Friedrich-Schiller-Universität Jena
Fakultät für Biowissenschaften
Abteilung für Molekulare Lebenswissenschaften

seit 1558

Production and characterization of a genetically modified *Drosophila* line which expresses a modified odorant receptor protein (Orco CaM)

Masterarbeit
zur Erlangung des Grades eines
Master of Science
vorgelegt von

Kalpana Jain
aus Indien

Jena, Februar 2019

Gutachter:

PD Dr. Dieter Wicher

Prof. Dr. Jürgen Bolz

Table of Contents

Abbreviation

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
dsDNA	Double stranded deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
CO_2	Carbon dioxide
Fwd	Forward
Kb	Kilo bases
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
Rev	Reverse
RNase	Ribonuclease
Dm	*Drosophila melanogaster*
RT	Room temperature
WT	Wild type
TAE	Tris acetate buffer
dNTP	Deoxynucleotide triphosphate
DEPC	Diethyl pyro carbonate
DNase	deoxyribonuclease
OSN	Olfactory sensory neurons.
IRs	Ionotropic receptors
GRs	Gustatory receptors
ORs	Odorant receptors
CaM	Calmodulin
CBS	Calmodulin binding site
UAS	Upstream activating sequence
AC	Adenylyl cyclase

N-terminus	Amine -terminus
C-terminus	Carboxyl terminus
GPCR	G-protein coupled receptor
cAMP	Cyclic adenosine monophosphate
$G_{\alpha s}$	Stimulatory G-protein
ATP	Adenosine triphosphate
HEK293	Human embryonic kidney
CHO	Chinese hamster ovary
CNG	Cyclic nucleotide gated
PLC	Phosolipase C
PKC	Protein kinase C
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
RYR	Ryanodine receptor
cpGFP	Circular permutated green fluorescent protein
M13	Myosin domain (calmodulin binding protein)
XP2	Binding buffer
GCaMP	genetically encoded calcium indicator (GECI)
EtBr	Ethyl bromide
Etb	Ethyl butyrate
GAL4	yeast specific transcription factor

List of figures

List of tables

Abstract

The olfactory system perceives volatile chemical signals from the environment. It allows animal and human beings to detect food, harmful substance, pheromones due to the presence of olfactory receptors on olfactory sensory neurons (OSNs). Insect olfactory receptors belong to the three different families, ionotropic receptors (IRs), gustatory receptors (GRs) and odorant receptors (ORs). ORs have been evolved to detect air borne odors with high sensitivity. They operate as ligand-gated non-selective cation channels and form a heteromeric complexes of an odor-specific OrX protein and a co-receptor protein Orco. The OR proteins have seven transmembrane segments with internal N-terminus and external C-terminus. The second intracellular loop of the Orco protein in *Drosophila melanogaster* bears a highly conserved calmodulin (CaM) binding site (CBS) ^{336}SAIKYWVER^{344}, where CaM binding to this motif modulates the Orco function. In this study we investigate how the point mutation K339N may affect the olfactory responses elicited by the synthetic agonist VUAA1 in an ex-vivo antenna preparation. Using Ca^{2+} imaging, we demonstrate that mutant flies show reduced olfactory responses. This reduction was similar to that observed in wild type OSNs upon CaM inhibition with the antagonist W7. We also investigated the role of CBS in OSN sensitization. Our results show that in mutant there is no OSN sensitization. Finally, we also asked how the mutation might affect the fly performance in odor localization. Using a free flight assay we show that the odor localization in mutant flies is severely affected by this point mutation. Taken together, the results show that CaM binding to Orco protein plays an essential role in olfactory responses, sensitizing ORs and the odor localization of the whole animal.

Zusammenfassung

Das olfaktorische System dient der Wahrnehmung chemischer Signale aus der Umwelt. Es erlaubt Mensch und Tier die Identifizierung von Nahrung, gefährlichen Substanzen oder Pheromonen. Deren Düfte werden von olfaktorischen sensorischen Neuronen (OSN) mittels olfaktorischer Rezeptoren detektiert. Diese Rezeptoren gehören drei Familien an, ionotropen Rezeptoren (IR), gustatorischen Rezeptoren (GR) und Odorantrezeptoren (OR). OR sind entstanden um volatile Duftmoleküle mit hoher Sensitivität zu detektieren. Sie fungieren als ligandengesteuerte, nichtselektive Kationenkanäle und bilden heteromere Komplexe, bestehend aus duftspezifischem OrX-Protein und Korezeptorprotein Orco. Diese Proteine besitzen sieben Transmembransegmente, der N-Terminus ist cytoplasmatisch und der C-Terminus ist extrazellulär. Der zweiten intrazellulären Loop von Orco bei *Drosophila melanogaster* trägt eine hochkonservierte Calmodulin (CaM)-Bindestelle CBS "^{336}SAIKYWVER^{344}". CaM-Bindung an dieses Motiv moduliert die Funktion von Orco. In dieser Studie untersuchen wir, wie die Punktmutation K339N in diesem Motiv die Duftantwort von OSNs in einer ex-vivo Antennenpreparation auf Stimulation mit dem synthetischen Agonisten VUAA1 beeinflusst. Unter Anwendung von Ca^{2+}-Imaging kann man zeigen, daß mutierte Fliegen eine reduzierte Duftantwort aufweisen. Diese Reduktion war der vergleichbar, die bei Wildtypfliegen unter CaM-Inhibition mit W7 beobachtet wird. Wir untersuchten ebenso den Einfluß der CBS auf die OSN-Sensitisierung. Für Mutanten wurde keine Sensitisierung gefunden. Abschließend wurde gefragt, wie die Mutation die Fähigkeit Duftquellen zu lokalisieren beeinflußt. In einem Freiflugassay gelang es zu zeigen, daß die Punktmutation diese Fähigkeit drastisch einschränkt. Zusammengenommen haben wir gefunden, daß CaM-Bindung an Orco von zentraler Bedeutung für Duftantwort, Sensitisierung und die Lokalisierung von Duftquellen ist.

1 Introduction

1.1 Olfaction

Olfaction is a sense of smell that allows us to perceive an odor coming from our environment. This enables us to identify various odors, mating partners or danger. Animals are able to detect an odor through their sophisticated olfactory system. In fact, the olfactory system is challenged to detect and to identify a myriad of different volatile chemicals with the help of olfactory receptors expressed by olfactory sensory neurons (OSNs) (Kaupp, 2010). The OSNs transfer the odor information to second-order neurons in which information is integrated and processed to the higher brain centres. There the odor information is further processed to initiate an appropriate behavior. Thus, to understand the complex mechanisms involved in olfaction, the vinegar fly *Drosophila melanogaster*, the insect model organism, has been studied. Olfaction in *Drosophila* has been intensively studied due to its less complex olfactory system as compared to other organisms (Vosshall and Stocker, 2007). Though the morphology of the insect nose is entirely different from the nose of other organisms, the organisation of the olfactory system is quite similar (Kaupp, 2010). Therefore, by studying olfaction process in *Drosophila*, it allows us to identify olfactory mechanisms involved in other insect species like agricultural pests that damage crops and mosquitos that transmit diseases in human beings like yellow fever, chikungunya, dengue, or malaria.

1.2 *Drosophila melanogaster* as insect model organism

Drosophila melanogaster is an extensively studied organism as it has a short life cycle, the genome sequence is available, is ease of maintaining, and has a small nervous system (Olsen and Wilson, 2008). The complete genomic sequence of *Drosophila* (Adams et al., 2000) allowed researchers to examine its genetic, physiological and behavioral aspects. Moreover, its olfactory system helps us to understand the underlying mechanisms of odor sensitivity and behavioral responses. Therefore, with the development of genetic tools, mechanism of insect olfaction became quite popular to study. One such genetic tool is the GAL4-UAS system (Brand and Perrimon, 1993). GAL4 has obtained from yeast *Saccharomyces cerevisiae*. The GAL4-UAS system allows to express a specific gene in a tissue-specific manner. The GAL4 gene has a tissue-specific

driver and can be expressed in a particular tissue. The GAL4 protein is a transcriptional activator (Fig. 1A) and can bind to upstream activator sequence (UAS) of any other gene (Fig. 1B). The binding of GAL4 protein causes the activation of gene downstream of the UAS sequence (Fig. 1C). In this way, the UAS sequence can be combined with any protein sequence. For example, GAL4 can be combined with calcium sensor proteins like GCaMP6f and causes the activation of GCaMP6f in that particular gene or tissue. The combined approach of GCaMP6f and GAL4-UAS system allows to study specific neuron populations.

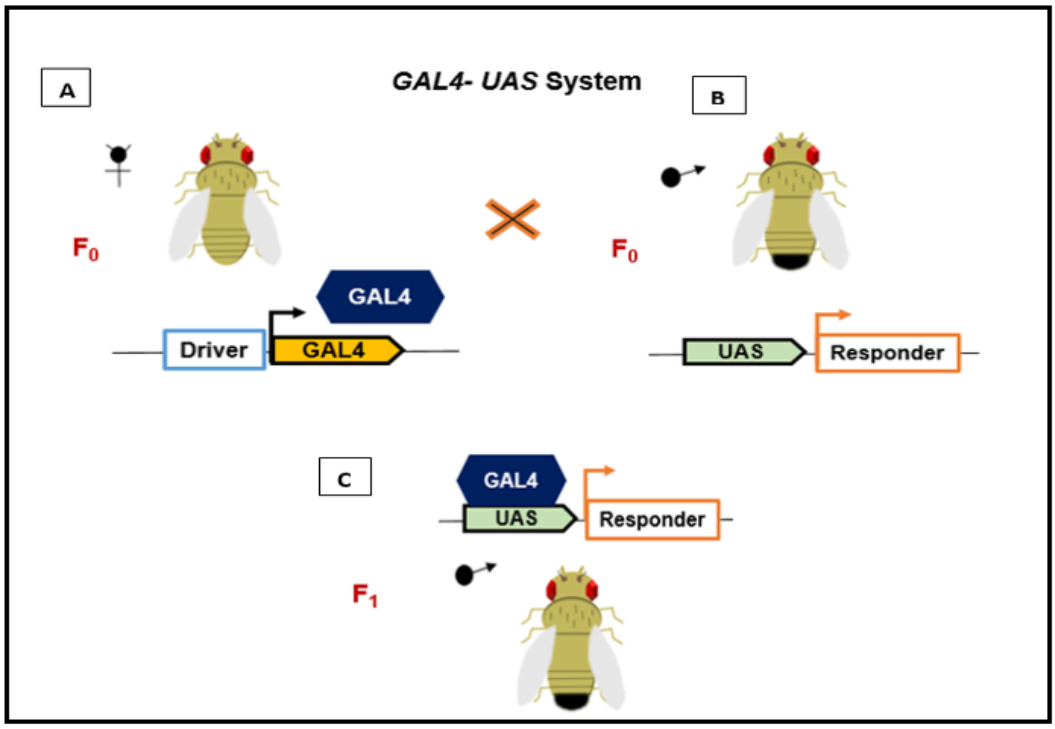

Figure 1: Genetic scheme of the GAL4-UAS system in *Drosophila melanogaster.* F_0 represents the parental generation. A. The mother carries a gene for GAL4 protein (a yeast transcriptional activator), located after a tissue specific driver. B. The father carries a gene of interest in responder, inserted downstream of the upstream activating sequence (UAS). X represents: mating between father and mother flies. $F_{1:}$ represent progeny. C. The progeny expresses the GAL4 protein which binds to the UAS sequence and activates the gene of interest in tissue specific manner.

1.2.1 Architecture of *Drosophila* Olfactory Organs.

Antenna and maxillary palps are two bilateral olfactory organs covered by a sensory hair-like structure called sensillum (Vosshall, L. B., & Stocker, R. F. 2007). The antennae are present on the forehead, whereas the maxillary palps are extended from the proboscis

(Fig. 2A). Sensilla are classified into four different types depending on the size and morphology (Stocker, 1994); (Shanbhag et al., 1999). The third antennal segment called funiculus is equipped with trichoid, coeloconic, basiconic, and intermediate sensilla (Fig. 2B). The maxillary palp is restricted to the basiconic type of sensilla. Each sensillum comprises one to four OSNs enclosed with supporting cells. The OSNs are typically bipolar, the outer dendritic region is located within the sensillum while the axon projects towards the antennal lobe to specific glomeruli shown in black circle (Fig. 2 C2). The projection neurons (PNs) send their axons from the antennal lobe to higher brain centers such as mushroom bodies and lateral horn (Fig. 2 C3).

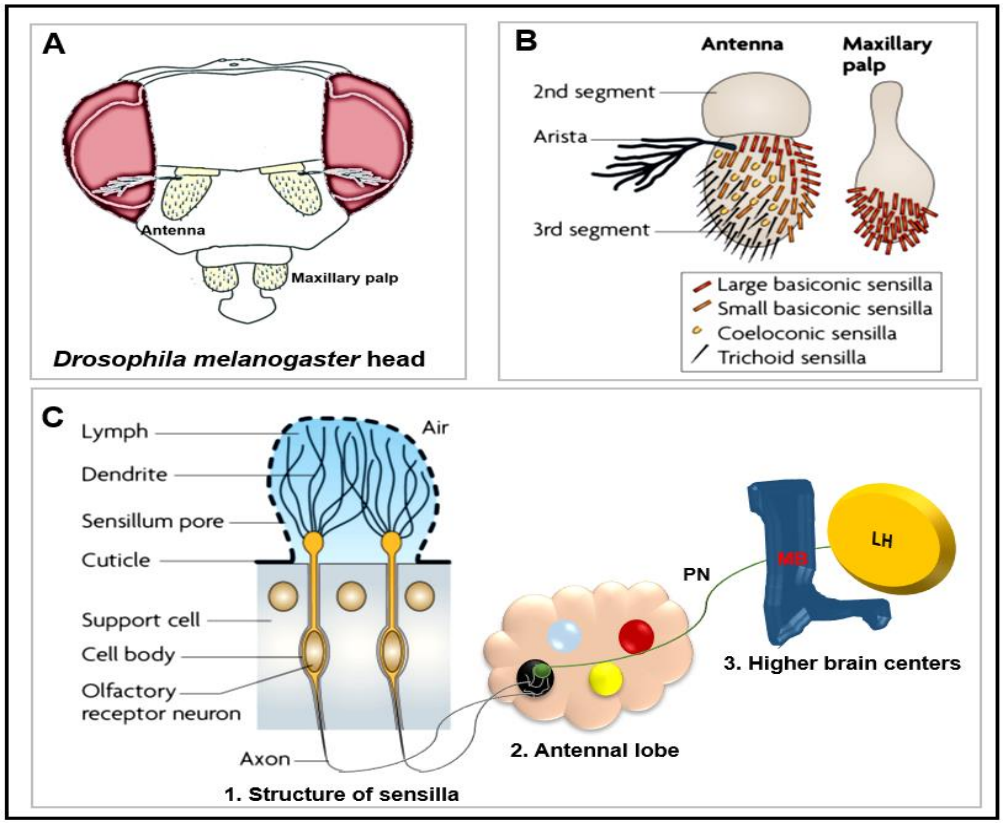

Figure 2: The olfactory system of *Drosophila melanogaster*. A. Head section of *Drosophila* indicating two bilateral organs: antenna (above) and maxillary palp (below). **B**. 3rd antennal segment with sensilla types: trichoid: pointy tip, basiconic: blunt shape, coeloconic: peg shape. **C1.** Structure of a sensillum comprise OSNs, supporting cells and sensillum lymph.**C2**. Antennal

lobe with specific receptor type glomeruli in coloured circles. **C3**. Higher brain centres. Picture **B** and **C1** are taken from (Kaupp, 2010).

