Internal State-dependent Neuromodulation in *Drosophila melanogaster*

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Erklärung

Diese Dissertation wurde angefertigt unter der Leitung von Dr. Ilona Grunwald Kadow am Max Planck Institut für Neurobiologie.

Eidesstattliche Versicherung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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Mo Zhang

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Abbreviations

AL	antennal lobe
ANOVA	analysis of variance
AA	acetic acid
biVPN	bilateral V-glomerulus projection neuron
CO_2	carbon dioxide
CNS	central nervous system
EB	ellipsoid body
GFP	green fluorescent protein
GRN	gustatory receptor neuron
GPCR	G protein-coupled receptor
iACT	inner antennocerebral tract
IR	ionotropic receptor
iGluRs	ionotropic glutamate receptors
KC	Kenyon cell
LH	lateral horn
LN	local interneuron
mACT	medial antennocerebral tract
MB	mushroom body
MBON	mushroom body output neuron
oACT	outer antennocerebral tract
OBP	odorant binding protein
OSN	olfactory sensory neuron

ORCO	olfactory receptor co-receptor
ORN	olfactory receptor neuron
OA	octopamine
PI	performance index
PN	projection neuron
PAM	protocerebral anterior medial
PNS	peripheral nervous system
ROI	region of interest
sNPF	short neuropeptide F
SEZ	subesophageal zone
SP	sex peptide
SPR	sex peptide receptor
SOD	Superoxide Dismutase
TRP	transient receptor potentials
UAS	upstream activation sequence
VNC	ventral nerve cord

Summary

Animals perceive environmental stimuli via sensory systems including the senses of smell and taste. The behavioral responses to odors and tastes are modulated by an animal's external world and internal states. Depending on internal state, such as starvation, pregnancy and aging, the behavioral responses of animals to odor and taste stimulation are modulated and adapted to its particular needs. This state-dependent neural modulation of chemosensory systems has been investigated in previous studies. The modulation included recruiting alternative neural circuits or changes in neuronal excitability (Root, Ko et al. 2011, Bargmann 2012, Bracker, Siju et al. 2013). Neuromodulators, including neurotransmitters and ligands for specific receptors, play an essential role in representing information of internal states. This thesis aimed at characterizing the neuronal and molecular changes underpinning chemosensory modulation in mainly two different states, the reproductive state and aging.

In the first part of this dissertation, in collaboration with additional members of the lab, I focused on the neural processing and neuromodulation of a particular class of odors and tastes, the polyamines. Polyamines are pungent-smelling chemicals highly contained in insects' habitat sites. Insects, such as some flies and mosquitos, prefer fermenting fruits and decaying organic materials, which contain polyamines, for egg laying (Okamoto, Sugi et al. 1997, Takeda, Yoza et al. 1997, Atiya Ali, Poortvliet et al. 2011). Previous studies reported that polyamines play important roles in cell survival and proliferation. They participate in fundamental cellular processes such as DNA replication, RNA translation and protein synthesis (Wallace, Fraser et al. 2003, Kusano, Berberich et al. 2008, Lefevre, Palin et al. 2011). Furthermore, polyamine levels are also associated with neurodegenerative diseases and aging (Kalac 2014, Ramani, De Bandt et al. 2014). A deficiency or excess of polyamines can be detrimental to health and lifespan (Ramani, De Bandt et al. 2014). The ability to find a nutrient-rich diet and its uptake in a proper amount is essential for survival and reproduction. Thus, it is interesting to understand the underlying mechanism of polyamine detection in *Drosophila melanogaster*. The body pool of polyamines is maintained by endogenous biosynthesis, intestinal bacteria production and exogenous supply through diets. Dietary intake provides an important source of polyamines (Atiya Ali, Poortvliet et al. 2011, Kalac 2014). However, how flies detect polyamines is still elusive. I used a combination of electrophysiology and *in vivo* calcium imaging showing that flies can detect polyamines using taste system. The results showed that a specific ionotropic receptor – IR76b – and a bitter taste receptor – GR66a – are involved in taste associated polyamine detection. Next, I confirmed that

the sensitivities of polyamine taste neurons are enhanced in mated females. Moreover, I showed that the sensitivity of polyamine taste neurons to polyamines is regulated by a G-protein coupled receptor – sex peptide receptor (SPR) and its neuropeptides – myoinhibitory peptides (MIPs) in a mating state-dependent manner. The data showed that an inner state-dependent behavioral preference to polyamines is mediated by SPR and MIPs pathway directly in the chemosensory neurons. Taken together, the data revealed a novel neuropeptide-mediated mechanism how reproductive state adjusts the sense of taste to the physiological needs of a gravid female.

In the second part of my dissertation, I analyzed the underlying mechanism of olfactory deficiency associated with another physiological context – aging. Again, in collaboration, I showed that olfactory driven choice behaviors largely declined with aging. A similar decline is also observed in humans, which appears to be a result of aging and neurodegenerative diseases. Interestingly, using behavioral genetics, anatomical and electrophysiological analyses, I found that the activity of olfactory receptor neurons (ORNs) declines only marginally, which suggests that another neuron type is affected by aging. Mechanistically, oxidative stress caused by loss of mitochondrial superoxide dismutase – SOD2 – leads to similar behavioral declines as aging in olfactory perception. Furthermore, genetic experiments demonstrated that SOD2 acts primarily on the secondary projection neurons, whereas the ORNs are not affected. Therefore, these data indicated that aging might affect primarily central neurons such as projection neurons. However, the underlying cellular mechanism and the potential circuit kinetics that contribute the olfactory deficiency with aging are still unclear and will be topics of future studies in the lab. In summary, the data suggested that flies could be a suitable genetic model system to unravel the genetic and neuronal mechanisms underpinning aging-related olfactory decline. Studies on flies potentially help to delve deeper into the reasons behind olfactory deficiency associated with aging and additional neurodegenerative diseases.

Altogether, in this dissertation, I described two mechanisms of sensory processing modulated by internal state. The data provided insights into the underlying mechanisms of how internal state affects chemosensory processing and ultimately choice behavior. These mechanisms also might shed light on the understanding of choice behaviors in humans.

Zusammenfassung

Tiere nehmen Reize aus der Umwelt über sensorische Systeme einschließlich des Geruchs- und Geschmackssinnes wahr. Die Verhaltensantwort auf Geruch und Geschmack eines Tieres wird allerdings auch durch externe und interne Zustände moduliert. Je nach internem Zustand, wie Hunger, Gravidität oder Altern, regeln neuronale Veränderungen die Verhaltensantwort der Tiere bei einer Geruchs- oder Geschmacksstimulation. Zustandsabhängige neuronale Modulationen chemosensorischer Systeme wurden bereits in früheren Studien untersucht. Diese Modulationen beinhalten sowohl die Integration alternativer neuronaler Schaltkreise, als auch die Entwicklung und Veränderung neuronaler Erregbarkeit (Root, Ko et al. 2011, Bargmann 2012, Bracker, Siju et al. 2013). Neuromodulatoren, einschließlich Neurotransmitter und Peptidliganden für spezifische Rezeptoren, spielen eine wesentliche Rolle bei der Repräsentation interner Zustände. Ziel der vorliegenden Arbeit war es, die zellulären und molekularen Mechanismen adaptiver chemosensorischer Prozessierung in zwei spezifischen Zuständen zu untersuchen – Gravidität und Altern.

Im ersten Teil dieser Dissertation habe ich in Zusammenarbeit mit Kollegen anhand eines bestimmten Duft- und Geschmacksmoleküls, dem Polyamin, die Rolle von Neuromodulation und adaptivem Verhalten während der Gravidität untersucht. Polyamine sind penetrant riechende Chemikalien, die besonders im Lebensraum von Insekten, aber auch von anderen Tieren zu finden sind. Insekten, wie manche Fliegen oder Moskitos, bevorzugen gärende Früchte und verfaulende organische Materialien, die Polyamine enthalten, zur Eiablage (Okamoto, Sugi et al. 1997, Takeda, Yoza et al. 1997, Atiya Ali, Poortvliet et al. 2011). Polyamine spielen aber auch eine wichtige Rolle beim Menschen. Frühere Studien haben gezeigt, dass Polyamine an vielen grundlegenden zellulären Prozessen teilnehmen und eine wichtige Rolle bei Zellüberleben und -proliferation spielen (Wallace, Fraser et al. 2003, Kusano, Berberich et al. 2008, Lefevre, Palin et al. 2011). Desweiteren ist ein niedriger Polyamin-Spiegel auch mit neurodegenerativen Krankheiten und Altern verbunden (Ramani, De Bandt et al. 2014). Mangel oder Überschuss an Polyaminen können sowohl die Gesundheit, die Fortpflanzung, als auch die Lebensdauer beeinträchtigen. Die Fähigkeit, eine nährstoffreiche Ernährung in geeigneter Menge aufzunehmen, ist essentiell für das Überleben und die Fortpflanzung. Daher ist es von großem Interesse, den zu Grunde liegenden Mechanismus der Polyamin-Erkennung in *Drosophila melanogaster* als Modellsystem zu verstehen. Obwohl Polyamine endogen biosynthetisiert werden, kann die Aufnahme über die Ernährung eine größere Ressource, gerade bei

abnehmender Polyaminkonzentration im alternden Körper darstellen (Atiya Ali, Poortvliet et al. 2011, Kalac 2014). Doch wie genau Fliegen Polyamine erkennen können, war bisher ungeklärt.

Um das zu klären, habe ich eine Kombination aus Elektrophysiologie und *in vivo* Kalzium Imaging benutzt, um zu zeigen, dass Fliegen Polyamine über ihren Geschmackssinn erkennen. Die Ergebnisse zeigen, dass ein bestimmter ionotroper Rezeptor – IR76b – und ein Bittergeschmacksrezeptor – GR66a – den Geschmack von Polyaminen erkennen. Da zusätzliche Polyamine im Futter die Anzahl der Nachkommen signifikant erhöhen, habe ich untersucht, ob die Polyamin-Erkennung und Wahrnehmung abhängig vom inneren Zustand ist. In Zusammenarbeit mit meinen Kollegen habe ich herausgefunden, dass und warum Weibchen nach der Paarung sehr viel stärker von Polyaminen angezogen werden als vorher. Das Verhalten zu Polyaminen wird abhängig vom Paarungszustand über den Signalweg vom Sex-Peptid-Rezeptor (SPR) und seinem Peptidliganden dem myoinhibitorischem Peptid (MIP) reguliert. SPR und MIP vermitteln das veränderte Verhalten direkt auf Ebene der chemosensorischen Neurone. Zusammengefasst zeigen meine Daten und die Daten meiner Kollegen einen neuartigen, über Neuropeptide vermittelten Mechanismus auf, der erklärt, wie der reproduktive Zustand den Geschmackssinn an die physiologischen Bedürfnisse eines graviden Weibchens anpasst.

Im zweiten Teil meiner Dissertation habe ich gefragt, wie das Altern die Verarbeitung von Duft verändert. Wieder in Zusammenarbeit habe ich gezeigt, dass geruchsgesteuertes Entscheidungsverhalten mit dem Altern der Fliegen zurückgeht. Diese Situation ist ähnlich dem Menschen, wo Alter und Krankheit zum Verlust des Geruchsinns führen können. Ich konnte durch Verhaltensgenetik, anatomische und elektrophysiologische Analysen zeigen, dass die Aktivität der olfaktorischen Rezeptorneuronen nur geringfügig sinkt, was darauf hindeutet, dass ein anderer Neuronentypus vom Altern betroffen ist. Mechanistisch gesehen, führt oxidativer Stress, verursacht durch den Verlust von „mitochondrial superoxide dismutase 2“ – SOD2, zu ähnlichen Verhaltensrückgängen wie die olfaktorische Wahrnehmung beim Altern. Ferner zeigen meine genetischen Experimente, dass SOD2 in erster Linie auf sekundäre Neurone, sogenannte Projektionsneurone wirkt, während olfaktorische Rezeptorneurone nicht betroffen sind. Daher legen diese Daten nahe, dass das Altern in erster Linie zentrale Neurone, wie Projektionsneurone, beeinflussen könnte. Jedoch ist der zu Grunde liegende zelluläre Mechanismus, der zum Abbau des Geruchssinnes im Alter führt noch unklar. Zukünftige Studien im Labor sollen das klären. Zusammenfassend deuten die Daten darauf hin, dass Fliegen ein geeignetes genetisches Modellsystem sein können, um die genetischen und neuronalen

Mechanismen des alterungsbedingten Geruchssinnsrückgangs zu erforschen. Studien an Fliegen helfen potenziell, die Gründe des Geruchssinnsrückgangs sowohl beim Altern, als auch bei zusätzlicher neurodegenerativer Erkrankung zu finden.

Insgesamt beschreibe ich in dieser Dissertation zwei Beispiele sensorischer Verarbeitung, die durch den internen Zustand moduliert werden. Die Daten gewähren Einblicke in die zu Grunde liegenden Mechanismen, wie sich der interne Zustand auf die chemosensorische Verarbeitung und letztlich auf Entscheidungsverhalten auswirkt. Diese Mechanismen könnten auch Aufschluss über den Prozess der Entscheidungsfindung bei Menschen geben.

1 Introduction

1.1 The effect of internal state on behavior

Animals in their natural environment are surrounded by a myriad of odors. These odors are a rich source of information, and are perceived by sophisticated olfactory systems. The sense of smell benefits species to localize oviposition places, avoid predators, explore food and recognize potential mates. In addition, behavioral responses of animals are modulated by external context as well as internal cues. Depending on internal environment, for instance, blood glucose of mammals could be regulated by secreting insulin or glucagon. In the nervous system, there are complex neural networks detecting, processing and inducing specific behavior to environmental stimuli. These innate behaviors are determined by neural circuits, which are dynamic and reversible. Neural circuits are networks of neurons heavily connected through a variety of synapses that allow for different types of signal processing (Bargmann 2012). The mechanisms of state-dependent modulation provide an opportunity to better understand the function of neural networks. In this dissertation, I will focus on how animals modulate their behavioral and neural responses according to their internal states.

Recently, several studies have demonstrated that behavior can be adapted to internal metabolic demands or physiological states through the modulation of neural processing (Harris-Warrick and Marder 1991). Various inner sensors and effectors provide a stable and constant homeostasis to regulate the internal environment of animals. Depending on internal state, neural circuits can be modulated by stress, hunger or sleep. Accordingly, the changes of neural circuits can have an impact on relevant behaviors (Fields 2004, Mousley, Polese et al. 2006, Root, Ko et al. 2011, Bracker, Siju et al. 2013, Wang, Pu et al. 2013, Oh, Yoon et al. 2014). Flies use diverse patterns of innervating pathways to drive various behaviors. For instance, the fruit fly *Drosophila melanogaste*r uses different CO_2 projection pathways, which can be switched depending on its satisfactory state (Bracker, Siju et al. 2013). However, it is unclear how the different pathways have been regulated in different states. In state-dependent modulation, neuromodulators such as neuropeptides were shown to play a role in representing internal changes. A good example is food-deprived flies who exhibited stronger preference for food odors than fed flies. This preference switch was induced by neuromodulation through the short neuropeptide F (sNPF) and its receptor sNPFR, which showed increased expression and activity on olfactory neurons (ORNs) induced by insulin signaling upon starvation (Root, Ko et al. 2011). The analysis of the activity of the ORNs and projection neurons (PNs) revealed that the starvation-dependent behavioral

change was dependent on presynaptic facilitation through this neuropeptide and its receptor. In this way, sNPF/sNPFR signaling enhanced preference to food odors through directly increasing sensitivity of ORNs upon starvation (Root, Ko et al. 2011). In addition to the sNPF/sNPFR signaling, up-regulation of neuropeptide F (NPF) and its mammalian homologs neuropeptide Y (NPY) can also increase food intake by regulating chemosensory processing at different levels (Mousley, Polese et al. 2006, Nassel and Winther 2010, Wang, Pu et al. 2013). Similarly, the inner state can evoke preference changes in gustatory systems. Starvation can enhance the sensitivity of sugar gustatory receptor neurons (GR5a) through dopaminergic signaling and metabolic hormones. Accordingly the food intake is increased by facilitated feeding in hungry flies (Bharucha, Tarr et al. 2008, Inagaki, Ben-Tabou de-Leon et al. 2012).

This state dependent regulation enables animals to adjust behavioral responses to their nutritional, sexual and other needs. However, the mechanisms underpinning the relationship between innate behavioral changes and inner states are not well understood. Studies using *Drosophila melanogaster* will contribute to a better understanding of the flexible neuromodulation mechanisms, which could also link between internal state and decision-making behaviors in higher organisms including humans.

1.2 The chemosensory system of adult *Drosophila melanogaster*

The chemosensory system includes the olfactory and the gustatory system. They convey chemical information from the environment to the central nervous system of the animal. The chemosensory system enables animals including the fly *Drosophila melanogaster* to seek food, recognize harmful substances and detect pheromone cues from potential mating partners. In this dissertation, I use the fruit fly *Drosophila melanogaster* as a study model to understand the changes of chemosensory systems in different innate states.

1.2.1 The olfactory organs and receptors of adult *Drosophila*

Adult flies sense odors with two olfactory organs on the head, the third antennal segment and the maxillary palp (figure 1A). Both these external organs are covered with specialized sensory hairs called sensilla, which host and protect olfactory receptor neurons (ORNs) (figure 1B). Around 1100 – 1250 ORNs are found on each antenna, whereas only around 120 ORNs are found on each maxillary palp (Stocker 1994, de Bruyne, Clyne et al. 1999). Like most of the olfactory neurons ORNs are bipolar neurons and extend a sensory dendrite into the shaft of the sensillum

and project a single axon to a so-called olfactory glomerulus in the antennal lobe (AL) in the brain (figure 1C).

The sensilla on the antenna can be subdivided into three distinguishable morphological types: basiconic, trichoid and coeloconic (figure 1B), whereas the maxillary palp contains only basiconic sensilla (Shanbhag, Muller et al. 2000). Each sensillum is a hollow, fluid-filled structure. It contains up to four ORNs, which are surrounded by support cells (figure 1C). The support cells secrete sensillum lymph and keep each sensillum electrically insulated from others. When odorants enter the sensillum through pores in the cuticular wall and dissolve in the sensillum lymph, the ORNs are activated. The structure of the sensillum makes it possible to measure the activity of ORNs by extracellular methods. Thus, the odor coding of individual ORNs is measured by functional analysis in vivo. The basiconic sensilla are found to respond mostly to food odors (Goldman, Van der Goes van Naters et al. 2005, Hallem and Carlson 2006). The trichoid sensilla are involved in pheromone detection (Ha and Smith 2006). And the coeloconic sensilla respond to acid, ammonia and water vapor (Yao, Ignell et al. 2005).

This translation and the specific response of ORNs are determined by olfactory receptors (ORs), ionotropic receptors (IRs) or gustatory receptors (GRs) expressed on the dendrites of ORNs (figure 1D). The ORs are a family of seven transmembrane domain proteins. They are morphologically and functionally different from G protein-coupled receptors (GPCRs) in mammals. The somewhat still controversial experimental evidence indicates that ORs might work as both GPCRs and ion channels (Vosshall, Amrein et al. 1999, Benton 2006, Wicher, Schafer et al. 2008, Nakagawa and Vosshall 2009). The 60 OR genes encode 62 ORs that are expressed in ORNs of basiconic and trichoid sensilla. The sole exception is OR35a, which is housed in coeloconic sensilla (Vosshall and Stocker 2007). A single receptor Orco (olfactory receptor co-receptor, formerly known as OR83b) is co-expressed with all other ORNs as a heterodimer which is necessary for the function of all basiconic and trichoid ORs (Vosshall, Amrein et al. 1999, Benton 2006). In parallel, the IRs are a family of ionotropic glutamate receptors (iGluRs) encoded by 66 genes that have been shown to sense small amines, acids and humidity in coeloconic sensilla (Yao, Ignell et al. 2005, Benton, Vannice et al. 2009). These IRs may function as heterodimers with two broadly-expressed co-receptors IR8a and IR25a (Abuin, Bargeton et al. 2011). Besides ORs and IRs, two co-expressed GRs, GR21a and GR63b were found to be CO_2 receptors. They were both required in specific antennal basiconic olfactory neurons for CO_2 detection (Jones, Cayirlioglu et al. 2007, Kwon, Dahanukar et al. 2007).

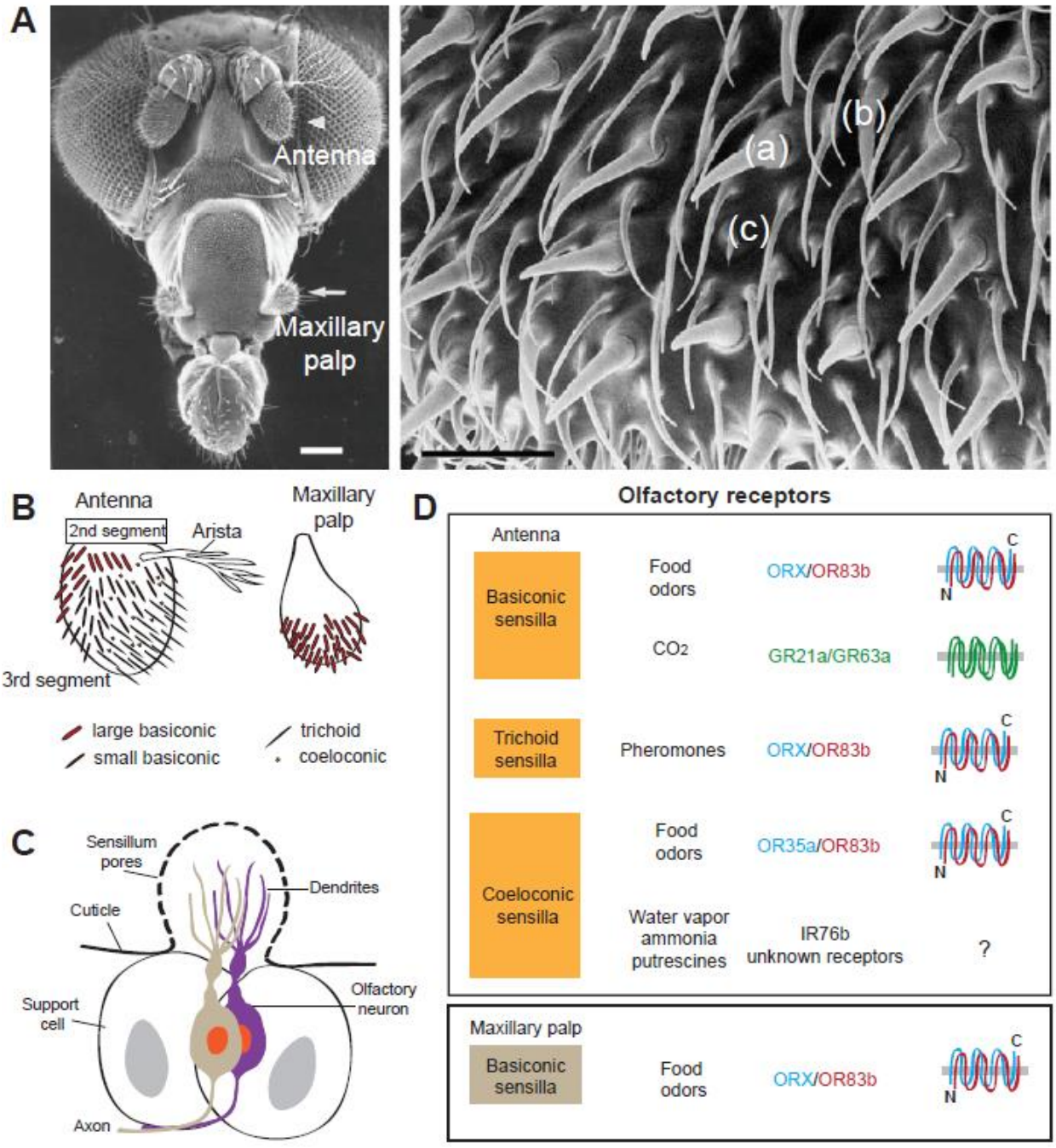

Figure 1 The *Drosophila* olfactory organs and receptors

(A) The head of an adult fly with the antenna and maxillary palp. Left scale bar =100µm. The morphological three types of olfactory sensilla on the surface of an antenna: (a) basiconic, (b) trichoid and (c) coeloconic. Right scale bar=10 µm. (B) Schematic of the sensilla of the olfactory organs. (C) Scheme of an olfactory sensillum housing two olfactory neurons. (D) A table of each morphological type of olfactory receptors (ORs) expressed in the olfactory organs. ORs are subdivided to functional classes. The image of receptors shows that ORs are seven transmembrane domain proteins with intracellular amino-termini, which may also be characterized by gustatory receptors. (A) and (B) are adapted from Hallem and Carlson et al. 2004. (C) and (D) are adapted from Vosshall et al. 2007.

1.2.2 The gustatory organs and receptors of adult *Drosophila*

Unlike the olfactory system, flies possess distributed taste organs over the whole body including their proboscis with the labellum, wings, legs, and even potentially the ovipositor organ (Stocker 1994). The proboscis, analogous to the vertebrate tongue, contains 31 taste bristles and around 30 taste pegs in each labellum (figure 2A and B). The taste sensilla on the labellum can be classified into three types by their sizes: small (S-type), intermediate (I-type) and long (L-type) sensilla (figure 2C). In addition, three internal taste organs are lined in the pharynx of the fly. Thus, a total of 69 taste sensilla are contained on each side of the proboscis (Vosshall and Stocker 2007), and each sensillum contains up to four gustatory receptor neurons (GRNs) (figure 2D). On the wing, forty taste sensilla decorate the wing margin, hosting four GRNs for each sensillum. On the legs, the number of sensilla shows sexual dimorphism on the first pair of tarsae. The female has 37, 30 and 32 taste sensilla, respectively, on the first, second and third legs, hosting up to four GRNs for each sensillum (figure 2E). In contrast, the male has around 50 taste sensilla on the first leg. The extra sensilla are involved in sexual behaviors (Bray and Amrein 2003, Park, Mann et al. 2006). Additionally, the female has around 10 sensilla on the vaginal plate, which may be involved in the egg-laying behavior (Vosshall and Stocker 2007). However, no formal proof of the existence of gustatory neurons on the ovipositor exists so far.

The studies of the function of taste organs are mostly focused on the labellum. Each of the L-type and S-type sensilla of the labellum contains four neurons that respond to different tastants: one is responsive to sugars, one to bitter and high salt, one to low salt and another to water. I-type sensilla include only two neurons: one responds to bitter and high salt and the other responds to sugar and low salt (Hiroi, Marion-Poll et al. 2002, Hiroi, Meunier et al. 2004). The taste pegs sensilla, which rows between and lateral to the pseudotracheal, may respond to carbonated water although the receptors are unclear (Fischler, Kong et al. 2007). The sensilla on legs can be characterized into six types. One responds broadly to bitter as well as sugar, whereas the others show little or no response (Ling, Dahanukar et al. 2014). The role of gustatory sensilla on the wings and the ovipositor organ remains elusive. According to the type of stimulus, taste neurons have been identified as sweet, bitter, water, salt and pheromone sensing neurons that express different taste receptors.

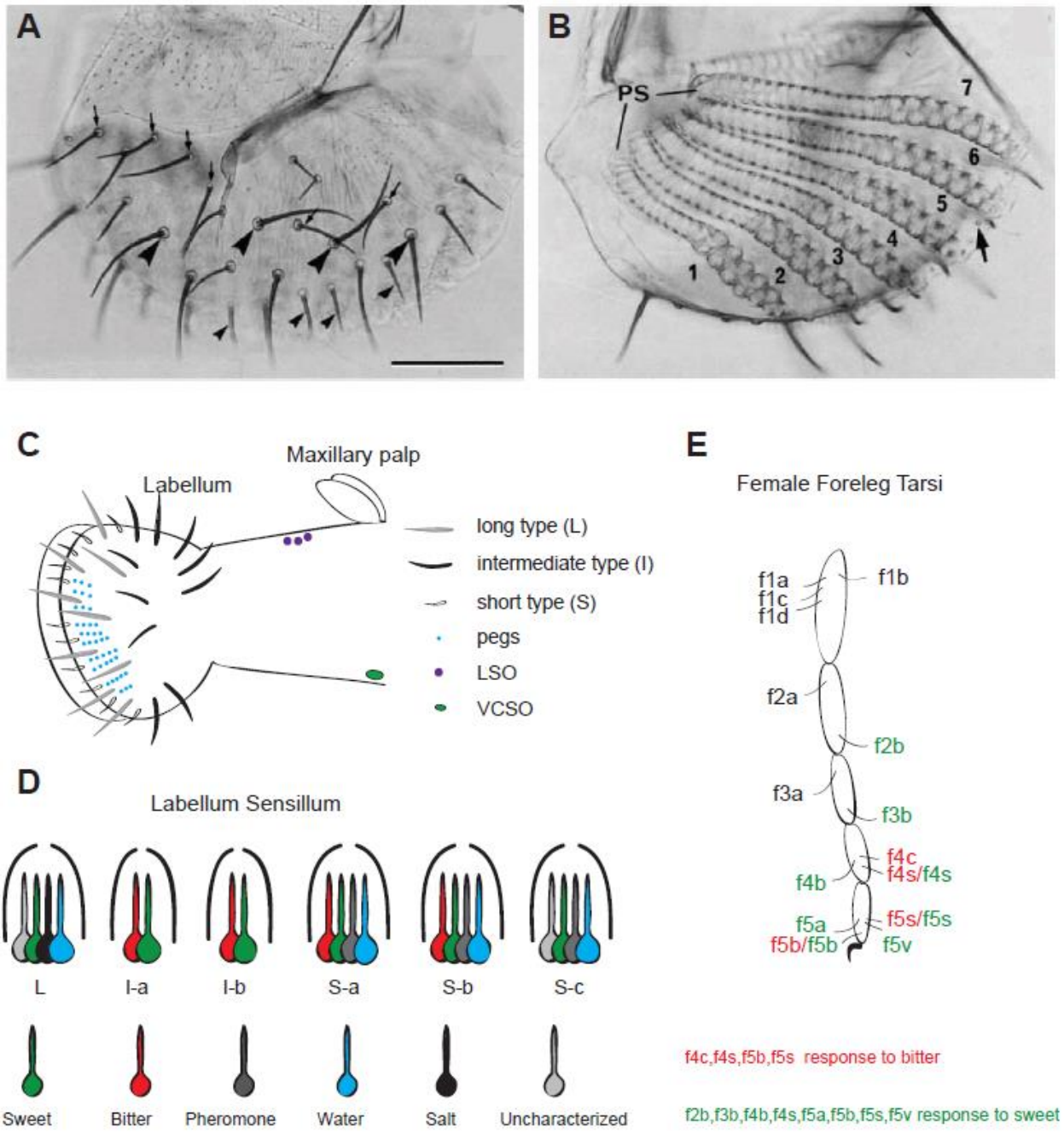

Figure 2 The *Drosophila* gustatory organs and receptors

(A) Surface of the labial palp of an adult fly. Anterior is to the left and dorsal is on the top. Arrows and arrowheads marked taste bristles and stars marked mechanosensory sensilla. Three morphological types of taste bristles are long (large arrowheads), intermediate (arrows) and short (small arrowheads). Scale bar =50μm. (B) Internal surface of the labial palp showing pseudotracheae (PS). 1 – 7 rows of peg neurons are located on the lateral sides of PS. (C) Schematic of three types of taste bristles and taste pegs in the labellum. Also indicating the location of other labral sense organs. (D) Diagram of functional subdivided classes of taste bristles on the labellum. (E) Tarsi sensilla in a female left foreleg, f=female. (A) and (B) are adapted from Shanbhag et al. 2001. (C), (D) and (E) are adapted from Freeman and Dahanukar et al. 2015 and Ling et al. 2014.

The taste receptors are comprised of gustatory receptors (GRs), IRs, pickpocket (*ppk*) family of epithelial sodium channels and transient receptor potentials (TRPs) family of cation channels, which are required for the activation of GRNs (Cameron, Hiroi et al. 2010, Kim, Lee et al. 2010, Weiss, Dahanukar et al. 2011, Zhang, Ni et al. 2013). The GRs are a family of 68 seven-transmembrane receptors which are encoded by 60 genes through alternative splicing (Clyne, Warr et al. 2000, Scott, Brady et al. 2001, Robertson, Warr et al. 2003). The GRs are related to ORs, and may form ionotropic receptors as well (Sato, Pellegrino et al. 2008). According to taste coding, GRs can be divided to two main groups: one mediates sweet or attractive compound perception and another mediates bitter or aversive perception (Thorne, Chromey et al. 2004, Marella, Fischler et al. 2006, Liman, Zhang et al. 2014). A large number of GRs are expressed on the bitter GRNs on the labellum and legs and mainly contribute to bitter and repulsive odors detection (Kwon, Dahanukar et al. 2011, Weiss, Dahanukar et al. 2011, Ling, Dahanukar et al. 2014). The ‘’core-bitter GRs’’, comprise of GR32a, GRR33a, GR66a, GR89a and GR39a.a, and have been suggested to function as co-receptors (Moon, Lee et al. 2009, Lee, Kim et al. 2010, Weiss, Dahanukar et al. 2011). In addition, the response to bitter is not only limited to GRs but also contributed by TRP channels (Kim, Lee et al. 2010). In contrast, a few of GRs belong to sweet receptors including GR5a, GR64a and GR64f (Dahanukar, Foster et al. 2001, Dahanukar, Lei et al. 2007, Jiao, Moon et al. 2007, Slone, Daniels et al. 2007, Jiao, Moon et al. 2008). And a single GR, GR43a, responds specifically to fructose (Miyamoto, Slone et al. 2012). Furthermore, it has been shown that a member of the IRs, IR76b, is required in low-salt sensing (Zhang, Ni et al. 2013). In addition, *ppk* members can mediate salt detection in larvae, along with detection of pheromones and water (Liu, Leonard et al. 2003, Cameron, Hiroi et al. 2010, Pikielny 2012).

1.2.3 The chemosensory projection pathway in adult *Drosophila*

In the olfactory system, ORNs in peripheral organs that express either ORs or IRs project their axons to a morphologically characterized structure in the central nervous system (CNS) – the AL (figure 3). The AL is comprised of approximately 50 morphologically defined glomeruli. The ORNs expressing the same OR converge onto one or rarely two glomeruli (Gao, Yuan et al. 2000, Vosshall, Wong et al. 2000). Genetic tracing confirmed that about 30 types of ORNs converge onto 46 glomeruli (Couto, Alenius et al. 2005, Fishilevich and Vosshall 2005). It has been found that 9 glomeruli are innervated by IRs (Benton, Vannice et al. 2009). A study showed that silencing or activating of specific glomeruli can dramatically change the food odor preference (Semmelhack and Wang 2009). This suggested that the olfactory preference is determined by the olfactory

glomerulus. The DM1 and VA2 glomeruli are required for odor attractions whereas the DM5 glomerulus mediate the odor aversion responses (Semmelhack and Wang 2009).

