | n=49 | n=49 | n=50 |
| | OLC15 | 1 | 185,6 | 0,02525 | 14 | 22 | 299 |
| | | n=50 | n=50 | n=50 | n=50 | n=50 | n=50 |
| Rest | DMSO | 1 | 205,8 | 0,01503 | 15 | 22 | 381 |
| | | n=45 | n=45 | n=43 | n=45 | n=45 | n=45 |
| | MIA | 0,7399 | 177 | 0,02953 | 12 | 19 | 353,5 |
| | | n=50 | n=50 | n=49 | n=50 | n=50 | n=50 |
| | HMA | 0,6176 | 112,4 | 0,03234 | 6 | 17 | 106 |
| | | n=50 | n=50 | n=48 | n=48 | n=48 | n=50 |
| | OLC15 | 1 | 180,2 | 0,02318 | 13 | 19 | 348,5 |
| | | n=50 | n=50 | n=50 | n=50 | n=50 | n=50 |
| **B** | **End** | **norm. SPA** | **APF** | **Latency** | **PSTH 0-150 ms** | **PSTHs 0-1000 ms** | **LLPR** |
| Activity | DMSO | 0,9596 | 192,7 | 0,033 | 10 | 18 | 304 |
| | | n=50 | n=50 | n=50 | n=45 | n=45 | n=45 |
| | MIA | 0,374 | 0 | 0,4646 | 0 | 3 | 135,5 |
| | | n=49 | n=49 | n=42 | n=50 | n=50 | n=50 |
| | HMA | 0,4639 | 1,361 | 0,4293 | 0 | 3 | 48,5 |
| | | n=43 | n=43 | n=36 | n=45 | n=45 | n=50 |
| Rest | DMSO | 1,021 | 126,7 | 0,0609 | 6 | 18 | 112 |
| | | n=45 | n=45 | n=43 | n=45 | n=45 | n=50 |
| | MIA | 0,5509 | 10,89 | 0,2115 | 0 | 3,5 | 64 |
| | | n=50 | n=50 | n=38 | n=50 | n=50 | n=50 |
| | HMA | 0,5888 | 0 | 0,2828 | 0 | 2 | 23 |
| | | n=41 | n=41 | n=34 | n=45 | n=45 | n=44 |

**Tab. A4 - Medians and numbers for Orco blocker experiments. A-B** For each agent and both ZTs the median values and numbers of experiments with MIA, HMA, OLC15 and associated control recordings are shown.

## Statistics for G-protein blocker GDP-β-S

| A | Begin | | SPA | AP frequency | Latency | # APs 0-150 ms | # APs 0-1000 ms | LLPR |
|---|---|---|---|---|---|---|---|---|
| **Activity** | DMSO | GDP-β-S | **0,0041** | 0,3039 | 0,0868 | 0,1825 | 0,3514 | 0,6174 |
| **Rest** | DMSO | GDP-β-S | **0,0007** | **0,0017** | **< 0.0001** | 0,4619 | 0,2358 | 0,9492 |
| **Activity vs. Rest** | DMSO | DMSO | 0,9957 | 0,0825 | 0,871 | 0,3006 | 0,6583 | **0,0415** |
| | GDP-β-S | GDP-β-S | 0,6426 | **0,0223** | **0,0312** | 0,7169 | **0,0196** | 0,6282 |
| **B** | **Begin vs. End** | | **SPA** | **AP frequency** | **Latency** | **# APs 0-150 ms** | **# APs 0-1000 ms** | **LLPR** |
| **Activity** | DMSO | DMSO | 0,5727 | **0,0136** | **0,0005** | 0,4944 | 0,7241 | **< 0.0001** |
| | GDP-β-S | GDP-β-S | **0,0031** | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0,2168 | **0,0002** |
| **Rest** | DMSO | DMSO | 0,9654 | **< 0.0001** | **< 0.0001** | **< 0.0001** | **0,0299** | **< 0.0001** |
| | GDP-β-S | GDP-β-S | 0,7986 | **0,0001** | **< 0.0001** | **< 0.0001** | **0,0005** | **0,0023** |
| **C** | **End** | | **SPA** | **AP frequency** | **Latency** | **# APs 0-150 ms** | **# APs 0-1000 ms** | **LLPR** |
| **Activity** | DMSO | GDP-β-S | **0,0157** | **< 0.0001** | **< 0.0001** | **< 0.0001** | **0,0007** | 0,395 |
| **Rest** | DMSO | GDP-β-S | **0,0431** | **0,0006** | **0,0004** | 0,3809 | **0,0054** | 0,4076 |
| **Activity vs. Rest** | DMSO | DMSO | 0,2858 | **0,0032** | **0,0143** | **< 0.0001** | 0,0872 | 0,1371 |
| | GDP-β-S | GDP-β-S | 0,0694 | 0,051 | 0,4905 | 0,5958 | 0,599 | 0,7668 |