1.2.2 Olfactory receptors

In *Drosophila*, olfactory receptors are expressed in ~1200 OSNs (Stocker, 1994) of the antenna and ~120 OSNs of the maxillary palps. Insects detect odor via three types of olfactory receptors, gustatory receptors (GRs), ionotropic receptors (IRs) and odorant receptor (ORs). IRs and GRs evolved early and are thus present in a many organisms (Croset et al., 2010); (Peñalva-Arana et al., 2009). IRs occur in coeloconic sensilla (Benton et al., 2009) and are involved in acid or amine sensing. They form heterotetrameric complexes composed of IRX proteins and a co-receptor protein IRcoY protein (Abuin et al., 2011) (Fig. 3A).GRs are expressed in basiconic sensilla and are involved in carbon dioxide perception. GR proteins are seven transmembrane-proteins like G protein-coupled receptors (GPCRs). In contrast to GPCRs, GRs adopt an inverted topology within the membrane, i.e. the N-terminus is intracellular and the C-terminus extracellular (Fig. 3B). GRs form heteromeric complexes of Gr1/2 and the co-receptor protein Gr3.

The main focus of this study is on odorant receptors, they are presented in next paragraph.

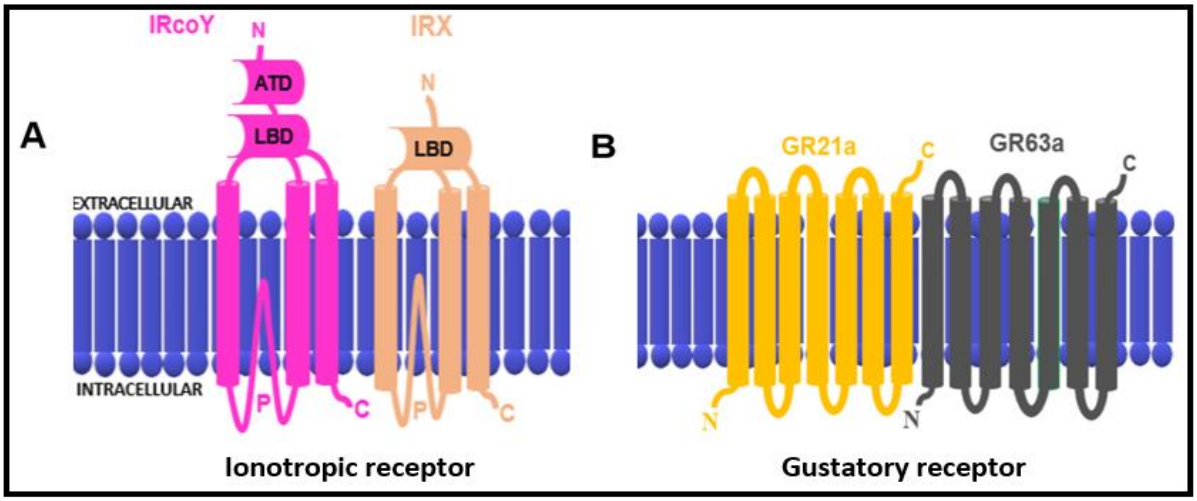

Figure 3: Schematic view of insect olfactory receptors. A. Ionotropic receptors (IRs) form heterotetramers of IRX and co receptor IRcoY. The ligand binding domain (LBD) and an extended amino-terminal domain (ATD) are located at the N-terminal region. The pore region (P) is located in the membrane and the C-terminus is intracellular. **B.** Gustatory receptors (GRs) form

heteromeric complexes of Gr1/2 and co receptor Gr3. In *Drosophila* olfaction the GRs form Gr21a and Gr63a. Picture is modified from (Wicher, 2015)

1.2.2.1 Odorant receptors (ORs)

During 1990's, odorant (OR) genes were discovered in various animals like mouse, channel cat fish, *Xenopus laevis*, *C. elegans* (Buck and Axel, 1991;Ngal et al., 1993;Freitag et al., 1995;Sengupta et al., 1996). The mammalian ORs form G-protein coupled receptors (GPCRs) that are expressed in the ciliated region of OSNs (review (Kaupp, 2010)). ORs bind odor molecules and activate the stimulatory G-protein, $G\alpha_{olf.}$ $G\alpha_{olf}$ stimulates the synthesis of the intracellular signaling molecule cAMP by adenylyl cyclase III (ACIII). The increase in cAMP concentration activates cyclic nucleotide-gated (CNG) channels and causes calcium influx into the cell. The channel opening increases the intracellular calcium concentration $[Ca^{2+}]_i$ which activates Ca^{2+} activated Cl^- channels. Their opening causes Cl^- efflux which leads to depolarizing of sensory neuron. On the other hand, insect ORs are genetically and structurally different from mammalian ORs. In the year 1999, the discovery of a large family of genes encoding the insect odorant receptors (ORs) were first identified in *Drosophila* (Clyne et al., 1999;Gao and Chess, 1999;Vosshall et al., 1999). ORs are present in all the insects living on land except non-hexapods (Brand et al., 2018).

Insect ORs are expressed in basiconic sensilla, trichoid sensilla and only one in coeloconic sensilla of the antennae (Vosshall and Stocker, 2007). These ORs form a heteromeric complex of a conventional odor-specific OrX and a universal co-receptor protein Orco (Neuhaus et al., 2004) (Larsson et al., 2004) (Fig. 4A). Insect OR proteins show a inverted seven transmembrane topology with intracellular N-terminus and extracellular C-terminus (Fig. 4A).

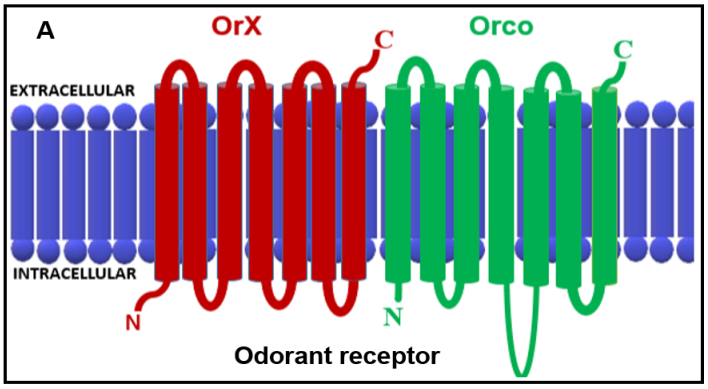

Figure 4: Structure of Odorant receptor (OR). Odorant receptors (ORs) are heteromeric complexes of two receptor proteins with seven transmembrane inverted topology (extracellular C-terminus and intracellular N-terminus): odor specific receptor protein (OrX) responsible for odor binding and a ubiquitous co-receptor Orco. Picture is modified from (Wicher, 2015).

Electrophysiological studies carried out with heterologously expressed insect ORs gave rise to two models of OR function (Sato et al., 2008;Wicher et al., 2008). According to the first model insect ORs are ionotropic receptors activated upon odor binding to the receptor (Sato et al., 2008) (Fig. 5A). The second model suggests that insect ORs proposes combined ionotropic and metabotropic signaling (Wicher et al., 2008) (Fig. 5B). At high odor concentration, odor binding to the receptor triggers activation of the Orco channel and flow of cations into the cells. However, at low odor concentration, odor binding to the receptor activates stimulatory G-protein ($G\alpha_s$). $G\alpha_s$ activates adenylyl cyclase (AC) that converts adenosine triphosphate (ATP) to cyclic 3`-5´ adenosine monophosphate (cAMP) through catalytic activity. The increase in cAMP level activates Orco protein which induces calcium influx into the OSNs and depolarizes the OSNs. solely expressed Orco protein in human embryonic kidney cells (HEK293) activated by intracellular cAMP or cGMP resulted in form of functional ion channels (Wicher et al., 2009). Similarly, heterologous expression of Orco dimers in Chinese hamster ovary (CHO) cells resulted in calcium conducting ion channels (Mukunda et al., 2014a).

The OSNs lacking Orco protein, called Orco mutant, resulted in loss of OrX in the ciliated dendritic region of OSN (Larsson et al., 2004;Benton et al., 2006;Vosshall and Hansson, 2011). Extracellular single sensillum recordings performed on a particular sensilla type of Orco mutant flies showed no odor evoked action potentials (Larsson et al., 2004).The behavior of the Orco mutant flies performed in trap assay severely impaired odor

responses (Larsson et al., 2004). Thus, Orco is chaperone protein that is involved in trafficking and tuning OR. The Orco sequence is conserved across insect species and it is highly expressed in trichoid and basiconic sensilla of an antenna and maxillary palps (Larsson et al., 2004). The Orco may form either homomers or heteromers ion channel with unknown stoichiometry (Neuhaus et al., 2004)(German et al., 2013). But recently, the cryo electron microscopy structure of Orco was identified as tetramer with central ion-conducting pore present in the middle (Butterwick et al., 2018). Orco is not involved in direct odor binding but can be activated by the synthetic OR agonist VUAA1 (2-(4-Ethyl-5-(pyridin-3-yl)-4H-1, 2, 4-triazol-3-ylthio)-N-(4-ethylphenyl) acetamide) that belongs to the VUAA family (W Taylor et al., 2012).

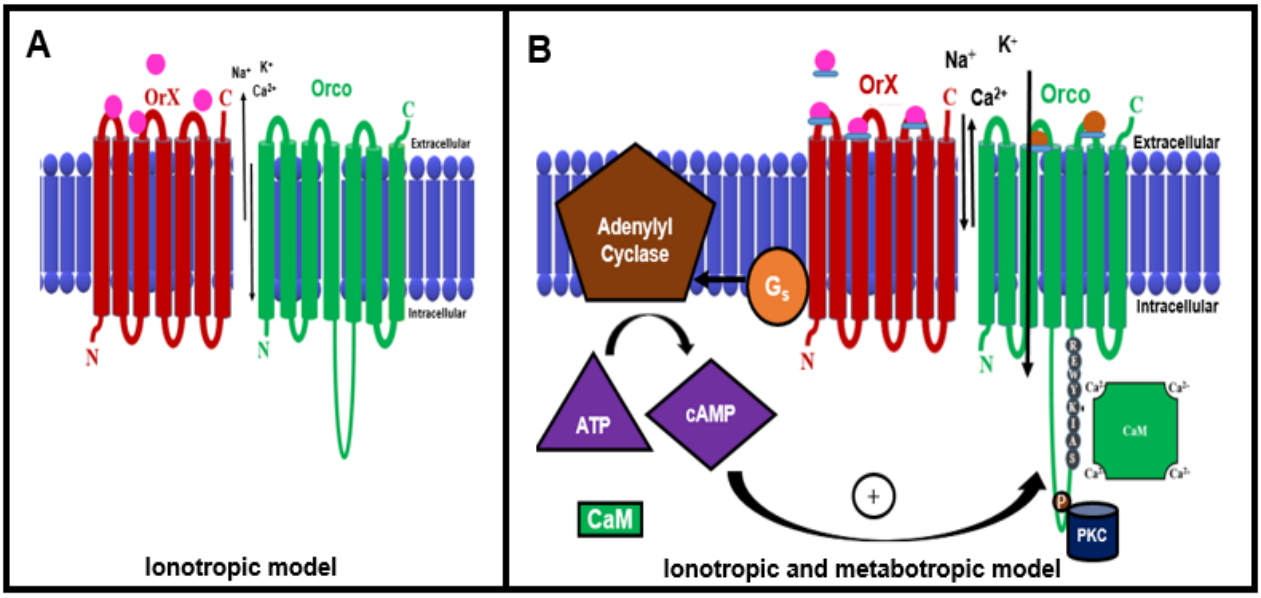

Figure 5: The schematic view of olfactory signalling cascade in insects. A specific odorant receptor (OrX) and ubiquitous co-receptor (Orco) form a ligand activated cation channel in OSNs A. Ionotropic model: ligand (pink) binds to OrX protein leads to activation of channel and flow of cations (in arrows) (Sato et al., 2008). B. Ionotropic and Metabotropic model: ligand (pink) binds to the OrX causes activation of channel and also stimulatory G-protein (G_s). G_s further activates intracellular signalling cascade that leads to activation of Orco channel (Wicher et al., 2008) Figure was referred from (Pellegrino and Nakagawa, 2009).

1.2.2.2 Sensitivity of ORs

Flying insects are capable of detecting volatile compounds at very low concentrations during flight. In addition, insect ORs are fine-tuned that allow an insect to track odor with high sensitivity (Getahun et al., 2012;Wicher, 2018). The odor sensitivity of the OR

complex expressed in HEK293 cells was reduced upon inhibition of G protein (Wicher 2008). The activation of Orco by cAMP requires basal Phospholipase C (PLC) activity mediated via G-proteins and the basal activity of PLC depends upon the Ca^{2+} level present within the cells (Sargsyan et al., 2011). Similarly, protein kinase C (PKC) has role in maintaining the OR sensitivity (Sargsyan et al., 2011). Extracellular single sensillum recordings performed on a large basiconic sensilla expressing Or22a/Orco receptor complex resulted in reduction of odor response after inhibiting intracellular PLC or PKC or adenylyl cyclase (AC) (Sargsyan et al., 2011;Getahun et al., 2013). And also by genetic manipulation for example, the five amino acid point mutation in PKC phosphorylation sites of Orco protein abolished the sensitivity to cAMP (Sargsyan et al., 2011).

The process of sensitization occurs when near threshold odor stimulation is repeatedly presented within a suitable time window. For example, repeated subthreshold of Or22a/Orco expressing OSNs with ethyl butyrate resulted in OR sensitization (Getahun et al., 2013). Pharmacological inhibition of AC/PKC abolished sensitization in Or22a/Orco complex expressing neurons. It was also shown that the OSNs expressing a Orco PKC mutant do not sensitize. The OSNs expressing mutant PKC subtypes reduced the sensitivity of ORs to odor stimulation (Getahun et al., 2016). The behavior of Orco PKC flies in odor tracking was severely impaired. Therefore, modulations in the metabotropic signalling pathway may affect the sensitivity of ORs and odor tracking performance in insects.

Furthermore, *in vitro* and *ex vivo* studies have shown that the highly conserved putative CaM binding site (CBS) ^{336}SAIKYWVER^{344} located within the second intracellular loop of Orco is necessary for activation of this channel and for olfactory responses (Mukunda et al., 2014b). CaM is essential for facilitating the sensitization process in insect ORs (Mukunda et al., 2016). The manipulation of the cAMP level in heterologous expression system resulted in reduced odorant responses (Smart et al., 2008). Moreover, odor stimulations presented to the heteromeric OR complex in OSNs lead to cAMP production (Miazzi et al., 2016). In the absence of the OrX subunit these cAMP responses were totally diminished.

Disruption of intracellular signaling molecules, genes or proteins may alter functional properties like sensitivity of OR expressing OSNs and the behavior of *Drosophila*.

Therefore, regulation of insect olfactory responses by intracellular signaling including cAMP, PLC, G-proteins, PKC, inositol triphosphate (IP_3) plays an essential role.