In the AL, the ORNs connect to the local interneurons (LNs) and the projection neurons (PNs). The LNs provide horizontal connections among glomeruli, comprising mostly GABAergic neurons which receive excitatory inputs and establish inhibitory synapses with the other LNs and PNs (Mori, Nagao et al. 1999, Sachse and Galizia 2002, Wilson and Laurent 2005). However, cholinergic excitatory LNs were also identified to elicit inter glomerular excitation of the PNs (Shang, Claridge-Chang et al. 2007), which was suggested to help balance between excitation and inhibition (Chou, Spletter et al. 2010, Yaksi and Wilson 2010). Of note, in addition to classical neurotransmitters, several neuropeptides made by the LNs and the ORNs are suggested to regulate the olfactory perception (Ignell, Root et al. 2009, Nassel and Winther 2010, Root, Ko et al. 2011).

The second class of connecting neurons, the PNs, connect by excitatory synapses from the ORNs, vertically link the glomeruli to two higher olfactory centers via different tracts, the inner (iACT), the outer (oACT), and the middle antennocerebral tract (mACT). Approximately 180 PNs project to the mushroom bodies (MB) and the lateral Horn (LH) (Margulies, Tully et al. 2005, Masse, Turner et al. 2009). The results from whole cell patch clamp demonstrated that the PNs were more broadly tuned than the ORNs and display a temporally more complex firing pattern. This finding indicated a transfer of activities between glomeruli within the LN circuits, called ‘’cross-talk’’ at the AL level (Ng, Roorda et al. 2002, Sachse and Galizia 2002, Wilson, Turner et al. 2004). Taken together, the evidence shows that a modification takes place before the olfactory information is transferred to higher brain centers, the mushroom body (MB) and the lateral horn (LH).

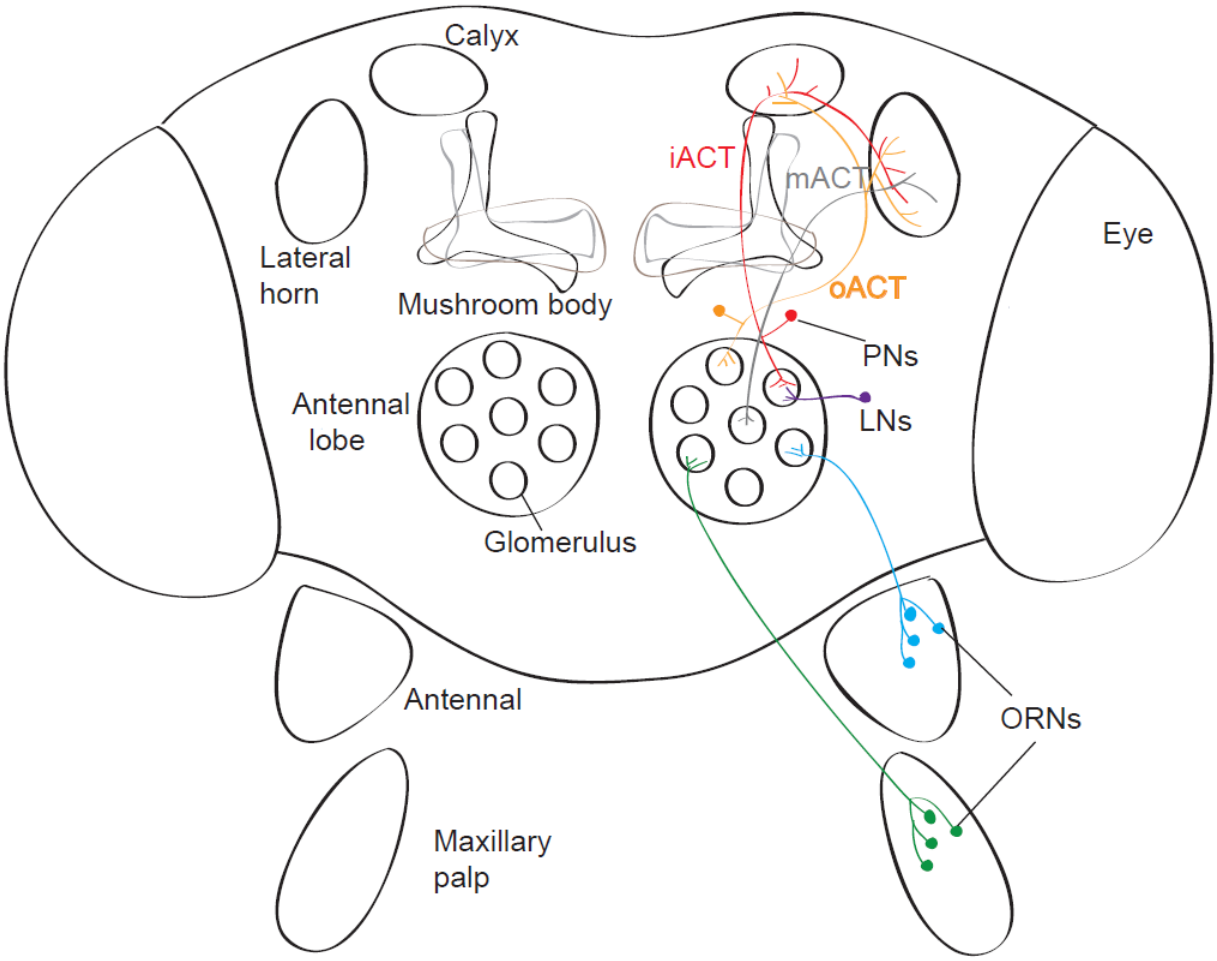

Figure 3 The olfactory projection pathway in adult *Drosophila*

Schematic of the fly olfactory circuits for odor processing. Olfactory stimuli are detected by the olfactory receptor neurons on the antenna and the maxillary palp, projecting to specific glomerulus in antenna lobe. Projection neurons (PNs) project to higher brain center the mushroom body and the lateral horn via different types of antennocerebral tracts (ACTs). The GABAergic local neurons (LNs) innervate glomeruli and suppress PN outputs. Adapted from Su and Wang et al. 2014.

The LH has been suggested to process innate olfactory information independent of experience (de Belle and Heisenberg 1994, Heimbeck, Bugnon et al. 2001, Tanaka, Awasaki et al. 2004). In contrast, previous studies showed that the MB is required for olfactory learning and memory (Heisenberg 2003, Davis 2004, Keene and Waddell 2007). However, recent studies indicate that the MB is also involved in processing innate olfactory behaviors in different contexts (Siju, Bracker et al. 2014, Cohn, Morantte et al. 2015, Lewis, Siju et al. 2015). The MB is comprised of intrinsic neurons, Kenyon cells (KCs). The dendrites of KCs that make up the calyx receive information from the PNs. The axons of KCs project to MB lobes, innervating 21 types of MB output neurons (MBONs). It has been shown that the KCs output is required for the innate CO_2 avoidance

behavior upon starvation (Bracker, Siju et al. 2013). A further study illustrated that the food odors activated dopaminergic neurons of the protocerebral anterior medial (PAM) which innervated in the specific MB lobe and suppressed the innate CO_2 avoidance behavior (Lewis, Siju et al. 2015).

The primary gustatory center of *Drosophila* is the subesophageal zone (SEZ), which is located on the ventral part of the brain (figure 4). The gustatory neurons from different gustatory organs terminate to distinct areas of the SEZ. The proboscis GRNs project to the central SEZ. The leg GRNs project to the posterior SEZ. And the internal taste organs target to the anterior dorsal SEZ (Thorne, Chromey et al. 2004, Wang, Singhvi et al. 2004). In addition, the neurons that express the same receptor project to the same area although the neurons themselves may locate in the different parts of the body. For instance, a genetic tracing study revealed that the GR5a neurons project to the ipsilateral side of the SEZ, whereas GR66a neurons project to a ring-like web in the medial SEZ (Thorne, Chromey et al. 2004, Marella, Fischler et al. 2006).

More evidence showed that the projections of other bitter receptor neurons overlap with the GR66a projections while projections of food-associated GRNs overlap with the GR5a projections (Wang, Singhvi et al. 2004, Inoshita and Tanimura 2006). The attractive and repulsive inputs have been shown to separate at the level of the primary taste center (Marella, Fischler et al. 2006). The taste neural modulation also happens in the primary taste center. For example, a class of interneurons in SEZ, TH-VUM (tyrosine hydroxylase positive, ventral unpaired medial neurons) can release dopamine to activate the sensitivity of sugar taste neurons under starvation. Other neurons expressing the dopaminergic receptors are also activated to promote the feeding behaviors (Marella, Fischler et al. 2006). Conversely, the aversive tastants can depress the activity of sweet neurons directly through Obp49a, an odorant binding protein (OBP), or indirectly through the GABAergic interneurons that connect bitter neurons (Jeong, Shim et al. 2013, Chu, Chui et al. 2014, French, Sellier et al. 2015).

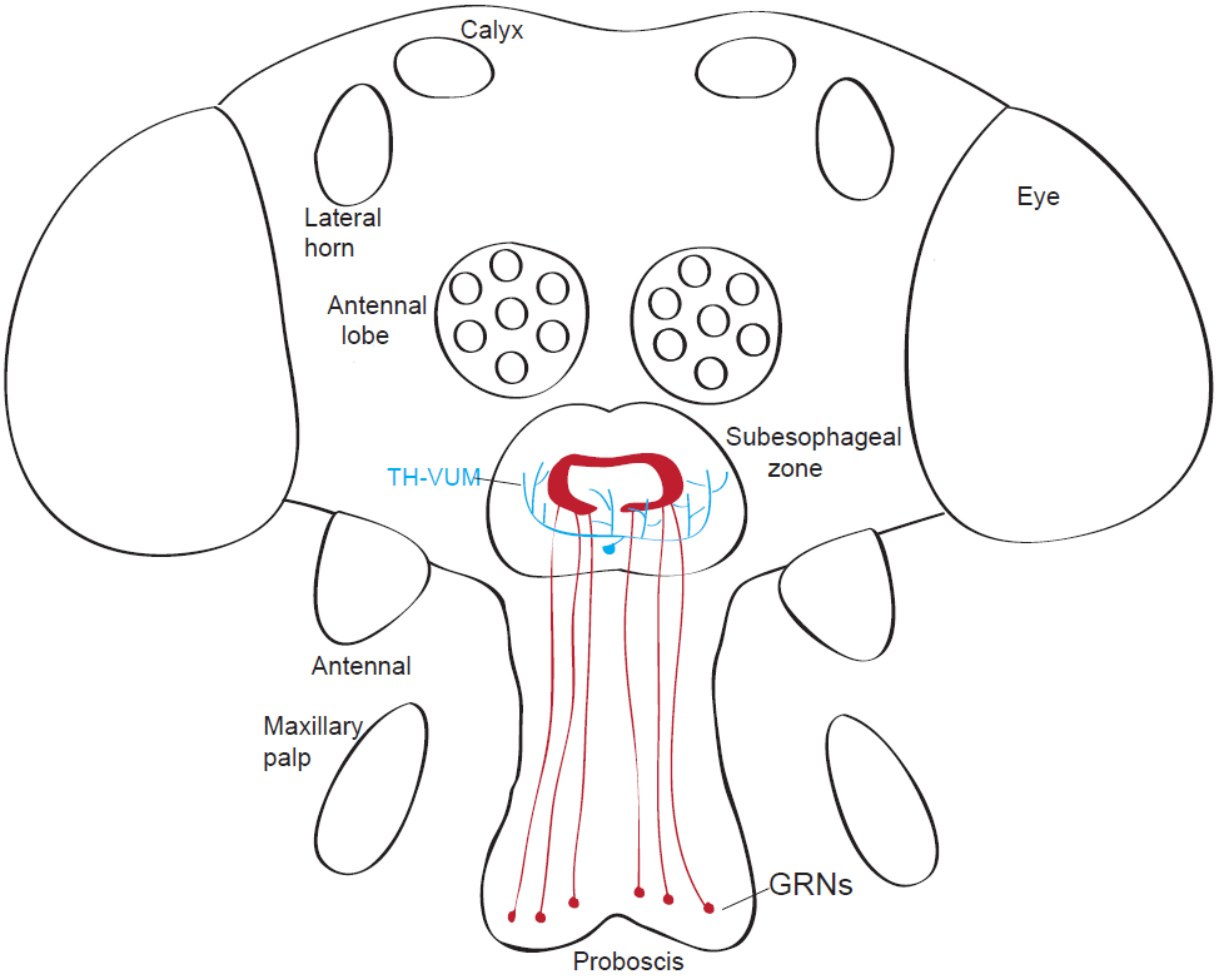

Figure 4 The gustatory projection pathway of adult *Drosophila*

Schematic of the fly gustatory circuits for taste processing. Gustatory afferents from the labellum, legs and other taste organs terminate to distinct areas of the subesophageal zone (SEZ) through different pathways. Interneurons TH-VUM can enhance the activity of sweet taste neurons. Adapted from Su and Wang et al. 2014.

1.2.5 The importance of chemosensation for fly behavior

Flies exhibit innate behaviors in response to their environment. For instance, both larvae and adult flies are attracted to the sources of food odors and exhibit aversion to the odors related to harmful sources (Hallem and Carlson 2006, Louis, Huber et al. 2008, Semmelhack and Wang 2009, Stensmyr, Dweck et al. 2012). Female flies will choose an appropriate site for egg laying in response to particular odors and tastes (Ruiz-Dubreuil, Burnet et al. 1994, Greenspan and Ferveur 2000, Hallem and Carlson 2006). Many of these behaviors require the involvement of the

chemosensory system. Chemosensory cues convey essential information that can evoke robust innate behaviors such as feeding, sexual and navigational behaviors (Greenspan and Ferveur 2000, Hallem and Carlson 2006, Louis, Huber et al. 2008). Moreover, flies can learn and remember odors and tastes associated with food sources or potential dangers (Tully and Quinn 1985, Dubnau and Tully 1998).

To study how the perception of odors is translated into appropriate behaviors, several behavioral assays have been developed in laboratories with the goal to measure the olfactory responses. For instance, behavioral assays such as the trap assays measure naïve attractive responses (Woodard, Huang et al. 1989, Larsson, Domingos et al. 2004). Another simple olfactory assay is the T-maze. This assay can measure the odor preference and avoidance by allowing the fly to choose between two arms filled with different odors or an odor and a control (Suh, Wong et al. 2004, Hallem and Carlson 2006). Furthermore, coupled with electric shock, the T-maze assay is used to condition flies to particular odors and to measure odor learning and memory (Quinn, Harris et al. 1974).

In the gustatory systems, the labellum as well as other gustatory organs provides information about whether to ingest or reject certain potential food sources. Sensilla on the legs are also involved in the initial decision about whether a substrate is a palatable food source. Additionally, the extra taste sensilla on male forelegs play a role in the sex pheromone detection (Stocker 1994, Singh 1997). Taste sensitivity triggers innate behaviors towards or away from food. This behavior can be measured in a number of simple behavioral assays in the laboratory. Associated with the feeding behavior, the proboscis extension is driven by the preference for food (Dethier 1976). The proboscis extension reflex assay is used to measure the appetitive or aversive to a stimulus (Wang, Singhvi et al. 2004). In addition, the two-way choice assay is also used for taste studies. In this assay, starved flies chose between two substrates. The feeding acceptance is measured by the intensity of the dye in the fly abdomen, which correlates with the consumed food (Amrein and Thorne 2005).

Pheromone detection is associated with the behaviors of copulation and oviposition. When pheromones are detected by the specialized sensors, the signals are integrated into the chemosensory information triggering different behaviors, such as aggregation, male courtship and female post-mating responses (Yapici, Kim et al. 2008, Joseph, Devineni et al. 2009, Dweck, Ebrahim et al. 2015). The copulation assay and egg-laying assay are used to measure female behavioral changes after the post-mating switch (Yapici, Kim et al. 2008).

1.3. The relationship between nutrient detection and mating state

Copulation leads to a series of behavioral changes in females, including the search to find a suitable oviposition place to benefit their offspring. Polyamines, a class of specific nutrients, are essential for growth and survival (Minois 2014). The detection of polyamines is unclear in flies. In the first part of my dissertation, I present a study on the underling neural mechanisms of polyamine perception in gravid females. This work could provide better understanding about the modulation of mating state on chemical substrate perception.

1.3.1 The post-mating behavior and molecular mechanisms

After mating, the behaviors of females change dramatically and quickly in many animals. In *Drosophila*, virgin females are highly receptive to males. They will slow down, cease rejecting behaviors, open vaginal plates and copulate with males rapidly. Virgin females lay few eggs and these eggs are non-fertilized. By contrast, mated females are unreceptive to copulation but instead start to look out for the appropriate sites to deposit their eggs. The egg-laying rate of mated females increases considerably (Connolly and Cook 1973, Ejima, Nakayama et al. 2001, Villella and Hall 2008). The post-mating changes, however, do not only affect behavior but also the physiology of the female leading to increased ovulation and sperm storage (Dickson 2008, Avila, Sirot et al. 2011).

During copulation, the seminal fluid is transferred from males to females. Sex peptides (SPs), a component of the seminal fluid proteins, are likely the primary trigger of the post-mating switch (Chen, Stumm-Zollinger et al. 1988, Kubli 2003, Carvalho, Kapahi et al. 2006, Ribeiro and Dickson 2010, Krupp and Levine 2014). Consequently, females don't show post-mating behaviors if they have mated with the SP deficient males (Chapman, Bangham et al. 2003, Liu and Kubli 2003). However, virgin females injected with SPs are unreceptive to males (Yapici, Kim et al. 2008). Sex peptide is detected by the sex peptide receptor (SPR) – a G protein-coupled receptor broadly expressed in the female reproductive tract and the nervous system (Yapici, Kim et al. 2008). The females lacking of SPR keep showing virgin behaviors even after mating (Yapici, Kim et al. 2008). Activating the SPR in a set of six to eight sensory neurons on the reproductive tract is sufficient to mediate the majority of post-mating behaviors including the change of receptivity and increase in egg numbers. These SPR sensory neurons are part of the neurons that express the sex-determinate gene *fruitless* (*fru*) and the proprioceptive neuronal marker *pickpocket* (*ppk*) and are found in the female reproductive system (Hasemeyer, Yapici et al. 2009, Yang, Rumpf et

al. 2009). Previous studies indicated that these *fru*$^+$/*ppk*$^+$ sensory neurons project to the abdominal ganglion of the ventral nerve cord (VNC) and the SEZ in the central nervous system. They appear to involve in regulating post-mating behaviors (figure 5). Recently, it was shown that *dsx*$^+$ neurons, expressed the sex-determinating gene *doublesex* (*dsx*), are also involved in mediating post-mating behaviors (Rezaval, Pavlou et al. 2012). The *dsx*$^+$ neurons and the *fru*$^+$/*ppk*$^+$ sensory neurons share the same downstream circuits with the SP-responsive sensory neuron in the reproductive system. Therefore, both the *dsx*$^+$ neurons and the *fru*$^+$/*ppk*$^+$ sensory neurons appear to transmit information to higher centers in the brain and generate the post-mating behavior (Hasemeyer, Yapici et al. 2009, Yang, Rumpf et al. 2009, Rezaval, Pavlou et al. 2012).

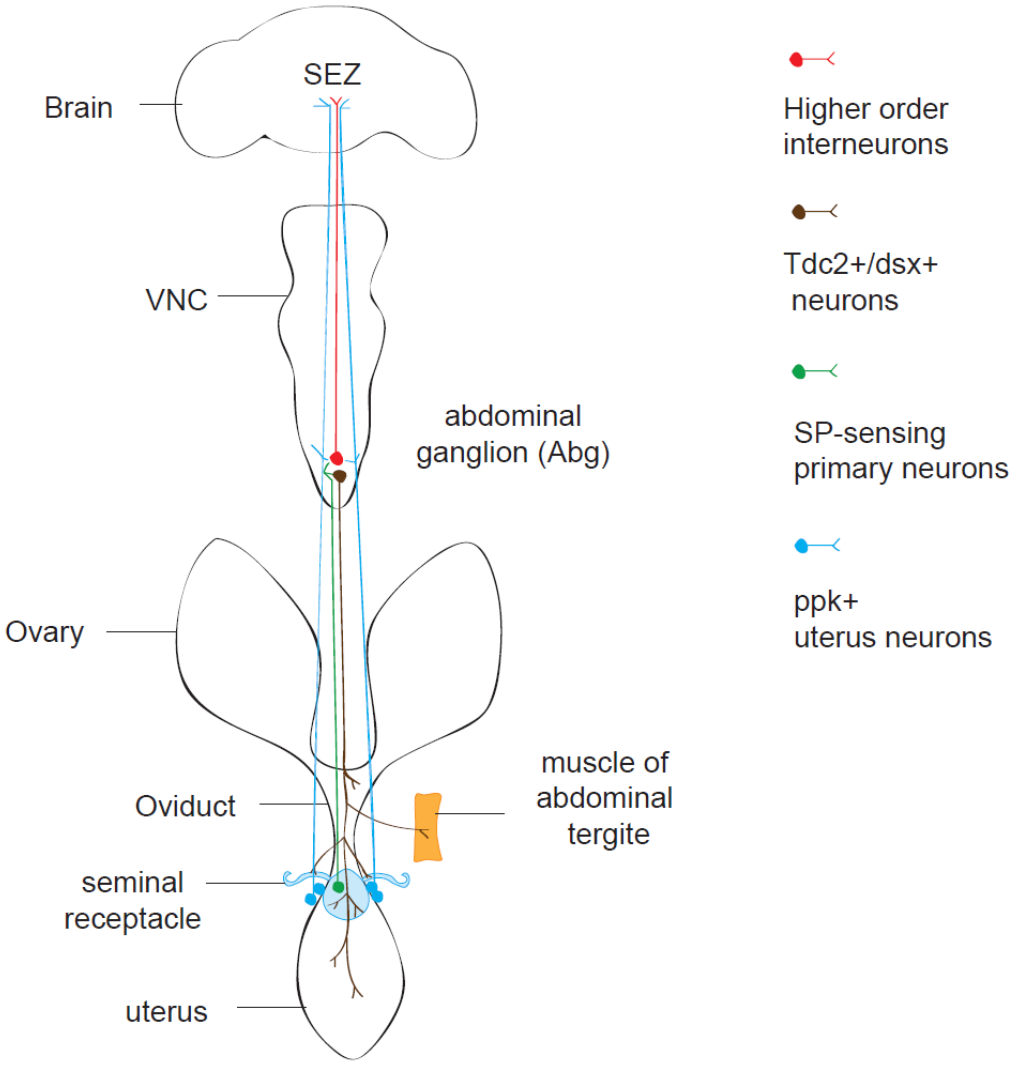

Figure 5 The neuron circuits mediating post-mating responses

Scheme of the central projections of *Tdc2/dsx*+ neurons and *ppk*+ uterus neurons. SPR neurons are part of the *Tdc2/dsx*+ neurons and the *ppk*+ neurons. *Tdc2/dsx*+ neurons are octopamine expressing neurons, which are only

found in the abdominal ganglion (Abg). In the Abg, signals are likely transferred by the other neurons projecting to the brain. Adapted from Krupp and Levine et al. 2014, and Häsemeyer et al. 2009.

Furthermore, the neuromodulator octopamine (OA) was found to modulate female behavior and physiology quickly after copulation. The increase of OA can induce the post-mating behavior in virgin females, whereas lacking of OA disrupts the post-mating responses in mated females (Rezaval, Pavlou et al. 2012, Heifetz, Lindner et al. 2014). Given that OA is secreted by the *dsx*$^{+}$ neurons in the female abdominal ganglion, OAs appear to play a role as neurotransmitter to transmit signals that mating took place to the higher brain centers.

Nevertheless, SPRs are implicated in the other function. Additional ligands to SP that bind and activate SPR exist. The myoinhibitory peptides (MIPs) have been found in many other insect species (Kim, Bartalska et al. 2010). The function of MIPs in the brain remains mostly elusive, although MIPs are broadly expressed in the brain, including the olfactory and gustatory systems (Kim, Bartalska et al. 2010). Only one very recent example showed that MIPs are involved in sleep control in *Drosophila* (Oh, Yoon et al. 2014). This function, interestingly, was not sexually dimorphic and was found in both males and females.

1.3.2 The senses of smell and taste affect the choice of egg-laying site

It has been reported that the senses of smell and taste are affected by pregnancy leading to increased food intake in human as well as other mammals (Faas, Melgert et al. 2010, Cameron 2014). In humans, the perception of odors and taste in pregnant women, especially in the early months of pregnancy, is reported to be stronger or different than in non-pregnant females (Bowen 1992, Duffy, Bartoshuk et al. 1998, Nordin, Broman et al. 2004, Cameron 2007). Previous studies showed that pregnant women preferred salty taste compared with non-pregnant women, which could be associated with additional needs for salty food (Faas, Melgert et al. 2010). Similar studies in rats also showed an increased preference to salt taste during pregnancy (Di Lorenzo and Monroe 1989). In contrast, the taste to bitter is more sensitive in pregnant women than non-pregnant women, which suggests an avoidance of bitter-tasting toxic substances (Duffy, Bartoshuk et al. 1998). These changes are regulated by sex hormones which are necessary to the growth and health of the progeny (Faas, Melgert et al. 2010). For egg laying animals, the changes in smell and taste can facilitate selecting an appropriate oviposition site that is essential for better survival of the offspring. Female flies will judge the condition of egg-laying sites depending on the benefits of their offspring. For instance, females preferred high nutrient

containing media and oviposition sites with low potential threats from predators and other species (Richmond and Gerking 1979, Chess and Ringo 1985). In order to identify appropriate egg-laying sites, the senses of smell and taste are crucial to evaluate environmental conditions (Yang, Belawat et al. 2008, Joseph, Devineni et al. 2009, Schwartz, Zhong et al. 2012, Stensmyr, Dweck et al. 2012, Dweck, Ebrahim et al. 2013).

Gravid *Drosophila melanogaster* females selectively lay eggs in fermenting fruit (Griffith and Ejima 2009). Although females show an innate preference for certain sites to lay their eggs, how flies find these sites and which cues they follow are not well understood yet. Initially, female flies exhibit search-like behaviors and probe chemosensory information on the substrate with legs, proboscis and ovipositor. As one of the main metabolites of fermentation, ethanol could act as the long-distance cue to attract flies to fermenting fruits. Indeed, in an oviposition site choice assay, female flies did show a strong egg-laying preference for low concentrations of ethanol but avoided higher concentrations (Stokl, Strutz et al. 2010, Azanchi, Kaun et al. 2013). The sensory mechanism has not been identified. Another example is citrus fruits, which are preferred by flies as the oviposition substrate. This preference is mediated by the olfactory cue terpenes. Among the olfactory neurons OR19a ORNs are necessary and sufficient for oviposition selection on citrus fruit (Dweck, Ebrahim et al. 2013). In addition to terpenes and ethanol, more sensory cues are involved as oviposition stimulant. For instance, acetic acid (AA) is attractive to female flies for egg-laying (Joseph, Devineni et al. 2009). Again, the receptors involved in detection of AA in this context remain unclear. In contrast, geosmin a strong aversion cue for egg-laying serves as an indicator of harmful microbes and is detected by the receptor OR56a (Stensmyr, Dweck et al. 2012). It is likely that additional cues, in particular the ones that distinguish overripe fruits from ripe or unripe fruits, are involved in female choice behavior.

Interestingly, the egg-laying preference is affected by internal state. It has been suggested that this change in chemosensory perception could meet specific needs for the developing embryo. Furthermore, the mechanism of how gravidity and pregnancy influence chemosensory processing at the neuronal level is unknown. However, in vertebrates several neuromodulators such as noradrenaline and serotonin are found in the chemosensory systems. They could be involved in modulating chemosensory perception (Brunton and Russell 2008, Palouzier-Paulignan, Lacroix et al. 2012, Linster and Fontanini 2014). In fact, a recent report in mouse showed that progesterone inhibits the response of olfactory neurons in the female vomeronasal organs to male pheromones when the female is not receptive (Dey, Chamero et al. 2015). Similar to mammals, gravid flies also showed an increased preference to salt taste than virgins. This preference is

driven by male's SPs acting on the SPR in female reproductive tract neurons (Walker, Corrales-Carvajal et al. 2015). In fruit flies, it shows that distinct subsets of dopaminergic neurons, which innervate the MB and ellipsoid body (EB), are required in modulating the egg-laying behavioral preference to ethanol (Azanchi, Kaun et al. 2013). Furthermore, in fruit flies, the egg-laying preference is modulated by mechanosensitive neurons in the female reproductive tract. The oviposition preference to AA is increased after egg delivery in the tract, which indicated that mechanical stretch of the internal reproductive tract is essential for triggering the preference to acetic acid (Gou, Liu et al. 2014). However, the mechanism of how the reproductive state and egg laying modulate the neural circuits that process odors and taste is still unknown.

1.3.3 The role of polyamines in egg-laying decisions and mating state-dependent behavior

In humans, pregnant women often report a changed perception of the intensity or valence of odors or tastants. The reason for this change in perception is unknown, but it is possible that it relates to the changed nutritional requirements (Faas, Melgert et al. 2010). Similarly, due to their different physiological needs, gravid female flies may search for additional nutrients. For instance, protein-deprived mated females show a strong preference to a protein-rich food source, whereas virgin females and males continue to prefer sugar (Ribeiro and Dickson 2010). Among variety of alternatives, polyamines are potential nutrients that gravid females require. The compounds play an essential role in reproductive processes and embryo development (Lefevre, Palin et al. 2011, Ramani, De Bandt et al. 2014).

The polyamines are small water-soluble polycationic molecules including putrescine, cadaverine, spermine, and spermidine (Lefevre, Palin et al. 2011). Spermine and spermidine were discovered in human semen, whereas cadaverine and putrescine were found as compounds of decaying flesh of corpse resulting from bacterial decomposition (Kusano, Berberich et al. 2008). In the cell, polyamines can interact with polyanionic molecules, such like DNA, RNA and phospholipids, play a role in multiple aspects of cell physiology such as DNA stabilization, regulation of gene expression, ion channel function, cell growth and proliferation, as well as cell death and apoptosis (Kusano, Berberich et al. 2008, Ramani, De Bandt et al. 2014). Moreover, in the reproductive system, polyamines are required in testicular development and spermatogenesis in males as well as in ovarian follicle development and ovulation in females. Studies also indicate that polyamines make function in embryo implantation and development (Lefevre, Palin et al. 2011). However, the mechanisms of how polyamines modulate the reproductive process are still elusive.

Given that polyamines are essential in reproductive functions and embryo development, gravid females may require additional polyamines from dietary. Polyamines can be generated from amino acids such as arginine, proline and methionine by endogenous biosynthesis or produced by intestinal microorganisms (Tabor and Tabor 1984, Lefevre, Palin et al. 2011). Additionally, polyamines can come from the diet. Given endogenous biosynthesis of polyamines is only 1-2 nmol per hour per gram of tissue in most active organs, diets are an important source of polyamines (Atiya Ali, Poortvliet et al. 2011). Good sources of polyamines are animal products, soybeans and certain fruits such as oranges and grapefruit which are rich of polyamines (Kalac 2014). Importantly, the intake of polyamines is essential for the survival of animals. Low levels of polyamines are related to neurodegenerative disease, decreased fertility, and aging, whereas high levels of polyamines are found in cancer cells in humans (Minois, Carmona-Gutierrez et al. 2011).

The fruit fly *Drosophila melanogaster* is attracted to overripe and fermenting fruits (Ashburner 1998, Dweck, Ebrahim et al. 2013). Similarly, mosquito *Aedes aegypti* is also attracted to lay eggs in decaying organisms (Ponnusamy, Xu et al. 2008, Wong, Stoddard et al. 2011). Previous studies reported that the amount of polyamines dramatically increases in those fermenting fruits and decaying meat or corpse (Okamoto, Sugi et al. 1997). The requirement to polyamines for feeding and oviposition could explain the behavioral preference of insects to those special places. A recent study showed that the zebrafish could detect polyamines by the olfactory receptor trace amine-associated receptor 13c (TAAR13c) (Hussain, Saraiva et al. 2013).

However, whether and how flies detect polyamines is still elusive. Given that polyamines play important roles in the reproductive process, we wonder whether gravid females exhibit preference to polyamines and use polyamines as cues for estimating the quality of oviposition places. Accordingly, given that flies show dramatic changes in the senses of smell and taste after mating, I wondered whether gravid females also exhibit increased sensitivity to the smell and taste of polyamines. If flies do show a different sensitivity to polyamines in a mating state-dependent manner, what are the physiological changes underlying this switch?

1.4 The relationship between olfaction and aging

Several studies focused on the mechanisms of aging. One aim of aging studies is to improve the qualities of life, especially in the late stages of life. The olfactory loss or anosmia is not only a feature of aging, but also an early symptom accompanied with aging-related neurodegenerative

diseases such as Parkinson's, Alzheimer's and Huntington diseases (Kovacs 2004, Murphy 2008, Doty 2009). Since aging is thought to be the largest risk factor for neurodegenerative diseases, the study on aging-associated declines can also benefit the pathological basis of age-related neurodegenerative diseases. Loss of olfaction correlates with neuronal and molecular disorders in the olfactory nervous systems. So far, the cellular and molecular mechanisms of how olfactory loss related to aging or neurological disease are unclear. In another part of my dissertation, I use the power of *Drosophila* genetics to analyze in depth the relationship of aging and the loss of smell.

1.4.1 The process of aging

Aging is the process of intrinsic deterioration that ends in death (Lopez-Otin, Blasco et al. 2013). The effect of aging includes the accumulation of cellular damage and weakening of the repairing capacity of the body (Kirkwood 2003, Lopez-Otin, Blasco et al. 2013). Aging is thought to be a biggest risk factor for many diseases including cancer, obesity, diabetes, cardiovascular diseases and neurodegenerative diseases (Hindle 2010). To achieve a longer lifespan and a better quality of life, a large number of studies focus on interpreting the process of aging. Instead of being a passive or random deterioration, aging is thought to be regulated by multiple internal (changes in homeostasis including reproductive signals, DNA replication failure and endocrine signals) and external factors (stress, sensory stimulation and diet) (Lithgow, White et al. 1995, Shama, Lai et al. 1998, Antebi 2004, Grotewiel, Martin et al. 2005, Libert and Pletcher 2007, Lee and Kenyon 2009, Kenyon 2010). Furthermore, with the expansion of genetic and molecular studies, it has been suggested that all these internal and external factors regulate the process of aging by signaling pathways and transcription factors (Kenyon 2001, Kenyon 2005, Kenyon 2010). For instance, the change of a few regulatory genes can largely change lifespan (Kenyon 2010). Importantly, the mechanisms appear conserved in many species including yeast, worms, flies and mice (Guarente and Kenyon 2000, Takahashi, Kuro et al. 2000, Boulianne 2001, Kenyon 2001, Helfand and Rogina 2003).

Although many aspects about the aging process are still elusive, different hypotheses are being tested to explain the regulation of aging. It is well known that caloric or dietary restriction extends lifespan in many organisms, conserved from yeast to mammals (Lakowski and Hekimi 1998, Mair, Goymer et al. 2003, Colman, Anderson et al. 2009, Katewa and Kapahi 2010). The concrete mechanism of how dietary restriction affects lifespan is unknown. Nonetheless, lifespan extension by dietary restriction indicates a regulation between metabolic rate and aging (Kenyon 2010).