**Tab. A5 - Significant differences within the time course of recordings or between different ZTs for G-protein blocker experiments. A-C** Data groups were compared using Mann-Whitney-test (α=0.05). Corresponding p-values are shown (significant differences in fat letters).

| A | Begin | norm. SPA | APF | Latency | PSTH 0-150 ms | PSTHs 0-1000 ms | LLPR |
|---|---|---|---|---|---|---|---|
| Activity | DMSO | 1 | 253,4 | 0,01347 | 11 | 15 | 573 |
| | | n=35 | n=35 | n=35 | n=33 | n=33 | n=33 |
| | GDP-β-S | 0,918 | 223,8 | 0,01793 | 13 | 16 | 535 |
| | | n=45 | n=45 | n=44 | n=45 | n=45 | n=45 |
| Rest | DMSO | 1 | 234,8 | 0,01358 | 14,5 | 22 | 447 |
| | | n=40 | n=40 | n=39 | n=36 | n=35 | n=35 |
| | GDP-β-S | 0,8655 | 173 | 0,02997 | 13 | 19 | 366 |
| | | n=40 | n=40 | n=40 | n=40 | n=40 | n=40 |
| **B** | **Begin** | **norm. SPA** | **APF** | **Latency** | **PSTH 0-150 ms** | **PSTHs 0-1000 ms** | **LLPR** |
| Activity | DMSO | 0,9982 | 196,7 | 0,03569 | 11 | 20 | 260 |
| | | n=35 | n=36 | n=36 | n=33 | n=33 | n=35 |
| | GDP-β-S | 0,7965 | 46,91 | 0,1253 | 2 | 14 | 204 |
| | | n=40 | n=43 | n=37 | n=40 | n=40 | n=28 |
| Rest | DMSO | 1,05 | 144,5 | 0,05178 | 6 | 18 | 176 |
| | | n=40 | n=40 | n=40 | n=36 | n=36 | n=35 |
| | GDP-β-S | 0,8347 | 67,72 | 0,1052 | 4,5 | 13,5 | 239 |
| | | n=32 | n=32 | n=31 | n=40 | n=40 | n=25 |

**Tab. A6 - Medians and numbers for GDP-β-S recordings. A-B** Median values and numbers for analyzed parameters in GDP-β-S recordings and associated control recordings are shown.

# Zusammenfassung

Neben der Verbreitung von gefährlichen Krankheiten sind Insekten für enorme agrarwirtschaftliche Schäden verantwortlich. Ein Großteil der Verhaltensweisen bei Insekten wird über den Geruchssinn gesteuert, der somit einen möglichen Angriffspunkt zur Bekämpfung von Schadinsekten darstellt. Hierzu ist es allerdings nötig, die Mechanismen der olfaktorischen Signalübertragung im Detail zu verstehen. Neben den duftstoffbindenden olfaktorischen Rezeptoren spielt hier auch ein konservierter Korezeptor (Orco) eine entscheidende Rolle. Inwieweit bei diesen Proteinen ionotrope bzw. metabotrope Prozesse involviert sind ist bislang nicht vollständig aufgeklärt. Um weitere Einzelheiten aufzuklären wurden daher Einzelsensillenableitungen am Tabakschwärmer *Manduca sexta* durchgeführt. Orco-Agonisten und Antagonisten wurden eingesetzt, um die Funktion des Korezeptors besser zu verstehen. Bei dem Einsatz des Orco-Agonisten VUAA1 konnte keine Verstärkung der Pheromonantworten bzw. eine Sensitivierung beobachtet werden, wie im Falle einer ionotropen Signalweiterleitung zu erwarten gewesen wäre. Ein ionotroper Signalweg über den OR/Orco-Komplex in *M. sexta* ist daher unwahrscheinlich. Der Orco-Antagonist OLC15 beeinflusste die gleichen Parameter wie VUAA1 und konnte die von VUAA1 generierte Spontanaktivität blocken. Daher ist es wahrscheinlich, dass dieser einen spezifischen Orco-Blocker darstellt. Sowohl VUAA1 als auch OLC15 hatten großen Effekt auf die langanhaltende Pheromonantwort, welches die Vermutung nahelegt, dass Orco modulierend auf die Sensitivität der Nervenzelle einwirkt. Von OLC15 abweichende Effekte durch die getesteten Amiloride HMA und MIA auf die Pheromonantwort lassen nicht auf eine spezifische Wirkung dieser Agenzien auf Orco schließen und zusätzliche Wirkorte sind anzunehmen. Um die These eines metabotropen Signalwegs zu überprüfen wurde ebenfalls der G-Protein-Blocker GDP-β-S eingesetzt. Alle Parameter der Pheromonantwort die innerhalb der ersten Millisekunden analysiert wurden wiesen eine Reduktion der Sensitivität auf. Im Gegensatz dazu hatte GDP-β-S keinen Effekt auf die langanhaltende Pheromonantwort. Somit scheint ausschließlich die schnelle Pheromonantwort über einen Ligand-bindenden G-Protein-gesteuerten Rezeptor gesteuert zu werden.

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