1.2.2.3 Calcium homeostasis

The intracellular calcium (Ca^{2+}) management plays an important role in maintaining the activity of cells. Within the neurons free cytosolic calcium $[Ca^{2+}]_i$ is 50 to 100 nM but during neural activity calcium concentration may raise 100 fold (Berridge et al., 2000). The plasma membrane is equipped with different channels like voltage operated, receptor operated or store-operated Ca^{2+} channels. The activation of these channels initiates a Ca^{2+} influx into cells which rises $[Ca^{2+}]_i$ to 100 times. Besides this, internal stores may release Ca^{2+} by IP_3R and ryanodine receptors. In order to maintain cellular homeostasis, an overload with cytosolic Ca^{2+} has to be avoided for example by Ca^{2+} extrusion via the sodium calcium exchanger (NCX) (P. Blaustein and Lederer, 1999). In *Drosophila* OSNs, CALX is the homolog to NCX and extrudes Ca^{2+} extrusion upon odor stimulation (Halty-deLeon et al., 2018). Additionally, Ca^{2+} is extruded by plasma membrane Ca^{2+}. Moreover, the sarco/endoplasmic reticulum calcium ATPase pumps excessive Ca^{2+} into the sarco/endoplasmic reticulum. In addition, mitochondrial uniporter channels transport Ca^{2+} into the mitochondrial lumen. Moreover, there are specific proteins that bind free Ca^{2+} in nM range, as they contain low affinity binding sites with selectivity for Ca^{2+}. One of the sensory protein is the Ca^{2+} sensor protein calmodulin (CaM) that is ubiquitously expressed in cytosol. CaM has several functions to maintain Ca^{2+} in moderate amount. For example, it maintains the inflow and outflow of Ca^{2+} and helps to deliver the important information coming from Ca^{2+} to the target protein. CaM is a calcium sensing protein, it is mostly conserved and ubiquitously expressed in the cytoplasm of the eukaryotic organisms. It appears in the form of dumbbell shape in which N-terminal and C-terminal domain connected via a central linker (Babu et al., 1985; Babu et al., 1988; Barbato et al, 1992). At each domain it has Ca^{2+} binding EF hands where Ca^{2+} binding causes a conformational change in protein structure. This allows CaM in binding to peptide motifs on target proteins like receptors and ion channels.

1.2.2.4 Role of calcium in monitoring OR function

The regulation of Ca^{2+} levels within the OSNs is essential to transfer a reliable odor information from the external environment to the brain (Menini, 1999). With the discovery of imaging technique measuring the activity of olfactory responses by indirect calcium sensors became possible (Leinders-Zufall et al., 1997). Since ORs are Ca^{2+} permeable channels, their activity can be monitored by observing intracellular calcium concentration $[Ca^{2+}]_i$ when calcium sensor proteins like genetically encoded calcium protein (GECI) are expressed in the cells.

GCaMP6f is an ultra-sensitive calcium sensor and belongs to the GECI family. It has a circularly permutated fluorescence protein (cpGFP) that is bound to CaM and the M13 domain of a myosin light chain kinase (Nakai et al., 2001). At low $[Ca^{2+}]_i$, CaM don't interact with M13 of cpGFP, which results in a low basal fluorescence level (Fig. 6A). At high $[Ca^{2+}]_i$, Ca^{2+} binds to CaM and interacts with M13 peptide (Fig. 6B). Binding of Ca^{2+}-CaM-M13 complex to cpGFP causes conformational changes in cpGFP that in turns leads to a rise in fluorescence. With the help of the GAL4-UAS system, GCaMP6f can be expressed in specific subpopulations of neurons. By this way, changes in $[Ca^{2+}]_i$ linked to neural activity can be measured using Ca^{2+} imaging. Moreover, GECI can be used to measure the activity of various proteins like ion channels or GPCRs. Besides, it can also measure the single action potential responses and allows the measurement of synaptic calcium signals.

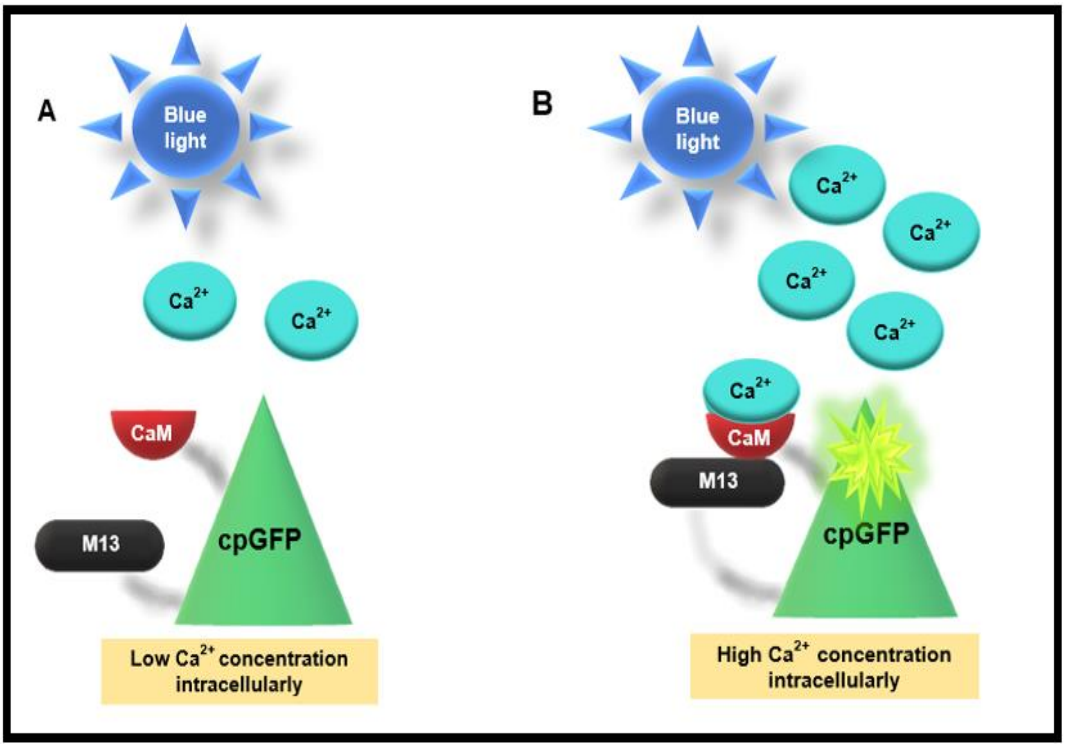

Figure 6: The GCaMP6f fluorescent indicator. GCaMP6f comprises circularly permutated green fluorescent (cpGFP), a CaM protein and M13 domain of the myosin light chain kinase (M13). **A.** At low $[Ca^{2+}]_i$ CaM and M13 do not interact with cpGFP leading to low basal fluorescence level when stimulated with blue light at 475 nm **B.** At high $[Ca^{2+}]_i$ constituent CaM and M13 interact with cpGFP and cause reversible conformational change of the cpGFP protein, this leads to fluorescent light emission.

1.2.3 Flying behavior in insects

The capability of an animal to smell myriads of substances depends on the performance of its olfactory system and determines the behavioral responses. Insects fly towards the air borne odors by navigating along the odor plume (Cardé and Willis, 2008). Flying insects display flight behaviors like upwind, take off, orientation and aversion depending on the type of presented odor stimuli. For example, *Drosophila melanogaster* (fruit flies) avoid flying towards fermented fruits which are intoxicated by microorganisms (Stensmyr et al., 2012). The odor geosmin is released from toxin-producing microbes and activates only one class of OR complex called Or56a/Orco in OSNs. The geosmin perception initiates an immediate avoidance behavior. fruit flies follow an odor plume in upwind direction by initiating take-off behavior to reach to the attractive odors like banana (Budick and Dickinson, 2006). Moreover, in the absence of a food source, they showed

anemotactic orientation. The hungry fruit flies show upwind flight behavior to reach towards food odors like vinegar in wind tunnel experiments (Becher et al., 2010). The yellow fever mosquito (*Aedes aegypti*) reaches the host by tracking CO_2 from long distances (Dekker et al., 2005).

The olfactory driven behavior in flying insects requires the optimal activity of OSNs in terms of OR sensitivity and speed to track odors in long distances. A study demonstrated that inhibition of Orco by the airborne OX1w antagonist impacted the olfactory behavioral responses in fruit fly larvae (Kepchia et al., 2017). In fruit fly larvae chemotaxis assay, the larvae were attracted by ethyl acetate, but in the presence of OX1w this attraction was abolished. Manipulation of intracellular *Drosophila* OR complex affected the odor localization in flying insects like fruit flies (Getahun et al., 2016). The authors show that Orco PKC mutant or mutation in PKC subtypes affects the odor localization in flies. The Orco lacking flies (Orco-/-) showed upwind flight, orientation behavior but failed to reach the odor source. Taken together, odor-guided behavior is essential for survival and reproduction in most animals like insects, however the genetic manipulation in the olfactory circuit affects the performance of fly in perceiving odor.

1.3 Goal of the thesis

Previous experiments using genetic and pharmacological approaches under *in vitro* and *ex vivo* condition showed that modulation of CaM affects the olfactory response in *Drosophila* OSNs (Mukunda et al., 2014b) (Mukunda et al., 2016) Therefore, the main aim of this study is to generate the Orco CaM flies using GAL4-UAS system and a genetic crossing scheme. The next task is to investigate the overall effect of CBS mutation within Orco protein of these flies by calcium imaging and behavioral experiments. By means of the calcium imaging method, the olfactory responses of these mutant flies are tested and compared with those of WT OSNs. As a control, WT OSNs are stimulated in the presence of the potent CaM inhibitor W7. In addition, sensitization experiments are performed on both WT and mutant OSNs. Finally, a wind tunnel bioassay is used to test the performance of Orco CaM flies in detecting and approaching the odor source.

2 Methods

2.1 Fly breeding

In this study, 4-8 days old *Drosophila melanogaster* were used. The flies were kept in vials containing cornmeal agar as the food source, where they feed, mate, lay eggs and become adult. To maintain many healthy generations, flies were flipped into new vials after every 7 days. The fly vials were maintained in an incubator, adjusted to 25˚C with 70% humidity and an alternating cycle of light and darkness, for 12h each.

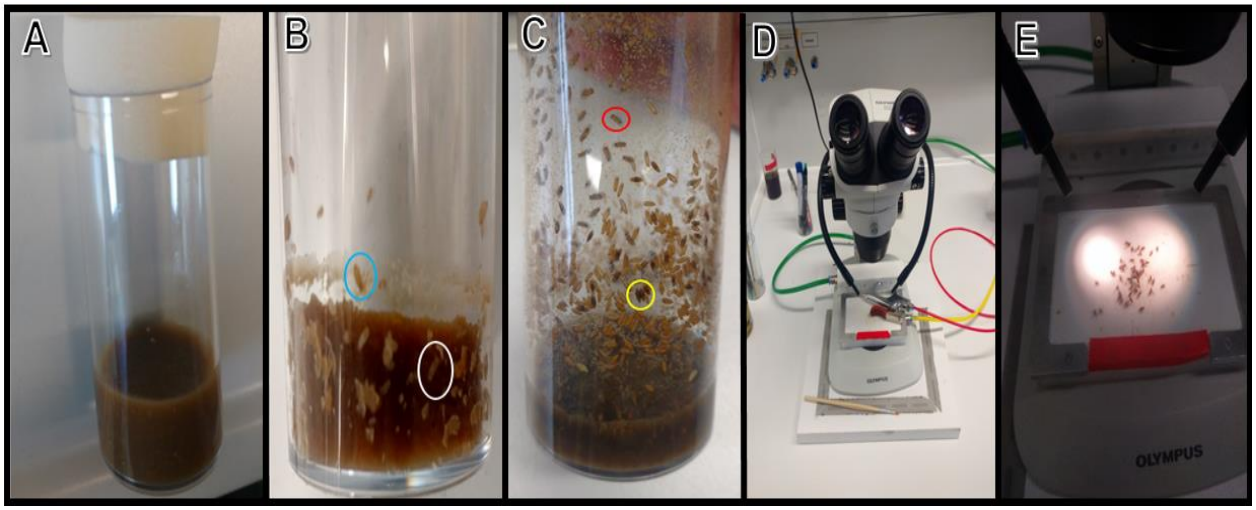

Figure 7: General view of Drosophila breeding. A. Fly vial with cotton plug. **B.** Developmental stages of *Drosophila* in a vial, for example: white circle indicates 3rd instar larval stage; blue circle shows pupal stage. **C.** Last stage of pupa shown in yellow circle, fly marked in red circle **D.** Stereo microscope to sort the flies according to sex, phenotype. **E.** Flies are anesthetized on CO_2 dispensing porous pad.

2.2 Generation of Orco CaM flies

The Orco CaM fly line was generated from (Table 1A). **1344** or **1345** that carry the OrcoK339N mutation, **593** had the GAL4 gene where Orco PKC was expressed in a modified Orco gene that was Orco-GAL4,Orco^{1}, **81** had double balancers which helped to segregate flies according to phenotypes (Bl; CyO; TM2;TM6B), **435** carried GCaMP6f which was important to mark neuronal populations. The transgenic fly line **1242** (Table 1B) which carried GCaMP6f in Orco expressing neurons was Orco wild type (Orco WT) used for Ca^{2+} imaging flies. The **Orco -/-** was a complete loss of Orco expression in the fly (Table 1C) , **Canton-S** fly lines that had Orco wild type expressed in their neurons was

Orco WT flies and Orco CaM fly line was generated using (Table 1A, except **435**), these fly lines were used to perform behavioral experiments.

Table 1: List of transgenic flies used in this study

	Stock number	Expression	source
A	1344	*w; UAS-OrcoK339N; +*	Lab stock
	1345	*w;UASOrcoK339N/CyO ; +*	Lab stock
	593	*W;UAS-OrcoPKC;Orco-GAL4,Orco^{1}*	Lab stock
	81	*W; BI/CyO; TM2/TM6B*	BL3704 (Bloomington)
	435	*W; UAS-GC6f; TM2/TM6B*	Lab stock
B	1242	*+ ; GCaMP6f/CyO ; Orco-Gal4/TM6B*	Chen(2013) Nature
C	99	*yw; +; Orco^{2}*	Larsson(2004)
D	Canton-S	*Orco WT*	Lab stock

2.2.1 Generation of Orco CaM flies for calcium imaging experiments

To understand the generation of Orco CaM fly line using genetic crossing scheme and GAL4-UAS system (Fig 8). Here two transgenic parental line (F_0), 593 and 81 (Fig. 8A) were taken. Red represents mother and blue represents father. These fly line carried certain phenotypic markers that were genetically linked and would been seen in progeny. The phenotypic markers were Bristle (short hair), TM2 (hair present on the halteres), TM6B (many hairs on their shoulders), CyO (curly wings). The 593 fly line was crossed with 81 fly line and the progeny obtained was selected with particular phenotypic marker to obtain progeny fly line F1 (Fig. 8A). Later progeny F1 (Fig. 8A) is crossed with F_0 435. The following F_2 (Fig. 8A) fly line was obtained with selected phenotypic markers like CyO; TM6B. Thus, the F_2 has a (w/w; UAS-GCaMP6f/CyO; Orco-GAL4, orco^{1}/TM6B). Likewise, two transgenic lines (593x81) and (1344x81) were crossed to obtain F_2 generation (Fig. 8B). The F2 transgenic fly line obtained from (Fig. 8B) was required gene of interest (w/w; UAS-OrcoK339N/CyO; Orco GAL4, orco^{1} / TM6B).

Later, F_2 virgin females (Fig 8 F_2A) (435x593x81) was crossed with males (Fig 8 F_2B) (593x81x1344x81) carrying the gene of interest. Finally the progeny F_3, OrcoK339N (point mutation in CBS of Orco protein) was expressed in modified Orco protein (*Orco-Gal4, Orco*^{1}) along with calcium sensor dye (GCaMP6f). The GCaMP6f was expressed in all the neurons carrying CBS mutation. Therefore, expression of GCaMP6f in particular tissue or gene allowed us to perform calcium imaging.