Recently, it was demonstrated that the longevity response to dietary restriction is regulated by nutrient sensing pathways including Insulin/IGF-1 signaling (IIS), kinase target of rapamycin (TOR), AMP kinase and sirtuins (Rogina and Helfand 2004, Wood, Rogina et al. 2004, Kaeberlein, Powers et al. 2005, Greer, Dowlatshahi et al. 2007, Arum, Bonkowski et al. 2009). Additionally, other nutrient sensors also extend lifespan in response to dietary restriction (Greer and Brunet 2009). Besides eating less, smelling less or tasting less extends the lifespan as well (Apfeld and Kenyon 1999, Libert, Zwiener et al. 2007, Pletcher 2009, Poon, Kuo et al. 2010). In fact, interestingly, dietary restriction cannot extend lifespan, if the animal was exposed to the olfactory or gustatory sensory stimuli (Libert, Zwiener et al. 2007). Notably, lifespan extension by sensory deprivation is also regulated by decreased insulin/IGF-1 signaling (Antebi 2004, Kenyon 2010, Ostojic, Boll et al. 2014).

The Insulin/IGF-1 signaling (IIS) is best-known genetic mechanisms of aging (Kenyon 2005, Kenyon 2010). The Insulin/IGF-1 pathway was first described in *C.elegans* and later found to be conserved in flies and mammals (Kenyon, Chang et al. 1993, Tatar, Bartke et al. 2003, Bartke 2008). The lifespan of worms was extended largely with a mutation *daf-2*, which is a mammalian insulin/IGF-1 receptor orthologue (Kenyon, Chang et al. 1993). The lifespan extension needed the decreased activity of *daf-2* but the sustained activity of *daf-16*, which is the orthologue of FOXO transcription factor (Kenyon, Chang et al. 1993, Henderson and Johnson 2001, Lee, Hench et al. 2001, Lin, Hsin et al. 2001). In addition, life extension depended on the insulin receptor substrate *chico* in flies and transcription factors HSF-1, AAK-2 and SKN-1 in worms (Clancy, Gems et al. 2001, Hsu, Murphy et al. 2003, Apfeld, O'Connor et al. 2004, Tullet, Hertweck et al. 2008). In summary, inhibition of the insulin receptor, the IGF-1 receptor, upstream genes that upregulate insulin and IGF-1 and downstream effectors all can extend lifespans (Bartke 2008). Because the insulin/IGF pathway senses nutrients, this pathway is suggested to be a cascade to trigger cell protection and maintenance that mediate lifespan in response to nutrient limitation in harsh environment (Kenyon 2010). On the other hand, nutrient and stress sensors are also suggested to mediate lifespan under harsh environment by shifting animals to a protective physiological state (Kenyon 2010).

The target of rapamycin (TOR) pathway by contrast is another nutrient sensing pathway distinct from that of insulin/IGF-1. Inhibition of TOR kinase extends lifespan in many species, including yeast, worms, flies and mice (Jia, Chen et al. 2004, Kapahi, Zid et al. 2004, Kaeberlein, Powers et al. 2005, Harrison, Strong et al. 2009). This inhibition extends lifespan by activating the ribosomal subunit S6 kinase and inhibiting a translation inhibitor, 4E BP (Kapahi, Zid et al. 2004,

Hansen, Taubert et al. 2007, Pan, Palter et al. 2007, Selman, Tullet et al. 2009). TOR inhibition also increases resistance to environmental stress (Hansen, Taubert et al. 2007). Thus, TOR was suggested to be another pathway that triggers cell protection and maintenance in food limitation (Kenyon 2010). Additionally, there are other genetic interventions that can affect lifespan significantly. Such as overexpressing AMP kinase and NAD^+-dependent protein deacetylases, the sirtuins, can increase lifespans (Apfeld, O'Connor et al. 2004, Kenyon 2005, Berdichevsky, Viswanathan et al. 2006, Greer, Dowlatshahi et al. 2007, Anisimov, Berstein et al. 2008). Loss of G-protein coupled receptor methuselah and a mitochondrial co-transporter encoded by gene *I'm Not Dead Yet* (*Indy*) can extend lifespan as well (Lin, Seroude et al. 1998, Rogina, Reenan et al. 2000, Rogina and Helfand 2013).

Oxidative stress and damage have been proposed as one of the major causes of aging (Harman 1956, Harman 1972, Hekimi and Guarente 2003, Harman 2006). Oxidative damage is also related to progressive pathological changes that include various forms of neurodegeneration (Botella, Ulschmid et al. 2004, Llorens, Navarro et al. 2007). Free radicals derived from the electron transport chain in mitochondria, are the main source of reactive oxygen species (ROS) within cells, which progressively damage and lead to an age-related pathology (Harman 1956, Harman 1972). Superoxide Dismutase 2 (SOD2) is the particular enzyme in mitochondrial matrix, which catalyzes the reaction of superoxide ($O_2^{\cdot-}$) into a harmless molecule (Weisiger and Fridovich 1973, Fridovich 1998). Together with cytoplasmic SOD (SOD1) and extracellular SOD (SOD3), the SOD family plays an important role in preventing the damage from the by-products of the respiration chain (Landis and Tower 2005, Murphy 2009). In fact, overexpressing SOD can extend lifespan in *Drosophila* (Sun, Folk et al. 2002). And flies with a SOD2 null mutation have a dramatically reduced lifespan (Duttaroy, Paul et al. 2003, Paul, Belton et al. 2007). However, although there is a clear relationship between oxidative resistance and longevity, the two phenomena are sometimes uncoupled as revealed by other mutations (Kenyon 2005, Gems and Doonan 2009).

There are other mechanisms that enhance longevity by activating conserved cell-protective pathways or by changing animal fertility or reproduction (Copeland, Cho et al. 2009, Cristina, Cary et al. 2009). Removing germ cells from the reproductive system of *C.elegans* could extend animals' lifespan dramatically, which required the activity of DAF-16/FOXO (Hsin and Kenyon 1999, Kenyon 2010). Another distinctive regulative way could be telomeres, although the mechanism is unclear. Though normally telomeres shorten with age, engineered mice with longer telomere gained longer lifespan (Tomas-Loba, Flores et al. 2008).

1.4.2 The aging-associated functional decline

Many aging studies are focused on trying to understand the molecular and genetic mechanisms of why the organism ages and how such aging could be prevented or slowed down. Measuring the lifespan is a straightforward method to assess aging. Nonetheless, the lifespan does not equal the time the organism and its organs function to a satisfactory degree. For example, some organs that deteriorate with age may not be directly involved in survival of the organism. The functional decline, however, will affect the quality of life but not the length of life. On the other hand, it is known that many genetic or environmental factors can extend lifespan, as mentioned above. However it is unclear how and where these factors affect the process of aging. Functional studies aim to identify the key organs and physiological basics involved in aging associated decline. Furthermore, these studies try to identify potential biomarkers of age for predicting the lifespans (Cook-Wiens and Grotewiel 2002, Grotewiel, Martin et al. 2005). Therefore, it is essential to first identify the pathophysiological changes in different organs that occur during aging. This final aim is to improve our understanding of the mechanisms that connect the lifespan and the function of individual organs or even cells.

The fruit fly, *Drosophila melanogaster*, is a particularly suitable model organism for functional studies (Grotewiel, Martin et al. 2005). In addition to a relatively short lifespan and inexpensive maintenance, adult flies contain mostly post-mitotic cells except for a few cells in the gonads and gut (Bozcuk 1972, Ito and Hotta 1992). After hatching from pupal stage, flies are sexually matured and been considered as an adult starting aging. In the absence of cells replacement and division, post mitotic cells of adult flies directly reflect the changes of deteriorations until death (Helfand and Rogina 2003). Moreover, the fly genome has been sequenced and many genes in flies are homologues to mammals (Adams, Celniker et al. 2000). Additionally, many genetic tools have been well established in flies which make further studies convenient (Brand and Perrimon 1993, Lee and Luo 2001, Osterwalder, Yoon et al. 2001, McGuire, Roman et al. 2004). For instance, a genome-wide transgenic RNAi library creates arguably unmatched opportunities for genetic manipulations and studies (Minois, Sykacek et al. 2010). Taken together, these advantages make the fly an excellent model to study aging. Given that aging also associate with neurodegeneration, organism such as *Drosophila*, which is almost entirely post mitotic, are ideal model system for age-related neurodegenerative disease. A physiological study on flies' the giant fiber neuron indicated an age-related decline of the number of synapses between dorsal longitudinal muscles and its interneurons that contribute to a reduced climbing ability in old flies (Martinez 2007). In old flies, various studies on different systems demonstrated a declined function associated with aging. Such as the copulation success of males and their reproduction abilities decrease with age.

Similarly, the quality of sperm and egg decreases with age (Iliadi and Boulianne 2010). Additionally, based on the molecular and genetic studies, functional senescence has also been found in homeostasis. In the circulatory system, the resting heart rate decreased when flies were aged (Grotewiel, Martin et al. 2005). Finally, the immune system and a number of metabolic processes also declined with age (DeVeale, Brummel et al. 2004, Grotewiel, Martin et al. 2005).

Studies on the behavioral decline are used as a sign of age-related functional declines of the nervous system (Camicioli, Moore et al. 1999). Many reports present behavioral changes associated with age on a variety of model systems including the fly *Drosophila* (Ingram 2000, Yeoman and Faragher 2001). In addition, many aspects of the age-related functional decline were also observed in humans (Winter, Patla et al. 1990, Rittweger, Schiessl et al. 2004, Shkuratova, Morris et al. 2004). Different behaviors of adult flies can be quantitatively assessed in the laboratory. These behavioral assays make it possible to observe locomotion, geotaxis, fast photo taxis and chemotaxis declines associated with age (Simon, Liang et al. 2006). Negative geotaxis and exploratory activity are two locomotor behavior assays, which are frequently used to assess the motor activity of aged flies. Numerous studies showed a decline in negative geotaxis and exploratory activity in aged flies without an observed gender difference (Le Bourg 1983, Minois, Khazaeli et al. 2001, Cook-Wiens and Grotewiel 2002). Furthermore, research demonstrated that the decline of both negative geotaxis and exploratory activity are accelerated at high temperature compared to low temperature, indicating the aging of motor activity related to the physiological inner state (Helfand and Rogina 2000, Gargano, Martin et al. 2005). In spontaneous locomotor behavior, flies exhibit circadian rhythms. Like reduced motor activity, the circadian circling of aged flies showed a delay compared with younger flies (Joshi, Barnabas et al. 1999, Driver 2000). Additionally, older flies not only exhibited reduced innate preference to some odors (Cook-Wiens and Grotewiel 2002), but also the learning and memory of odor associations were affected (Tamura, Chiang et al. 2003). Several studies revealed that older flies had defects in olfactory learning and associative memory (Tamura, Chiang et al. 2003, Simon, Liang et al. 2006). In contrast, some behaviors are not or less affected by age. Flies continued to avoid electric shocks and were attracted to light (Cook-Wiens and Grotewiel 2002, Simon, Liang et al. 2006, Martinez, Javadi et al. 2007). These preserved behavioral abilities in aged flies indicated that some neurons or neural circuits are more susceptible to aging than others.

1.4.3 The aging-associated olfactory decline

The abilities to identify and discriminate odors deteriorate with age. This change can also lead to a perceived decrease of the quality of life (Murphy 2008, Rawson, Gomez et al. 2012). This olfactory loss or anosmia is a primary feature of aging but also of several neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Doty 2009). Decreased olfactory function is accompanied by structural abnormalities in the olfactory system, but the underlying cellular and molecular mechanisms are largely unclear (Kovacs 2004, Mobley, Rodriguez-Gil et al. 2014). Olfactory decline in aging humans has been extensively studied at the behavioral level. They indicate a decrease in the odor detection threshold as well as in the discrimination of different odors (Patel and Larson 2009, Iliadi and Boulianne 2010). The cellular and molecular studies on animal models could provide important clues of the underlying mechanisms of the aging of olfactory system on the one hand, and the relationship of olfactory aging and neurodegenerative diseases on the other hand.

Unlike in flies, neurogenesis in the olfactory bulb and epithelium in mammal is maintained. However, the replacement of older neurons by newer neurons, in aged mice is less efficient than in younger mice (Weiler and Farbman 1997, Bermingham-McDonogh and Reh 2011). Therefore, the less efficient replacement led to a decreased number of olfactory sensory neurons (OSNs) on the surface of the olfactory epithelium in humans, and similarly reduced number of OSNs in the mice epithelium during aging has been shown (Maresh, Rodriguez Gil et al. 2008, Suzukawa, Kondo et al. 2011). However, a study in mice showed that the sensitivity of the sensory neurons seems comparable to younger mice in aged mice. In contrast, the dynamics of OSNs seems to decrease in aged humans (Lee, Tian et al. 2009, Rawson, Gomez et al. 2012). One study in *Drosophila*, measuring transcriptional activity of genes on the antenna failed to show any changes in aged flies (Rogina, Vaupel et al. 1998). Apart from the periphery, there was no difference in the number of interneurons in the olfactory bulb in aged mice. In addition, the number and diameter of glomeruli remained stable during aging (Richard, Taylor et al. 2010). However, the morphology of neurons and the density of synapse were affected (Burke and Barnes 2006, Richard, Taylor et al. 2010, Livneh and Mizrahi 2011). There was a decrease in dendritic length and dendritic structure in aged mice compared to young mice (Livneh and Mizrahi 2011). Furthermore, synapse densities decreased on both axons and dendrites (Richard, Taylor et al. 2010).

In the mouse brain, the sub-ventricular zone (SVZ) and the rostral migratory stream (RMS) generate new interneurons migrating to the olfactory bulb. However, age-associated effects led to lower neurogenesis in both SVZ and RMS and decreased the proliferation of new-born

interneurons. This may contribute to the age-related deficits in olfactory behavior (Jin, Sun et al. 2003, Mobley, Bryant et al. 2013). A decrease in cell proliferation was also recognized in amygdala and the entorhinal cortex of older monkeys (Zhang, Cai et al. 2009). Nonetheless, age-associated structural changes were not found in those areas. These data indicate fewer adult-born interneurons in the olfactory bulb and the olfactory cortices in older animals compared to younger ones (Zhang, Cai et al. 2009, Mobley, Bryant et al. 2013). A recent study reported a loss of glutamatergic synaptic receptors in the piriform cortex of aged mice (Gocel and Larson 2013). Nevertheless, it is not understood, whether and how these changes contribute to the loss of the sense of smell during aging. They indicate that the synapses in the olfactory system, e.g., the glomeruli could be more susceptible to the effects of aging. However, the changes on synapses may not be enough to fully explain the behavioral changes with age. Therefore, it is important to identify both the molecular as well as the neural mechanisms underlying the aging-associated olfactory decline. A better understanding of aging–associated olfactory decline could also help to comprehend the relationship between olfaction and neurodegeneration. This may lead to better and more directed therapies to these diseases.

1.5 The aims of the dissertation

Chemosensory systems extract chemical information from the external and internal environments. According to their external environment and internal needs, animals execute behaviors controlled by their nervous system. The underlying neural circuits driving innate behaviors are not well understood. In particular, how mechanistically internal states affect sensory processing and innate behaviors is unclear. In chemosensory processing, several studies have shown that starvation can affect odor and taste processing at several levels. However, the knowledge about other physiological states is limited. A better understanding of the internal state-dependent modulation would not only enrich our knowledge of olfactory processing, but also serve to understand more complex processes such as decision making. Therefore, the goal of this dissertation is to explore the underlying mechanisms of internal state-dependent neural modulation.

To address this goal, *Drosophila melanogaster*, which is a powerful genetic model organism and shares many homologous genes with humans, was used. I focused on two different internal states, reproductive state and aging, which both impact on the perception of and behavior to odors and tastes.

In the first case, I addressed the question of whether reproductive state changes chemosensory processing using the circumstance that flies are attracted to a beneficial nutrient, the polyamine. While previous studies have reported a mating state-dependent change in the odor or taste perception, the underlying mechanisms were not understood. In collaboration with two colleagues in the laboratory, we asked whether the taste-dependent preference for polyamines is modulated by mating state. To this end, we explored how polyamines are detected by the taste system of the fly. I conducted experiments using a combination of tip recordings and *in vivo* calcium imaging. I tested the neural response to polyamines in the peripheral taste neurons of flies and the primary gustatory center, the SEZ. Importantly, to understand how mating state affects these neural responses, I compared mated and virgin female flies using these approaches.

For the second case, I addressed the question whether and how aging affects olfactory processing. The loss of olfactory sensitivity is not only associated with aging but also with neurodegenerative disease, such as Alzheimer's and Parkinson's. However, little is known regarding the underlying mechanism. In cooperation with my colleague, I complemented a series of experiments to explore the potential neural and molecular mechanisms of olfactory defects associated with aging with the aim of establishing *Drosophila* as a genetic model system for aging-associated olfactory decline.

To explore the aging associated mechanisms of olfactory decline, I first conducted experiments using a combination of anatomy, electrophysiology and behavioral analysis. I compared young and old flies in several parameters and for a panel of odorants. I conducted single sensillum recordings on young and old flies to understand the basis of the observed behavioral decline.

Furthermore, to address the potential molecules involved in olfactory decline, I implemented a small scale screen of genes involved in organismic aging. These aging-associated genes were reported to regulate lifespan in previous studies. Using different genetic approaches, I aimed at pinpointing not only the genetic basis but also the neuronal basis of this decline.

Taken together, the overall aim of this dissertation was to explore the genetic and neuronal mechanisms involved in internal state-dependent chemosensory processing and behavior. I anticipate that results of my work could have broader implications for chemosensory processing and decision making in other animals including humans.

2 Results

2.1 Polyamine taste perception of female flies is enhanced by mating state

As adult female flies prefer to lay eggs into fermenting or decaying fruits (Griffith and Ejima 2009, Azanchi, Kaun et al. 2013), the polyamines, a class of byproducts of fermentation, with strongly pungent smell and present in decaying and fermenting fruits are alternative cues attracting egg laying (Okamoto, Sugi et al. 1997, Takeda, Yoza et al. 1997). In addition, polyamines are important nutrients for reproductive processes and embryo development in mammals (Igarashi and Kashiwagi 2010, Lefevre, Palin et al. 2011). Therefore, I collaborated with a postdoctoral fellow, Dr. Ashiq Hussain to ask whether and how polyamines play a role in oviposition site selection. To put my results into the necessary context, I will show behavioral and anatomical experiments and results that have been carried out by Dr. Hussain and other colleagues..

2.1.1 Polyamines detection in *Drosophila*

Firstly, to analyze whether polyamines are required as nutrients for reproduction, we asked whether the dietary polyamines could benefit the success of oviposition and the survival of offspring. Ashiq Hussain crossed single males to single females on standard fly food mixed with polyamine solution (putrescine or cadaverine ~2.5 mmol polyamine/l of food), controlled with same crosses on standard fly food. After four days parental flies were discarded and the number of eggs was quantified. Later the number of hatched adult flies were also counted (figure 6). The quantification showed that the eggs laid on polyamine-rich diets were about three times more than those laid on the control diets. Similarly, the offspring hatched on polyamine-rich diets were about three times amount of flies compared to those hatched on standard food. Thus, the result indicated that polyamines-rich food could benefit the reproduction of flies.

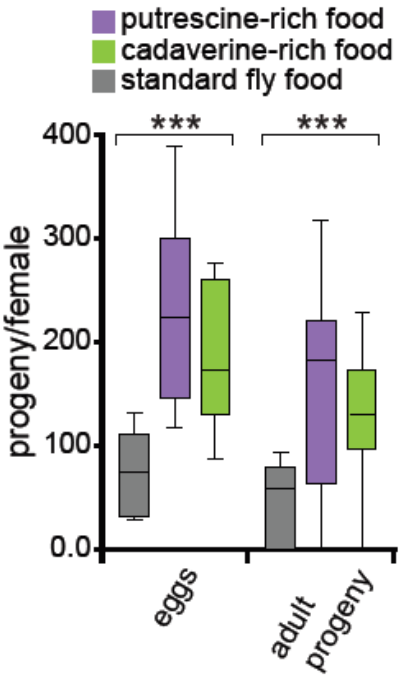

Figure 6 A polyamine-rich diet increases reproductive success

The eggs and adult progeny were produced by single females crossed with single males in different conditions after 4 days. The polyamine-rich food was standard fly food with added putrescine or cadaverine solution (~2.5 mmol polyamine/l of each bottle). The quantification of eggs and offspring are shown by box plot with median and upper/lower quartiles and whiskers show minimum/maximum values (n=8± SEM). The p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Adapted from Figure 1 of Hussain, Zhang et al. PLoS Biology 2016.

Previous work suggested that female flies use their sense of taste to identify putative egg laying places (Joseph, Devineni et al. 2009, Azanchi, Kaun et al. 2013). Therefore, female flies might also taste polyamine as hallmark to ensure proper amount and food quality for egg laying. To analyze this, Ashiq Hussain tested the preference of females to polyamines in an oviposition assay. The oviposition assay is in a round 60mm petri dish with 1% low melting agarose. Polyamines were spread on one-half of the dish or mixed directly into the agarose (figure 7A). Female flies were tested with the odor of different polyamines of variable chain lengths (spermine, spermidine, putrescine, cadaverine, diaminohexane, diaminoheptane, diaminooctane, and diaminodecane) (figure 7B). Surprisingly, comparison between the two sides indicated that flies avoided to lay eggs on polyamine-rich side and laid most of the eggs on the polyamine-free side (figure 7B). This result was different from the beneficial result of reproduction on polyamine-rich substrates. However, as previous study suggested that female flies avoided laying eggs directly into feeding substrates, this avoidance was consistent with the previous data (Yang, Belawat et

al. 2008). Moreover, in natural decaying fruits, polyamines should be detected with other combined food cues. When polyamines were mixed with other food substrate such as apple juice, flies preferred the mixture of polyamines and apple juice to apple juice alone (figure 7C). These data suggested polyamines did play a role as landmarks in oviposition site selection and enhanced the preference.

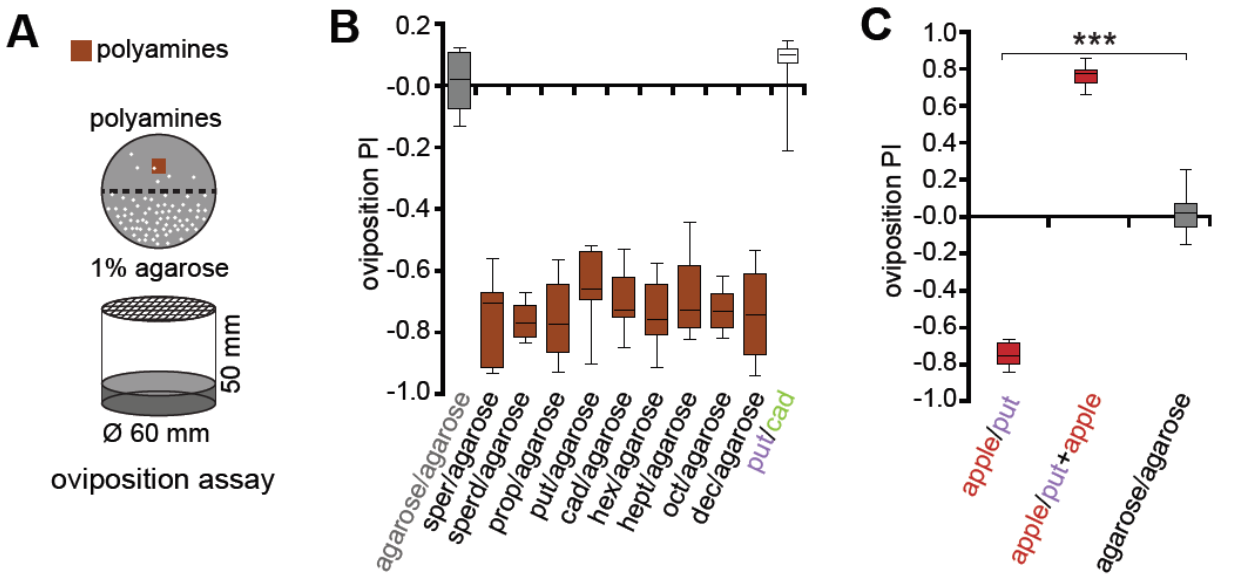

Figure 7 Oviposition preferences to polyamines

(A) Schematic illustration of the oviposition assay (bottom). A sample of egg laying plate shows aversive preference of females to polyamines during egg laying choices (top). The egg laying plate contains 1% low melting agarose and specific polyamine applied in one side (brown box). (B) Oviposition results of the females' preference to different polyamines at 1mM compared with agarose alone. Flies showed no choice difference between two of polyamine chemicals putrescine and cadaverine (n=8± SEM). (C) Additional putrescine mixed with apple juice is more attractive than apple juice alone (n=8± SEM). All the box plots show median and upper/lower quartiles and whiskers show minimum/maximum values. All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Adapted from Figure 3 of Hussain, Zhang et al. PLoS Biology 2016.

Then, we asked which chemosensory organs and chemosensory receptors mediated polyamine sensation in oviposition. Antenna ablated flies showed normal oviposition avoidance to polyamines (figure 8A). When removing the first, the second or third pair of tarsae or wings no change in polyamines choice was found. By contrast, ablation of the labellum of the proboscis completely abolished female's choice behavior and they became indifferent to polyamines in oviposition assay (figure 8A). Similarly, taste impaired *Poxn* mutant flies completely lost their aversion to lay eggs on polyamines-rich substrates (figure 8B). The transcription factor Poxn can specify the taste organs during development. In *Poxn* mutant flies, most taste organs were transformed into mechanosensory organs except some taste sensors in the pharynx

(Damblychaudiere, Jamet et al. 1992, Boll and Noll 2002). This abolished preference in oviposition can be rescued by re-expressing a full-genomic *Poxn* construct that rescued all taste neurons (Boll and Noll 2002) (figure 8B). Taken together, these data suggested that taste organs on the labellum were sufficient to mediate polyamines sensation during oviposition site selection.

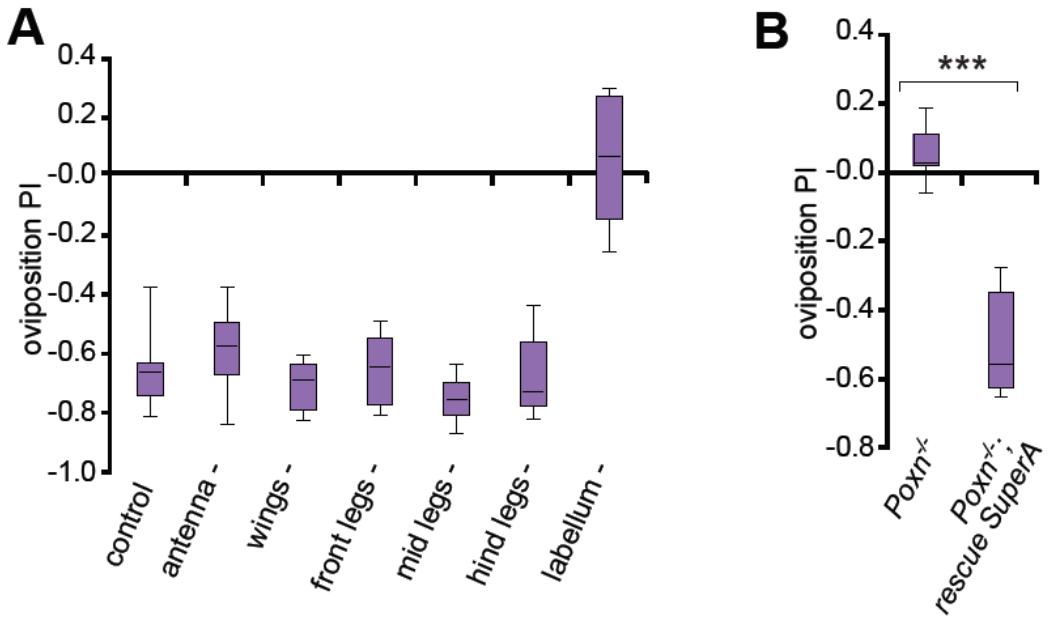

Figure 8 Flies taste polyamines by gustatory organs

(A) Oviposition results of intact females and females without the antenna, wings, labellum or different pair of tarsae (legs) separately. Females missing labellum loss the preference to the putrescine or cadaverine applied site compared with the control site (agarose alone). While all other ablations didn't affect the preference (n=8± SEM). (B) Loss of function *Poxn* (*Poxn*$^{-/-}$) females showed no preference to putrescine or cadaverine applied site in the oviposition assay. The *Poxn* rescue construct (SuperA-158) rescued the preference of *Poxn* females to polyamines (n=8± SEM). All the box plots show median and upper/lower quartiles and whiskers show minimum/maximum values. All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Adapted from Figure 3 of Hussain, Zhang et al. PLoS Biology 2016.

Next, Ashiq Hussain determined the taste receptor of polyamines using genetic screen experiments. Out of all olfactory receptor mutants tested in oviposition behavior, he identified the requirement of IR76b receptor by testing *IR76b* mutant flies (figure 9). The requirement of IR76b were confirmed by genetically silencing the activity of IR76b neurons with the expression of the inward-rectifier potassium channel Kir2.1 (Paradis, Sweeney et al. 2001). The flies (*IR76b-Gal4; UAS-Kir2.1*) lost the preference to polyamines completely (figure 9). Therefore, IR76b appeared to be the polyamine receptor in gustatory neurons.

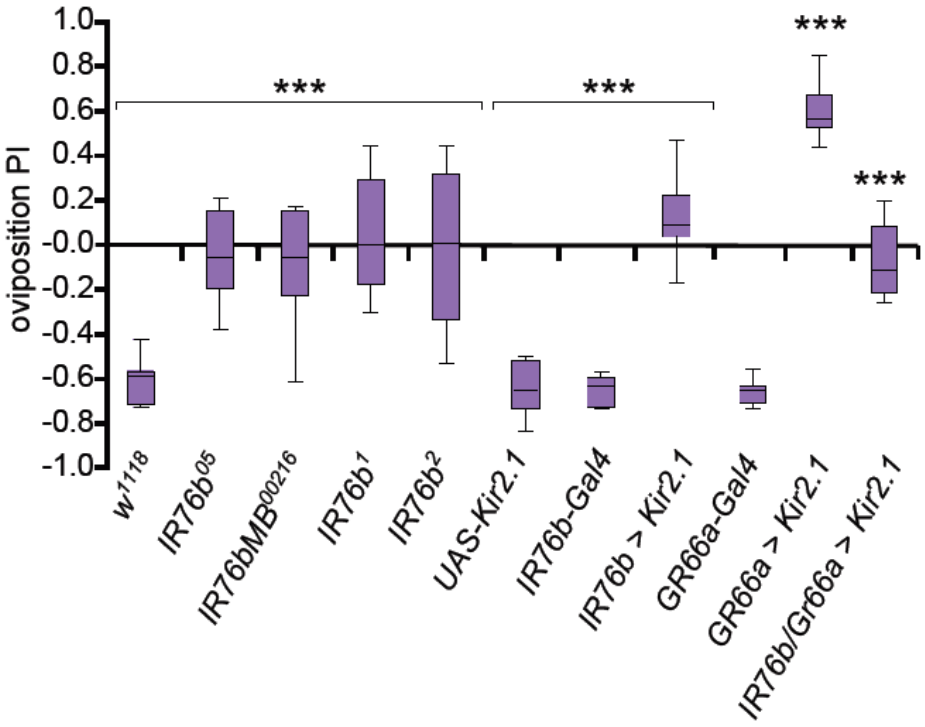

Figure 9 IR76b is required for polyamines taste detection

IR76b mutant females lost their preference to putrescine or cadaverine in the oviposition assay. Flies with Kir2.1 silenced IR76b neurons also lost the polyamines preference in the oviposition assay. Silencing bitter taste receptor neurons (GR66a neurons) with Kir2.1 made polyamines attractive. The box plots show median and upper/lower quartiles (n=8± SEM). The p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). In the figure, asterisks above boxes refer to p-values of comparison to the wild type control. Lines joining multiple boxed denote all other comparisons. All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Adapted from Figure 3 of Hussain, Zhang et al. PLoS Biology 2016.

Interestingly, putrescine and cadaverine are bitter compounds to humans (Kelleher et al,1992). Given that behavioral results showed that flies avoided to lay eggs on polyamine-rich sites but turned to polyamine-rich sites when they were mixed with apple juice, the bitter taste could mediate the avoidance when polyamines were applied alone. Thus, Ashiq Hussain tested the involvement of the bitter receptor GR66a in polyamine aversion during oviposition behavior. The flies with Kir2.1 silenced GR66a neurons (*GR66a-Gal4; UAS-Kir2.1*) started to lay eggs on polyamine-rich substrate in oviposition behavior (figure 9). When silencing GR66a and IR76b neurons simultaneously, the flies lost their preference for any side of the substrate (figure 9). These results confirmed the role of IR76b in mediating polyamine sensation in oviposition behavior. In addition, the result of oviposition choice between pure putrescine and putrescine with apple juices suggested that polyamines were attractive to egg laying sites, if the bitter taste was

masked by the taste of sugar or sweet. Thus, it appeared that GR66a neurons could inhibit or counteract this attractiveness.

To confirm the IR76b receptors were required in taste perception of polyamines, Ashiq Hussain carried out rescue experiments in *IR76b*[1] mutant females (figure 10). Re-expressing IR76b receptors in IR76b neurons (*IR76b-Gal4, UAS-IR76b; IR76b*[1]) indeed fully rescued oviposition behavior (figure 10). By contrast, re-expressing IR76b in GR66a neurons did not rescue the choice behavior in oviposition assays (figure 10). This result confirmed that the IR76b receptor is critical for polyamines taste detection and suggested that IR76b do not co-expressed in GR66a neurons.

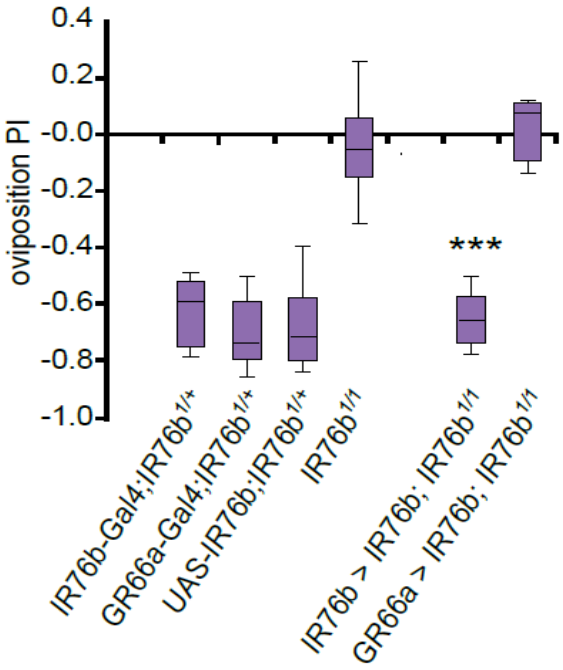

Figure 10 Re-expression of IR76b receptor could rescue the taste preference to polyamines

Re-expression of IR76b in IR76b neurons of *IR76b* mutants can fully rescue the lost preference to polyamines in oviposition behavior. But re-expression of IR76b in GR66a neurons can't rescue the defect. Asterisks above boxes refer to p-values of comparison to wild type control. All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Adapted from Figure 3 of Hussain, Zhang et al. PLoS Biology 2016.

Anatomical experiments carried out by Dr. Laura Loschek in our laboratory showed that IR76b receptors and GR66a receptors were both expressed on the labellum and the primary gustatory center subesophageal zone(SEZ) of the brain but not in the same neurons (figure 11). In

summary, the data suggested that flies use two neurons to sense one molecule and integrate two types of information to reach a final decision on where to lay their eggs.

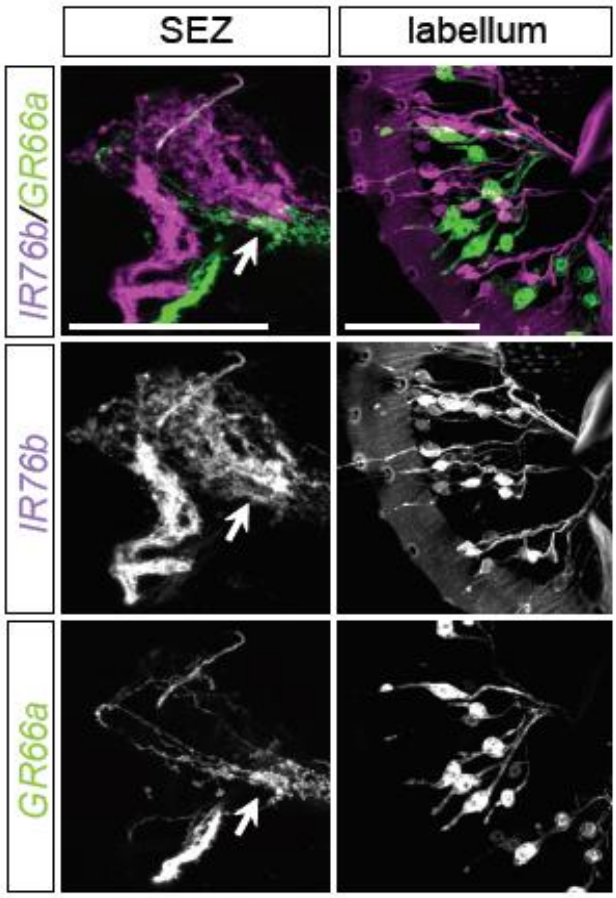

Figure 11 Expression of IR76b taste receptors and bitter taste receptors in the SEZ and the labellum

Expression of IR76b (*IR76b-QF; QUAS-mtdTomato-3xHA*, magenta) and bitter receptors GR66a (*GR66a-Gal4; UAS-mCD8GFP*, green) receptors in the subesophageal zone (SEZ) and the labellum. IR76b taste receptors are not co-expressed with bitter receptors in the labellum. IR76b neurons are not innervate the same region as GR66a neurons in the SEZ (arrow). Figure is adapted from Figure 3 of Hussain, Zhang et al. PLoS Biology 2016.

My role in this joint project was to characterize the physiological response and the role of IR76b neurons in polyamine sensing by using electrophysiology and functional imaging. To directly test whether taste neurons on the proboscis responded to polyamines, I carried out tip recordings on the labellum (figure 12A). The labellum is covered with three types of taste sensilla: small (S-type), intermediate (I-type) and long (L-type) sensilla, which host different kinds of taste neurons (Montell 2009). Using tip recordings, I found robust responses to putrescine of S-type sensilla but not of L-type sensilla (figure 12B). A previous study showed that the detection of salt was also

mediated by IR76b, but in L-type sensilla (Zhang, Ni et al. 2013). Facing this discrepancy I asked whether IR76b receptor mediated putrescine detection in S-type sensilla. To this aim, I tested *IR76b* mutant flies and found that IR76b abolished flies still showed a strong response in S-type sensilla to putrescine (figure 12C). This result suggested IR76b did not mediate this S-type sensilla response to putrescine. Given that bitter receptors including GR66a were expressed in these S-type sensilla, I proposed that this response of S-type sensilla likely came from bitter receptors. On the other hand, we wondered which type of IR76b-expressing GRNs response to putrescine. Using Gal4 reporter (*IR76b-Gal4; UAS-mCD8GFP*) we found the IR76b also expressed in taste peg neurons (figure 12D). It is likely the peg neurons involved in putrescine responses.

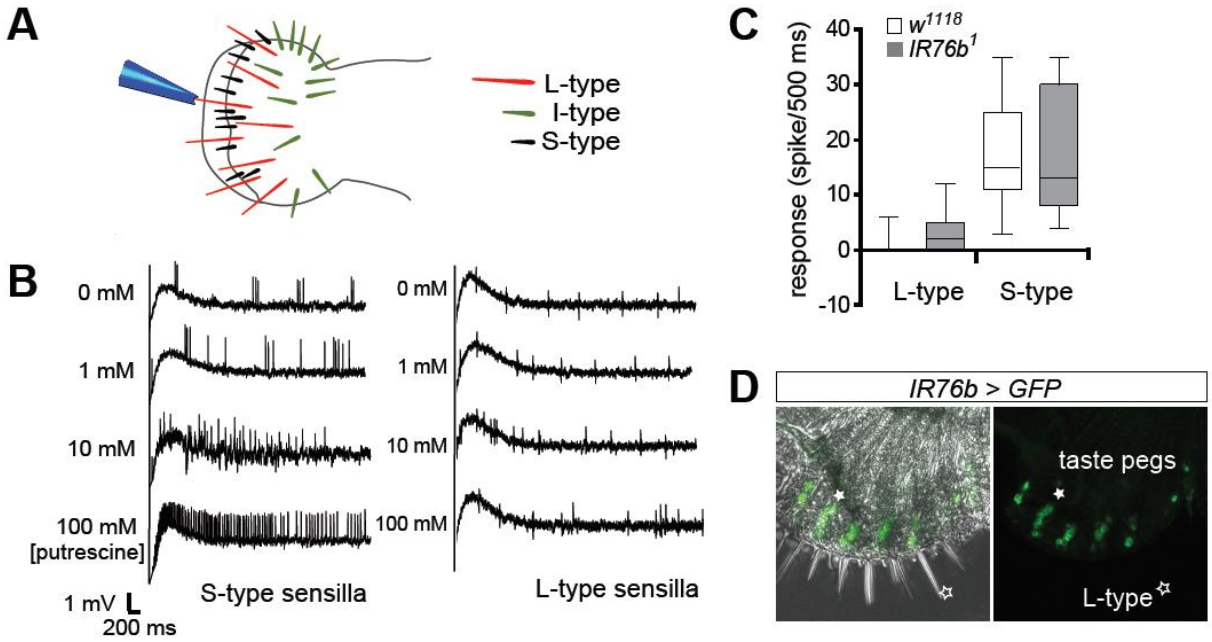

Figure 12 Taste sensilla on the labellum response to polyamines

(A) Schematic drawing of *Drosophila* labellum with three types of taste sensilla. (B) Responses of S-type and L-type senilla to putrescine with tip recording at different concentrations (0–100 mM, n=8 ± SEM). (C) Quantification of responses of S-type and L-type senilla to putrescine in *IR76b* mutant and wild type control (100mM, n=8 ± SEM). (D) Expression of IR76b (*IR76b-Gal4; UASmCD8GFP*) in peg taste sensilla on the labellum. Filled star indicates a peg taste neuron and open star indicates an L-type sensillum. Figures are adapted from Figure 4 of Hussain, Zhang et al. PLoS Biology 2016.

Although the IR76b neurons in L-type sensilla did not respond to putrescine, the anatomical results showed that additional IR76b neurons were broadly present in the gustatory system (Zhang, Ni et al. 2013). Since it is technically very difficult to record electrophysiological polyamine responses from peg type sensilla, I decided to use *in vivo* calcium imaging to record the response

of all IR76b neurons directly from the SEZ, where axons of IR76b neurons projected to (figure 13A). The calcium indicator GCaMP6f was expressed in IR76b neurons, which enabled me to assess the activity of IR76b neurons by the change of calcium-dependent fluorescence (Fiala and Spall 2003, Tian, Hires et al. 2009). With putrescine stimulation of different concentrations (1mM to 100mM) on the labellum, GCaMP-fluorescence was significantly increased in two areas of the SEZ of flies (*IR76b-Gal4; UAS-GCaMP6f*) (figure 13B-D). Accordingly, I calculated the different responses in two regions of interest (ROI) of SEZ separately. The responses of two ROIs in the SEZ were dose-dependent (figure 13B). One of two regions (ROI1) of SEZ reached the highest response already at 10mM putrescine and decreased at higher concentrations (figure 13B-D). In contrast, the response in ROI2 increased with the increase of putrescine concentration and reached highest responses to 100mM putrescine (figure 13B-D). It seems that ROI1 responded stronger at the concentration (1mM) used for oviposition behavior.

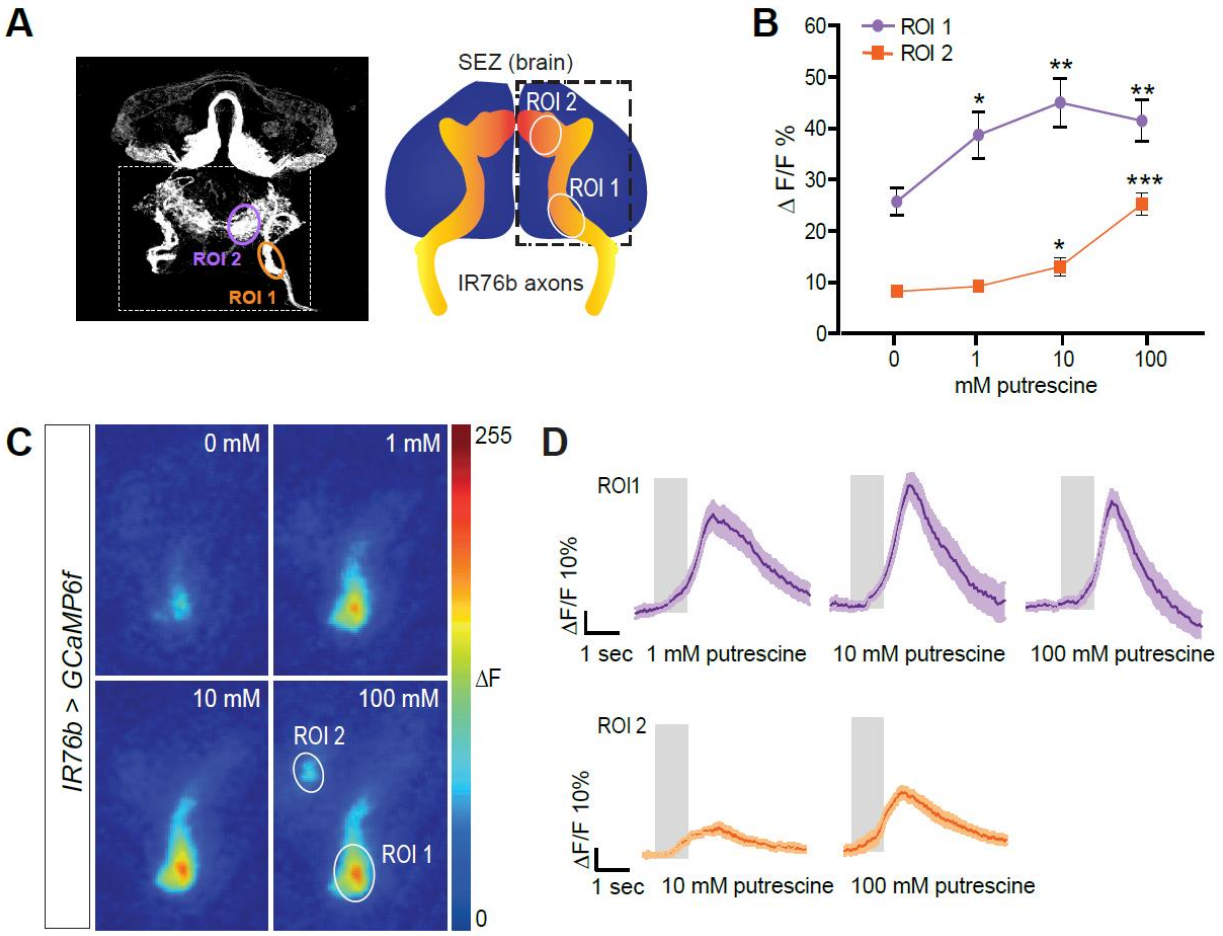

Figure 13 IR76b taste neurons response to polyamines in *in vivo* Calcium imaging

(A) Scheme of the fly brain and regions of interest (ROI) in the SEZ in *in vivo* calcium imaging. The ROIs were used to quantify the relative changes in GCaMP-fluorescence (%ΔF/F). (B) Average trace of GCaMP6f-fluorescence responses to different concentrations of putrescine in the ROI1 and ROI2 areas of females respectively (n=11 ± SEM). Statistics are compared with the control. (C) Representative images of SEZ imaging of *IR76b-Gal4; UAS-GCaMP6f* females stimulated with different concentrations of putrescine (1–100mM) and distilled water as control (0mM).(D) Quantification of GCaMP6f-fluorescence peak responses in ROI1 and ROI2 areas of females to putrescine from 1mM to 100mM (n=11 ± SEM). All GCaMP6f-fluorescence responses were calculated in %ΔF/F. All p-values were calculated via one-way ANOVA (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure 4 of Hussain, Zhang et al. PLoS Biology 2016.

To strengthen the evidence for IR76b as polyamine receptor, I tested the fluorescence changes in *IR76b*^{1} mutants with GCaMP6f expressed under the control of IR76b-Gal4 (*IR76b-Gal4, UAS-GCaMP6f; IR76b*^{1}). The responses to putrescine in both ROI1 and ROI2 area of SEZ were significantly reduced (figure 14A–C). This result demonstrated that IR76b plays a role as polyamine receptor in taste neurons.

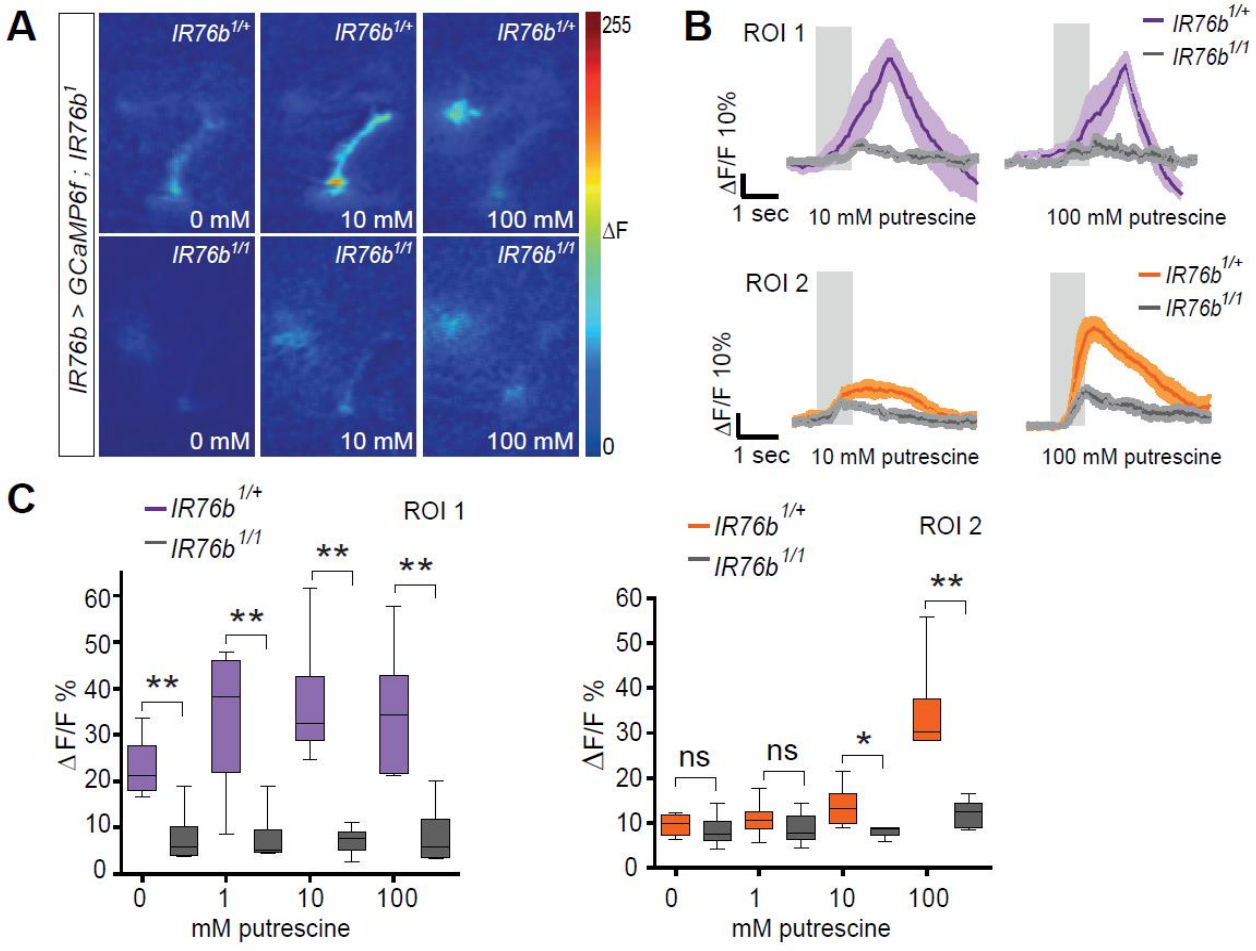

Figure 14 IR76b mutants show largely reduced taste neuronal responses to polyamines in *in vivo* calcium imaging

(A) Representative images of SEZ of *IR76b-Gal4, UAS-GCaMP6f; IR76b*^{1} and heterozygous control females stimulated with different concentrations of putrescine (10–100mM) and distilled water as control (0mM). (B) Average traces in the ROI1 and ROI2 areas respectively of *IR76b* mutant and control females stimulated with different concentrations of putrescine (n=6 ± SEM). (C) Quantification of peak responses in the ROI1 and ROI2 areas respectively to different concentrations of putrescine in *IR76b* mutant and control females (n=6 ± SEM). All GCaMP6f-fluorescence responses were calculated in %ΔF/F. All p-values were calculated via Student's T-test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure 4 of Hussain, Zhang et al. PLoS Biology 2016.

In addition, a previous study showed that IR76b mediates salt detection. I also observed the response of ROI1 neurons to salt (50mM $NaCl_2$) but not ROI2 neurons (figure 15A). The result was consistent with the previous report that implicated the receptor IR76b in a low concentration of salt detection (Zhang, Ni et al. 2013). Consequently, the response to salt was also largely reduced in *IR76b*^{1} mutants (figure 15B).

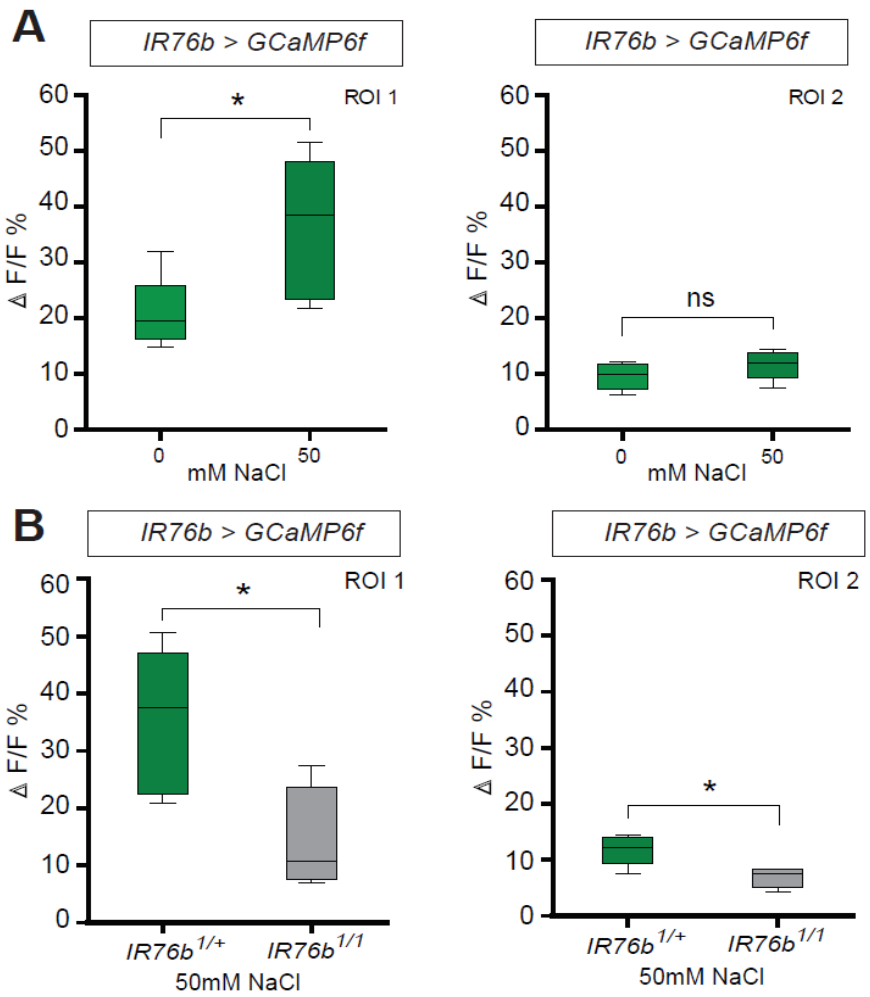

Figure 15 IR76b taste neurons also response to a low concentration of salt

(A) Quantification of GCaMP6f-fluorescence peak responses to distilled water and 50 mM $NaCl_2$ in the ROI 1 and ROI 2 areas respectively (n=6 ± SEM). (B) Quantification of peak responses to distilled water and 50 mM $NaCl_2$ in the ROI1 and ROI2 areas of *IR76b* mutants and control females respectively (n=6 ± SEM). All GCaMP6f-fluorescence responses were calculated in %ΔF/F. All p-values were calculated via Student's T-test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure S6 of Hussain, Zhang et al. PLoS Biology 2016.

I demonstrated that the sensilla on the labellum mediate the taste detection to polyamines. However, the IR76b neurons on legs project to the ventral nerve cord but not the brain (Zhang, Ni et al. 2013). Although the leg ablation experiment showed that flies ablated one pair of legs at a time showed the normal polyamine preference, flies without legs could not freely walk and choose in the oviposition assay and hence, were not tested. To understand whether tarsae IR76b neurons are involved in polyamine detection or at least could detect polyamines, I adapted the setup of *in vivo* calcium imaging and recorded the responses of tarsae neurons to putrescine directly on ablated tarsae. I recorded GCaMP-fluorescence changes of IR76b neurons on ablated tarsae of forelegs (figure 16A-C). These data suggested that tarsal IR76b neurons also respond to polyamines. Therefore, it is possible that tarsal IR76b neurons also contribute to oviposition choices. Of note, I only observed significant response to high concentration of putrescine but not to low concentrations (figure 16C). This result may indicate that the response of tarsae is less important than labellum in behavioral choices.

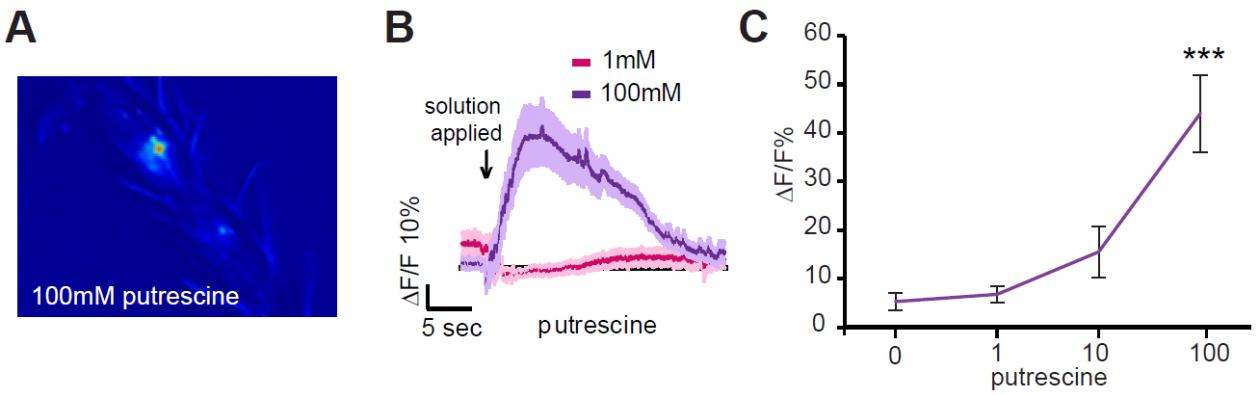

Figure 16 Tarsal neurons response to putrescine in *in vivo* Calcium imaging

(A) A representative image of a fore tarsae of *IR76b-Gal4; UAS-GCaMP6f* females stimulated with putrescine (100mM). (B) Average trace of GCaMP6f-fluorescence responses of tarsal neurons to 100mM putrescine compared with control (n=8 ± SEM). (C) Quantification of GCaMP6f-fluorescence peak responses in tarsal neurons of females to putrescine from 1mM to 100mM (n=8 ± SEM). Statistics are compared with the control (0mM). All GCaMP6f-fluorescence responses were calculated in %ΔF/F. All p-values were calculated via one-way ANOVA (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure S6 of Hussain, Zhang et al. PLoS Biology 2016.

In summary, my SEZ imaging data strongly supported the conclusions reached by behavioral experiments and showed that IR76b receptors were specifically required for polyamine sensation in gustatory receptor neurons found primarily on the labellum.

2.1.2 Mating state modulates the perception of polyamines at the level of taste neurons

Previous studies in *Drosophila* suggested that the perception of sensory information depends on the internal state (Ignell, Root et al. 2009, Root, Ko et al. 2011, Bargmann 2012, Bracker, Siju et al. 2013). The internal state and changed physiological needs of gravid females alter their behaviors to provide better conditions for their offspring (Carvalho, Kapahi et al. 2006, Ribeiro and Dickson 2010). Given that female flies were attracted to polyamines and polyamines worked as landmarks for choosing egg laying places, we asked whether the mating state would influence the perception of polyamines.

At first, to compare the difference in polyamines perception, Ashiq Hussain tested virgin and mated females in oviposition behaviors. In the oviposition assay, although virgin females laid fewer eggs (10-15 eggs/each test), the eggs distributed equally between the polyamine-rich side and the control side (figure 17). The result indicated that the perception of polyamines depended on females' mating state. Mated females showed a higher preference to polyamine compared with virgin females.

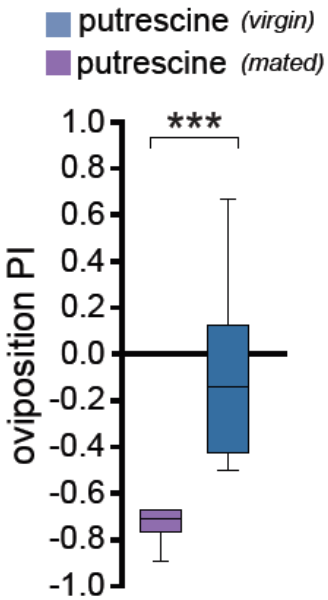

Figure 17 The polyamine preference in oviposition assay is mating state dependent

Virgin females show no preference to putrescine (1mM) compared with mated females in oviposition assay (n=8± SEM). Box plots show median and upper/lower quartiles, while whiskers show minimum/maximum values. All p-values were calculated via Student's T-test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figure is adapted from Figure 1 of Hussain, Ucpunar et al. PLoS Biology 2016.

To understand the mechanism of mating state-dependent modulation in polyamine perception, we asked whether the modulation happened at the level of the peripheral taste neurons. Thus, I used calcium imaging to assess the response of IR76b neurons in virgin and mated females respectively. The mated females were tested at 5 – 7days after mating. There was no difference in two ROIs of SEZ between virgin and mated females (figure 18). In contrast to ROI2, the response of ROI1 in mated females was little higher than the response of ROI1 in virgin females but not significantly (figure 18).

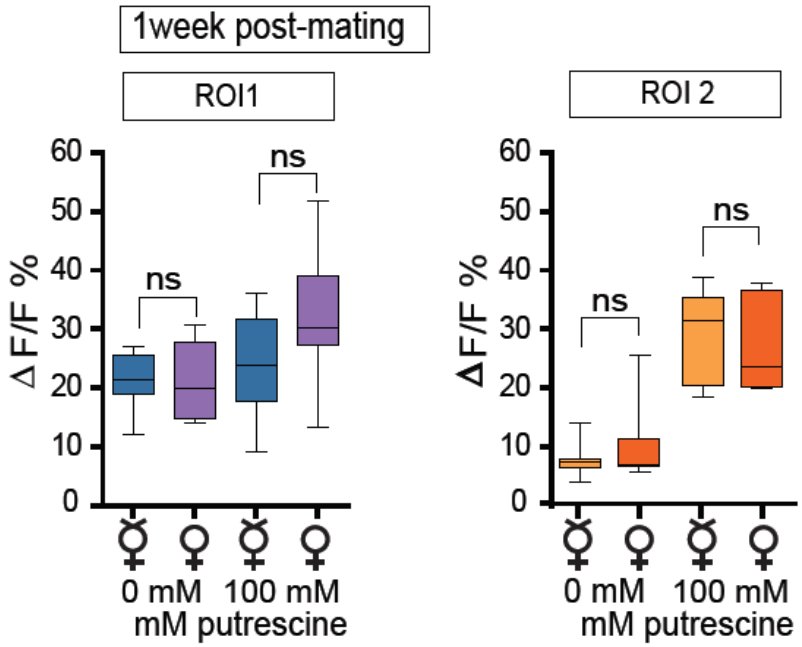

Figure 18 Mated females after one week showed no significant difference in neural sensitivity than virgin females

IR76b neuron terminals in the SEZ of 1 week post-mated females show no significant difference in response to putrescine (100mM) in either ROI1 or ROI2 area (n=7± SEM). Box plots show median and upper/lower quartiles, while whiskers show minimum/maximum values. All p-values were calculated via Student's T-test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

Because mating lead to long-term (~ 1 week) and short-term (< 24 hours) effects, the mating induced changes in ORN lasted for short time (Chapman, Bangham et al. 2003, Heifetz, Lindner et al. 2014). Thus, we asked whether time scales affected the modulation. I performed the same

experiment on mated female immediately after mating. The response of ROI1 in mated 1-3 hours females was significantly higher than virgin females (figure 19A–D). Interestingly, the response in ROI2 of SEZ, which responded to high concentrations of putrescine (10–100mM), was no difference between mated and virgin females (figure 19A–D). The data indicated that the sensitivity of polyamine responses in IR76b neurons was transiently changed after mating. In ROI1 of SEZ, IR76b neurons might interact with other molecules that were triggered by the post-mating switch. In behavior, the modulation of polyamine perception after mating is a result of the change in sensitivity of polyamine detecting GRNs, which is induced by a switch in mating state.

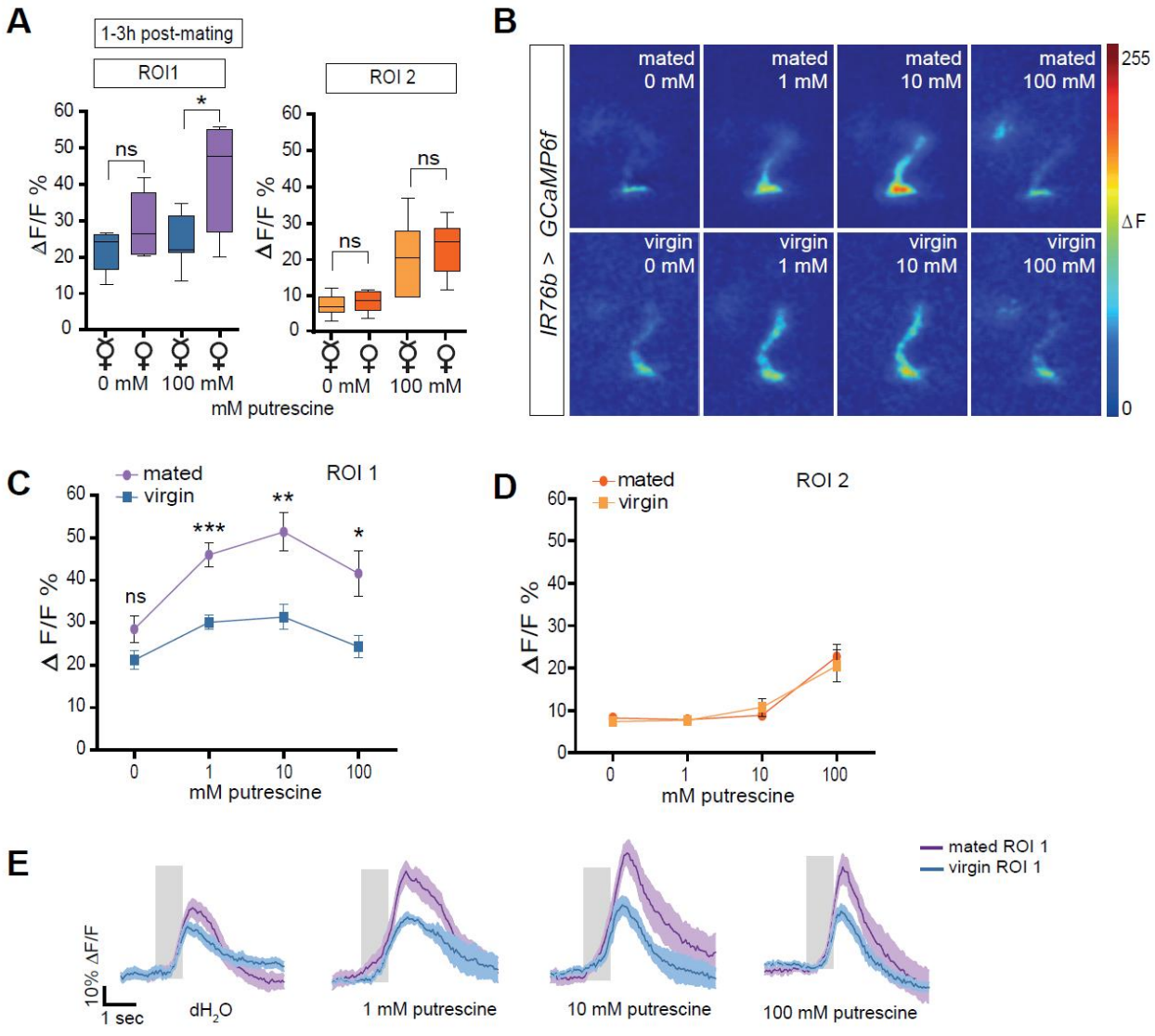

Figure 19 Mated females showed higher neural sensitivity than virgin females

(A) Females at 1–3 hours post-mating show a significantly higher response to putrescine (100mM) in ROI1 of IR76b neuron terminals (n=7± SEM). (B) Representative images of SEZ of 1–3 hours post-mated females and virgin females (*IR76b-Gal4, UAS-GCaMP6f*) stimulated with different concentrations of putrescine (1–100mM) and distilled water as control (n=7± SEM). (C) Average trace in the ROI1 areas of females at 1–3 hours post-mating compared with virgin females stimulated with different concentrations of putrescine (1mM to 100mM, n=7 ± SEM). (D) Average trace in the ROI2 areas of 1–3 hours post-mating females at 1mM to 100mM (n=7 ± SEM). (E) Quantification of peak responses of ROI1 and ROI2 areas respectively to different concentrations of putrescine in 1–3 hours post-mating females and virgin females (n=7 ± SEM). All GCaMP6f-fluorescence responses were calculated in %ΔF/F. Box plots show median and upper/lower quartiles, while whiskers show minimum/maximum values. P-values of box plots were calculated via Student's T-test. P-values of concentration traces were calculated via one-way ANOVA (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure 3 of Hussain, Ucpunar et al. PLoS Biology 2016.