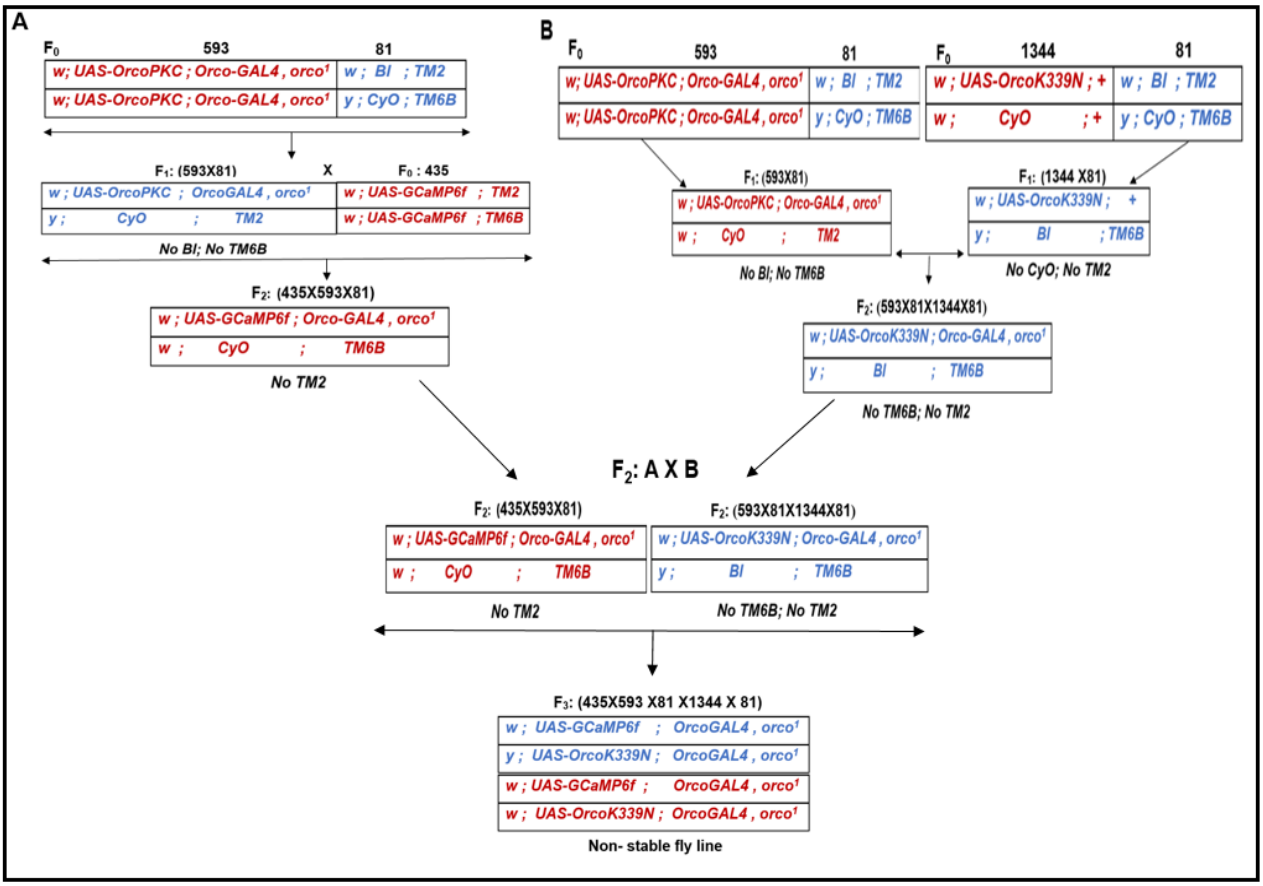

Figure 8: **Crossing scheme of Orco CaM flies for calcium imaging experiments**. Flies from **A** and **B** upon crossing generated F_2 flies. F_2 flies carry gene of interest. The virgin female flies of F_2 (A) (red) that carry UAS-GCaMP6f driven by Orco-GAL4, orco^{1} is crossed with male flies of F_2 (B) (blue) that expresses UAS-OrcoK339N driven by the Orco-Gal4, Orco^{1}. F_3 shows one copy of gene carry UAS- GCaMP6f driven by Orco-Gal4; Orco^{1} and another copy of gene carry UAS-GCaMP6f driven by Orco GAL4, orco^{1}. F_0= parent, F_1= 1st progeny, F_2= second progeny F_3= 3rd progeny. Mother (red), father (blue). CyO, TM6B, Bl, TM2 represents phenotypic markers. w= white eye. Y= male. UAS= upstream activating sequence. GAL4= a protein from yeast. OrcoK339N= mutation in putative CBS within Orco protein. orco^{1}= no presence of Orco. GCaMP6f a calcium indicator. Non-stable fly line represent final line cannot be crossed for next generation.

2.2.2 Generation of Orco CaM flies for wind tunnel experiments

The Orco CaM fly line was generated using (Fig. 9). F_0 593 line was crossed with F_0 81 and flies were selected against BI and TM6B in F1 generation. In similar way F0 1344 was crossed with F_0 81 and flies were selected against CyO and TM2 in F_1 generation. F_1 fly lines of (593x81) and (1344x81) were crossed together and were selected with CyO and TM6B in F_2 generation (593x81x344x81). Then males and females of F_2 (593X81x1344x81) carrying (UAS-OrcoK339N/CyO; Orco-GAL4, $orco^1$/TM6B) are crossed together. Finally, F_3 flies were collected without CyO and TM6B. This transgenic line carried overexpression of OrcoK339N in the modified Orco protein which were used in wind tunnel free fly bioassay.

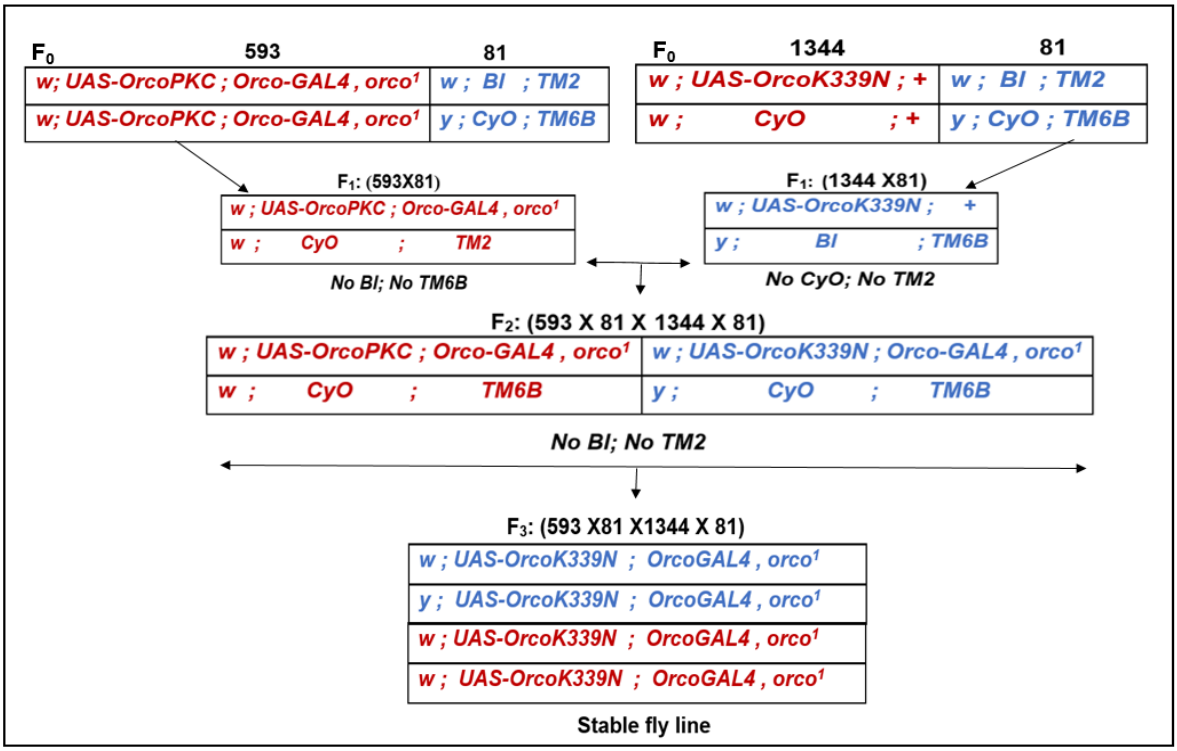

Figure 9: Crossing scheme of Orco CaM fly line for wind tunnel experiments. From F_0 flies generated carry gene of interest. Female flies of F_1 (593x81) expresses UAS-OrcoPKC driven by the Orco-Gal4, $Orco^1$ is crossed with male flies of F_1 (1344x81) that carry only UAS-OrcoK339N, +. F2 males and females obtained from F1 are crossed together, males and females carry UAS-OrcoK339N driven by OrcoGAL4, $orco^1$. F3 flies contains both copies of UAS-orcoK339N driven by OrcoGal4, orco1. F_0= parent, F_1= 1st progeny, F_2= second progeny F_3= 3rd progeny. Mother (red), father (blue). CYO, TM6B, BI, TM2 represents phenotypic markers. w= white eye. Y= male. UAS= upstream activating sequence. GAL4= a protein from yeast. OrcoK339N = mutation in putative CBS within Orco Protein. $Orco^1$= no presence of Orco. Stable fly line represent crossing are stable and kept for longer time.

2.3 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR is a molecular biological method that is used to detect the RNA transcript levels in the genome. The starting genetic material in the PCR reaction is RNA obtained from RNA extraction and purification methods (described in 2.3.1, 2.3.2). The RNA is first transcribed into its complementary DNA (cDNA) sequence by the reverse transcriptase enzyme (2.3.3). The new cDNA containing the reversed transcription is amplified using real-time PCR (2.3.4). The amplified gene product is visualized by the presence or absence of RNA fragment on agarose gel electrophoresis (2.3.5, 2.3.6). Furthermore, the presence of CBS mutation in the gene of Orco CaM flies is confirmed using DNA sequencing (2.3.7).

2.3.1 RNA extraction

30 WT and Orco CaM flies were placed in two empty vial. They were anesthetized by placing them on ice. Heads were separated using forceps in a chamber containing PBS solution. Next, they were transferred in a 1.5 ml micro centrifuge tube containing 1 ml of TriZol solution and washed gently using TriZol reagent. After that, brains were lysed in an appropriate volume of TRiZol reagent (600 µl) or a similar acid-guanidium-phenol reagent. They were homogenized by centrifuging at 10,000 x g for one minute and then the supernatant was transferred into an RNase-free tube.

2.3.2 RNA purification

An equal volume of ethanol (600 µl) was added to the supernatant solution and later it was mixed thoroughly. Then the mixture was transferred into a Zymo-spin^{TM} IIC Column in a collection tube. Subsequently, the mixture was centrifuged at 10,000 x g for 30s and solution was discarded. Next, the column was placed into a new collection tube. Subsequently, genomic DNA was removed using DNase I treatment and 400 µl of RNA wash buffer was added to the column matrix. Next, the mixture was centrifuged at 10,000 x g for 30s. After that, a mixture of 5 µl of DNase I (6 U/µl) and 75 µl of DNA digestion buffer was added in an RNase-free tube. Afterwards, the mixture was added to the column matrix and incubated at room temperature (RT) (20-30°C) for 15 min. Next, 400 µl of Direct-zol^{TM} RNA prewash buffer was added to the column, then it was centrifuged for

30s at 10,000 x g and later flow through was discarded. Later, 700 µl of RNA wash buffer was added to the column and centrifuged at 10,000 x g for 2 min. The column containing RNA was transferred to RNase-free tube. Further, RNA was extracted by adding 50 µl of DNase/RNase-free water to the column matrix. Finally, RNA column was centrifuged at 10,000 x g for 30s. RNA was used immediately for further steps. In table 2, A260/280 or A260/230 determines nucleic acid purity.

Table 2: RNA measurement using NanoDrop.

RNA	ng/µl	A260/280	A260/230
Range	1ng-5 µg	>1.80	>1.80
A. *Canton-S*	122.10 ng	2.09	2.19
B. *Orco CaM*	444.30 ng	2.18	2.59

2.3.3 First-strand cDNA synthesis system

Before starting the following protocol, all the components were briefly mixed and centrifuged (Table 3). The reaction mixture was prepared in 0.5 ml tube without adding RNA (Table 3). Later, 3 µl of isolated RNA from Canton-S (Table 2A) was added to remaining reaction mixture. Similarly, the protocol was followed for Orco CaM (Table 2B, Table 3). The RNA mixture of both the sample was incubated at 65°C for 5 min and kept on ice for 1 min.

Table 3: Components used for preparation of RNA reaction mixture.

Component	Amount
RNA	*n* µl
10mM dNTP mix	1 µl
Primer (0.5µg/µl$(Dt)_{12\text{-}18}$, or 2µM gene-specific primer)	1 µl
DEPC-treated water	to 10 µl

Table 4: 2X reaction mixture components.

Component	1 Rxn
10X RT buffer	2 µl
25mM $MgCl_2$	4 µl
0.1M DTT	2 µl
RNaseOUT™ (40 U/ µl)	1 µl

9 µl of 2X reaction mixture was prepared (Table 4) and then it was added to 10 µl of RNA mixture prepared previously in 2.3.3 (Table 3). It was mixed thoroughly and collected by brief centrifugation. Then, PCR reaction was followed by incubating it for 2 min at 42°C. Subsequently, 1 µl of SuperScript™ II RT was added to each sample and incubated for 50 min at 42°C. Finally, the sample reaction was terminated at 70°C for 15 min and kept immediately on ice. After brief centrifugation of samples, 1 µl of RNase H was added and incubated at 37°C for 20 min. Finally, cDNA samples were stored at -20°C.

2.3.4 PCR

Initially, all the components (Table 6) were brought down to RT except Advantage Taq polymerase which was a sensitive enzyme that would degrade easily. Then, the components were briefly vortexed and kept on ice. Next, PCR mixture was prepared in a sequential order (Table 6) except Advantage Taq polymerase. Later 1 µl of cDNA was added to 23.75 µl of PCR mixture and finally 0.25 µl of Advantage Taq polymerase was added.

Table 5: Orco gene primers (purchased from eurofins genomics)

Oligoname	Sequence (5'-> 3')	T_m [°C]
DmOrcoIntrSpan_fwd	GAAGAATTTCTACAGAACATTGAATATATGGAAC (34)	62,20
DmOrcoIntrSpan_rev	GCACCCAGAACCGAAGCAAACAAATC (26)	64,80

Table 6: PCR components

Component	Amount
H_2O	18.75 µl
buffer	2.50 µl
DNTPs	0.50 µl
Advantag *taq* polymerase	0.25 µl
DmOrcoIntrSpan_fwd	1 µl
DmOrcoIntrSpan_rev	1 µl

Furthermore, the PCR reaction mixture was placed in a thermocycler, the program was set to 35 cycles. The initialization step was started at 94°C for 5 min, which was followed by denaturation of cDNA at 94°C for 30s. The annealing of primers was carried out at 51°C for 30 s. Followed by elongation step at 68°C for 1.5 min. Final elongation was at 68°C for 5 min. Finally, holding temperature for PCR samples was at 4°C.

2.3.5 Gel electrophoresis

Gel electrophoresis was performed to separate size of each DNA/ RNA molecule. First, 1.5% agarose gel was prepared by dissolving 1.125g of Agarose in 75 ml of 1XTAE buffer. Subsequently, the agarose gel was heated at 800 watts to dissolve agarose completely and finally it was brought down to RT. Next, 7.5 µl of ethidium bromide (EtBr) (temperature sensitive) was added to the agarose gel and poured in an agarose chamber. Impression of wells were made on agarose gel and allowed the gel to set for 15 min. Then agarose gel was casted on PeQLab gel electrophoresis with 1XTAE buffer. Then, 4 µl of DNA ladder was added in the first well of agarose gel. Consequently, 25 µl of PCR reaction mixtures was loaded along with 5 µl of gel loading dye. The samples were run at 120 Volts for 30 min and visualized using UV trans illuminator.

2.3.6 Gel extraction

The gel fragments were excised from agarose gel using a scalpel by visualizing them under UV trans illuminator. Then, the gel fragment was placed in a clean 1.5 ml micro centrifuge tube and weighed by inferring the density to 1 g/ml. To it 1 volume of binding buffer (XP2) was added and incubated at 60°C for 7 min. Then HiBind DNA mini column

was placed into a 2 ml collection tube. Subsequently, 700 µl of gel fragment solution obtained from above step was added to the HiBind ^{R} DNA Mini column and was centrifuged at 10,000 x *g* for 1 min at RT. Then the filtrate was discarded and the above step was repeated. Later, 300 µl of binding buffer was added to the sample and was centrifuged at maximum speed (≥ 13,000 x *g*) at RT for 1 min. Then, filtrate was discarded and it was reused again. Further, 700 µl of SPW Wash buffer was added to the samples and they were centrifuged at maximum speed for 1 min. Later, the filtrate was discarded and collection tube was reused. SPW wash buffer step was repeated to transfer the samples completely. Finally, column was dried by centrifuging at maximum speed for 2 min. Then, column was placed on a clean 1.5 ml micro centrifuge tube and 30-50 µl of elution buffer was added. Incubated at RT for 2 min and centrifuged at maximum speed for 1 min.

Table 7: dsDNA measurement using NanoDrop

dsDNA	ng/µl	A260/280	A260/230
Canton-S	8.50	2.01	0.94
Orco CaM	29.20	1.88	1.12

Above two samples were prepared separately in 0.5 µl tube.120 ng of dsDNA, with 0.5 µl of gene specific primer was added in this tube. Finally, volume was made up to 6 µl using water (aqua bidest). These prepared samples were sent for Sangers sequencing to Eurofins MWG operon.

2.3.7 Confirmation of Orco CaM mutation (UAS-OrcoK339N; Orco-GAL4, orco^{1})

The presence of point mutation in the Orco CaM flies was confirmed using Sangers sequencing as shown in (Fig. 10) The consensus sequence was used as reference sequence (Fig. 10A). The black box represents the CaM binding site (SAIKYWER) which is a highly conserved amino acid sequence (Fig. 10A). The black circle from consensus sequence indicates lysine (K). Lysine was replaced by asparagine (N) shown in red circle in (Fig. 10B) using site directed mutagenesis. This further allowed me to perform behavioral experiments.

Figure 10: Genomic sequence of Orco CaM flies**. A. Consensus sequence B. The template strand. The black box indicates putative amino acid motif ^{336}SAIKYWER^{344} within the second intracellular loop of the Orco protein. The red box indicates mutant putative amino acid sequence. K = lysine (black circle). N = asparagine (red circle).**

2.4 Antenna Preparation

2.4.1 Stimulus solution and dilution

1. VUAA1 a known synthetic OR agonist was used in the experiments. It was synthesized by the group Mass Spectrometry/Proteomics of the Max-Planck Institute for Chemical Ecology (Jena, Germany).
2. W7 hydrochloride is a CaM inhibitor. It was purchased from Tocris Bio-science (Wiesbaden-Nordenstadt, Germany)

   A 100 mM of VUAA1 stock solution was prepared by dissolving it in dimethyl sulfoxide. Working solution (µM) range was prepared by diluting VUAA1 stock solution in the Ringer solution.

2.4.2 Preparation of *Drosophila* antenna

The Orco WT flies were collected in an empty vial using a transparent pipe and were used anesthetized by placing them on ice. A dissection microscope was used to visualize and dissect the flies accordingly. Initially in a petri dish, the head was sectioned using a needle, then antennae were excised and placed immediately in *Drosophila* Ringer solution containing (5 mM HEPES; 130 mM NaCl; 5 mM KCl; 4 mM MgCl2 .6H2O; 2 mM CaCl2 and 36 mM sucrose), 323 mOsmol/l and pH 7.3. All the reagents used in *Drosophila* Ringer solution were purchased from Sigma-Aldrich (Steinheim, Germany). On a glass slide, antennae were fixed in a vertical position using a two silicon component glue. Subsequently, 200 µl of *Drosophila* Ringer solution was added on to the antennae present on the glass slide. A micro scissor was used to section the funiculus of the antennae to the half of its length. Funiculus sectioning of the antenna allows us to view different types of olfactory sensory neurons (OSNs). OSNs can be observed using dissecting microscope by means of fluorescence light. The same procedure of antenna preparation was carried out for Orco CaM flies.

2.4.3 Ca^{2+} imaging method

Since ORs are also Ca^{2+} permeable channels, the Ca^{2+} imaging method can be used to monitor OR function. The changes in $[Ca^{2+}]_i$ were measured with the help of GECI (see 1.2.2.3). Excitation of Orco WT or Orco CaM OSNs was observed with an Axioskop FS microscope (Carl Zeiss, Jena, Germany).

Axioskop FS microscope (Carl Zeiss, Jena, Germany) was connected to a monochromator (Polychrome V, TILL Photonics, Gräfelfing, Germany) equipped with epifluorescence condenser having an objective lens (LUMPFL 10xW/IR/0.8; Carl Zeiss, Jena, Germany) and a water immersion objective (LUMPFL 60xW/IR/0.8; Olympus, Hamburg, Germany). It was controlled by an Imaging Control Unit (ICU, Till Photonics). The objective lens was used to locate the antennae that were embedded in two component silica with 200 µl of *Drosophila* ringer solution. After locating the antennae, 600 µl of *Drosophila* Ringer solution was added to visualize the internal structure of antenna using the water immersion objective lens (LUMPFL 60xW/IR/0.8; Olympus, Hamburg, Germany). With the help of a 490 nm dichroic mirror and a 515 nm long-pass

filter, emitted light was separated and filtered. A cooled CCD camera was controlled by TILL Vision 4.0 software (TILL Photonics) that captured fluorescent images of OSNs containing cellular compartments, somata, inner dendrite and outer dendrite shown in (Fig. 11). The size of each image was 640 x 480 pixels in a frame of 175 x 130 µm. GCaMP6f was excited at a 0.2 Hz frequency with an exposition time of 50 ms per cycle with 475 nm light. The response magnitude was calculated as the average $\Delta F/F_0$ in percentage as described in (Mukunda et al., 2014b).

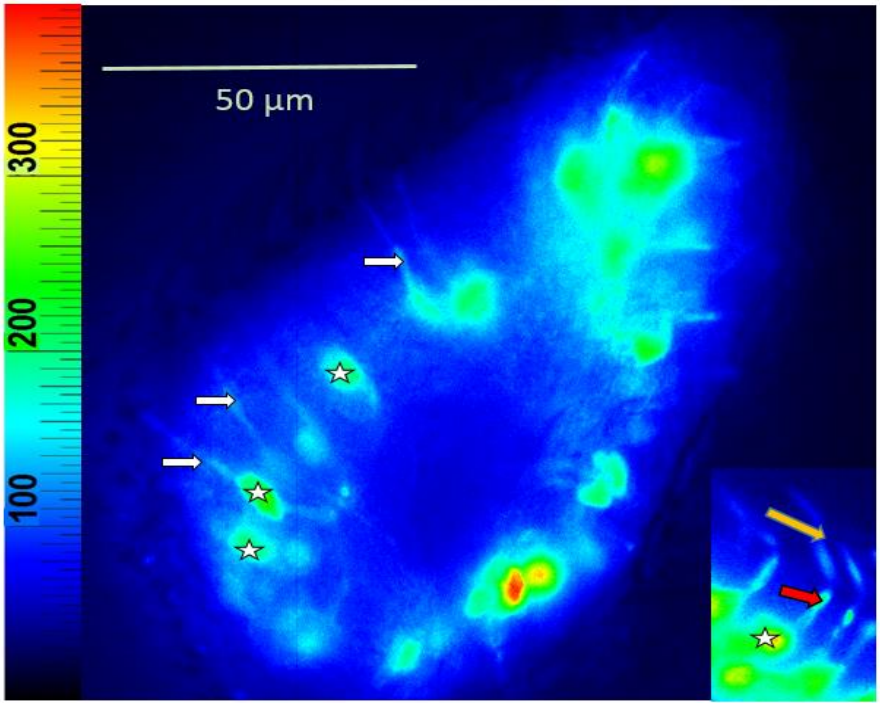

Figure 11: Fluorescent image of antennal 3rd segment (funiculus). Antenna preparation with GCaMP6f excitation by 475 nm light. White arrow indicates dendrites extending towards sensilla. Yellow star indicates somata (cell bodies of OSNs). The small picture on right corner shows three different cellular compartments of OSNs. Yellow arrow indicates outer dendrite, red indicates inner dendrite and yellow star indicates somata.

The TILL vision 4.0 software protocols (Table 8) were followed to stimulate/inhibit the Orco WT OSNs using agonist VUAA1 and antagonist W7 solutions (2.4.1). The protocol generally runs for 180 cycles comprising 5 seconds for 1 cycle. Orco WT OSNs were stimulated with VUAA1 at cycle 50. To perform dose response experiments (Table 8A) in the presence of CaM inhibitor W7 on Orco WT OSNs, W7 was applied to Orco WT OSNs at cycle 40, then VUAA1 was applied at cycle 50. The sensitization protocol was followed

for WT OSNs and mutant OSNs with VUAA1 stimulation at 50th and 65th cycle (Table 8B). For the dose response curves experimental chamber was a glass slide, where Ringer solution and stimulus solution were manually pipetted. For performing sensitization experiments, experimental chamber was a perfusion chamber (RC-27, Warner Instruments Inc., Hamden, CT, USA). Ringer solution was perfused continuously till the end of the protocol, but the stimulus solution was added manually.

Table 8: Ca^{2+} imaging protocols

Protocol types	Frames	Agonist solution applied at the frame	Ringer solution (µl)	VUAA1 concentration (µM)	Time s between stimulations
A Dose response curve	180	50th cycle	800	0 , 0.1 , 1 , 3 , 10 , 30 , 100 , 300 , 1000 , 3000	Stimulated only once
B Sensitization	180	50th cycle 65th cycle	1000	3	75

2.5 Free-flight behavior

2.5.1 Insects

The wild type flies (Orco WT) and negative control flies Orco$^{-/-}$(yw; +; Orco^{2}) were available to perform wind tunnel experiments. Orco CaM fly line (w; UAS-OrcoK339N; Orco-GAL4, orco^{1}) was generated (Fig 10). In this study, four days old flies irrespective of their sexes were anesthetized by puffing brief carbon dioxide into the fly vial and then they were collected in an empty vial. On the fifth day females were separated in a new food vial. A few h later female flies were put in an empty food vial stuffed with moist cotton and starved at RT for 18 h.

2.5.2 Odors

Two types of attractive fruit odor mixtures were used in this experiments.

Vinegar (Aceto balsamico di modena 1.G.P (500 ml)): vinegar was diluted at different concentrations using water. Grape juice (Merlot-Traubensaft): pure grape juice was used in my experiments.

2.5.3 Wind tunnel

The rectangular shaped wind tunnel assay has a flight section of 30 × 30 × 100 cm in size, made up of glass material. Wind tunnel chamber was placed in daylight bioassay room at a temperature of 28°C, 42% of relative humidity and bright light (LED) in the room. The upwind and downwind end of the wind tunnel was covered with polyamide mesh (pore size 0.5 × 0.5 mm; Sintab, Oxie, Sweden). Polyamide mesh allowed passage of laminar air flow at 0.3 m/s throughout the tunnel by fan (Fischbach GmbH, Neukirchen, Germany) and air was filtered through activated charcoal (14.5 cm diam. × 32.5 cm long; Camfil, Trosa, Sweden). In this study 15 replicates of each fly line, WT flies, Orco CaM flies and orco -/- flies using grape juice and vinegar were performed. Before starting wind tunnel experiments, the starved flies were kept for one h in daylight assay room to introduce them into new RT conditions. One h later, ten flies were placed in an empty vial that is considered to be one replicate. Subsequently, 100 µl of diluted odor was pipetted on to a filter paper. Then, the filter paper was placed on a 12 cm tall metallic stand using two magnets that can hold filter paper tightly. From upwind end of the wind tunnel, filter paper stand was placed on one side. At the other side, ten female flies in a vial (fly replicate) were placed on metallic stand which was 70 cm away from filter paper stand. Each fly replicate was released into the chamber and number of flies reaching the odor source was counted in period of ten min. Flies were removed from the wind tunnel chamber using vacuum cleaner after the end of every replicate. Then the number of flies reaching the odor source was calculated in terms of percentage. If five flies reached the odor source out of 10 flies, then landing percentage of flies will 50%. 15 replicates were calculated in terms of average of every replicate scores multiplied with 100. The obtained values were plotted on Graph Pad Prism 4.

2.6 Data analysis

Data were analyzed using MS Excel and Prism 4 software (Graph Pad Software, Inc.; LaJolla, CA, USA).

3 Results

3.1 Odor response sensitivity in Orco CaM OSNs

In a first attempt, Ca^{2+} imaging experiments were performed on OSNs in an open antenna preparation of *Drosophila melanogaster* (2.4.2 & 2.4.3). As ORs are Ca^{2+} permeable channel, their opening causes Ca^{2+} influx and rises $[Ca^{2+}]_i$. The GCaMP6f labelled OR expressing OSNs were stimulated with VUAA1 that caused changes in GCaMP6f fluorescence response ($\Delta F/F_0$). The GCaMP6f which upon agonist stimulation thus reflects channel opening.

The fluorescence responses were determined for Orco WT (Fig 12A in blue) and Orco CaM (Figure 12A in orange) OSNs after the application of VUAA1. The fluorescence peak was reached within few seconds after agonist stimulation. Few seconds later the fluorescence response declined which could be described by a mono exponential decay characterized by time constant τ. Compared to Orco WT the maximum fluorescence response (Fig. 12B) was significantly reduced in the Orco CaM OSNs, but the decay was not affected (Fig. 12C).

The next approach was to investigate the stimulus concentration-dependence of the OR response (Fig. 13). Compared to Orco WT OSNs (Fig. 13 in blue), stimulation of Orco CaM OSNs with VUAA1 did not significantly shift the sensitivity (Fig. 13 in orange) but the efficacy of the fluorescence response was affected.

Finally, we have compared the effect of CBS mutation on Orco WT OSNs in the presence of pharmacological CaM inhibitor (W7). Stimulation of Orco WT OSNs with VUAA1 in the presence W7 (Orco WT-W7), reduced the fluorescence response similar to Orco CaM OSNs . Then, stimulus concentration-dependence of the OR response in Orco WT, Orco WT-W7 was compared. Compared to Orco WT OSNs, there was no significant shift in sensitivity of Orco WT-W7 OSNs with VUAA1 stimulation (Fig. 13 in black) but the efficacy of fluorescence response was decreased.