As discussed above, flies use two types of gustatory neurons for polyamine perception. The receptor GR66a detects the bitterness of polyamines and IR76b recognizes the polyamine itself. To test whether the sensitivity of bitter neurons changed with mating, I used tip recordings and analyzed the responses of S-type sensilla in virgin and mated females, respectively. There was no difference between virgin and mated females of S-type sensilla response (figure 20). The result indicated that this putrescine responding sensilla on the labellum were not essential in mating state-dependent modulation.

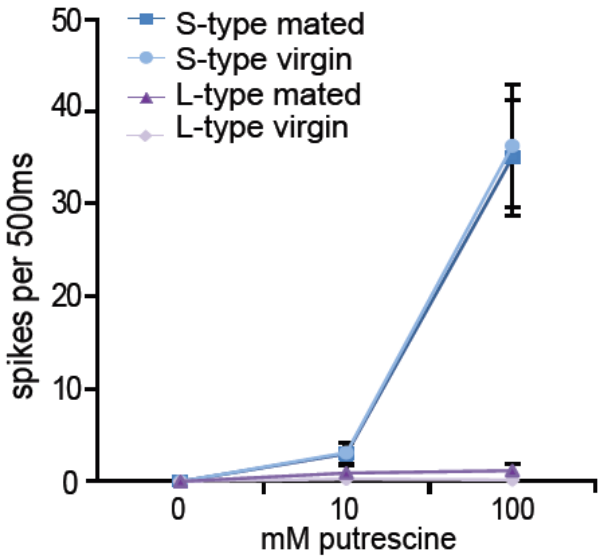

Figure 20 Mated females showed no higher neural activity in bitter neurons than virgin females

Quantification of the responses of S-type and L-type sensilla on the labellum to different concentrations of putrescine in mated and virgin females (1–100mM) (n=8 ± SEM). There are no differences between sensilla responses of mated and virgin females.

2.1.3 A G-protein coupled receptor regulates the sensitivity of polyamine sensory neurons

From previous studies, the classic post-mating switch is regulated by SPR and its ligand sex peptides (SPs) (Yapici, Kim et al. 2008). We next asked whether SPR was involved in modulating post-mating polyamine taste perception. To understand the role of SPR, Ashiq Hussain tested the oviposition behaviors of *SPR* mutant flies. Mated *SPR* mutant flies displayed a strongly reduced egg laying aversion in oviposition assay (figure 21). Although *SPR* mutant females laid more eggs (30–40 eggs/each test) than its wildtype control, they showed abolished behavioral preference to polyamine in oviposition choice similar to that of virgins. Downregulating the SPR expression selectively in IR76b neurons of mated females (*IR76b-Gal4; UAS-SPRi*), he observed similar reduced oviposition preference to polyamine in *SPR* mutants (figure 21B). Re-expression of SPR construct using IR76b driver (*IR76b-Gal4, UAS-SPR; SPR*$^{-/-}$) can rescue the polyamine preference of *SPR* mutant females in oviposition choices. However, re-expression of SPR in bitter neurons (*GR66b-Gal4, UAS-SPR; SPR*$^{-/-}$) did not rescue the polyamine preference in behavior (figure 21C). These results suggested that the expression of SPR in IR76b taste neurons plays an important role in modulation of polyamine perception after mating.

Since we demonstrated that SPR was required for modulation of polyamine perception after the post-mating switch, we asked whether SPR is sufficient to modulate the sensation. Ashiq Hussain re-expressed SPR in IR76b neurons and bitter taste neurons in *SPR* mutant females separately. The test in oviposition behavior showed that the re-expression of SPR in IR76b neurons could fully rescue the absent preference in egg laying (figure 21C). By contrast, re-expression of SPR in GR66a neurons did not rescue the SPR mutant phenotype (figure 21C). This result demonstrated that SPR signaling was sufficient in IR76b neurons for the mating state-dependent polyamine perception modulation.

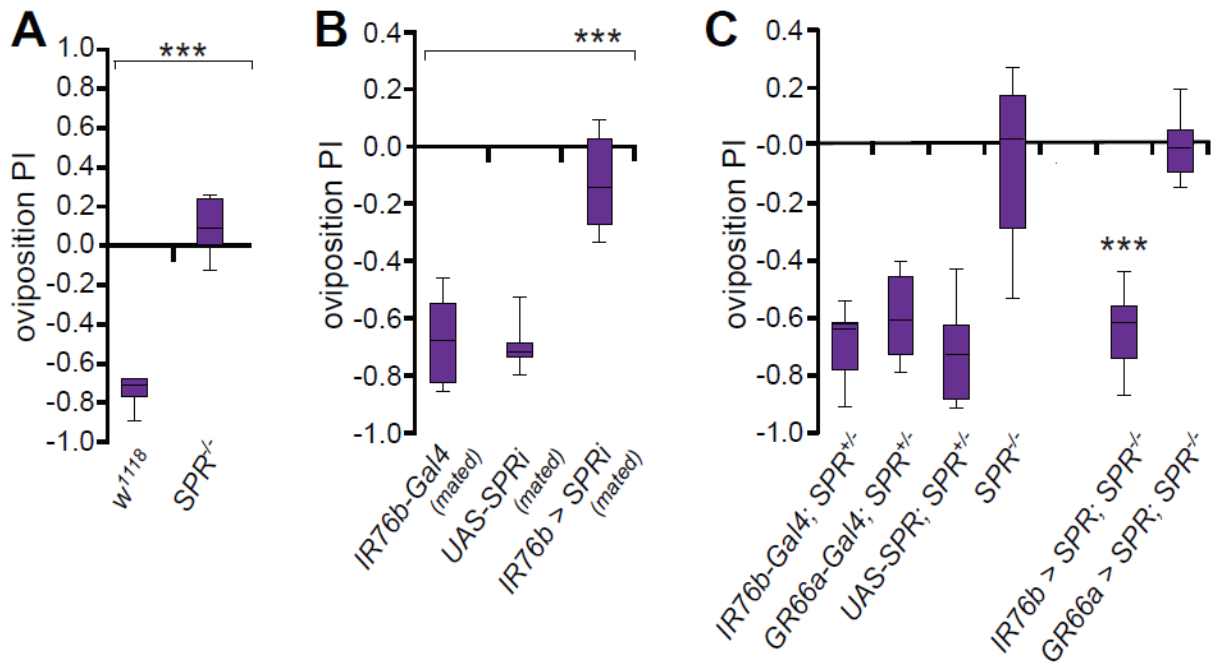

Figure 21 Mating state regulate polyamine detection by SPR signaling

(A) Sex peptide receptor mutant (*SPR*$^{-/-}$) females display a similar preference index as compared to virgin females in oviposition behavior (1mM putrescine, n=8± SEM). (B) Knockdown of SPR in IR76b polyamine taste neurons using RNAi (*IR76b-Gal4; UAS-SPRi*) significantly reduce oviposition preference to putrescine in mated females as compared to mated controls (1mM putrescine, n=8± SEM). (C) *SPR* mutant phenotype can be fully rescued in mated females in oviposition behavior by re-expression of *SPR* using IR76b-Gal4 in IR76b taste neurons. However, re-expression of SPR in GR66a bitter taste neurons did not rescue the preference of oviposition behavior (n=8± SEM). Box plots show median and upper/lower quartiles, while whiskers show minimum/maximum values. All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure 2 of Hussain, Ucpunar et al. PLoS Biology 2016.

Then we asked whether SPR signaling might act directly in the chemosensory peripheral neurons. Recent studies on hungry flies showed that the olfactory sensitivity to food odors could be increased by increased expression of sNPFR. Its ligand is the ORN-resident short neuropeptide F, sNPF (Ignell, Root et al. 2009, Root, Ko et al. 2011). A similar mechanism could also affect the mating state-dependent polyamine perception. Given that IR76b is necessary and sufficient to polyamine perception and expressed broadly in gustatory neurons, we asked whether SPR was required directly in the sensitivity of IR76b neurons. Using driver IR76b-Gal4, I expressed a previously characterized SPR-RNAi construct in IR76b gustatory neurons (Yang, Rumpf et al. 2009).

To assess the influence of SPR signaling on taste neurons of mated females, I used calcium imaging to test the neuronal changes of labellar IR76b neurons in mated females with

downregulated SPR in IR76b neurons (*IR76b-Gal4, UAS-SPRi; UAS-GCaMP5*) and in mated control females of the relevant genetic background (*IR76b-Gal4i; UAS-GCaMP5*). The mating strategy was same as the one used for immediately mated females I tested before. The response of ROI1 in mated *IR76b-SPRi* females was significantly reduced compared to the response of ROI1 in mated control females (figure 22A). This reduced response of IR76b neurons in *IR76b-SPRi* females was similar to the reduced response of virgin female neurons as compared to that of mated control females (figure 22B–C). This result confirmed the involvement of SPR on IR76b neurons in modulating post-mating polyamine perception directly on the level of the peripheral gustatory neurons.

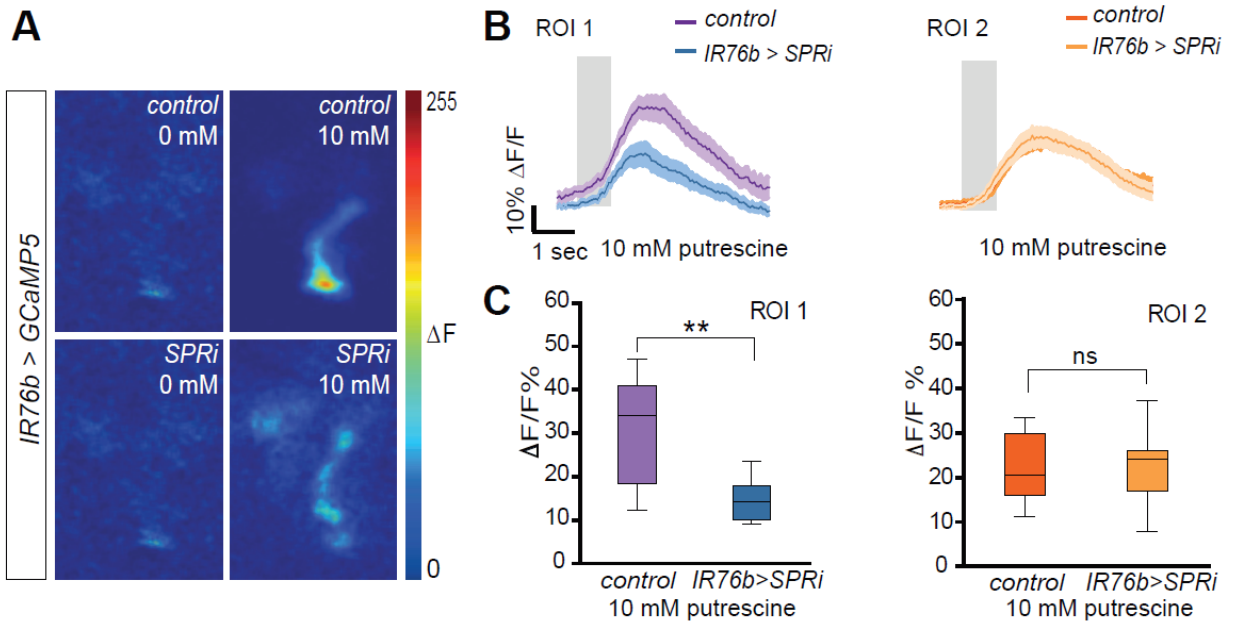

Figure 22 SPR is necessary for modulation of polyamine detection after mating

(A) Representative images of IR76b neuron terminals in SEZ of IR76b RNAi females (*IR76b-Gal4, UAS-SPRi; UAS-GCaMP5*) and control females (*IR76b-Gal4; UAS-GCaMP5*) at 1–3 hours post-mating stimulated with 10mM putrescine and distilled water as control (n=8± SEM). (B) Average trace in the ROI1 and ROI2 areas of IR76b RNAi females compared with the control females at 1–3 hours post-mating stimulated with 10mM putrescine and distilled water as control (n=8± SEM). (C) Quantification of responses in the ROI1 and ROI2 areas of IR76b RNAi females compared with control at 1–3 hours post-mating stimulated with 10mM putrescine respectively (n=8 ± SEM). All GCaMP6f-fluorescence responses were calculated in %ΔF/F. All p-values were calculated via Student's T-test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure 3 of Hussain, Ucpunar et al. PLoS Biology 2016.

We next asked which ligand triggered the SPR signaling. The classical ligands of SPR are SPs – a component of male's ejaculate (Hasemeyer, Yapici et al. 2009). When SPs were transferred from males to females, they triggered the behavioral post-mating switch (Yang, Rumpf et al. 2009). Given that mating state regulated the signaling of SPR to modulate polyamine perception,

we asked whether SPs were required to trigger SPR leading to modulation of polyamine sensitivity. Ashiq Hussain crossed wildtype virgin females with SP^0 mutant males, which lacked of SPs in males' semen (Liu and Kubli 2003). Wildtype virgin females mated with wildtype males were used as control. The females mated the SP^0 mutant laid few eggs (10–15 eggs/each test) and exhibited dramatically reduced taste preference similarly as virgin in oviposition assay (figure 23A). However, since wildtype flies were attracted to odorant polyamines in olfactory assay, females mated with the SP^0 mutant did not been affected in the attractive preference to polyamines in olfactory behavioral choices. These results indicate another candidate could involve in mating state-dependent polyamine perception modulation. Furthermore, alternative candidate, myoinhibitory peptides (MIPs), has been recognized as additional SPR ligands (Kim, Bartalska et al. 2010). The expression of MIPs was broad in the brain covered primary gustatory center the SEZ (Kim, Bartalska et al. 2010). Importantly, when Ashiq Hussain tested the involvement of MIPs using MIP-RNAi construct downregulating MIPs expression in IR76b neurons (*IR76b-Gal4; UAS-MIPi*), only females but not males showed reduce attractive preference to polyamines in olfactory assay. This indicated that MIPs were involved in mating state-dependent modulation together with SPR. In oviposition behavioral assays, females with MIPs downregulated on IR76b neurons showed similarly reduced preference to lay eggs on polyamine-rich site similarly as SPR downregulated females (figure 23B). The result suggested that the mating state-dependent polyamine taste perception was regulated by the SPR/MIPs signaling pathway.

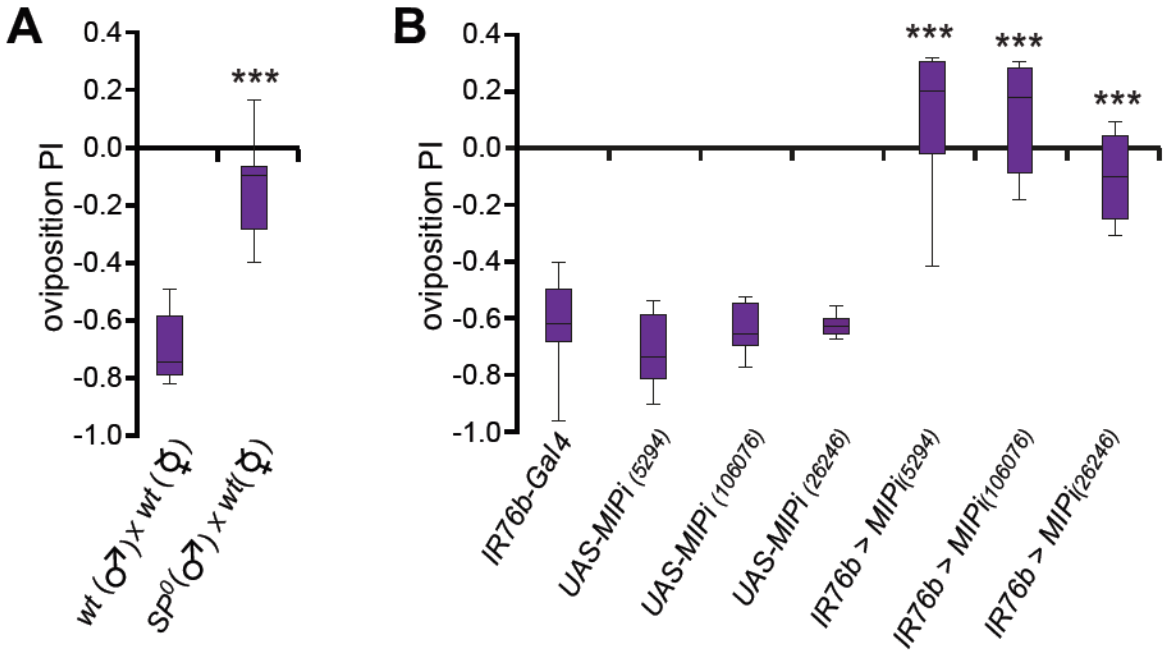

Figure 23 Myoinhibitory peptides as the ligand of SPR regulate polyamine taste sensitivity

(A) *SP^{0}* male-mated Canton S females show no preference to polyamines in oviposition behavior (n=8± SEM). (B) Mated females with RNAi knockdown of MIPs in IR76b neurons (*IR76b-Gal4; UAS-MIPi*) loss the preference to polyamines in oviposition behavior (n=8± SEM). Box plots show median and upper/lower quartiles, while whiskers show minimum/maximum values. All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Figures are adapted from Figure 5 of Hussain, Ucpunar et al. PLoS Biology 2016.

2.1.4 A summary of the mating state-dependent polyamine taste preference

In summary, we demonstrated that *Drosophila* females use polyamines to find and evaluate egg laying sites. We showed that polyamines are beneficial and increase the reproduction of offspring significantly. Furthermore, we first show that polyamine taste perception is modulated by mating state. Moreover, the modulation of polyamine taste perception depends on the SPR/MIPs signaling pathway that acts directly at the level of gustatory neurons. Using functional calcium imaging, I showed the role of IR76b neurons in polyamine detection in the gustatory system. Importantly, I showed that IR76b neurons of mated females had a significantly higher sensitivity than virgin females. I showed modulation of neural sensitivity on the taste peripheral neurons. In addition, I demonstrated that SPR signaling in IR76b neurons was necessary and sufficient in the function of this modulation. The result showed that the sensitivity of polyamine taste neurons are modulated by mating state. This increased sensitivity to polyamines of IR76b neurons on mated females was regulated by SPR signaling directly on neural level transiently after mating.

2.2 Identification of neural mechanisms of aging associated olfactory decline

The loss of smell is one of the earliest symptoms of aging and neurodegenerative diseases (Kovacs 2004). To understand the cellular and molecular mechanism of aging-associated olfactory decline, I collaborated with Dr. Ashiq Hussain and performed behavioral, electrophysiology and anatomical analysis in *Drosophila* to explore the effect of aging and aging-regulated genes in the function of the olfactory system. As above, I will first introduce his behavioral results in figure 24 to 26 to put my results into the necessary context.

2.2.1 *Drosophila* shows olfactory behavioral decline with aging

Several studies have analyzed behavioral decline associated with aging in flies (Grotewiel, Martin et al. 2005, Iliadi and Boulianne 2010). One study suggested that avoidance of an aversive odor is reduced in aged flies (Cook-Wiens and Grotewiel 2002). However, it is unknown whether this aging-associated olfactory preference is general for odors and how and where it happens in the whole olfactory system. To establish the phenomenon of olfactory decline with age, Dr. Hussain tested naïve flies of different ages (figure 24). During the 1-minute test in T-maze behavioral assay, flies made binary choices between the odor solvent and different odors. Three attractive and five aversive odors were tested (table 1). Out of all the odors, younger flies showed stronger attractive or aversive preferences than older flies (figure 24). The results demonstrated that flies exhibit gradual aging-associated olfactory decline to different odors. He used a wide array of chemosensory receptors that cover olfactory perception. These include polyamine-specific IR receptors (Hussain, Zhang et al., in press), the CO_2 receptors GR21a and GR63a (Jones, Cayirlioglu et al. 2007) and OR-type receptors for other odors (Hallem and Carlson 2006). These data showed that aging-associated olfactory decline happened in all types of olfactory receptors (figure 24).

Table 1 The list of odors used in behavioral tests

Odorant	Behavioral response	Candidate Receptors	Candidate Neurons
Pentanoic acid	attraction	OR42b, OR67a,OR92a	ab3b, ab10
2,3-Butanedione	attraction	OR42a,OR67a,OR92a	ab1a, ab1b
Putrescine (1,4-Diaminotutane)	attraction	unknown IR	ac2?
Benzaldehyde	aversion	OR7a, OR35a, OR45b, OR67a,OR24a	ac1b, ab4
4-methylcyclohexanol	aversion	OR67b	ab9b
3-Octanol	aversion	OR35a	ac1b, ac3b
Linalool	aversion	OR98a,OR19a,OR19b	at3b, ab7a
CO_2	aversion	GR21a, GR63a	ab1c

In *Drosophila*, Orco is thought to be the co-receptor for all the ORs (Vosshall 2000). The function of the OR system can be impaired by mutating Orco. To illuminate whether olfactory decline is dependent on the characteristic of Orco expressing neurons, *orco* mutant flies were tested in the

same assay. As expected, the *orco* mutant with different ages showed no responses to odors. The decline of the response to putrescine and CO_2 was similar to wildtype controls, since Orco is not required for IR- and GR-dependent odor detection (figure 25).

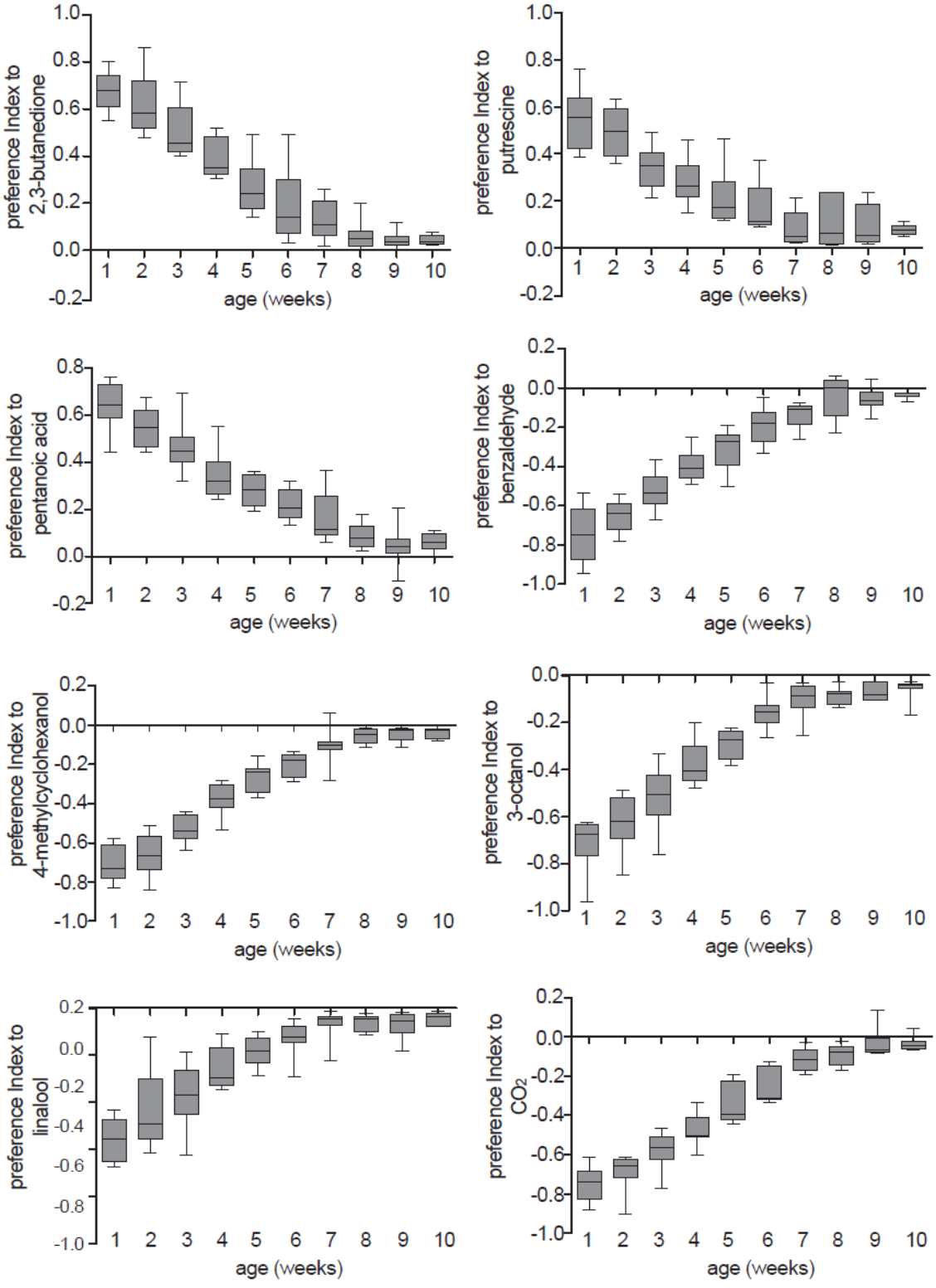

Figure 24 Aging associated olfactory behavioral decline

Wild flies (Canton S) show attraction and avoidance to different odors in behavioral tests. The responses associated with olfactory perception to attractive or aversive odors decline with aging from one to ten weeks old flies (n=8± SEM).

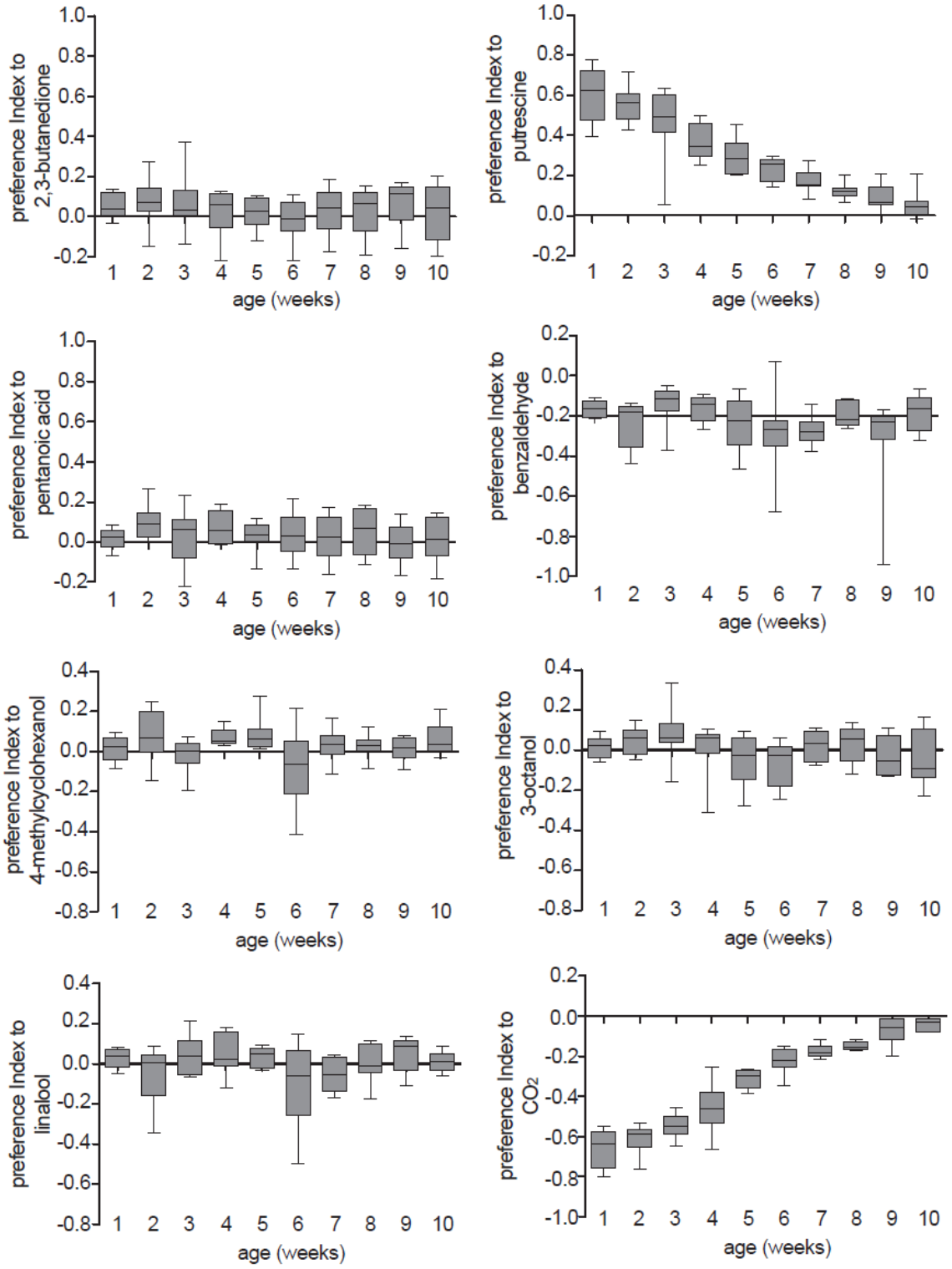

Figure 25 All types of ORNs show aging-dependent olfactory decline including Orco-independent ORNs

Olfactory receptor (OR) dependent responses are lost in o*rco* mutants to both attractive and aversive odors. While CO_2 is detected by gustatory receptors (GR21a and GR63a) and putrescine (1, 4-diaminobutane) is perceived by ionotropic receptors, the olfactory preferences mediated by GRs and IRs still decline with aging (n=8± SEM).

2.2.2 The visual preference behavior is only marginally affected by aging

Next, I joined this project and investigated the reason for this aging-associated olfactory decline. Given that the motility of old flies was reduced with aging, old flies exhibited a slower movement in the arms of the T-maze that could lead to a reduced preference index. Thus, we modified the behavioral assay to confirm the aging-associated motility decline. Flies show strong phototaxis or attractive preference to blue light. In the modified T-maze assay, flies were attracted to one side with a LED emitting a visible blue light (465–470 nm) while the opposite side was an invisible red light (625–630 nm). The preference of flies was recorded in a custom-made video tracking setup. The preference index was calculated by subtracting the number of flies walking to one side from the number of flies walking to the other side, divided by the total number of flies in the two sides during the 1-minute test. Flies of all age groups showed a strong attractive preference to blue light (Figure 26). Although the preference of old flies showed a little decline compared to younger flies, the decline was not as strong as the olfactory decline associated with aging. This result indicated that older flies maintained the mobility to make choices in T-maze test and that the aging-associated olfactory decline was not a result of reduced motility in old flies.

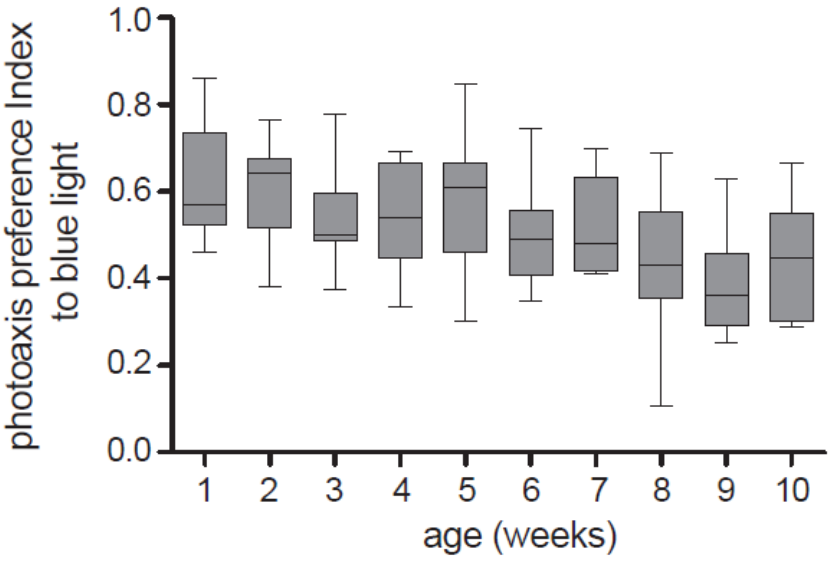

Figure 26 Light based motility is not changed with aging

Aged flies still show high attraction to blue light. The motility of aging flies is not significantly decreased compared to young flies (n=8± SEM).

2.2.3 *Drosophila* shows functional decline with age on olfactory receptor neurons

Our data above suggested that the cause of the olfactory behavioral decline affects olfactory detection or processing. In peripheral olfactory organs, the expression of particular receptors on olfactory neurons is essential for odor detection (Vosshall 2000). Receptor OR42b is the specific receptor to 2, 3-butandione, which was tested in behavior. Using GAL4/UAS system to label OR42b neurons with mCD8GFP, the number of OR42b neurons can be quantified under the fluorescence microscope. Using genetic GFP labelling, I compared the number of OR42b neurons in young and old flies. Old flies did not show a reduced number of OR42b neurons compared with younger flies (figure 27 A and B). The result suggested that the number of olfactory neurons was not affected by aging.

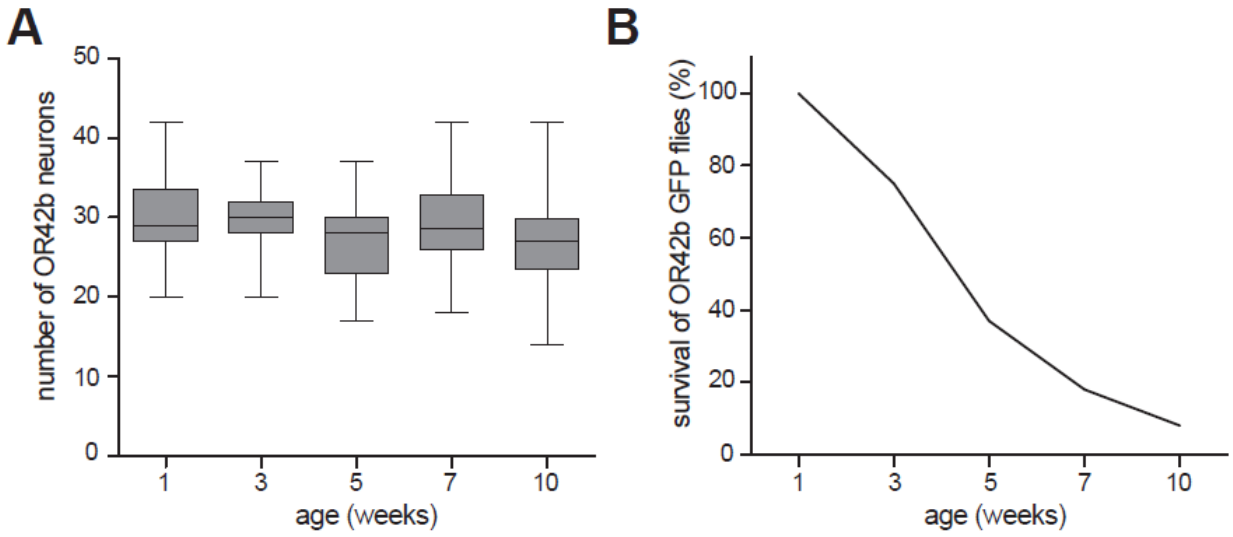

Figure 27 The number of ORN is not changed with aging

(A) Quantification of cell numbers of OR42b receptor neurons from 1 week to 10 weeks old flies (*OR42b-Gal4; UASmCD8GFP*) and (B) the survival curve of those flies. The number of GFP expressed OR42b ORNs did not show a significant decrease in aged flies compared to young flies, while the survival of flies was decreased (n=300 ± SEM).

However, survival of neurons in old flies did not mean the activity of cells stayed at the same level as in younger flies. A previous EAG study indicated that the decline in electrophysiological responses of olfactory neurons occurred with aging (Ayer and Carlson 1992). Thus, I performed single sensillum recording (SSR) to record the activities of the peripheral olfactory neurons on the antenna. The electrophysiological characteristics of ORNs can be precisely measured by SSR

(de Bruyne et al. 1999). Before the electrophysiological recording, flies were tested in the T-maze and sorted into two groups: 1. Responders; flies that behaved as expected to the odor and 2. Non-responders; flies that did not behave as expected (figure 28 A). Flies were attracted to 2, 3-butanedione and avoided benzaldehyde at a young age. These olfactory preferences declined with aging. Aged flies and young flies were tested. According to their choice flies were separated into “responders” and “non-responders”. The activities of neurons on 7 weeks old flies were compared with 1-week-old flies as controls. Two classes of neurons were recorded. One expressed 2, 3-butanedione receptors and the other expressed benzaldehyde receptors. For each odor, a total of four groups of flies was tested in single sensillum recording using different concentrations of the same odorant (figure 28 B).

The result showed that neural activities of old flies declined compared with young flies. The spiking activity of neurons decreased with age in response to both attractive odors and aversive odors (figure 28 B). Interestingly, even at the same age, neurons of the “responder” groups responded more than of the “non-responder” groups. The same phenomenon was observed in both the attractive odor and the aversive odor. The result not only indicated that the activities of ORNs declined with age, but also suggested that the behavioral responses of flies were related with the activities of responding neurons. Furthermore, ORN activity appears to vary in individual flies. Nevertheless, the small decrease in ORN responses did not explain the full decrease of olfactory preference behavior. Therefore, I continued to try to identify the full reason for the observed decline.

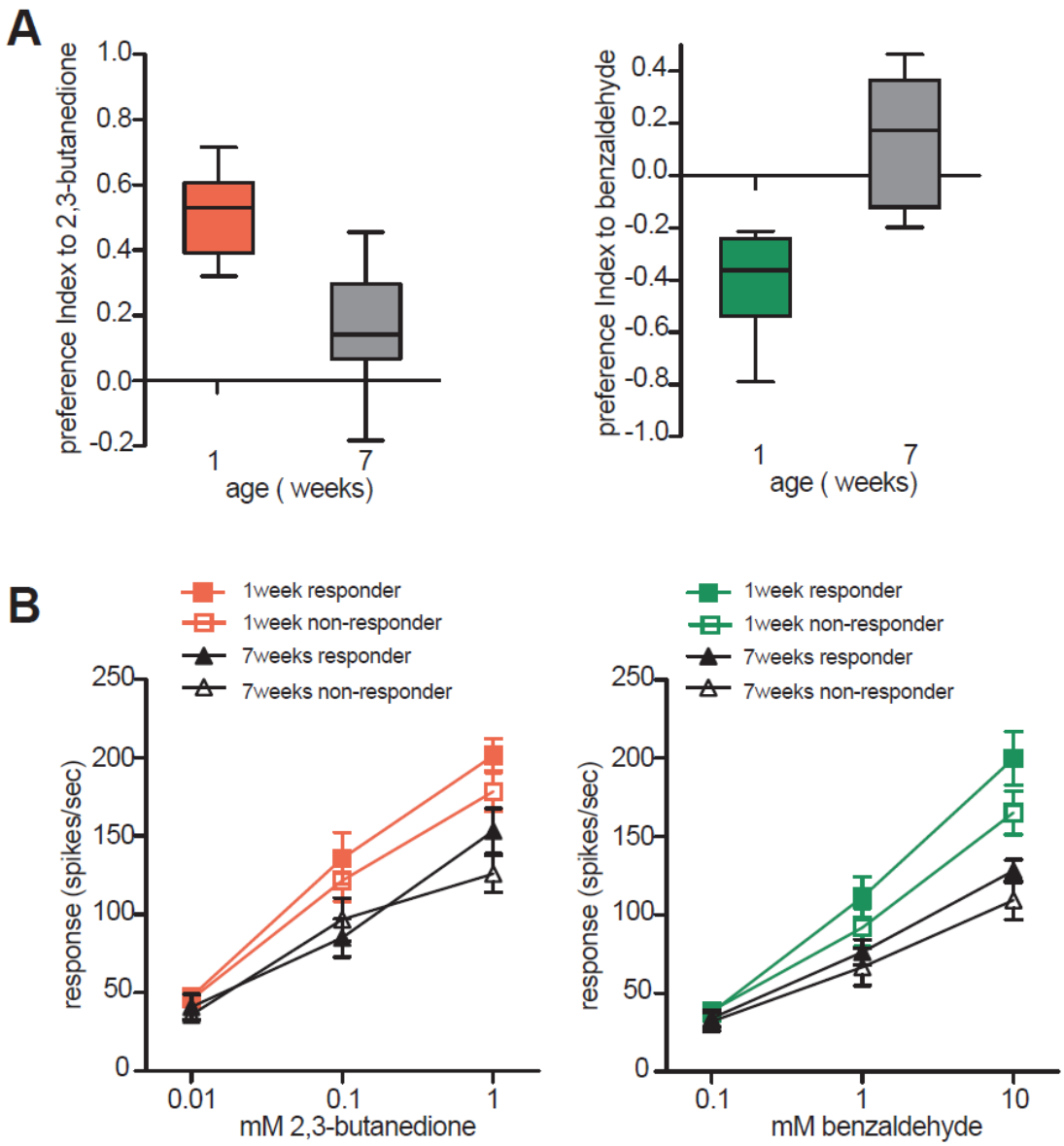

Figure 28 Electrophysiological responses of two olfactory neurons declined with aging

(A) The behavioral response 7weeks old flies lost strong preference to 2, 3-butanedione (attractive) and Benzaldehyde (aversive) compared to 1 week old flies (n=8 ± SEM). (B) Responses of ab1A and ab1B, which respond to 2, 3-butanedione, and responses of ab4A, which respond to Benzaldehyde were recorded by single sensillum recording. The responses of neurons showed a significant decline with aging (n=8 ± SEM).

2.2.4 Downregulating aging-related genes can mimic aging effect on lifespan and olfactory ability

Next, it is essential to understand the genetic and cellular mechanisms of aging-associated olfactory decline. To the end, I analyzed the role of candidate genes previously implicated in aging

mechanisms. To investigate the candidate genes, I identified a list of aging genes, which regulate lifespan in different model organisms from previous studies (table 2).

Table 2 Potentially aging genes used in RNAi screening

Gene	Function	Effect on lifespan	Effect on behavior
SOD1	Superoxide dismutase activity	Reduce	
SOD2	Superoxide dismutase activity	Reduce ~80%	Decline in negative geotaxis, olfactory behavior
FMR1	RNA-binding protein	Reduce ~60%	Decline in climbing
FOXO	Forkhead box, sub-group O		
Sirtuin	NAD^+- dependent protein deacetylases		
chico	Insulin-like growth factor receptor binding	Increase ~48%	Delayed senscence of nagative geotaxis
InR	Insulin-like receptor	increase	
mth	G-protein-coupled receptor activity	Increase ~35%	
Indy2	Sodium dicarboxylate cotransporter	Increase ~50%	Restricted decline in nagative geotaxis
EcR	Ecdysone receptor	increase ~45%	delayed decline in nagative geotaxis
mys	Beta-integrin (surface receptor)	increase ~20%	Delayed senscence of nagative geotaxis
Tor	Target of rapamycin	increase	

I used RNAi constructs expressed in transgenic flies to knockdown individual genes and analyzed the effects on olfactory preference (Dietzl, Chen et al. 2007). First, I used a pan-neural driver *elav-Gal4* to downregulate aging genes in all neurons (Robinow and White 1991). The offspring (*elav-Gal4; X-RNAi*) was tested in the T-maze with an attractive odor (2, 3-butanedione) and an aversive odor (benzaldehyde) at 10 days and 20 days of age. One of candidates, *mitochondrial superoxide dismutase2 (SOD2)* showed a significant effect on olfactory preference. Flies (*elav-Gal4; SOD2-RNAi*) showed reduced attraction and aversion preference in behavior (figure 29A). The flies were tested at younger age (3 days) to reduce the effect of SOD2 and to analyze its effect in an aging-dependent manner.

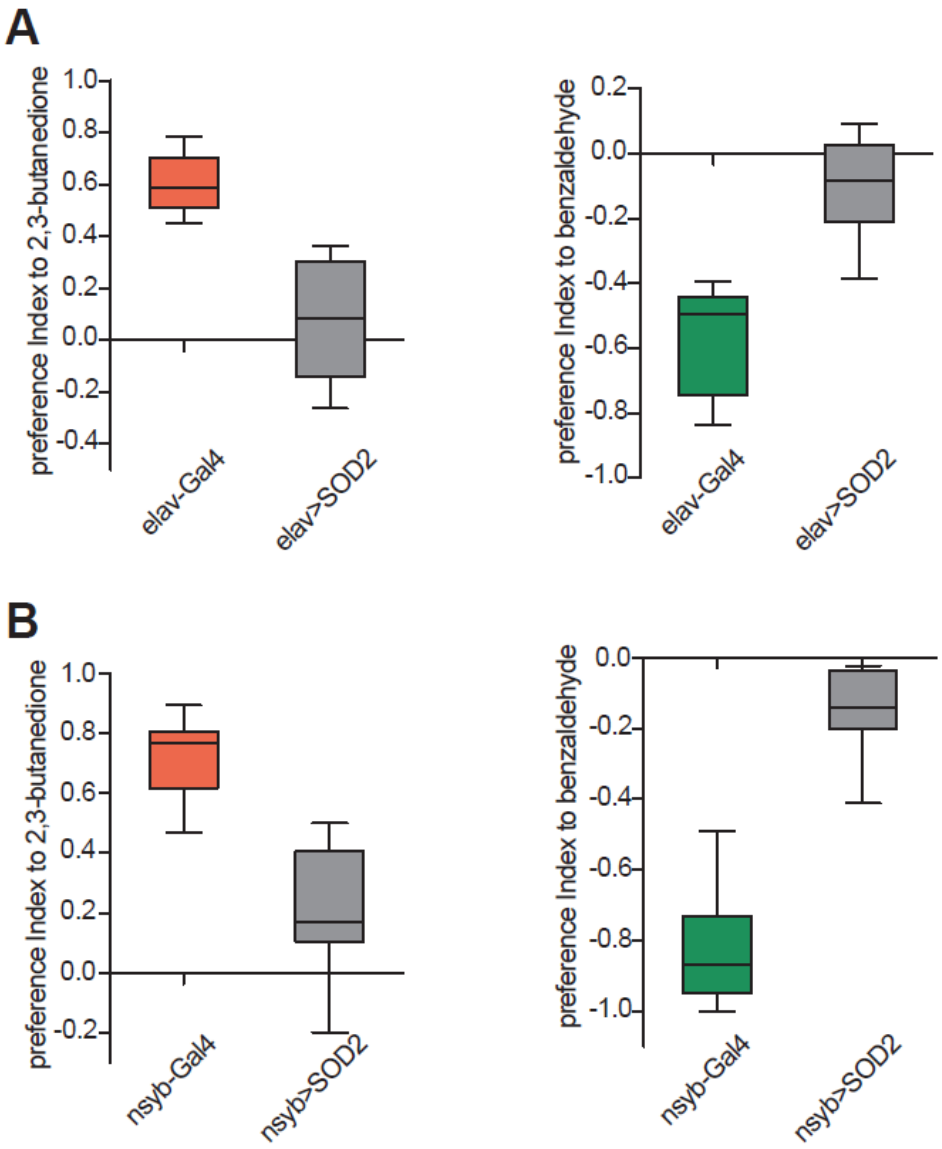

Figure 29 RNAi suppression of SOD2 in pan-neurons mimics aging olfactory decline

(A) Knockdown of *SOD2* using pan-neuron driver elav-Gal4 (*elav-Gal4; UAS-SOD2-RNAi*) caused olfactory decline to both attractive and aversive odors in young flies (n=8 ± SEM). (B) Knockdown of *SOD2* in nsyb-Gal4 (*nsyb-Gal4; UAS-SOD2-RNAi*) caused olfactory decline in young flies to both attractive and aversive odors (n=8 ± SEM).

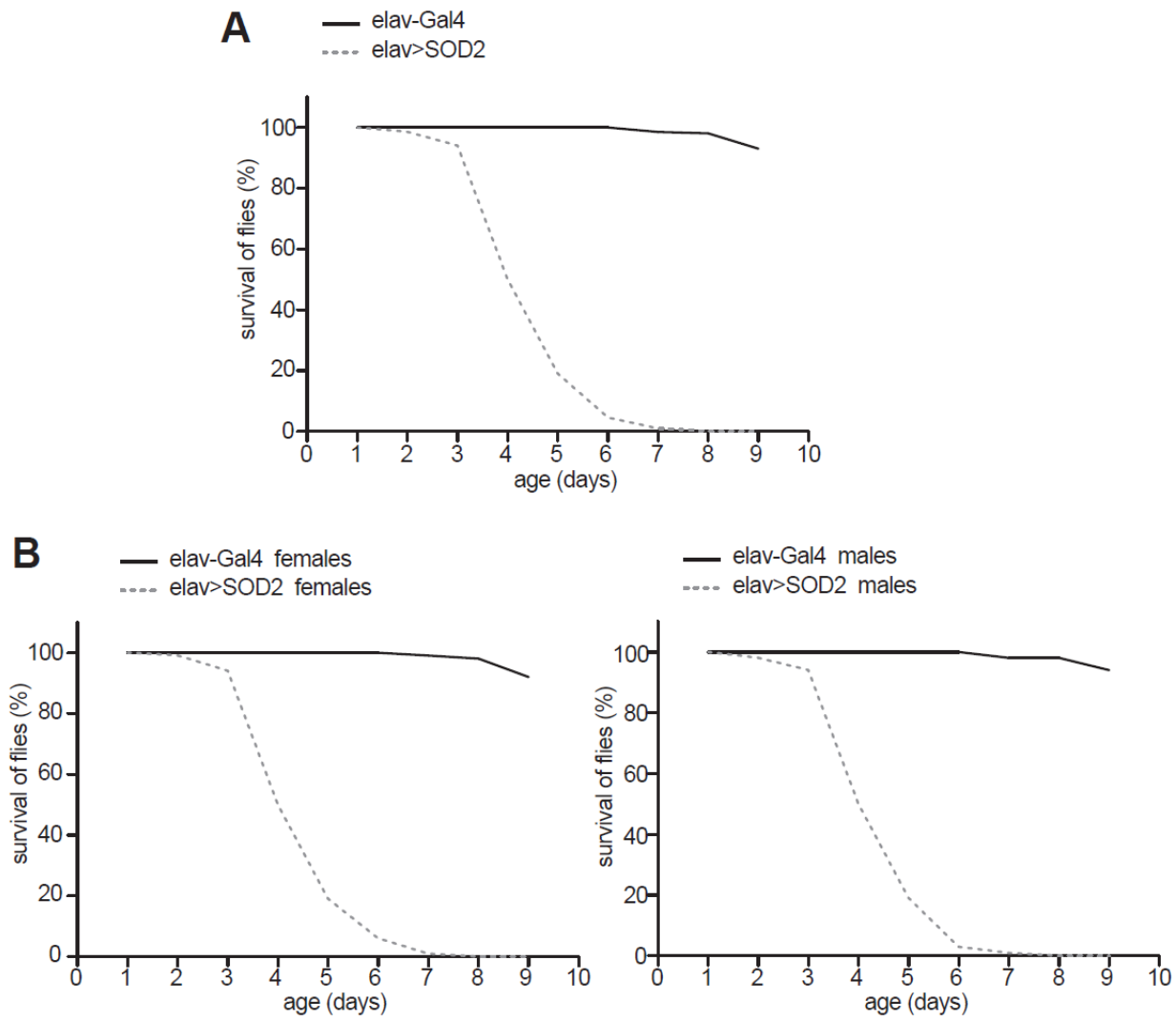

Figure 30 RNAi suppression of SOD2 in pan-neurons causes rapid mortality in young flies

(A) The survival of SOD2 downregulating flies (*elav-Gal4; UAS-SOD2-RNAi*) was largely reduced in the first week. (B) No gender difference in the survival curve of SOD2 downregulated flies (n=200 ± SEM).

As *elav-Gal4* was also broadly expressed during development, I asked whether this olfactory deficit was caused by developmental defects of the olfactory system or whether the gene was required to prevent aging. To this end, I used another pan-neural driver *nsyb-Gal4*. In contrast with *elav-Gal4*, *nsyb-Gal4* is only expressed in mature neurons (Yoshihara, Ueda et al. 1999). The SOD2 downregulated flies (*nsyb-Gal4; SOD2-RNAi*) showed a similarly reduced olfactory preference to odors compared with controls of the same age suggesting that SOD2 might indeed

play a role in olfactory aging (figure 29B). Of note, the lifespan of SOD2 downregulated flies (*elav-Gal4; SOD2-RNAi*) was also largely reduced and showed no gender difference (figure 30 A and B). This result indicated that the olfactory decline in SOD2 downregulated flies is associated with lifespan of flies. In summary, the results suggested that the adult flies showed a reduced olfactory preference at a young age when the expression of *SOD2* gene was downregulated. The reduced olfactory preference of SOD2 downregulated flies was similar to the reduced olfactory preference of aged flies.

2.2.5 Downregulating *SOD2* gene on projection neurons is sufficient to reduce olfactory preference

Next, I asked in which region of the olfactory system expression of *SOD2* was required to prevent olfactory aging. The receptor neurons on the peripheral olfactory organs were tested first. As mentioned above, I found that the activity of ORNs declined with age (figure 28). Given that Orco was required for both 2, 3-butanedione receptors and benzaldehyde perception (figure 25), I investigated the role of *SOD2* in ORNs. Therefore, I used *Orco-Gal4* to downregulate *SOD2* expression in ORNs. The *SOD2* downregulated flies (*Orco-Gal4; SOD2-RNAi*) were tested in T-maze for both attractive odor (2, 3-butanedione) and aversive odor (benzaldehyde). However, downregulating *SOD2* in ORNs showed no difference in olfactory preference compared with wild type controls (figure 31A). The result indicated that the ORNs were not the place where *SOD2* was involved to affect the olfactory preference in behavior.

In the olfactory system, the axons of peripheral ORNs innervate to the antennal lobe (AL) glomeruli. There, the olfactory information is processed by local interneurons (LNs) and projection neurons (PNs), and transfer to higher brain centers mushroom body (MB) and lateral horn (LH) (Vosshall and Stocker 2007). I asked whether a lack of *SOD2* expression in PNs affected the behavioral preference. I used driver *GH146-Gal4* to downregulate *SOD2* expression specifically in PNs. This *GH146-Gal4* labeled two-thirds of the projection neurons in adult flies (Jefferis, Marin et al. 2001). The *SOD2* downregulated flies (*GH146-Gal4; SOD2-RNAi*) were tested in the T-maze with both attractive (2, 3-butanedione) and aversive odors (benzaldehyde). Interestingly, the flies showed a dramatically reduced preference to attractive odor but not to aversive odor (figure 31B). In contrast to the strongly reduced attractive preference, the aversive preference to benzaldehyde was only slightly reduced (figure 31B).

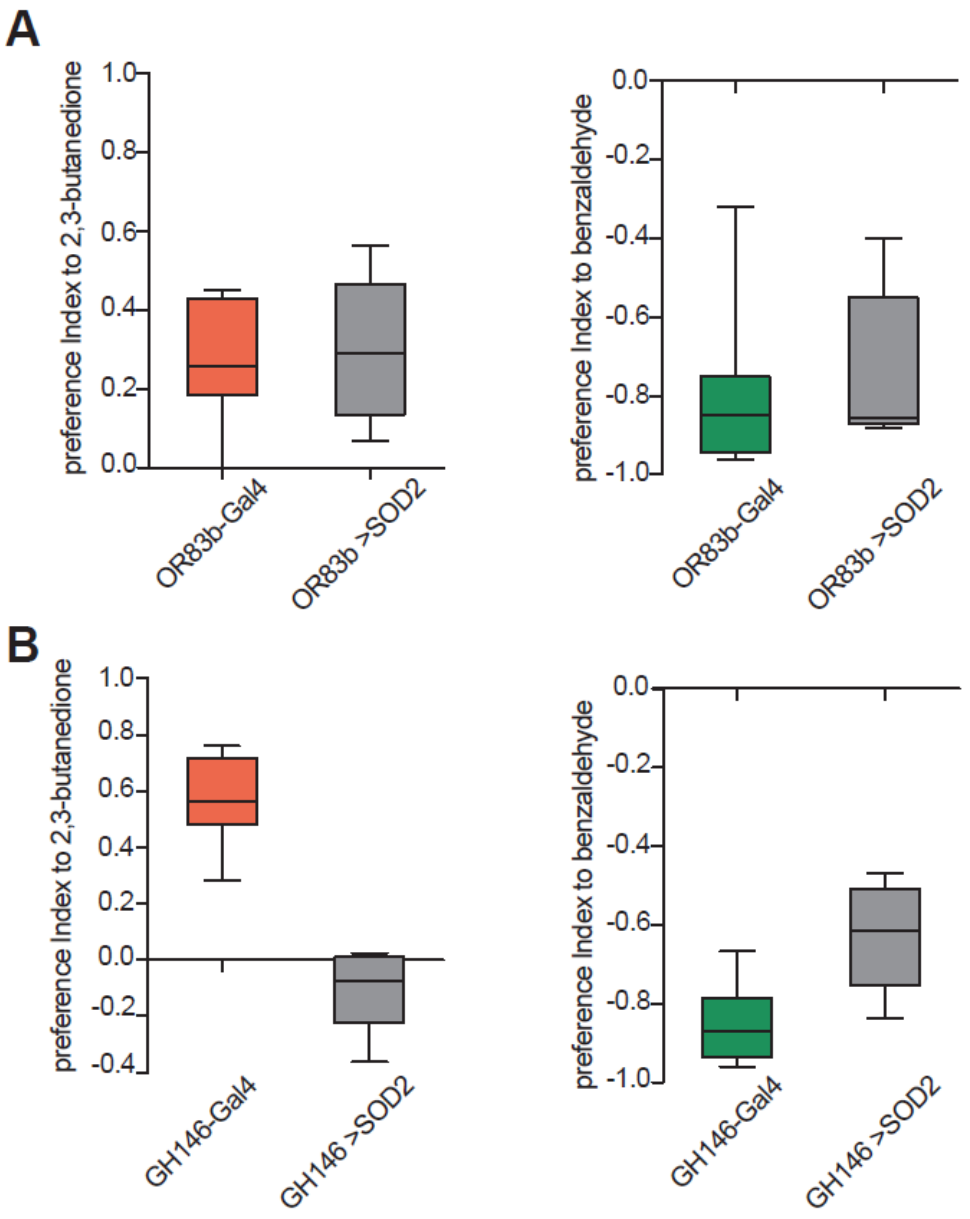

Figure 31 RNAi suppression of SOD2 in projection neurons but not olfactory neurons mediates the olfactory decline

(A) Knockdown of *SOD2* using OR co-receptor driver OR83b-Gal4 (*OR83b-Gal4; UAS-SOD2-RNAi*) caused no olfactory decline in young flies to neither attractive nor aversive odors (n=8 ± SEM). (B) *SOD2* downregulating on projection neurons (*GH146-Gal4; UAS-SOD2-RNAi*) leaded to strong olfactory decline in young flies (n=8 ± SEM). Flies were test at 1 week old.

Then I asked whether the effect of SOD2 missing was stronger in attractive PNs than aversive PNs. Thus, I tested more odors. Similarly, the flies (*GH146-Gal4; SOD2-RNAi*) showed reduced

attraction to attractive odors but less reduced avoidance to aversive odors (figure 32). The results suggested that this projection neuron Gal4 line might cover more attraction-mediating PNs than aversion-mediating PNs. Alternatively it is possible that aversion can be mediated by less PNs than attraction as previous publication suggested (Gao, Clandinin et al. 2015).

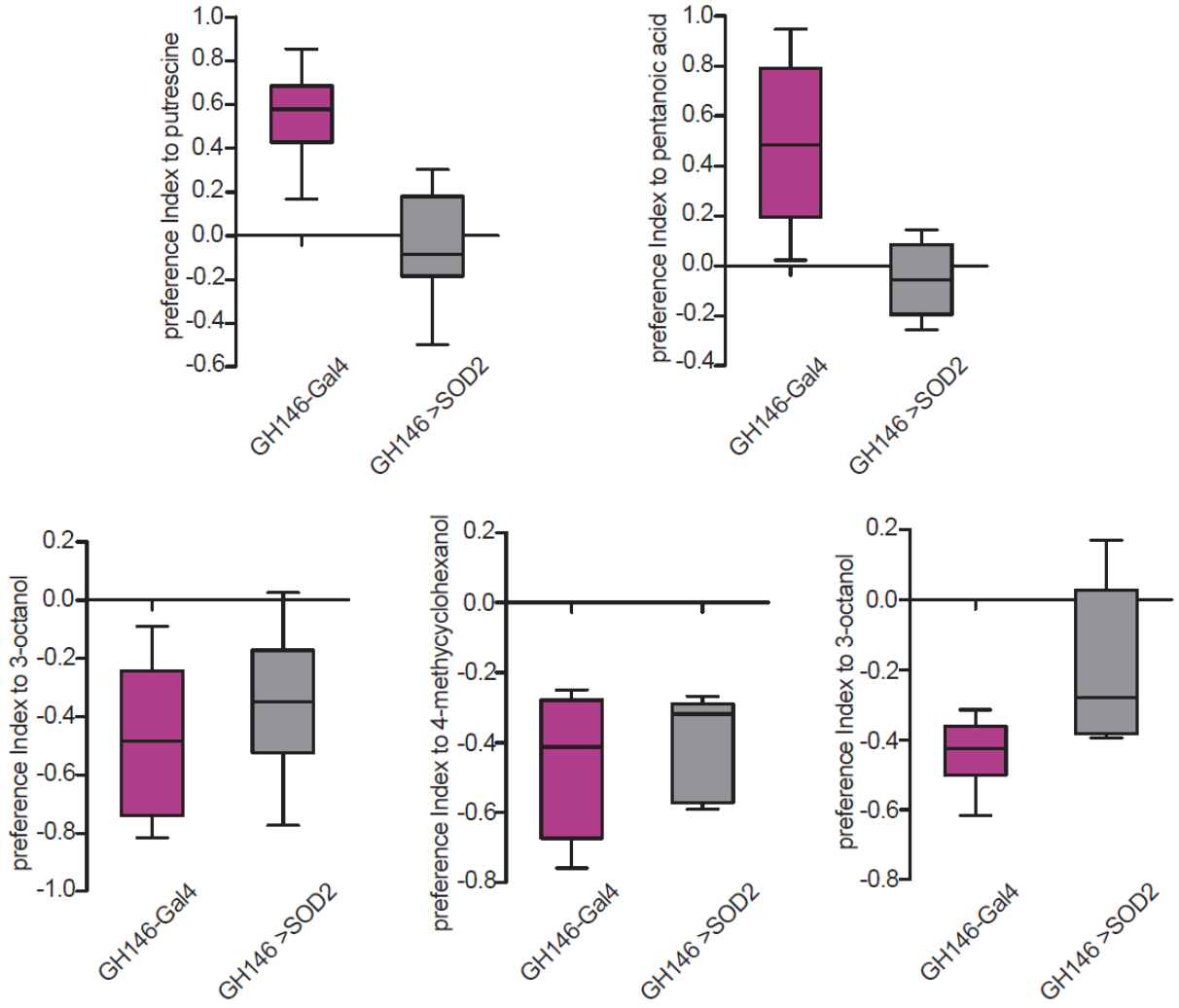

Figure 32 RNAi suppression of SOD2 in projection neurons broadly causes olfactory decline

One-week-old flies with SOD2 downregulating in projection neurons (*GH146-Gal4; UAS-SOD2-RNAi*) showed similar olfactory decline to other attractive odors (up row) and aversive odors (down row). However, flies did not show declines to all of aversive odors (n=8 ± SEM).

Importantly, the lifespan of these flies was not affected when *SOD2* expression was downregulated in PNs showing that the effect on olfactory behaviors was independent of other aging defects (figure 33). Therefore, the *SOD2* was required for behavioral preference in olfactory system and functions in PNs but not ORNs. This result also indicated that the aging process affects the neural substrates. Therefore, future analysis should be focused on the neuronal activities of PNs during aging.

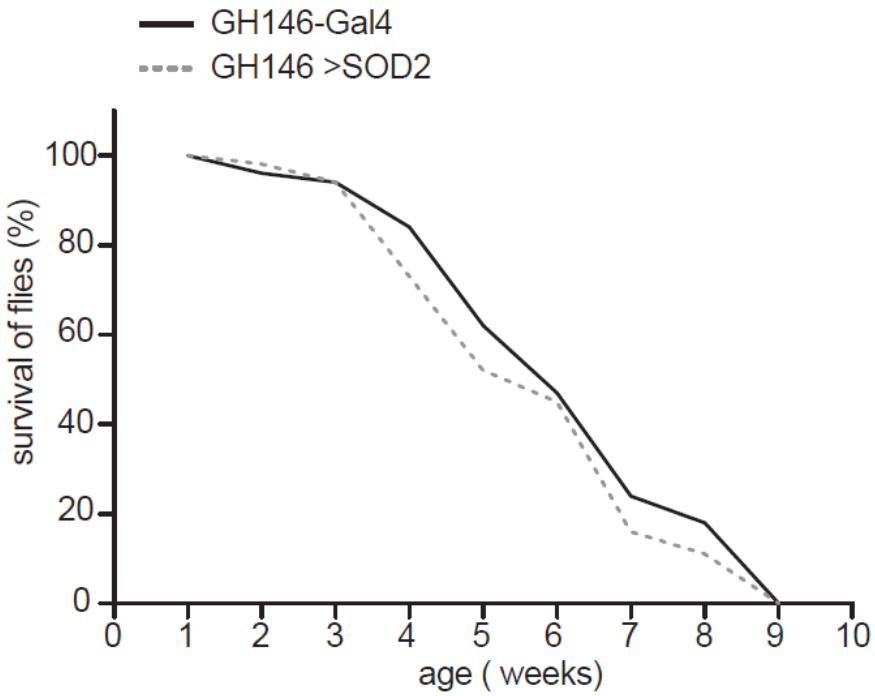

Figure 33 RNAi suppression of SOD2 in projection neurons did not affect lifespan

The survival curve of flies with SOD2 downregulating in projection neurons (*GH146-Gal4; UAS-SOD2-RNAi*) showed a similar lifespan compared with that of wildtype control (n=200 ± SEM).

2.2.6 A summary of the mechanism of aging associated olfactory decline

In summary, together with Dr. Hussain, we found that the decline of olfactory driven choice behaviors with aging is a general phenomenon across different types of odorant receptors. The loss of behavioral olfactory sensitivity is not related to motility defects. The visual preference dependent behavioral experiments suggested that olfactory system is more vulnerable to aging than visual system. Moreover, my anatomical and electrophysiological data suggested that the activities of peripheral ORNs were only mildly reduced with aging. Furthermore, the SOD2

defected flies with high oxidative stress showed behavioral olfactory decline similarly as aged flies. Mechanistically, genetic behavior experiments showed that loss of SOD2 in ORNs did not affect the behavioral choices. In contrast, the behavioral olfactory choices were affected by loss of SOD2 in PNs, which is consistent with previous reports in other model species such as *C. elegans* and suggests that second order central neurons are mainly affected by aging. This result implicated that accumulated oxidative stress in PNs with aging could be one reason of behavioral olfactory defects.

3 Discussion

3.1 Neuromodulation and mating state-dependent polyamine sensation

My experiments demonstrated that reproductive state could regulate the perception of taste by neuronal changes. In cooperation with other colleagues, we investigated a novel mechanism that tuned the choice behavior of gravid female flies to their increased nutritional needs (Hussain, Zhang et al., in press; Hussain, Üçpunar et al., in press). The mechanism directly modulated the sensitivity of taste neurons by SPR, a G-protein coupled receptor (GPCR), and its peptide ligands myoinhibitory peptides (MIPs). In addition, I demonstrated that polyamines, as particular nutrients, are detected by gustatory neurons ionotropic receptors IR76b and bitter receptor GR66a. In the behavior assays, polyamines were used as landmarks for choosing egg-laying sites. They were detected by gravid females using specific receptors of the olfactory and gustatory systems. While the mating state of females regulated this multisensory perception of polyamines, my work has focused on the taste sensory receptor neurons that detect polyamines in the gustatory system, as well as their modulation upon mating.

3.1.1 Polyamines benefit reproduction and are involved in egg-laying choices

Fermented food and certain fruits, which are rich in polyamines, are preferred by insects. Although previous studies showed that polyamines form an important part of male ejaculate and the role of polyamines in cell proliferation and embryonic development has been confirmed not only in insect but also in human, whether polyamines improve reproductive success of flies is unknown (Lefevre, Palin et al. 2011, Kalac 2014, Ramani, De Bandt et al. 2014). The behavioral experiments showed that this was indeed the case and polyamine feeding increased the number of offspring per female by 2–3 times. Polyamine played a role as a nutrient to benefit the reproductive success. The behavioral result also indicated that the mated females need polyamines for reproduction and therefore might use polyamines as a cue for choosing egg-laying sites. To test whether the role of polyamine in egg-laying choices is conserved between different species, we cooperated with Rickard Ignell's lab at SLU, Sweden, who works on mosquitos. They showed that *Aedes aegypti* mosquitoes were attracted to polyamines by smelling and also laid more eggs in polyamines-added water. Taken together, these experiments suggested that detecting nutrient polyamines as egg-laying cues could be conserved and the study of polyamines detection in flies could illustrate a similar mechanism conserved between different species. Alternatively, given that *Aedes aegypti* mosquitoes transmit pathogens of the deadly disease

dengue fever, polyamines could be used as a potential mosquito oviposition trap for mosquito control.