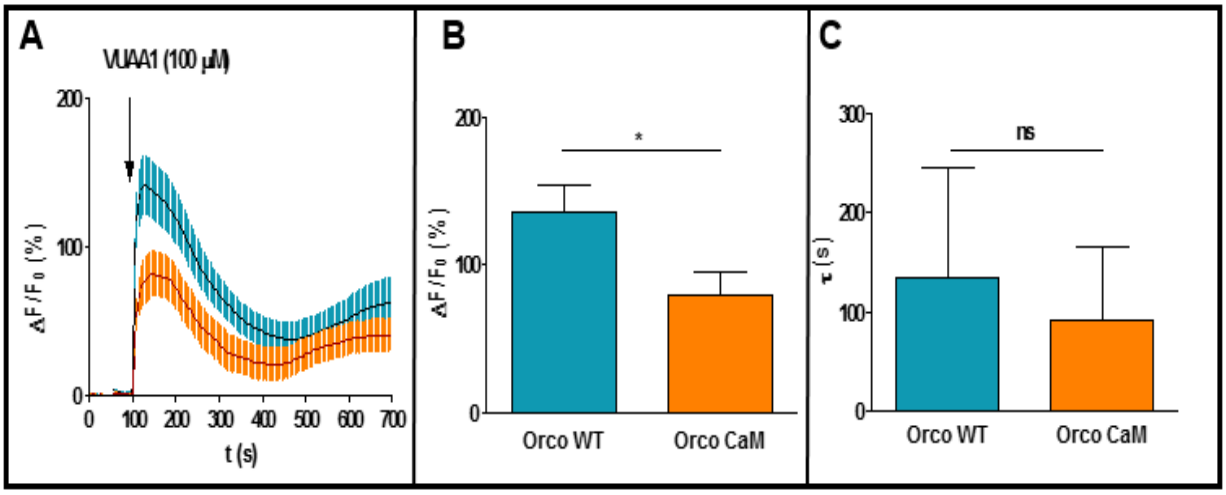

Figure 12: Time course, maxima and decay time constant of fluorescence responses for Orco WT (blue) and Orco CaM (orange) OSNs. A. Time course of OSN responses as change in GCaMPf6 fluorescence (%). Black arrow: 100 µM concentration of the VUAA1 application. **B.** Maximum fluorescence responses. **C.** Time course of fluorescence decay expressed in seconds τ (s). The maximum fluorescence response was obtained by subtracting the basal intensity from maximum intensity. Time course τ (s) fits mono exponential decay curve determined between t= 150 s and 400s. Graphs represent mean ± SEM. Two – tailed unpaired t-test *p < 0.05, ns not significant.

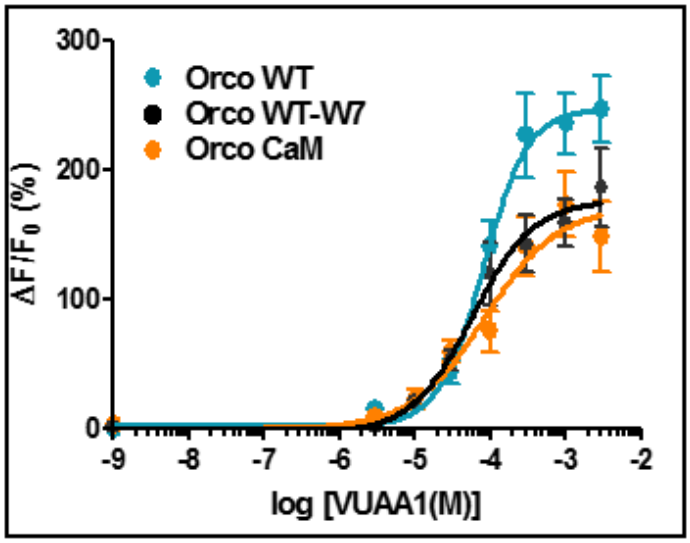

Figure 13: Concentration dependence of calcium response of the Orco WT (blue); Orco WT-W7 (black); Orco CaM (orange) upon VUAA1 stimulation. Best fitted curves shown in different colors are described by sigmoidal dose-response curves with given datasets. Hill coefficient of Orco WT: 1.473, Orco CaM: 0.8958, Orco WT-W7:1.074 EC_{50} for Orco WT: 81 µM, Orco CaM: 85 µM, Orco WT-W7: 5.8 µM. Log concentration of synthetic agonist (VUAA1) on X axis. The change in fluorescence response (ΔF/F0 (%)) on Y-axis. Dotted points are mean±SEM; 8≤ n≤ 10, n= number of antennae.

3.2 Sensitization in Orco CaM OSNs

The capability of OSN to sensitize, i.e. to respond to repeated weak stimulation with increasing strength of olfactory response was compared between Orco CaM and Orco WT flies. The stimuli used to sensitize OSNs have to be applied at near subthreshold concentration of VUAA1 (Mukunda et al., 2016). They were taken from the concentration response curve (Fig. 13). In addition, sensitization requires odor stimulation in a suitable time frame (Mukunda et al., 2016). VUAA1 was applied at cycles 50 and 65, the duration between two stimuli was 75 seconds. In Orco WT OSNs (blue) sensitization was observed in somata (Fig. 14 A and 14B), inner dendrite (Fig. 15A and 15B) and outer dendrite (Fig. 16A and 16B). However, no sensitization was seen in these compartments of Orco CaM OSNs. Which means sensitization was not observed in somata (Fig. 14C and 14D), inner dendrite (Fig. 15C and 15D) and outer dendrite (Fig. 16C and 16D). But the first fluorescence response in inner dendrite of Orco CaM OSNs (Fig.15C and 15D) was comparatively higher than first responses in other two cellular compartments of Orco CaM OSNs.

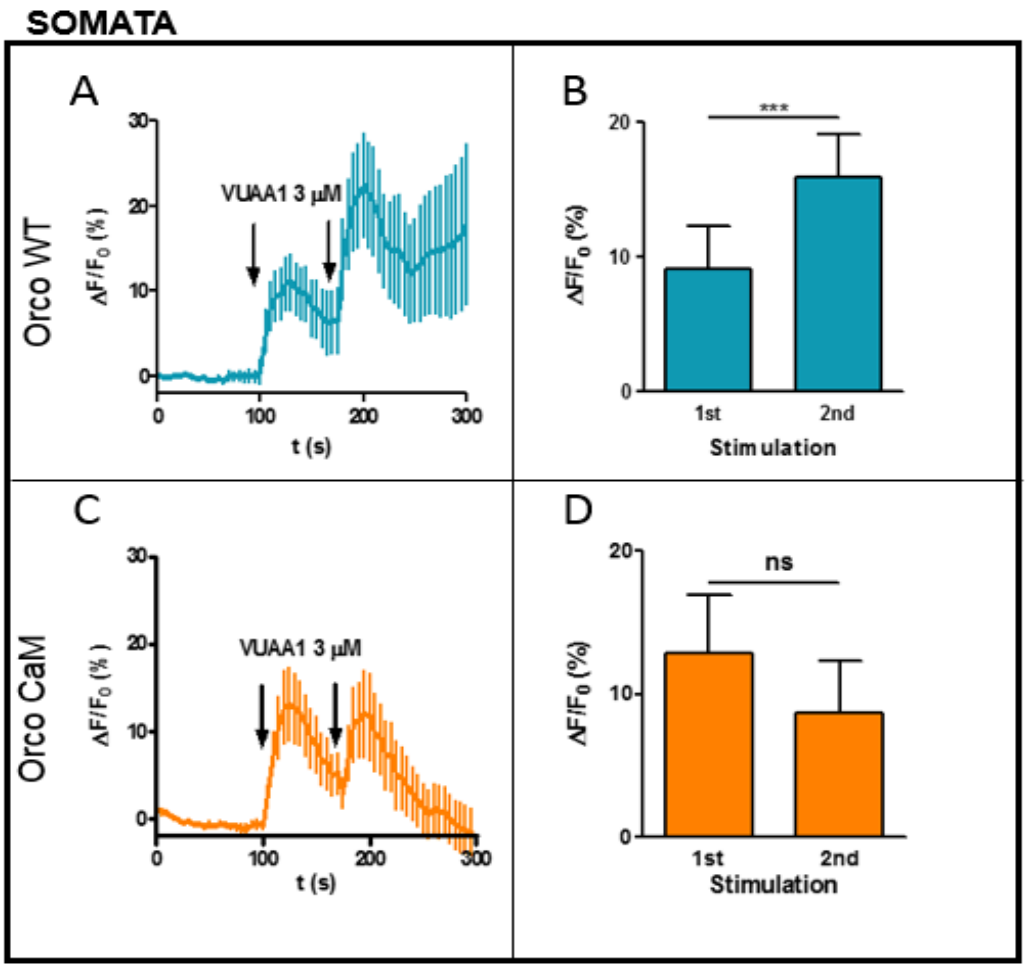

Figure 14: Sensitization in somata of Orco WT (blue) and Orco CaM (orange). A&C time course of maximum response. **B&D** maximum fluorescence response. Black arrow: 3 µM VUAA1 application at 50th cycle and 65th cycle. Graphs represent mean ± SEM. Two–tailed, unpaired t-test ***p < 0.001, ns= not significant.

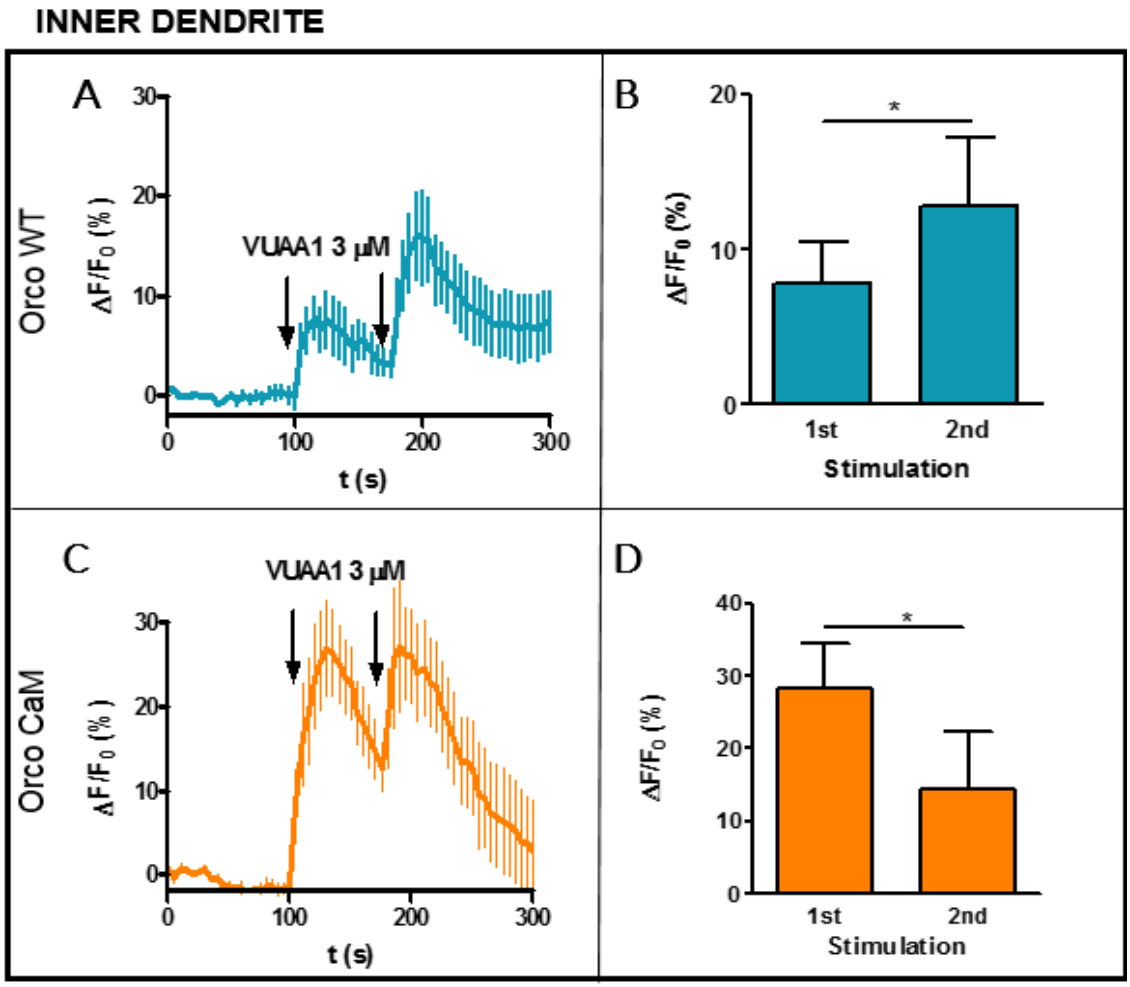

Figure 15: Sensitization in inner dendrite of Orco WT (blue) and Orco CaM (orange). A&C time course of maximum response. **B&D** maximum fluorescence response. Black arrow: 3 µM VUAA1 application at 50th cycle and 65th cycle. Graphs represent mean ± SEM. Two–tailed unpaired t-test *$p < 0.05$.

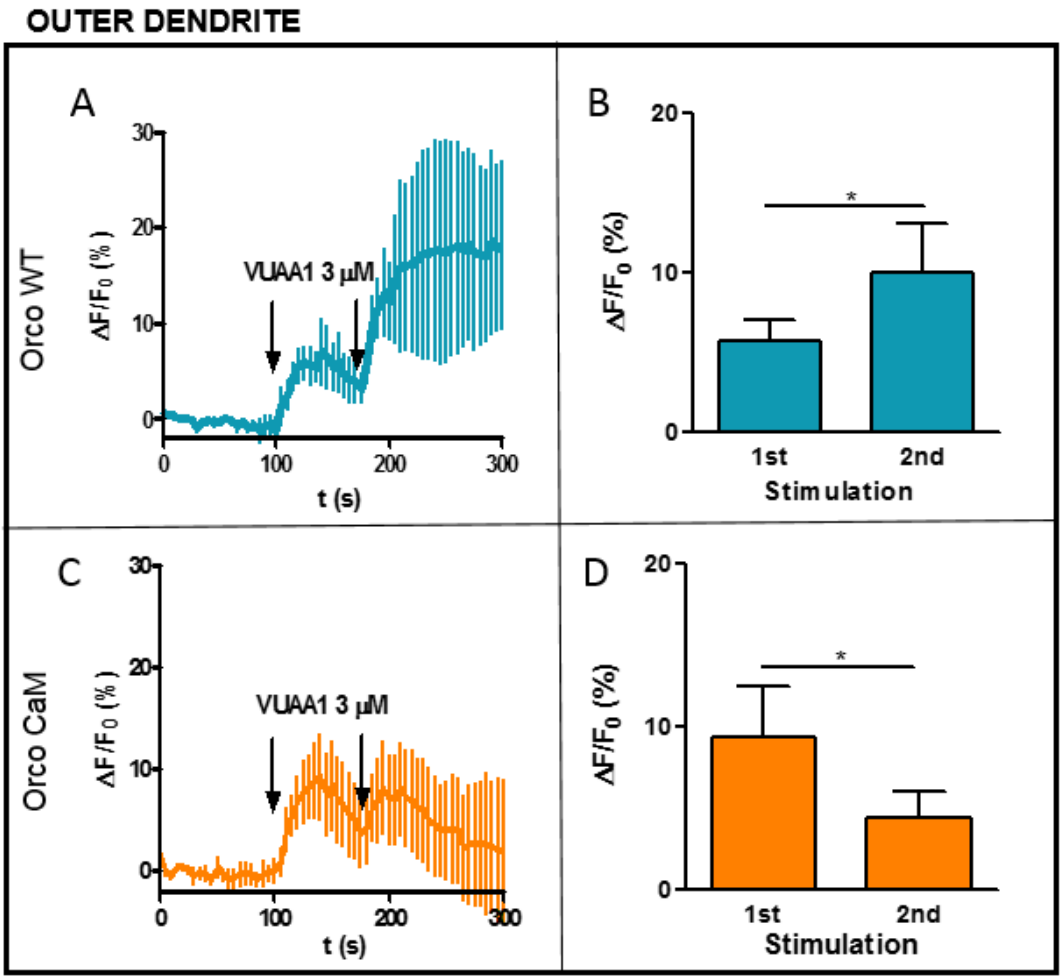

Figure 16 Sensitization in outer dendrite of Orco WT (blue) and Orco CaM (orange). **A&C** represents A&C time course of maximum response. **B&D** the maximum fluorescence response. Black arrow: 3 µM VUAA1 application at 50th cycle and 65th cycle. Graphs represent mean ± SEM. Two–tailed unpaired t-test *$p < 0.05$.