However, although previous studies showed that polyamines are important molecules involved in cell growth and proliferation (Lefevre, Palin et al. 2011), how polyamines improve the process of reproduction is still elusive. However, supplementation of polyamines could be also linked to other functions. Such as loss of polyamines related to aging-associated loss of memory and even lifespan (Minois, Carmona-Gutierrez et al. 2011). However, excess polyamine is related to the occurrence of cancer (Ramani, De Bandt et al. 2014). To understand how the intake of polyamines been regulated is essential to avoid either overtaking or deficiency. Thus, using as a model the physiological changes that occur upon mating, my study sought to elucidate state-dependent changes in polyamine sensory processing.

3.1.2 The detection of polyamines in *Drosophila*

The behavioral experiments found that flies use both the olfactory system and the gustatory system to detect polyamines. The parallel behavioral experiments tested by my colleague showed that flies were highly attracted to odor of polyamines. The ablation experiment showed that the oviposition choice to polyamine-rich site was taste organ dependent. Interestingly, although the diet polyamines could benefit the success of reproduction, the behavioral oviposition experiments illustrated that flies showed avoidance to lay eggs on the polyamine-rich site. However, the avoidance only happened when polyamines were applied alone in the behavioral assay. When polyamines were mixed with sweet substrates such as apple juice or sugar, the attraction to lay eggs on sweet substrates was strongly enhanced by polyamines. This result suggested that polyamines were attractive cues for egg laying. Together with the reproduction results, the data indicated that polyamines were actually preferred in relevant contexts in line with their requirement for reproduction.

To explain why flies avoided laying eggs on polyamines side, the avoidance to polyamine was connected with bitter taste. As assumed, when bitter receptor GR66a neurons were silenced, female flies strongly preferred to lay eggs on the pure polyamine side. This result suggested that both bitter neurons and IR76b neurons mediated polyamine taste detection. One hypothesis for the requirement of bitter taste is that this multisensory processing could be useful to evaluate the food quality in different aspects. Given that either overtaking or deficiency of polyamines can be detrimental to health and reproduction (Ramani, De Bandt et al. 2014), the final choice of egg-laying site could be judged by the balanced information of polyamines and other nutrients such

as apple juice. In addition, a previous study demonstrated that sweet neurons could be indirectly inhibited by bitter neurons through the GABAergic inhibitory neurons in the SEZ (Chu, Chui et al. 2014). In this case, bitter neurons potentially affected the polyamine detecting neurons following a similar mechanism.

In spite of integrating of two tastes in gustatory detection, the sense of smell to odor of polyamines is also required in detecting. With a video-monitor in the setup of oviposition assay, the mated females were found to spend more time on the polyamine-rich side but lay eggs on the control side. Experiments with mutants showed that the positional preference for the polyamine rich side was dependent on olfactory receptors. Nevertheless, the positional preference was decreased with time. By contrast, the preference to lay eggs on polyamine-free side was stable with time. These results indicated that polyamine detection was an integration of two sensory inputs. In long distance, flies trace the source of polyamines with the sense of smell. However, once flies find the food, and in short ranges, the decision of egg-laying place will depend on taste modalities. Although how odor and taste information of polyamines are integrated is unclear, this behavioral results illustrated a multisensory mechanism could be used for polyamines detection in complex environment.

3.1.3 The taste receptor of polyamines

Using ablation experiments, the labellum was found to be the essential organ for polyamine detection in gustatory perception. Then using genetic experiments the ionotropic receptor IR76b was demonstrated to be involved in polyamine detection in the gustatory system. Parallel genetic experiments in olfactory assay also illustrated that IR76b was co-expressed with IR41a as olfactory receptors for polyamine perception.

Behavioral results showed that the *IR76b* mutants had reduced attraction to polyamines in olfaction and were indifferent to lay eggs on the polyamine side. Rescue of IR76b expression could rescue the polyamine choice behavior in both taste and olfactory behavioral assays. Using SEZ calcium imaging, I found that the IR76b neurons responded to polyamines with taste stimulating on the labellum. In *IR76b* mutants, the responses of IR76b neurons did not respond to polyamines. Of note, the IR76b neurons in tarsals also responded to polyamine stimulation. The IR76b neurons on the legs possibly also contribute to the oviposition choices. Nevertheless, the labellum ablated flies totally lost the oviposition preference. The IR76b neurons on legs did not compensate for the lack of labellar neurons. This result again showed that the labellum was essential to mediate the choice for egg laying.

On the other hand, IR76b neurons also showed responses to water although the responses to water were very low compared with putrescine responses. IR76b receptors were also required for salt detection as a previous study showed (Zhang, Ni et al. 2013). This indicated that the IR76b receptors mediated the detection of more than one taste. The anatomy data showed that IR76b neurons were broadly innervated in the SEZ, the IR76b receptors could potentially play the role as a general co-receptor of more GRs. Thus, another co-expressed receptor may also be involved in polyamine taste detection. Alternatively, it is also possible that IR76b detects tastes of different kinds as a single receptor.

The tip recording result showed that putrescine elicited responses of S-type sensilla on the labellum. However, when I tested mutants of IR76b, the responses of S-type sensilla were not affected. Given the bitter receptors GR66a are located on the same region of the labellum, these responses possibly come from the bitter neurons. Of note, as a previous study showed, the IR76b receptors were required in L-type sensilla to respond to salt (Zhang, Ni et al. 2013). The IR76b neurons located in L-type sensilla could be not required for polyamine detection. Different IR76b-expressing GRNs appear to mediate responses of different substances. On the other hand, the anatomical data showed that IR76b receptors were also located deeply inside labellar pseudotrachea. This indicated that alternative IR76b neurons responding to polyamines could locate in peg neurons in labellar pseudotrachea.

3.1.4 The detection of polyamines is modulated by mating-state directly on peripheral taste neurons

Reproductive behaviors will change dramatically after mating such as a female's willingness to copulate again (Dickson 2008, Yapici, Kim et al. 2008, Ribeiro and Dickson 2010). Similarly, mating will initiate changes in metabolic state. This metabolic change will lead to additional nutritional requirements such as the protein-rich yeast that in turn benefit the female's offspring (Ribeiro and Dickson 2010, Chou, Hara et al. 2014). Accordingly, the taste preference for certain foods was regulated by mating state, which is reported in moth, flies, rat and human (Pike and Yao 1971, Duffy, Bartoshuk et al. 1998, Faas, Melgert et al. 2010, Saveer, Kromann et al. 2012, Itskov and Ribeiro 2013, Walker, Corrales-Carvajal et al. 2015). Consistently, our behavioral results in olfactory preference demonstrated that the mated females preferred to a higher concentration of polyamines than virgin females. Moreover, mated female showed high egg-laying preference to avoid polyamine side in oviposition behavior experiment but virgin female were indifferent to polyamines in egg-laying choice.

Although it was previously shown that mating state changes taste preference, how the sense of taste and gustatory processing are modulated at the molecular and neuronal levels remains unclear. A study on moth provided evidence that olfactory preference could be modulated after mating, owing to marked response changes of fluorescence labeled glomeruli in primary olfactory center (AL) (Saveer, Kromann et al. 2012). Recently another study in mice showed that the estrus cycle regulates pheromone sensitivity of ORNs (Dey, Chamero et al. 2015). Female mice that are not in estrus show significantly decreased behavioral responses to male pheromones. This decrease in behavior appears to be mediated partly by a modulation of the sensitivity of pheromone OSNs (olfactory sensory neurons) in the female. Progesterone and its receptor, which is expressed in OSNs desensitize pheromone sensitive OSNs when the female is not in estrus (Dey, Chamero et al. 2015). In this project, I demonstrated for the first time that the mating state directly modulated chemosensory perception in *Drosophila* by regulating the sensitivity of sensory neurons. The calcium imaging results suggested that the modulation of polyamine preference was the result of changes in the sensitivity of the sensory neurons themselves.

This mechanism of mating state modulation appears to be similar to what was previously observed in feeding state. The sensitivity of olfactory and gustatory neurons can be modulated by fed or starved conditions (Root, Ko et al. 2011, Inagaki, Ben-Tabou de-Leon et al. 2012, Dey, Chamero et al. 2015). Together with the feeding state modulation in *Drosophila*, our results demonstrated that internal state modulated behaviors directly by regulating the sensitivities of chemosensory neurons. We were the first to show that the mating state modulated nutrient perception directly by regulating the activities of chemosensory neurons.

Interestingly, the calcium imaging results I collected in SEZ showed the sensitivity of taste neurons were increased after mating. By contrast, the imaging results produced by another PhD student Habibe Ucpunar indicated that the sensitivity of olfactory neurons decreased after mating. The opposite neuronal modulation could be because of the two types of sensory neurons. Although the behavioral effects are similar, the mating state modulates the taste sensation by increasing the sensitivity of GRNs but regulates the olfactory sensation by decreasing the sensation of ORNs. In the next paragraph, I will illustrate the potential mechanisms and signaling pathways that are triggered by mating state and modulate the sensation of smell and taste.

3.1.5 The mechanism of mating state-dependent modulation on polyamine detection

Since the mating state can modulate both taste and smell dependent behavioral choices and even the sensitivities of chemosensory neurons to polyamines are affected, to understand the

relationship between mating state and chemosensation of polyamines is essential. Previous studies demonstrated that the classical post-mating switch (i.e., decrease in female receptivity and increase in egg laying after mating) is regulated by a specific receptor – SPR (sex peptide receptor). The principle ligand of SPR in the regulation of these female post-mating behaviors is the sex peptides (SPs), which reaches the female reproductive system via the males' seminal fluid (Kubli 2003, Yapici, Kim et al. 2008). Thus, the current view of mating regulation is that the signal of the mating status is transferred by SP/SPR signaling in the female reproductive tract into the central brain to trigger the classical post-mating responses (Yapici, Kim et al. 2008). The signal of mating is detected by local sensory neurons in oviduct that express SPR.

However, our results suggested that SPs were dispensable for the modulation of the behavioral preference to polyamines in olfaction. Instead, we show that the preference of polyamines is regulated by SPR and another conserved ligand – MIPs (myoinhibitory peptides) directly in chemosensory neurons themselves. Loss of the expression of SPR or MIPs in taste neurons could reduce the polyamine preference in mated females to the same level as virgin females or full *SPR* mutants. When the expression of SPR was reduced only in taste neurons by an RNAi construct against SPR, the activity of taste neurons as judged by calcium imaging in the SEZ in mated females was also reduced to the same level as in virgin females. The results are similar to comparison between virgin and mated females. This was the first time to show the role of SPR in mating state-dependent modulation of sensory neurons. The SPR/MIPs signaling pathway was shown to regulate the mating state-dependent sensory perception. Importantly, these results showed a role of the SPR and MIPs signaling pathway in modulating sensation preference directly in sensory neurons.

In *Drosophila*, a similar mechanism is modulated by feeding state. The sNPF/sNPFR signaling pathway was activated by signaling of starvation and be involved in modulating feeding behavior by regulating the activity of ORNs where sNPFR expressed (Root, Ko et al. 2011). The starvation increased the expression of sNPFR in ORNs by insulin signaling. The sNPFR and its ligand sNPF made a positive feedback loop to increase the activity of ORNs at starvation state (Root, Ko et al. 2011). Similarly, our imaging data suggested that the SPR and its ligand MIPs could target GRNs and ORNs by presynaptic facilitation in mated females as same as sNPF/sNPFR signaling modulates the activity of ORNs in hungry flies. Signaling of mating could activate the following SPR/MIPs signaling pathway leading to changes of sensory neurons and ultimate behaviors (figure 34).

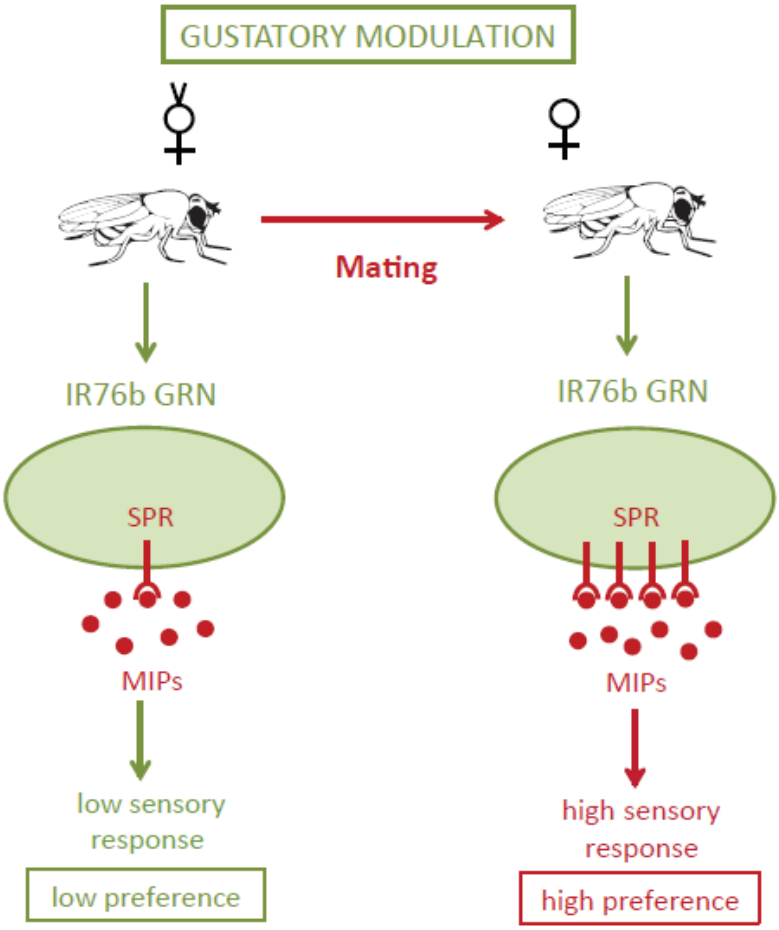

Figure 34 Model of mating state-dependent modulation in gustatory system via SPR/MIPs signaling

Virgin females show a low behavioral preference to polyamines (left). Upon mating, increased expression of SPR in polyamine taste sensory neurons enhanced the output of polyamine taste neurons. Accordingly, female's behavioral preference to polyamines is increased (right). Figure is adapted from Figure 7 of Hussain, Ucpunar et al. PLoS Biology 2016.

We found that the SPR/MIPs signaling pathway is involved in modulating chemosensation upon mating. If the SPR/MIPs signaling pathway is activated by mating, we should inspect an increase of the expression of SPR or MIPs after mating. Research technician Laura Loschek in our lab did antibody staining using previously published antibody (Yapici, Kim et al. 2008), but we didn't find difference between the brain of wildtype control and *SPR* mutant in quantification of SPR. However, using quantitative PCR, my collaborator Ashiq Hussain found that mRNA levels of SPR increased about 10-fold in the antenna and increased around 3-fold in brain upon mating.

However, the mRNA levels of MIPs in mated females only slightly increased compared to virgins (1–2 fold) in both antenna and brain. This result supported the model described above that the SPR expression is selectively increased in chemosensory neurons.

Using antibody staining, a significant increase of MIPs expression in the AL was also observed in mated females compared to virgin females. Unfortunately, in the SEZ, redundant neuronal tracts masked the MIPs-stained axons projecting from peripheral gustatory organs. The expression of MIPs cannot be selectively quantified in the gustatory primary center. Nonetheless, the result we observed in the AL indicated that the expression of MIPs was also modulated by mating state.

In parallel with experiments in the gustatory system, loss of the expression of SPR or MIPs in olfactory neurons also reduce the behavioral polyamine preference in mated females to the same level as virgin females or full *SPR* mutants. The olfactory neurons in mated females monitored by calcium imaging in the AL showed increased activities compared to virgin females. By contrast, loss of SPR or MIPs expression in taste neurons led to reduced polyamine preference in mated females to the same level as virgin females in behavior. In addition, taste neurons showed reduced activity of mated females to the level of virgin females. Two opposite effects on different sensation systems were regulated by the same receptor – SPR and its ligand MIPs. How the opposite neuronal mechanisms are regulated is unknown. However, as we known SPR is G protein-coupled receptor (GPCR). Signaling of SPR could recruit different G-protein subunits to either inhibit or activate downstream effectors leading inhibitory or exhibitory effect on the SPR expressed neurons.

I showed that the mating state modulated the taste preference by the SPR and MIPs signaling pathway. However, what active the SPR/MIPs signaling after mating on the chemosensory neurons is unclear. As the previous data showed that SPs could not involve in regulating olfactory post-mating modulation, the neuromodulator might be triggered by mating signaling. SPs could neither involve in regulation at the level of taste neurons. Therefore, the question is how the SPR/MIPs signaling affects the sensitivity of taste and olfactory neurons in response to mating. In other words, what regulates SPR/MIPs signaling activation in a mating state-dependent manner? To identify this mechanism, my colleague and I checked the sensitivity of taste and olfactory neurons at different time point as 1–6 hours immediately after mating and 7 days after mating. The effect of mating after 7 days is much weaker than after 1–6 hours immediately after mating. The internal mating state triggers the change of neural sensitivity transiently. As previous study reported that the level of octopamine rises steeply within 20 minutes after mating in the female reproductive tract and became very low at 90 minutes after mating (Heifetz, Lindner et al.

2014). The transient changes of octopamine might trigger the increase of SPR or increase of MIPs' secreting on sensory neurons. Further behavioral results done by Ashiq Hussain showed that mated female with silenced octopaminergic neurons displayed a reduced preference during egg laying assays. This result indicated that octopamine could be a signal induced by mating that modulates SPR/MIPs signaling pathway. However, how it interacted with SPR/MIPs signaling pathway is still unclear. Whether octopamine directly or indirectly affected on taste and olfactory neurons is unknown.

In summary, our results demonstrated a significant role of polyamine in reproductive success of flies. It was previously reported that polyamines are an important component of nutrition, which are not only required in development and reproduction, but also related with lifespan and diseases such as cancer in humans (Cerrada-Gimenez, Pietila et al. 2011, Minois, Carmona-Gutierrez et al. 2011, Kalac 2014). Thus, it is important to understand how polyamines interact with metabolic process and whether they can be detected by chemosensation. Here we provide a model on how polyamine perception can be modulated by internal state. The change could adapt the female behaviors for physiological or nutritional needs. The increased sensitivity of GRNs and decreased sensitivity of ORNs to polyamines are regulated by SPR/MIPs signaling. The sensitivity changes of sensory neurons could help to initiate changes in the higher brain centers, and in turn a memory mechanism might help to maintain enhanced sensitivity to important sensory cues for as long as they are required. In this point, transient sensory facilitation might induce more long-lasting behavioral changes.

3.2 Neural mechanisms of aging-associated olfactory decline

In this project, I investigated the neural and molecular mechanisms of aging-related olfactory impairment. In cooperation with another colleague, we used *Drosophila* as a model organism and determined that the declined olfactory perception is a general phenomenon in the olfactory system, which occurs independently of motor or visual system decline. I precisely measured the neurophysiological responses of ORNs. The electrophysiological data suggested that the age-associated olfactory impairment in behavior is only partly related to the sensitivity of ORNs. However, I observed that olfactory perception declined more rapidly in the aging-associated gene *mitochondrial superoxide dismutase2* (*SOD2)* loss of function flies. Using targeted knockdown, I showed that loss of SOD2 activity in olfactory projection neurons (PNs) was responsible for this decline. More generally, my results indicate that aging affects the olfactory system most strongly

at the level of PNs but not peripheral sensory neurons. To this end, we provide a genetic model to study the aging-associated olfactory defect.

3.2.1 Olfactory decline with aging is a general phenomenon across odors and receptor types

The sense of smell plays an essential function for maintaining a good quality of life. Previous works suggest a decline of olfactory ability in aged humans (Murphy 2008, Rawson, Gomez et al. 2012, Mobley, Rodriguez-Gil et al. 2014). Furthermore, the smell dysfunction is one of the first indicators of aging, but also of neurodegenerative diseases such as Alzheimer's and Parkinson's (Doty 2009). Similarly, declines in olfactory dependent behaviors are also evidenced in worms, insects and mammals (Iliadi and Boulianne 2010, Mobley, Rodriguez-Gil et al. 2014, Leinwand, Yang et al. 2015). A study on worm even indicated a better performance in olfactory behavior correlated with healthier physiological state and longevity (Leinwand, Yang et al. 2015). However, while the phenomena of olfactory defect were broadly reported, the neural and genetic mechanisms of aging-associated olfactory decline remain largely elusive.

Our results show that while olfactory performance quickly declines, visual choice behavior remained overall very stable even in very old animals. Thus, the aging-associated olfactory decline is not due to motility defects. Furthermore, this result is consistent with previous reports that the behavioral responses to electric shock and light are remained in aged flies (Cook-Wiens and Grotewiel 2002). Nevertheless, more tests such as the Drosophila Activity Monitoring (DAM) system will be used to address the role of general activity and circadian rhythm in aging-related olfactory behavior.

To identify the molecular and cellular mechanisms of aging-associated olfactory decline, we proposed to use high-throughput RNA sequencing of antenna and brains comparison between young and aged flies. If the expression of ORs and related molecules are regulated with aging, we hypothesized to generate a list of aging-regulated genes. Moreover, by deeply analyzing the role of aging-regulated genes in loss of olfaction, we could identify a potential conserved mechanism to interpret aging-associated olfactory functional declines in human. So far, an experiment of RNA sequencing on the third segment of antenna has been executed by Ashiq Hussain. The preliminary result indicated that the expression of 37 ORs out of 59 ORs that we found are downregulated in 7 weeks old flies compared to 1-week young flies but other 22 ORs are upregulated in old flies. And the expression of 42 IRs out of 61 IRs were downregulated in the antenna of old flies compared to that of young flies but the other 19 IRs were upregulated in old

flies. The data from the antenna show that the expression of olfactory receptors is up-or down-regulated with aging. Moreover, other receptors or proteins involved in regulation of biological processes were also found to be up-or down-regulated with aging. We suppose to analyze in depth the function of these potential candidates that could interpret how the loss of smell is regulated in the process of aging. As decline of olfactory sensitivity is also a common symptom of neurodegenerative diseases, we could also open a door for understanding how olfactory decline linked with neurodegeneration.

3.2.2 Neural activity of olfactory sensory neurons is only partly responsible for aging-dependent behavioral decline

We wished to identify the cellular and molecular reasons for the observed decline in olfaction. In primary olfactory organs, the changes during aging may be due to the distribution, density or function of specific ORs, GRs and IRs or even a result of a decrease in the amount of ORNs. The preliminary result of RNA sequencing indicates a change of the expression of olfactory receptors with aging. The expression of OR42b is downregulated during aging. As OR42b mediates the olfactory processing for 2, 3-butanedione which was tested in behavior, I was interested to check the amount of OR42b neurons on the antenna comparison between young and old flies. The mCD8GFP labelled OR42b ORNs didn't show number changes with aging. Similarly in epithelium of mice and human, a reduced number of OSNs has been shown (Maresh, Rodriguez Gil et al. 2008, Suzukawa, Kondo et al. 2011). Nevertheless, the numbers of other types of ORNs haven't been checked in aged flies. From my data I can conclude that the number of ORNs is not decreased with downregulated expression of ORs. Moreover, the activity of neurons can't be measured just by labelling the location of neurons using Gal4/UAS system.

Thus, characterizing the aging-associated neuronal activity of sensory neurons may provide the key for understanding the cellular basis for functional senescence. Although the cellular mechanism of aging is poorly understood, a few studies show that aging leads to weaken neuronal activation across invertebrate and vertebrates (Martinez, Javadi et al. 2007, Mobley, Rodriguez-Gil et al. 2014). To study the activities of ORNs, I performed neurophysiological analyses by single sensillum recording (SSR) to determine the characteristic of ORNs between young and old flies. A significant decline of ORN responses in aged flies was observed compared to young flies. The neural activity declines happened in both attractive and aversive olfactory responses. However, the neural activity of aged flies was not reduced as dramatically as the preference in behavioral

experiments. Therefore, the result indicates that the behavioral aging-associated olfactory decline is only partly caused by the deficits of ORNs.

As previous studies showed, aging-related changes are not only the properties of individual neurons, but also the synaptic communication between neurons and target cells (Janse, Peretz et al. 1999, Martinez, Javadi et al. 2007). For instance, in *Drosophila*, the aging-associated defect in climbing behavior is related to the synaptic transmission of motor interneurons (Martinez, Javadi et al. 2007). Thus, aging-related synaptic deficits are possibly linked with olfactory behavioral declines. In addition, a study on worms suggested that secondary neurons are most affected by aging but not primary sensory neurons, which is similar to our result (Leinwand, Yang et al. 2015). An investigation of the effect of aging on projection neurons is therefore important in the future.

3.2.3 The level of oxidative stress in PNs is crucial for olfactory behavior

Nowadays, massive studies show that the aging process similar to other biological processes is regulated by multiple signaling pathways rather than by simply passive degrading of tissues (Kenyon 2010). To study the molecular mechanism of aging, many aging studies in model animals identified candidate genes regulating lifespan (Kenyon 2005, Kenyon 2010). Among these, many candidate genes have human homologues (Grotewiel, Martin et al. 2005, Kenyon 2010). One conserved candidate is the SOD family, which code enzymes that provide resistance to oxidative stress in a cell (Zelko, Mariani et al. 2002, Landis and Tower 2005).

SOD2 is particularly studied because of its specific expression in mitochondria (Landis and Tower 2005). Experiments on *SOD2* mutants showed that these mutants are more vulnerable to oxidative stress and lead to a reduced lifespan in worms, flies and mice (Lebovitz, Zhang et al. 1996, Murakami and Johnson 1996, Duttaroy, Paul et al. 2003). Consistently, dietary antioxidants and overexpression of superoxide dismutase experiments do increase the lifespans of worms, flies and mammals (Melov, Ravenscroft et al. 2000, Sun, Folk et al. 2002, Agarwal, Gupta et al. 2005). Additionally, a *methuselah* mutant (*mth*) with enhanced oxidative stress resistance also increases lifespans in flies (Lin, Seroude et al. 1998). All these studies suggest a link between oxidative stress and regulation of lifespan. Furthermore, deterioration of organs was also related to oxidative damage (Botella, Ulschmid et al. 2004, Piazza, Hayes et al. 2009). Behavioral experiments with *SOD2* mutant flies indicate an accelerated aging-associated functional decline in locomotor ability, cardiac performance and aspects of olfactory choice behavior in flies (Cook-Wiens and Grotewiel 2002, Paul, Belton et al. 2007, Piazza, Hayes et al. 2009). Consistently,

dietary antioxidants could protect against age-associated behavioral declines in flies (Orr and Sohal 1994).

All these report suggest oxidative stress mediates functional senescence associated with aging. Therefore, aging is thought to be determined by damages accumulated of oxidative stress (Finkel and Holbrook 2000). However, the molecular mechanism of oxidative stress on aging process has not been proved. Which specific organs or tissues deteriorate under oxidative stress and how oxidative damages lead to aging-associated functional loss are still unknown. Thus, studying on defected or enhanced oxidative resistance transgenic models would be helpful to understand the mechanisms of aging-associated functional declines in particular organ system. Thus, I executed experiments to associated function of SOD2 with olfactory behaviors by reducing expression of SOD2 with RNAi construct in neural system. SOD2 missing flies showed similar aging-associated olfactory decline to both aversive and attractive odors at young age. This result confirms the role of SOD2 in keeping normal function of olfactory behaviors. In addition, the result provides new evidence in *Drosophila* that oxidative damages in olfactory neuronal circuits could be the reason of olfactory decline with aging. On the other hand, downregulating SOD2 in PNs only affect the olfactory preference but not reduce the lifespans of flies. This result supports the idea that oxidative stress in olfactory neural circuits could be a reason of aging-associated olfactory defects. To explore the underlying mechanism, further experiments will be implemented to check the relationship between oxidative stress and aging. For example, over feeding SOD2 or antioxidants might rescue or reduce the aging-associated olfactory defects.

Current data suggested that oxidative damage accumulates in parallel in multiple organ systems at different rates (Martinez, Javadi et al. 2007). This indicated that different organs may have a variable tolerance for oxidative damage before a functional loss. Of note, the result using *GH146Gal4; UAS-SOD2 RNAi* flies with downregulated expression of SOD2 exclusively in olfactory projection neurons showed that aging-associated behavioral decline in olfaction was due to a deterioration of these neurons. By contrast, downregulating expression of SOD2 in ORNs had no effect on behavioral preference. This result indicated that the PNs are more susceptible than ORNs, and are potential targets of aging in olfactory system. This result matched with the study in motor interneurons in flies, which showed a reduced synaptic responses of interneuron in aged animals (Martinez, Javadi et al. 2007). Additional relevant reports also showed reduced dendritic length of adult-born interneurons – periglomerular cells and decreased synaptic density in the olfactory bulb of aged mice (Mobley, Rodriguez-Gil et al. 2014). The oxidative damage may result from neurodegeneration in interneurons and its synapse. Furthermore, a recent

investigation in *C.elegans* also suggested that aging-associated olfactory behavioral are due to reduced activity of secondary neurons, but not the activity of primary sensory neurons, which remains robust in aged animals (Leinwand, Yang et al. 2015). This result in worm is similar to ours. My results provide another evidence that the projection neurons are more sensitive to aging-associated effect but not the peripheral ORNs.

Where the endogenous oxidative stresses come from is unclear. A study on mice suggested oxidative stress comes from the metabolism and nutritional processing itself. The activation of polyamine catabolism leading to elevated oxidative stress also associated with reduced lifespans in mice (Cerrada-Gimenez, Pietila et al. 2011). Thus, how polyamines play a role in metabolic process related with oxidative stress is of interest to me. Nutrients balance of inner state could also be associated with lifespan and other aging-associated diseases.

On the other hand, the flies with SOD2 missing in PNs provide a good model to study the mechanism of functional declines under oxidative stress, as well as a model to study the effect of exogenous protective measures against functional damages induced by oxidative stress.

In summary, I describe another example of sensory processing modulation by internal state. My work has focused on how aging impacts on odor processing. I showed that olfactory sensing is generally regulated by aging and identified the first step of a mechanism that potentially explains the effect of aging. This mechanism might also relate to olfactory defects caused by aging-associated neurodegenerative diseases. Further experiments are ongoing for deeper analyses. Together with the first example of modulation in reproductive state, the study on underlying mechanisms of chemosensory modulation by internal states could enrich our knowledge about sensory processing. Furthermore, these studies might also shed light on understanding how internal states influence relevant behavioral decisions even at higher neural levels.

4 Methods

4.1 Fly rearing

Drosophila melanogaster flies were reared on standard cornmeal medium at 25 ˚C and 60% relative humidity under a 12:12h light: dark period. For RNAi experiments, parent flies were reared at 25 ˚C. 0–2 day old offspring was transferred to 29 ˚C after eclosion to increase the efficacy of RNAi. For other behavioral experiments, 0–2 day old flies were collected after hatching and reared under the same conditions. 5–7 days old flies were tested in behavioral experiments of polyamine project.

4.2 T-maze Assay

The T-maze apparatus is a custom made non-aspirated assay adapted from a previous publication (Suh, Wong et al. 2004). Two test vials (15 ml) connected to opposite sides of the choice point in middle part. A loading elevator connected to the middle part allowed flies to be transferred to the choice point (figure 35 A). Stimulus odors were diluted in water or paraffin oil and applied on a piece of paper at the end of test vials. One test tube was applied with odor solutions and the other side was applied with odor solvent as control. The concentration of odors was 1mM. The amount of solution applied was 40 ml in both sides. The stimuli could be changed to gases by MFC (Mass Flow Controller, NATEC Sensors GmbH) and sealed using parafilm (Neenah, WI 54956). In the CO_2 case, one test tube was filled with 1% CO_2 as stimulus synthesized with compressed air and pure CO_2. And the other side was filled with compressed air as control. The final concentration of CO_2 was checked by GM70 Hand-Held CO_2 meter (Vaisala CARBOCAP).

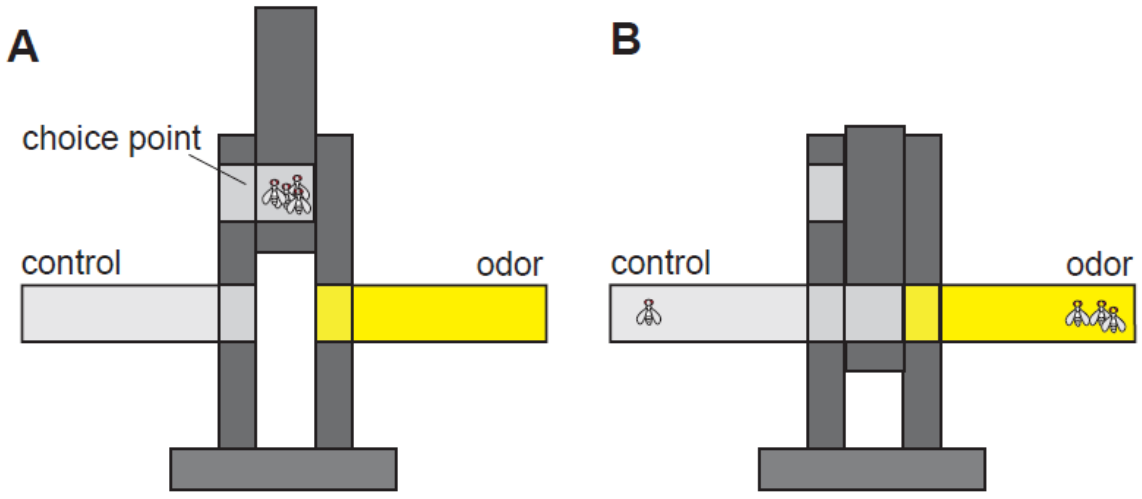

Figure 35 Scheme of T-maze assay

(A) Flies are loaded to choice point in the middle of two arms. (B) Flies have one minute to choose the preferred side according to odors.

The T-maze experiment was operated in a custom made climate box with designed temperature and humidity. A heating plate was set at the bottom of the climate box to increase air temperature. Two fans with a pipe system pump the air through a fog generator. In this way, water vapor is introduced into the climate box. The temperature and humidity are regulated to a desired range by a control panel via activing the heating plate and fog generator.

Before test, groups of 40–60 flies were collected into preparing vials to adapt the environment for 20 minutes (figure 35 A). Flies were transferred into the elevator of the T-maze apparatus tenderly. After placing odor applied test vials on two sides, the test started by pushing down the flies from elevator to the choice point. Then flies had 1 minute to respond to the stimulus (figure 35 B). The elevator would be pulled up after the 1-minute test. The whole test was tenderly carried out at 25 ˚C and 60% relative humidity in darkness. Two side vials were plugged after the test to collect flies.

The number of flies in each vial was counted separated by gender. The performance index (PI) was calculated by subtracting the number of flies on the test odor site from the number of flies on the control site and normalizing the result to the total number of flies. A zero PI indicated that the flies have no preference to the tested odor. A positive PI indicated that the flies are attracted to the odor. A negative PI indicated odor avoidance.