3.3 Flight performance of Orco CaM flies

In this approach we asked whether the change in the olfactory performance of the OSNs in Orco CaM flies would also have behavioral effects and might affect the flight performance. For this sake, wind tunnel bioassay was used to test long range attraction of Orco WT (Canton-S), Orco CaM and Orco -/- female flies.

The hungry Orco WT flies and Orco CaM flies forage towards the odor source by performing certain flight behavior. The hungry Orco WT female flies showed immediate upwind flight behavior in the direction of the vinegar plume, finally within few minutes nearly 50 % of flies reached the odor source (Fig. 17A in blue). Whereas impaired Orco CaM flies exhibited initial search behavior towards vinegar but only 12 percent of the Orco CaM flies were able to locate vinegar (Fig. 17A in orange).

The vinegar contains a high amount of acetic acid, a small amount of other chemical compounds and water. The fly might be able to sense acids present in vinegar due to the presence of IRs (Silbering et al., 2011). To exclude the contribution of IRs, fruit juice was chosen as it contain chemical compounds like esters, alcohol that activate ORs (Silbering et al., 2011). Therefore, in this study grape juice was used as an attractive odor which was tested for long range attraction. 62% of Orco WT female flies surged forward, turned upwind and increased their flight speed to reach towards the grape juice (Fig. 17B in blue). By contrast Orco CaM flies also showed similar behavior like WT flies, but only 10 percent of the Orco CaM flies were able to reach the odor source (Fig. 17B in orange).

The flies lacking Orco protein (orco-/-) exhibited anemotaxis behavior but only 4% of the flies were able to reach vinegar (Getahun et al., 2016). In this study (orco-/-) flies were tested using grape juice and only one percent of the fly reached grape juice odor (Figure not shown).

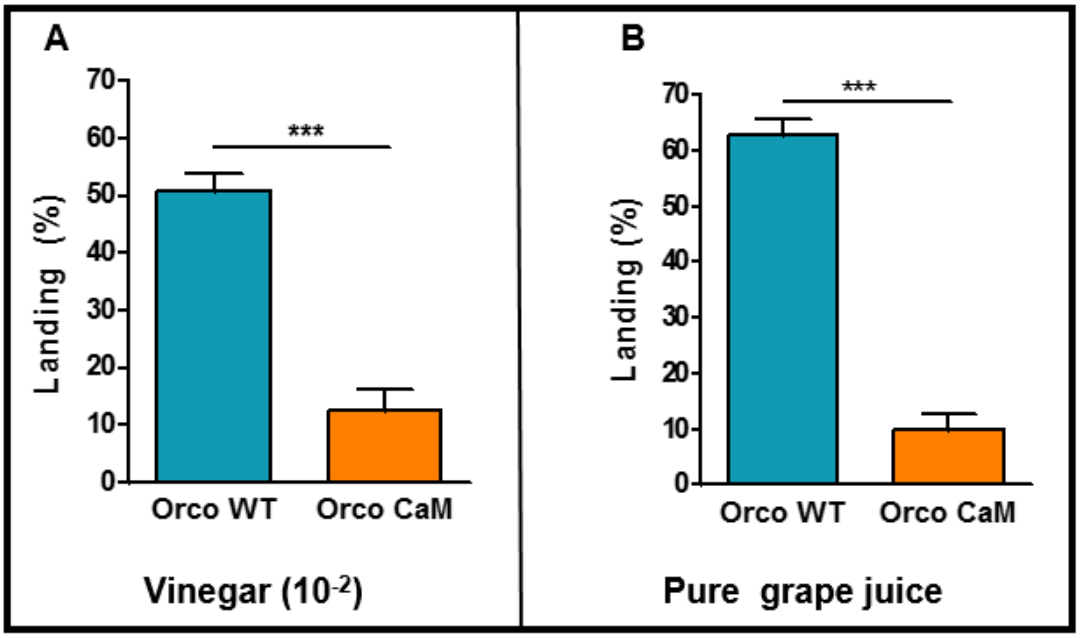

Figure 17: Flight performance of Orco WT flies (blue) and Orco CaM flies (orange). A. Landing percentage of Orco WT and Orco CaM on vinegar at 10^{-2} concentration **B.** Landing percentage of Orco WT and Orco CaM flies on pure grape juice. Graph represents mean±SEM, two tailed unpaired *t*-tests *** $p<0.001$. n = 15

4 Discussion

4.1 CBS mutation and odor responses

As presented earlier in chapter (1.2.2.2), several *in vitro* and *in vivo* studies demonstrated that intracellular signalling enzymes like AC, PKC, and PLC play an important role in maintaining the sensitivity of Orco. Abolishing the activity of these enzymes either by pharmacological means or genetic manipulation reduces the olfactory responses to odorant stimulation. Likewise, in another study it was shown that CaM plays a role in maintaining the function of Orco. Previous experiments with pharmacological CaM inhibition of heterologously expressed *Drosophila* ORs revealed quite variable effects on the odor response (Mukunda et al., 2014b). There were no significant differences in the responses for Or33a/Orco and Or47a/Orco receptor complexes whereas Or22a/Orco and Or56a/Orco were affected.

In the present study a fly line, Orco CaM was generated. The OSNs in this line were equipped with the point-mutated CBS within the Orco protein. Putative CaM binding sites in the OrX proteins which could have been affected by pharmacological CaM inhibition in the previous approach (Mukunda et al., 2014b) remained accessible as in Orco WT flies here.

That means, pharmacological CaM inhibition in *in-vitro* or *ex-vivo* studies can affect the whole *Drosophila* OR complex. For example, CaM inhibition of Or22a/Orco protein by W7 in heterologous expression system (Mukunda et al., 2014b) or in *ex vivo* preparations reduced the calcium responses to VUAA1.

By contrast, genetic manipulation in CBS of Orco solely affects the function of the Orco protein but not of the OrX protein. The stimulation of Orco CaM OSNs with VUAA1 showed reduction in calcium responses. This is surprising as OrX proteins are unaffected. On other hand, CaM affects OrX in complex and diverse manner (Mukunda et al., 2014b). So, these variable effects might neutralize each other in average. In line with this is the intriguing finding is the sensitivity of Orco CaM OSNs was not shifted. A possible reason might be the presence of different types of receptors in the OSNs where only some receptors might be affected by the CBS mutation in Orco.

Interestingly, the reduction in fluorescence responses in Orco CaM OSNs were similar to CHO cells solely expressing Orco upon CaM inhibition (Mukunda et al., 2014b). Since the CBS mutation in the Orco protein can cause dysfunction of Orco channel, this may finally lead to a decrease in olfactory responses.

Testing the effect of Orco CaM mutant in OR expressing flies to a specific odor can determine sensitivity of OrX protein. But the complexity arises due to the presence of mixture of OR expressing neurons in antennae of the animal. Therefore, genetic tools can be applied to express specific receptor type along with mutated Orco protein in that particular sensillum type of *Drosophila* antennae. By this way the effect of CaM can be studied on a specific OrX/Orco complex.

4.2 CBS mutation and sensitization

The process of sensitization which involves metabotropic signaling was previously described in (Getahun et al., 2013). The authors showed in extracellular single sensillum recordings on large basiconic sensilla expressing Or22a/Orco complex that repeated subthreshold odor stimulation led to an increase in OSN activity when the interval between stimuli was at least 10 s. But the injection of an AC inhibitor in Or22a/Orco expressing sensilla abolished this sensitization to repeated subthreshold odor stimulation. In the Orco PKC mutant, where mutations were carried out in the five PKC phosphorylation sites within the Orco protein (Orco PKC) (Sargsyan et al., 2011), these Orco PKC OSNs failed to show sensitization to repeated subthreshold odor stimulation (Getahun et al., 2016).

Another study demonstrated that incubation of Orco/Orco, Or22a/Orco and Or56a/Orco expressing CHO cells with the CaM inhibitor W7 abolished sensitization upon repeated VUAA1 stimulation. Similarly, in CBS mutated Orco/Orco expressing cells VUAA1 stimulation did not lead to sensitization (Mukunda et al., 2016). This shows the importance of the CBS within the 2nd intracellular loop of Orco protein for OR sensitization. Taken together, these studies show that proper Orco function is necessary for sensitizing ORs.

In addition, the role of CaM on OR sensitization was also tested on native *Drosophila* OSNs. The use of heterologous expression system can determine the function of receptor complexes but not the properties of native neuron. An *ex vivo* preparation was examined

to understand the role of sensitization in different cellular compartments of the native OSNs. Agonist stimulation (VUAA1) and ligand stimulation (Ethyl hexanoate) of OSNs expressing the Or22a/Orco complex in connection with pharmacological CaM inhibition was tested (Mukunda et al., 2016). They showed that repeated VUAA1 stimulation of Or22a/Orco expressing OSNs in the presence of W7 showed no sensitization in somata and in the outer dendritic region of OSNs, but a residual sensitization was seen in the inner dendrite. Contrastingly, ligand stimulation of the Or22a/Orco complex in the presence of W7 showed no sensitization in outer dendrite. That means properties of OSNs also depend on the type of ligand stimulation.

From the two studies it is known that OR sensitization is mediated via Orco activation and modulators of Orco is necessary for the process of sensitization. In (Mukunda et al., 2016) the authors show that the whole OR complex is affected upon pharmacological inhibition. Here we investigate the effect of repeated agonist stimulation in a situation where only the Orco protein is affected by CBS mutation. We show that repeated VUAA1 stimulation leads to sensitization in all three compartments of Orco WT OSNs. However, in Orco CaM OSNs sensitization was not seen in all three compartments. In the inner dendritic segment of Orco CaM OSNs, though sensitization was not seen but the first response was already very strong. So it cannot be excluded that the reduction of the second response might be caused by adaptation. The inner dendrite comprises intracellular compartments like mitochondria and endoplasmic reticulum. The release of Ca^{2+} from these internal stores could have resulted in increased calcium responses in inner dendrite segment (Mukunda et al., 2016).Taken together, the effect of CBS mutation is assumed to be different in all the cellular compartments of OSNs. Therefore, it will be interesting to further investigate whether this is the property of the neuron or the receptor type what affects the process of sensitization. Future investigation can be done to test the effect of CBS mutation on OSNs expressing only one type of OR complex. This would determine the role of CaM in sensitizing ORs.

4.3 CBS mutation and flight performance

In a final approach, a wind-tunnel bio assay was used to investigate the effect of CBS mutation on insect's ability to fly towards the food odors. Various studies have shown that IR expressing neurons are involved in detecting amines, food odors, sugar, salt, acids but ORs expressing neurons are only involved in detecting food odors (Gomez-Diaz et al., 2018). The one volatile compound from vinegar, acetic acid, induces upwind flight attraction in *Drosophila* flies in wind tunnel experiments (Becher et al., 2010). In this study, thus vinegar was used as a long range attractive odor.

Mated female Orco WT flies exhibited spontaneous take off and upwind flight behavior, where 52% of the Orco WT flies reached the vinegar source. But in case of mated female Orco CaM flies, they exhibited upwind flight behavior but only 12% of the flies reached the vinegar. Thus, the single amino acid replacement disrupted the performance of Orco CaM flies in reaching the odor. That still a few flies were able to reach to the odor might be due to the presence of IRs. Moreover, the odor-evoked behavioral response also depends upon internal state of animal, like extent of being hungry, thirst, stress, circadian period (Huetteroth and Waddell, 2011). Since mated female flies were carrying eggs, they needed energy to oviposit by feeding on food source. Compared to Orco WT flies, very few Orco CaM flies were hovering near the odor source to lay their eggs, thus this was also due to CBS mutation.

We have also tested pure grape juice in long range attraction because it was known that overripe fruits or fermented fruits activates OR in the OSNs (Stensmyr et al., 2003). Nearly 60% of Orco WT flies showed immediate flight response to reach to the grape juice. But the Orco CaM mutant flies showed anemotaxis but only 10% reached the grape juice. Further, a comparison was carried out in Orco WT flies for landing percentage in vinegar and grape juice but there was no significant difference between two odors. Thus we can exclude a disturbing role of IRs. As in both, grape juice and vinegar, the landing percentage of Orco CaM flies was significantly reduced compared to Orco WT flies, this reduction is due to the effect of CBS mutation.

A previous study showed the effect of PKC subtype mutation and Orco phosphorylation in odor evoked behavioral responses using different behavioral assays (Getahun et al., 2016).The mutation was carried out in five PKC binding site within the Orco protein (Orco

PKC) and two more transgenic lines were generated, PKC53E RNAi and PKC δ RNAi. PKC53E or PKC delta targets the odor specific protein OrX, PKC based Orco phosphorylation and they are important for OrX-Orco signal transduction. Orco mut, PKC53E, PKC delta flies showed upwind flight and were orienting towards the source but very few flies reached the odor source in comparison to WT flies. WT flies showed upwind anemotactic flight behavior and 50% of them reached the source. Therefore, disruption of PKC signaling and Orco phosphorylation in OR-expressing OSNs of *Drosophila* reduces the peripheral sensitivity to trigger odor evoked behavioral responses. Orco CaM flies also showed upwind flight and orientation behavior but very few of them reached the source. That means, the CaM binding region within the Orco protein is necessary for odor induced long range attraction.

Taken together, these findings suggest that CBS within the Orco protein of the fruit flies is also essential to localize the odor in long range attraction, like the role of PKC phosphorylation was seen for long range attraction. Intriguingly, a single point mutation (lysine (K) to asparagine (N) replacement) within the CaM binding region of the Orco protein is sufficient to impact the overall performance of Orco CaM flies in localizing odors. And this effect is even stronger compared to the five amino acid mutation in Orco phosphorylation sites (Getahun et al., 2016).

4.4 Conclusion and outlook

In this thesis we investigated the role of the conserved calmodulin binding site (CBS) SAIKWYVER present within 2nd intracellular loop of Orco protein in native *Drosophila* OSNs. In Orco CaM OSNs, activation of ORs by the synthetic agonist VUAA1 resulted in a decrease in olfactory response with respect to Orco WT OSNs. As well, repeated threshold stimulation on Orco CaM OSNs showed no sensitization in somata, inner dendrite and outer dendritic region. Moreover, odor localization of the mutant fly was extremely impaired in locating the odor source. Therefore, the intracellular regulation of Orco function plays a vital role in physiology and behavioral ecology. The results support the notion that the CBS within second intracellular loop of Orco protein is necessary for maintaining the activity of Orco channel, Ca^{2+} influx, olfactory responses, sensitizing ORs and behavior of the fly.

We further suggest to investigate the effect of CBS mutation in a specific OrX/Orco complex expressed in native *Drosophila* OSNs. In this way, we might know if the sensitivity of the specific CBS mutated OrX/Orco is affected to ligand stimulation and synthetic agonist stimulation. Previous sensitization experiments carried out in Or22a/Orco complex were restricted to pharmacological inhibition of the whole OR complex. In the suggested approach sensitization can be investigated in a CBS mutated specific OrX/Orco complex. By this way it can be determined, if the CBS mutation affects all three cellular compartments or not. Extracellular single sensillum recording (Pellegrino, M., Nakagawa, T., & Vosshall, L. B.2010) can be performed on CBS mutated OR complex expressing OSNs. This can show how CBS mutation can impair the fly's ability to smell by studying the electrophysiological properties of specific CBS mutated OrX/Orco complexes.

References

Abuin, L., Bargeton, B., Ulbrich, M.H., Isacoff, E.Y., Kellenberger, S., and Benton, R. (2011). Functional architecture of olfactory ionotropic glutamate receptors. *Neuron* 69**,** 44-60.

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., George, R.A., Lewis, S.E., Richards, S., Ashburner, M., Henderson, S.N., Sutton, G.G., Wortman, J.R., Yandell, M.D., Zhang, Q., Chen, L.X., Brandon, R.C., Rogers, Y.-H.C., Blazej, R.G., Champe, M., Pfeiffer, B.D., Wan, K.H., Doyle, C., Baxter, E.G., Helt, G., Nelson, C.R., Gabor, G.L., Miklos, Abril, J.F., Agbayani, A., An, H.-J., Andrews-Pfannkoch, C., Baldwin, D., Ballew, R.M., Basu, A., Baxendale, J., Bayraktaroglu, L., Beasley, E.M., Beeson, K.Y., Benos, P.V., Berman, B.P., Bhandari, D., Bolshakov, S., Borkova, D., Botchan, M.R., Bouck, J., Brokstein, P., Brottier, P., Burtis, K.C., Busam, D.A., Butler, H., Cadieu, E., Center, A., Chandra, I., Cherry, J.M., Cawley, S., Dahlke, C., Davenport, L.B., Davies, P., Pablos, B.D., Delcher, A., Deng, Z., Mays, A.D., Dew, I., Dietz, S.M., Dodson, K., Doup, L.E., Downes, M., Dugan-Rocha, S., Dunkov, B.C., Dunn, P., Durbin, K.J., Evangelista, C.C., Ferraz, C., Ferriera, S., Fleischmann, W., Fosler, C., Gabrielian, A.E., Garg, N.S., Gelbart, W.M., Glasser, K., Glodek, A., Gong, F., Gorrell, J.H., Gu, Z., Guan, P., Harris, M., Harris, N.L., Harvey, D., Heiman, T.J., Hernandez, J.R., Houck, J., Hostin, D., Houston, K.A., Howland, T.J., Wei, M.-H., et al. (2000). The Genome Sequence of Drosophila melanogaster</em&gt. *Science* 287**,** 2185.

Becher, P.G., Bengtsson, M., Hansson, B.S., and Witzgall, P. (2010). Flying the Fly: Long-range Flight Behavior of Drosophila melanogaster to Attractive Odors. *Journal of Chemical Ecology* 36**,** 599-607.

Benton, R., Sachse, S., Michnick, S.W., and Vosshall, L.B. (2006). Atypical Membrane Topology and Heteromeric Function of Drosophila Odorant Receptors In Vivo. *PLOS Biology* 4**,** e20.

Benton, R., Vannice, K.S., Gomez-Diaz, C., and Vosshall, L.B. (2009). Variant Ionotropic Glutamate Receptors as Chemosensory Receptors in Drosophila. *Cell* 136**,** 149-162.

Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. *Nature Reviews Molecular Cell Biology* 1**,** 11.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118**,** 401.

Brand, P., Robertson, H.M., Lin, W., Pothula, R., Klingeman, W.E., Jurat-Fuentes, J.L., and Johnson, B.R. (2018). The origin of the odorant receptor gene family in insects. *eLife* 7**,** e38340.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: A molecular basis for odor recognition. *Cell* 65**,** 175-187.

Budick, S.A., and Dickinson, M.H. (2006). Free-flight responses of Drosophila melanogaster to attractive odors. *Journal of Experimental Biology* 209**,** 3001.

Butterwick, J.A., Del Mármol, J., Kim, K.H., Kahlson, M.A., Rogow, J.A., Walz, T., and Ruta, V. (2018). Cryo-EM structure of the insect olfactory receptor Orco. *Nature* 560**,** 447-452.

Cardé, R.T., and Willis, M.A. (2008). Navigational Strategies Used by Insects to Find Distant, Wind-Borne Sources of Odor. *Journal of Chemical Ecology* 34**,** 854-866.

Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., and Carlson, J.R. (1999). A Novel Family of Divergent Seven-Transmembrane Proteins: Candidate Odorant Receptors in Drosophila. *Neuron* 22**,** 327-338.

Croset, V., Rytz, R., Cummins, S.F., Budd, A., Brawand, D., Kaessmann, H., Gibson, T.J., and Benton, R. (2010). Ancient Protostome Origin of Chemosensory Ionotropic Glutamate Receptors and the Evolution of Insect Taste and Olfaction. *PLOS Genetics* 6**,** e1001064.

Dekker, T., Geier, M., and Cardé, R.T. (2005). Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. *Journal of Experimental Biology* 208**,** 2963.

Freitag, J., Krieger, J., Strotmann, J., and Breer, H. (1995). Two classes of olfactory receptors in xenopus laevis. *Neuron* 15**,** 1383-1392.

Gao, Q., and Chess, A. (1999). Identification of Candidate Drosophila Olfactory Receptors from Genomic DNA Sequence. *Genomics* 60**,** 31-39.

German, P.F., Van Der Poel, S., Carraher, C., Kralicek, A.V., and Newcomb, R.D. (2013). Insights into subunit interactions within the insect olfactory receptor complex using FRET. *Insect Biochemistry and Molecular Biology* 43**,** 138-145.

Getahun, M.N., Olsson, S.B., Lavista-Llanos, S., Hansson, B.S., and Wicher, D. (2013). Insect Odorant Response Sensitivity Is Tuned by Metabotropically Autoregulated Olfactory Receptors. *PLOS ONE* 8**,** e58889.

Getahun, M.N., Thoma, M., Lavista-Llanos, S., Keesey, I., Fandino, R.A., Knaden, M., Wicher, D., Olsson, S.B., and Hansson, B.S. (2016). Intracellular regulation of the insect chemoreceptor complex impacts odour localization in flying insects. *The Journal of Experimental Biology* 219**,** 3428.

Getahun, M.N., Wicher, D., Hansson, B.S., and Olsson, S.B. (2012). Temporal response dynamics of Drosophila olfactory sensory neurons depends on receptor type and response polarity. *Frontiers in cellular neuroscience* 6**,** 54-54.

Gomez-Diaz, C., Martin, F., García-Fernández, J., and Alcorta, E. (2018). *The Two Main Olfactory Receptor Families in Drosophila, ORs and IRs: A Comparative Approach.*

Halty-Deleon, L., Hansson, B.S., and Wicher, D. (2018). The Drosophila melanogaster Na+/Ca2+ Exchanger CALX Controls the Ca2+ Level in Olfactory Sensory Neurons at Rest and After Odorant Receptor Activation. *Frontiers in Cellular Neuroscience* 12.

Huetteroth, W., and Waddell, S. (2011). Hungry flies tune to vinegar. *Cell* 145**,** 17-18.

Kaupp, U.B. (2010). Olfactory signalling in vertebrates and insects: differences and commonalities. *Nature Reviews Neuroscience* 11**,** 188.

Kepchia, D., Moliver, S., Chohan, K., Phillips, C., and Luetje, C.W. (2017). Inhibition of insect olfactory behavior by an airborne antagonist of the insect odorant receptor co-receptor subunit. *PloS one* 12**,** e0177454-e0177454.

Larsson, M.C., Domingos, A.I., Jones, W.D., Chiappe, M.E., Amrein, H., and Vosshall, L.B. (2004). Or83b Encodes a Broadly Expressed Odorant Receptor Essential for Drosophila Olfaction. *Neuron* 43**,** 703-714.

Leinders-Zufall, T., Rand, M.N., Shepherd, G.M., Greer, C.A., and Zufall, F. (1997). Calcium Entry through Cyclic Nucleotide-Gated Channels in Individual Cilia of Olfactory Receptor Cells: Spatiotemporal Dynamics. *The Journal of Neuroscience* 17**,** 4136.

Menini, A. (1999). Calcium signalling and regulation in olfactory neurons. *Current Opinion in Neurobiology* 9**,** 419-426.

Miazzi, F., Hansson, B.S., and Wicher, D. (2016). Odor-induced cAMP production in Drosophila melanogaster olfactory sensory neurons. *The Journal of Experimental Biology* 219**,** 1798.

Mukunda, L., Lavista-Llanos, S., Hansson, B.S., and Wicher, D. (2014a). Dimerisation of the Drosophila odorant coreceptor Orco. *Frontiers in Cellular Neuroscience* 8.

Mukunda, L., Miazzi, F., Kaltofen, S., Hansson, B.S., and Wicher, D. (2014b). Calmodulin modulates insect odorant receptor function. *Cell Calcium* 55**,** 191-199.

Mukunda, L., Miazzi, F., Sargsyan, V., Hansson, B.S., and Wicher, D. (2016). Calmodulin Affects Sensitization of Drosophila melanogaster Odorant Receptors. *Frontiers in Cellular Neuroscience* 10.

Nakai, J., Ohkura, M., and Imoto, K. (2001). A high signal-to-noise Ca2+ probe composed of a single green fluorescent protein. *Nature Biotechnology* 19**,** 137.

Neuhaus, E.M., Gisselmann, G., Zhang, W., Dooley, R., Störtkuhl, K., and Hatt, H. (2004). Odorant receptor heterodimerization in the olfactory system of Drosophila melanogaster. *Nature Neuroscience* 8**,** 15.

Ngal, J., Dowling, M.M., Buck, L., Axel, R., and Chess, A. (1993). The family of genes encoding odorant receptors in the channel catfish. *Cell* 72**,** 657-666.

Olsen, S.R., and Wilson, R.I. (2008). Cracking neural circuits in a tiny brain: new approaches for understanding the neural circuitry of Drosophila. *Trends in Neurosciences* 31**,** 512-520.

P. Blaustein, M., and Lederer, W. (1999). *Sodium/Calcium Exchange: Its Physiological Implications.*

Pellegrino, M., and Nakagawa, T. (2009). Smelling the difference: controversial ideas in insect olfaction. *The Journal of experimental biology* 212**,** 1973-1979.

Peñalva-Arana, D.C., Lynch, M., and Robertson, H.M. (2009). The chemoreceptor genes of the waterflea Daphnia pulex: many Grs but no Ors. *BMC evolutionary biology* 9**,** 79-79.

Sargsyan, V., Getahun, M., Lavista Llanos, S., Olsson, S., Hansson, B., and Wicher, D. (2011). *Phosphorylation via PKC Regulates the Function of the Drosophila Odorant Co-Receptor.*

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L.B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* 452**,** 1002.

Sengupta, P., Chou, J.H., and Bargmann, C.I. (1996). odr-10 Encodes a Seven Transmembrane Domain Olfactory Receptor Required for Responses to the Odorant Diacetyl. *Cell* 84**,** 899-909.

Shanbhag, S.R., Müller, B., and Steinbrecht, R.A. (1999). Atlas of olfactory organs of Drosophila melanogaster: 1. Types, external organization, innervation and distribution of olfactory sensilla. *International Journal of Insect Morphology and Embryology* 28**,** 377-397.

Silbering, A.F., Rytz, R., Grosjean, Y., Abuin, L., Ramdya, P., Jefferis, G.S.X.E., and Benton, R. (2011). Complementary Function and Integrated Wiring of the Evolutionarily Distinct Drosophila Olfactory Subsystems. *The Journal of Neuroscience* 31**,** 13357.

Smart, R., Kiely, A., Beale, M., Vargas, E., Carraher, C., Kralicek, A.V., Christie, D.L., Chen, C., Newcomb, R.D., and Warr, C.G. (2008). Drosophila odorant receptors are novel seven transmembrane domain proteins that can signal independently of heterotrimeric G proteins. *Insect Biochemistry and Molecular Biology* 38**,** 770-780.

Stensmyr, Marcus c., Dweck, Hany k.M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S., Wicher, D., Sachse, S., Knaden, M., Becher, Paul g., Seki, Y., and Hansson, Bill s. (2012). A Conserved Dedicated Olfactory Circuit for Detecting Harmful Microbes in Drosophila. *Cell* 151**,** 1345-1357.

Stensmyr, M.C., Giordano, E., Balloi, A., Angioy, A.-M., and Hansson, B.S. (2003). Novel natural ligands for Drosophila olfactory receptor neurones. *Journal of Experimental Biology* 206**,** 715.

Stocker, R.F. (1994). The organization of the chemosensory system in Drosophila melanogaster: a rewiew. *Cell and Tissue Research* 275**,** 3-26.

Vosshall, L.B., Amrein, H., Morozov, P.S., Rzhetsky, A., and Axel, R. (1999). A Spatial Map of Olfactory Receptor Expression in the Drosophila Antenna. *Cell* 96**,** 725-736.

Vosshall, L.B., and Hansson, B.S. (2011). A Unified Nomenclature System for the Insect Olfactory Coreceptor. *Chemical Senses* 36**,** 497-498.

Vosshall, L.B., and Stocker, R.F. (2007). Molecular Architecture of Smell and Taste in Drosophila. *Annual Review of Neuroscience* 30**,** 505-533.

W Taylor, R., Romaine, I., Liu, C., Murthi, P., Jones, P., Waterson, A., Sulikowski, G., and Zwiebel, L. (2012). *Structure-Activity Relationship of a Broad-Spectrum Insect Odorant Receptor Agonist.*

Wicher, D. (2015). "Chapter Two - Olfactory Signaling in Insects," in *Progress in Molecular Biology and Translational Science,* ed. R. Glatz. Academic Press), 37-54.

Wicher, D. (2018). Tuning Insect Odorant Receptors. *Frontiers in Cellular Neuroscience* 12.

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., and Hansson, B.S. (2008). Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* 452**,** 1007.

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M.C., Heller, R., Heinemann, S.H., and Hansson, B.S. (2009). dOr83b—Receptor or Ion Channel? *Annals of the New York Academy of Sciences* 1170**,** 164-167.

Acknowledgement

I would like to express my gratefulness to PD Dr. Dieter Wicher who gave me the opportunity to work on this project. I was always motivated by him during my project. He guided me all the time during the project and writing the thesis. He also gave me the opportunity to present my project during Institute symposium. I also want to express my deep gratitude to Professor Dr. Jürgen Bolz for the encouragement, support and taking out time for reviewing my thesis.

I am grateful to Dr. Sofia Lavista Llanos who always patiently guided and inspired me to work on genetic and behavioral part of this project. My sincere thanks also goes to Dr. Fabio Miazzi, Lorena Halty de-Leon, Sabine Kaltofen, Sarah Körte, Dr. Ana Depetris Chauvin, Mohammed A Khallaf, Silke Trautheim, Ibrahim Alali, Dr. Sudeshna Das Charkoborty, Megha Treesa Tom for their invaluable help. I am thankful to all member of the HAN department, MPI, for making my stay a memorable one.

Finally, I immensely thank my family for their enduring support throughout my years of study, without them this would not have been possible. Thank you.

Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt, nicht anderweitig zu Prüfungszwecken vorgelegt und keine anderen als die angegebenen Hilfsmittel verwendet habe. Sämtliche wissentlich verwendeten Textausschnitte, Zitate oder Inhalte anderer Verfasser wurden ausdrücklich als solche gekennzeichnet.

Jena, Februar 2019

_____

Kalpana Jain