Eight repetitions were done for each genotype or stimulus. Statistical analysis of different groups was performed using Prism GraphPad6 by one-way analysis of variance (ANOVA) to determine whether different groups have significant difference. To avoid false positive results, the Bonferroni multiple comparisons post-hoc test was used to adjust the probability threshold. The difference between two groups are accepted as significant if the *p*-value is below 0.05.

4.3 Fly tracking assay

Fly tracking assay was used for phototaxis experiment. This setup is custom-made and allows video-recording of fly behaviors in a light-tight climate box (figure 36). The climate box is 75x45x47 cm black box with infrared light and air flow circuiting system including two fans connected with a fog generator. A control panel can regulate the temperature and humidity to a designed range via

activing a heating plate and the fog generator. A modified transparent T-maze apparatus was placed flatly in middle of bottom. Two sides of the arms of T-maze were two LED emitting lights. One side is a visible blue light (465-470nm) and the other side is an invisible red light (625-630 nm). On top of the T-maze a camera can record the movement of flies in T-maze during experiments. A videoGUI, using the VLC media player and ctrax software, controls the camera setup.

The tests were similar as regular T-maze experiments. A group of 40-60 flies were transferred to the modified T-maze apparatus before experiment. After transferred, flies were allowed to acclimate the environment for at least 20 minutes. The preference index was calculated by the number of flies walking towards each side during 1-minute test.

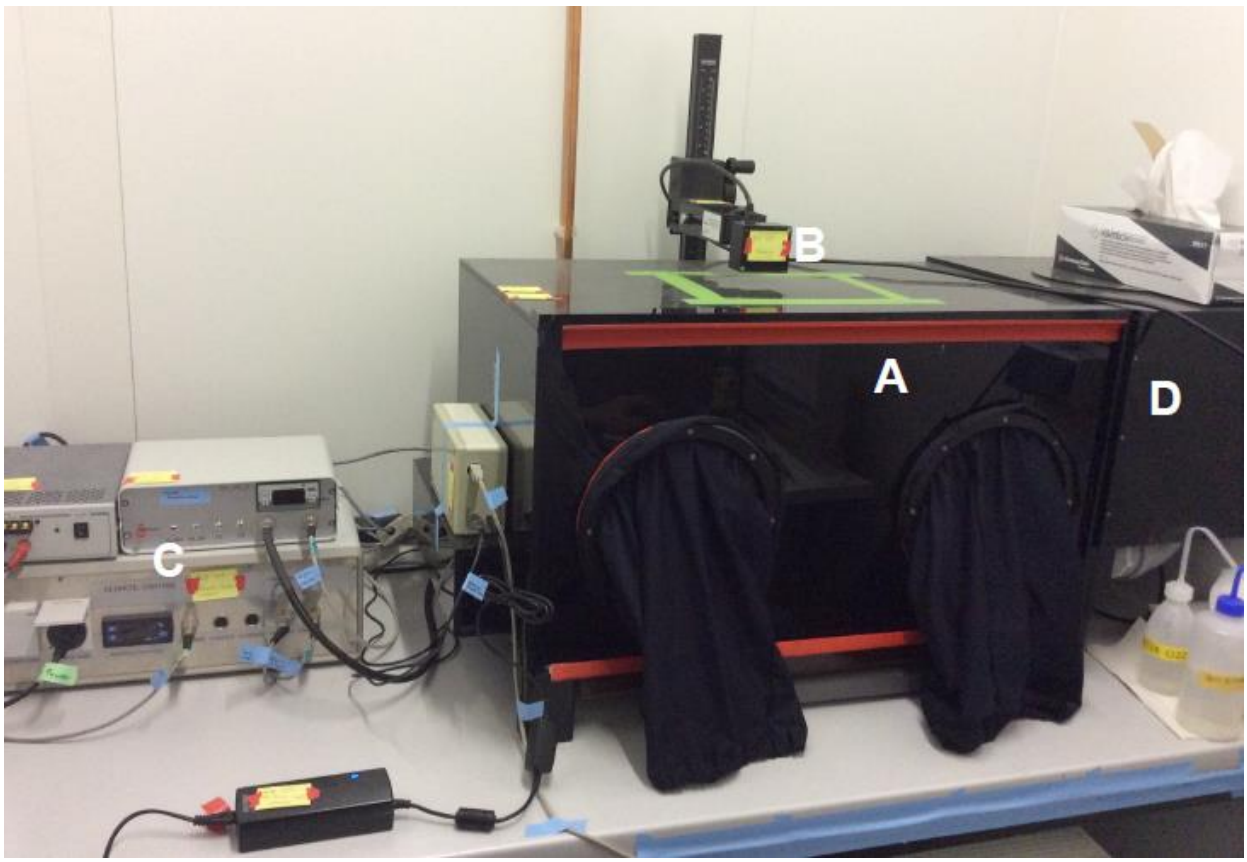

Figure 36 Fly tracking climate box

This light-tight climate box was used to record the movement of flies in infrared backlight with controlled temperature and humidity. A, light-tight box; B, camera; C, regulation panels; D, ultrasound fog generators.

4.4 Single sensillum recording

Extracellular single sensillum recordings were performed from antennal olfactory sensilla according to previous publication (de Bruyne, Clyne et al. 1999). A single fly was caught and expelled into a truncated 200 µl pipette tip with a mouth pump. The fly was blown sharply to trap the fly in the narrow neck of the tip. The fly was trapped in an orientation as heading to the narrow end of the tip and no legs or wings were trapped anterior to the head. From the narrow end, the fly's head was half exposed. From the wide end of the pipette tip, the tube was cut until ~6mm left for the abdomen of the fly. The fly was immobilized within the tip with a small plug of tissue. The fly was mounted on a glass slide with the antenna protruding on a glass coverslip. The fly head was adjusted so that the edge of the coverslip can be gently against the head just beneath the antenna. The antenna projected out over the top of the coverslip away from the rest of the head and fixed by a glass capillary on the side of the coverslip. The tip of the capillary was hooked in the joint between the second and third segments of the antenna closest to the edge of the coverslip (figure 37). The antenna was pulled down such that it becomes held gently but firmly between the coverslip and the capillary. The mounting was performed under ZEISS stereomicroscope.

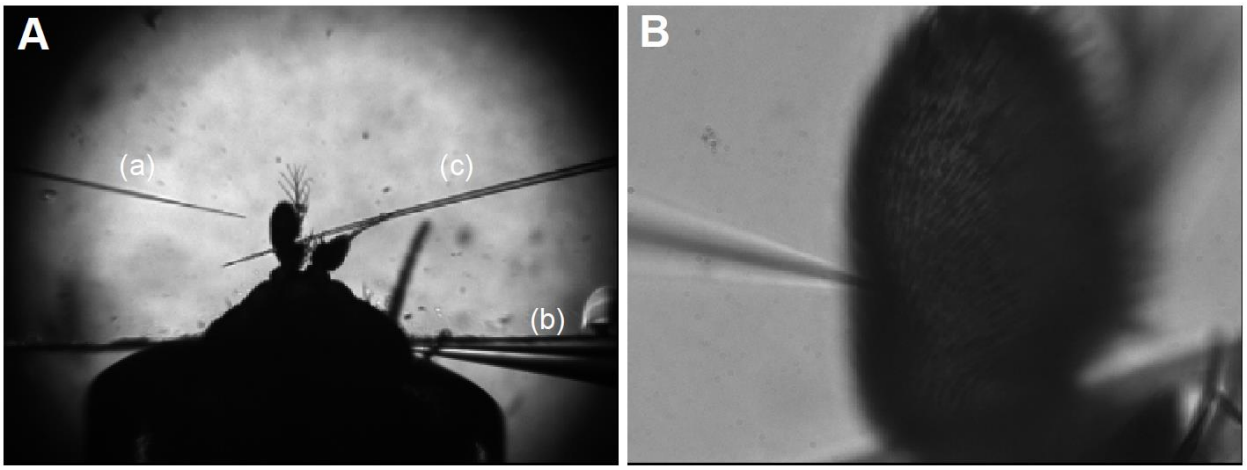

Figure 37 Scheme of Single sensillum recording

(A) Image of a mounted fly under 4X objective. (a) Recording electrode. (b) Reference electrode. (c) An empty electrode fixes the third segment of antenna. (B) A recording electrode is approaching one sensillum of the antenna under a 40X objective.

After mounting and stabilizing the antenna of the fly, the preparation slide was placed on the stage of microscope. The position of stage was adjusted until the fixed antenna was in focus in the center of the field of view (FOV) for objective. The odor stimulus delivery pipette was set facing

to the antenna of fly as close as possible so that the antenna was in the center of the airstream. A reference electrode filled with ringer (0.01 mM KCl) was inserted into the eye gently. The recording electrode was carefully brought into the FOV with micro manipulator (Sutter instrument). Recording was performed with 40X objective. The position of recording electrode was adjusted until its tip adjacent to the antenna. Both reference and recording electrode used are glass capillary.

The electrode was continually pushed against the sensillum until it pierced the cuticular wall of sensillum. Once stable spontaneous responses of olfactory neuron had been observed, odor deliver system (figure 38G) proceeded odor stimulus with a desired series. The AC signals (10 - 2800 Hz) of responses of particular stimulus were amplified by amplifier Multiclamp 700B and were recorded for 2-3 s, starting before stimulation, and analyzed by Clampex10.3 (Digidata 1440A). The responses of neuron firing were calculated by counting the number of action potentials from 200 to 700 ms after initial contact, as previously reported (Bracker, Siju et al. 2013). Eight repetitions were done for each genotype with each stimulus. Statistical analysis of different groups was performed using Prism GraphPad6 by one-way analysis of variance (ANOVA) to determine whether different groups have significantly difference.

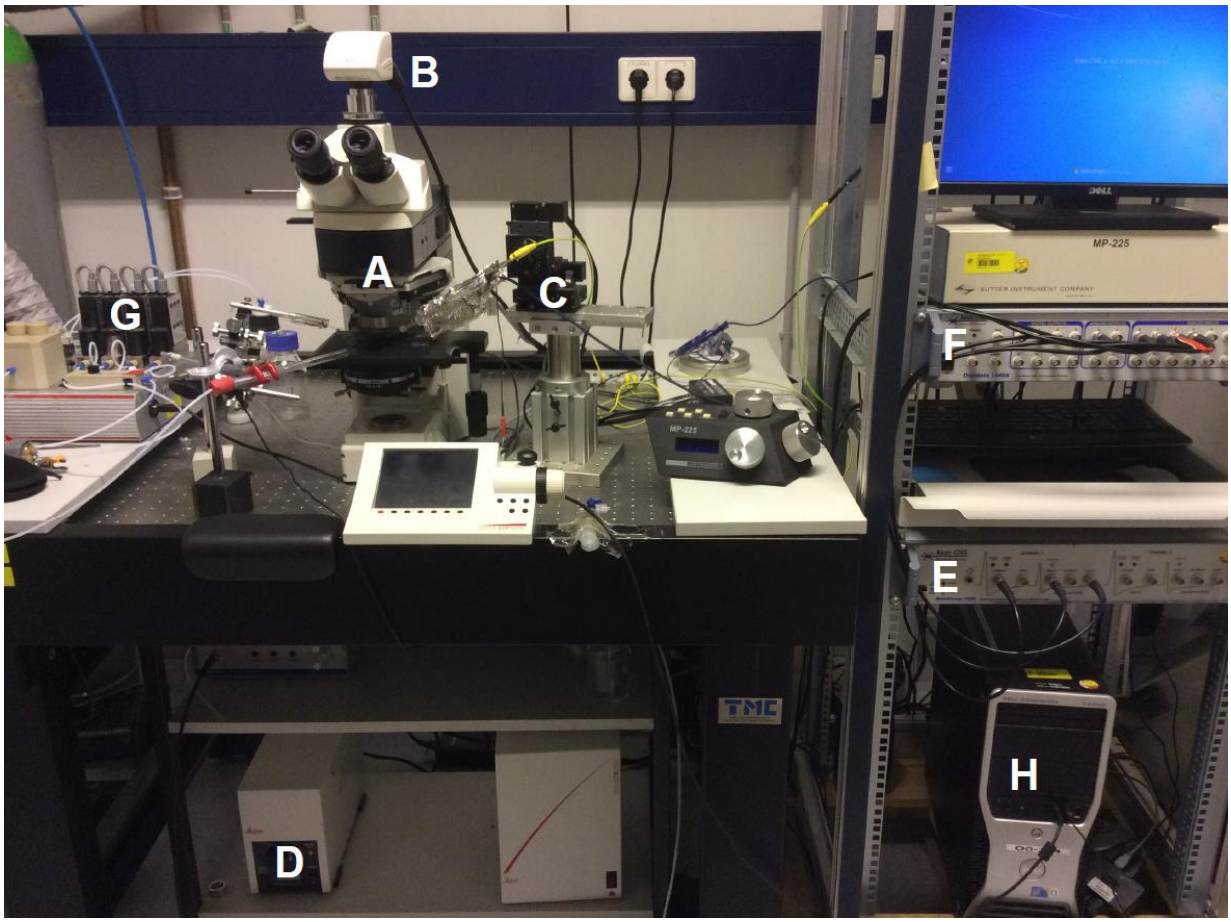

Figure 38 Electrophysiology equipment

Main devices for electrophysiology setup. A, fluorescent microscope; B, fluorescent camera; C, micro manipulator; D, fluorescent lamp; E, amplifier; F, digitizer; G, odor deliver system; H, computer.

4.5 Tip recording

Tip recordings were carried out as previously described with minor modifications (Hodgson, Lettvin et al. 1955). 5–7 days old female flies were used for all of experiments. The legs and wings of flies were removed during preparation to avoid extra noise in recording. For recording, flies were wedged into the narrow neck of a 200 µl pipette tip with head out. The proboscis was extended and pasted by double-sided tape on a cover slide. The tip of a glass micropipette was used to hold the proboscis in a stable position. A reference electrode containing 0.01 mM KCl was inserted into one eye of the fly. The recording electrode consisted of a fine glass pipette (10–15 µm tip diameter) and a silver wire connected to an amplifier. The recording electrode played the dual function of recording and container for the stimulus. Recording started the moment that the recording electrode contacted the tip of the sensillum. The polyamine solution used for

stimulation contained 30 mM tricholine citrate (TCC) as the electrolyte to suppress responses from the osmolarity-sensitive taste neuron. To avoid desensitization, stimuli were given at least 3 min apart. In all recordings concentrations were increased sequentially and a control stimulus without polyamine was applied first in all cases. Recordings were performed on L-type and S-type sensilla on the labial palp. The recording electrode was connected to an amplifier Multiclamp 700B and the AC signals (10 -2800 Hz) were recorded for 2–3 s, starting before stimulation, recorded and analyzed using Clampex10.3 (Digidata 1440A). The responses of neuron firing were calculated by counting the number of action potentials from 200 to 700 ms after initial contact, as previously reported (Weiss, Dahanukar et al. 2011).

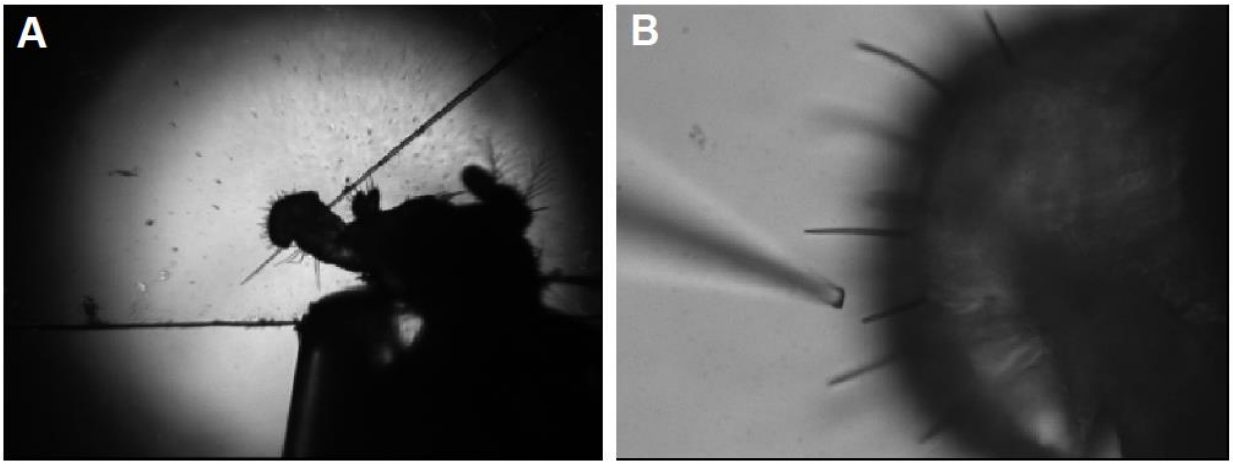

Figure 39 Scheme of tip recording

(A) Image of a mounted fly under 4X objective. The proboscis of the fly was fixed with an electrode. (B) A recording electrode is approaching one sensillum of the labellum under 40X objective.

4.6 *In vivo* SEZ Calcium imaging

For calcium imaging experiments in SEZ, flies expressed calcium indicator GCaMP6f or GCaMP5 under the IR41a-Gal4 or IR76b-Gal4. *In vivo* preparations of flies were performed in a modified setup according to a method previously reported (Yoshihara 2012). Flies were anesthetized by placing in a 15ml plastic tube on ice. Using forceps, the fly was inserted into a modified chamber described in previous publication (Yoshihara 2012). The fly chamber was a piece of plastic glued on a pipette tip. The head of fly was gently pushed out of the tube against the piece of plastic slide. The surrounding parts of the head were sealed using dental UV light-curing glue. A vacuum pulled out the proboscis of the fly carefully avoiding touching any glue to the labellum or making any damage in the taste sensilla. The top of proboscis was fixed by gluing to prevent it from going back into the head capsule. After fixing the head of fly, a small window was cut on top of head to

expose SEZ of the brain. The preparation was dissected with *Drosophila* Ringer solution and placed under ZEISS stereomicroscope (figure 40).

Using a Leica DM6000FS fluorescent microscope, preparations were imaged with a 40X water immersion objective and recorded by a Leica DFC360 FX fluorescent camera. All images were acquired with the Leica LAS AF E6000 image acquisition suit. Images were acquired for 20 s at a rate of 20 frames per second with 4 x 4 binning mode. During all measurements the exposure time was kept constant at 20 ms. For taste stimulation, taste stimuli were diluted in distilled water and delivered by a custom-build syringe delivery system to the proboscis. Distilled water (control), 1 mM, 10 mM, and 100 mM putrescine were applied respectively. Application of the stimulus was monitored by anther stereomicroscope. A drop of taste was delivered to touch the labellum. The stimulus was applied around 1s after the start of each measurement.

To measure the fluorescent intensity change in SEZ, the region of interest was delineated by hand and the resulting time trace was used for further analysis. To calculate the normalized change in the relative fluorescence intensity, we used the following formula: $\Delta F/F = 100(Fn-F0)/F0$, where Fn is the nth frame after stimulation and F0 is the averaged fluorescence of 15 frames before stimulation. The peak fluorescence intensity change is calculated as the mean of normalized trace over a 2 s time window during the stimulation period. The pseudocolored images were generated in MATLAB using a custom written program. All analysis and statistical tests were done using Excel and GraphPad6 Prism as described above.

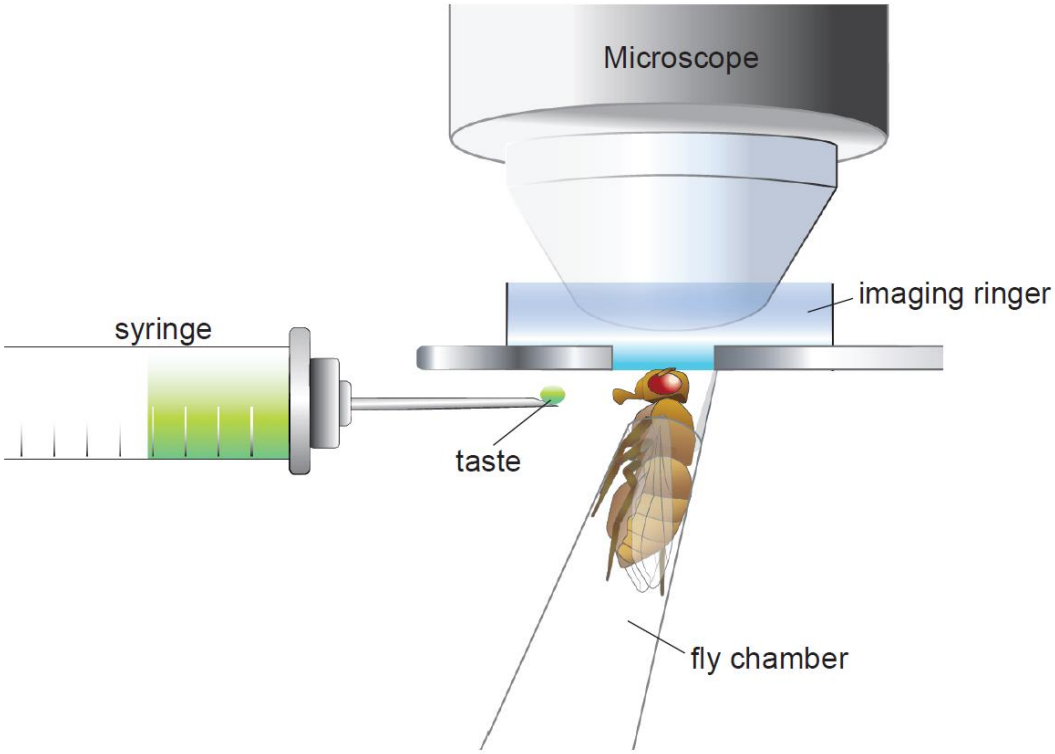

Figure 40 Schematic drawing of *in vivo* calcium imaging setup

The fly was fixed in fly chamber with extended proboscis. A drop of taste was delivered by a syringe. The responses of neurons in fly brain were recorded by fluorescent microscope.

4.7 Imaging on legs

The preparation of forelegs was performed as previously described (Miyamoto, Chen et al. 2013). Flies expressed calcium indicator GCaMP6f under the IR76b-Gal4. One foreleg of flies was cut between the femur and the tibia and fixed to a 76 x 26mm glass microscope slide. The tibia and the upper tarsal segments are covered and fixed with a double-sides stick tape. While the 4th and 5th tarsal segment are exposed and submerged in 100µl of imaging ringer, 100µl of test solution was applied by pipette directly to imaging ringer to stimulate the response of neurons. Images were acquired with the Leica LAS AF E6000 image acquisition suit. Images were acquired for 10 s before application and 30 s after application at a rate of 20 frames per second with 4 x 4 binning mode. *In vivo* imaging was performed with a Leica DM6000FS fluorescent microscope equipped with a 40X water immersion objective and a Leica DFC360 FX fluorescent camera. The measurement of max ΔF/F % was calculated in cell bodies. Average of ten frames immediately before the application of taste solution was defined as a baseline. The max ΔF/F % was calculated within 30 s after application.

4.8 Anatomy

Adult flies were anesthetized and placed on ice. The brain was dissected in cold PBS. Dissected brains were collected immediately and fixed with 4% paraformaldehyde (PFA) for one hour at room temperature. Brains were washed in PBS with 0.1% X-100 at least 3times and stained with the primary antibody in 0.1% X-100 overnight at 4˚C. After washed in PBS, the brains were stained with second antibody in 0.1% X-100 2 hours at RT. The primary antibodies were: chicken anti-GFP (molecular probes, 1:100) and rabbit anti-Dsred (Clontech, Living colors DsRed polyclonal AB, 1:200). The secondary antibodies were: anti-chicken Alexa 488 (molecular probes, 1:250) and anti-rabbit Alexa 549 (molecular probes, 1:250).

The brain was mounted in middle of a microscope slide. Mounting medium (VECTASHIELD H-1000) was applied on top of the brain after removing excess PBS with tissue. A coverslip was placed gently on top of the drop to cover the brain. Excess mounting medium was removed.

The adult fly antenna was dissected in cold PBS and mounted immediately in the same way.

Microscopic scanning was made by Olympus FV-1000 confocal microscope. Images were analyzed and processed using ImageJ and Photoshop.

5. Materials

Table 3 Buffers used calcium imaging, electrophysiology and anatomy

Name	Ingredients
Imaging ringer solution	182mM KCl 46mM NaCl 3mM $CaCl_2$ 10mM Tris-Cl adjusted to pH 7.2 in H_2O
Recording and reference electrode ringer solution	0.01mM KCl
Phosphate buffered saline (PBS)	137mM Na_2HPO_4 1.5mM KH_2PO_4 137mM NaCl 2.7mM KCl adjusted to pH 7.2 in H_2O
Phosphate buffered saline with Triton (PBT)	0.5% Triton X-100 in PBS

Table 4 Odors used for behavior and electrophysiology

Odors	Source
Carbon dioxide (pressured)	Westfalen (Germany)
Air (pressured)	Westfalen (Germany)
Paraffin oil	Sigma-Aldrich (USA)
Distilled water	Sigma-Aldrich (USA)
Pentanoic acid	Sigma-Aldrich (USA)
2,3-Butanedione	Sigma-Aldrich (USA)
Putrescine (1,4-Diaminotutane)	Sigma-Aldrich (USA)
Benzaldehyde	Sigma-Aldrich (USA)
4-methylcyclohexanol	Sigma-Aldrich (USA)
3-Octanol	Sigma-Aldrich (USA)
Linalool	Sigma-Aldrich (USA)

Table 5 Electrophysiology equipment

Devices	Name	Source
Micro manipulator	MP-225	Sutter instrument (USA)
Amplifier	MultiClamp 700B	Molecular Devices (USA)
Digitizer	Digidata 1440A	Molecular Devices(USA)
Stereomicroscope	ZEISS Stemi 2000	ZEISS (Germany)
Electrode puller	Sutter-97	Sutter instrument (USA)
Odor deliver system	SMARTEC	SMARTEC IngenieurBüro (Germany)

Table 6 Calcium imaging equipment

Devices	Name	Source
fluorescence microscope	DM6000FS	Leica Microsystems (Germany)
fluorescence camera	DFC 360 FX	Leica Microsystems (Germany)
Rotary Vane Pump	G24/08-30 Watt	Gardner Denver Thomas (Germany)
Custom made imaging holder	imaging holder	Max Planck Institute for Neurobiology workshop (Germany)
Dental LED Curing Light	M+W Superlite 1300	M+W Dental Müller & Weygandt GmbH (Germany)
Dental glue	M+W Permaplast LH Viscous Flow	M+W Dental Müller & Weygandt GmbH (Germany)
Voltage power supply	Voltcraft PPS-11360	Conrad Electronic SE (Germany)

Table 7 Other equipment

Equipment	Source
GM70 portable CO_2 meter	Vaisala (Finland)
Mass flow controller MC-500	NATEC Sensors GmbH (Germany)
Leica MZ 205 fluorescence stereomicroscope	Leica (Germany)
Custom made T-maze	Max Planck Institute for Neurobiology workshop (Germany)
Custom made climate box with humidity and heat controller	Max Planck Institute for Neurobiology workshop (Germany)

ZEISS Stemi 2000 stereomicroscope	ZEISS (Germany)
FV-1000 confocal microscope	Olympus (Japan)

Table 8 Consumables

consumable	purpose	source
Microscope slides (76x26mm)	mounting	Menzel Gläser (Germany)
Microscope cover glass (24x22mm)	mounting	Menzel Gläser (Germany)
Razor blade No.35010.20	mounting	Martor Solingen (Germany)
Blade holder	mounting	Fine Science Tools (Germany)
Dumont #55 forceps	dissecting	Fine Science Tools (Germany)
Drosophila vial (15ml, PS)	Behavior	VWR (Germany)
Facial tissue	Behavior	KiMTECH Science (US)
Parafilm	Behavior	Pechiney plastic packaging (USA)
Omnifix-F tuberculin syringe	Taste stimulating	B.Braun Melsungen(Germany)
Glass electrode GB150E TF-8P	recording	Science Products GmbH (Germany)
Pap pen	mounting	Kisker Products (Germany)

Table 9 Fly stocks

Referred to as	Genotype
Wild flies	Canton S
Genome background flies	w^{1118}
orco mutant	$w^{*};orco^{1}$
IR76b mutant	$w^{*};IR76b^{1}$
IR76b mutant	$w^{*};IR76b^{2}$
IR76b mutant	$y^{1}w^{*};P\{UAST\text{-}YFP.Rab39.S23N\}IR76b^{05}$
IR76b mutant	$y^{1}w^{67c23};Mi\{ET1\}IR76b^{MB00216}$
inward-rectifier potassium channel Kir2.1	$w^{-};+;$ UAS-Kir2.1::eGFP
IR76b-Gal4	$w^{*};P\{IR76a\text{-}Gal4.916\}$ 226.8;TM2/TM6B,Tb^{1}

IR76b-QF	w*;P{IR76b-QF.1.5}2
IR76b-RNAi	y^1v^1;P{TRiP.HMJ21583}attP40
UAS-IR76b	w*;P{UAS-IR76b.Z} 2/CyO;TM2/TM6B,Tb^1
GR66a-Gal4	w*; GR66a-Gal4/CyO;TM2/TM6B
UAS-mCD8GFP	yw, UAS-mCD8GFP; UAS-mCD8GFP/CyO; UAS-mCD8GFP
GCaMP5	W^{1118};PBac{20XUAS-IVS-GCaMP5G}VK00005
GCaMP6f	W^{1118};P{20XUAS-IVS-GCaMP6f}attP40
Poxn	w^{1118};Poxn[ΔM22-B5]/CyO
SPR-RNAi	P[KK103356]VIE-260B
OR42b-Gal4	w*;P{OR42b-Gal4.F}64.3
elav-Gal4	w*;P{elav-Gal4.L}3
nsyb-Gal4	y^1w*;P{nSyb-Gal4.s}3
OR83b-Gal4	w^{1118};P{OR83b-Gal4.K}97.1/TM3
GH146-Gal4	y^1w^{1118};P{GawB}GH146
SOD2-RNAi	w^1;P{UAS-Sod2.dsRNA.K}15/SM5
actin-Gal4	actin-Gal4/CyO
trp γ mutant	w^{1118};Mi{ET1}trpγ MB^{06664}
inaE mutant	w^{1118};P{EP}inaE^{EP1101}
globin1 mutant	y^1w^{67c23};ry506P{SUPor-P}glob1 ^{KG06649}
ttk mutant	ttk^1/TM6,Tb^+
ttk mutant	w*; ttk^{1e11}/TM3,ry*Sb^1
ttk mutant	y^1w*;P{lacW}64A ttk ^{E389}/TM6B,Tb^1
ttk mutant	y^1w*;Mi{MIC}ttk^{MI00087}
ttk deletion	w^{1118};Df(3R)BSC505/TM6C,sb^1cu^1
ttk deletion	w^{1118};Df(3R)ED6361,P{3^1,RS5+3.3^1}ED6361/TM6C, sb^1cu^1
ttk deletion	Df(3R)awd-KRB,ca^1/TM3,Sb^1Ser^1
UAS-ttk	w*;P{UAS-ttk.p69}2/CyO

6. Appendix

6.1 The author contributions form

Figures	Chapters	Author contributions
Figure 6	2.1.1	Ashiq Hussain
Figure 7	2.1.1	Ashiq Hussain
Figure 8	2.1.1	Ashiq Hussain
Figure 9	2.1.1	Ashiq Hussain
Figure 10	2.1.1	Ashiq Hussain
Figure 11	2.1.1	Laura F. Loschek
Figure 12 A-C	2.1.1	Mo Zhang
Figure 12 D	2.1.1	Anja B. Friedrich
Figure 13	2.1.1	Mo Zhang
Figure 14	2.1.1	Mo Zhang
Figure 15	2.1.1	Mo Zhang
Figure 16	2.1.1	Mo Zhang
Figure 17	2.1.2	Ashiq Hussain
Figure 18	2.1.2	Mo Zhang
Figure 19	2.1.2	Mo Zhang
Figure 20	2.1.2	Mo Zhang
Figure 21	2.1.3	Ashiq Hussain
Figure 22	2.1.3	Mo Zhang
Figure 23	2.1.3	Ashiq Hussain
Figure 24	2.2.1	Ashiq Hussain
Figure 25	2.2.1	Ashiq Hussain
Figure 26	2.2.2	Ashiq Hussain and Mo Zhang
Figure 27	2.2.3	Mo Zhang
Figure 28	2.2.3	Mo Zhang
Figure 29	2.2.4	Mo Zhang
Figure 30	2.2.4	Mo Zhang
Figure 31	2.2.5	Mo Zhang
Figure 32	2.2.5	Mo Zhang
Figure 33	2.2.5	Mo Zhang

6.2 The olfactory detection of CO_2 in adult *Drosophila*

Carbon dioxide (CO_2) is a ubiquitous gas present in the atmosphere at around 0.03% concentration (Guerenstein and Hildebrand 2008). CO_2 is also a product of respiration regulating breathing in vertebrates (Lahiri and Forster 2003). In insects, it has been shown to play an important role in ecology, such as locating a host, searching proper food and avoiding dangers (Faucher, Forstreuter et al. 2006, Guerenstein and Hildebrand 2008). The blood-feeding insects such as mosquitoes are attracted to CO_2 as a cue to human hosts. In contrast, fruit flies *Drosophila melanogaster* avoid CO_2 even at low levels (Suh, Wong et al. 2004, Faucher, Forstreuter et al. 2006). However, the reason for this aversion remains unclear. Given that CO_2 is a main component of the so-called *Drosophila* stress odor (dSO), which is emitted by stressed flies when stressed by shaking or electric shock, it was suggested to act as a signal to warn other flies (Suh, Wong et al. 2004, Kwon, Dahanukar et al. 2007). Moreover, another explanation correlates this avoidance response with CO_2 release from fruits. As flies prefer ripened fruits and unripe fruits emit more CO_2 than ripe ones, CO_2 may act as a signal to avoid unripe fruits during foraging (Faucher, Forstreuter et al. 2006). On the other hand, little is known about how flies overcome the aversion to CO_2 when it is produced during fruit ripening and fermentation by yeast in yeast-infested fruit, which are the fly's favorite food source. It has been shown that the behavioral avoidance to CO_2 can be inhibited by food odors directly acting on the CO_2 receptors (Turner and Ray 2009). Moreover, a recent study shows that the processing of food odors can suppress CO_2 avoidance by inhibiting CO_2 responsive MBONs in certain context (Lewis, Siju et al. 2015).

Unlike olfactory CO_2 sensing, the gustatory detection of flies exhibits an appetitive preference and high threshold to CO_2 (Scott 2011). Volatile CO_2 dissolved in water and detected as carbonate solution. Although the receptor has not been identified, it shows that carbonated water is sensed by the E409 neurons on the labellum of the proboscis (Fischler, Kong et al. 2007).

In *Drosophila*, volatile CO_2 is detected by two GRs, GR21a and GR63a, that are co-expressed in the antennal basiconic sensillum 1C (ab1C) (figure 41A) (Suh, Wong et al. 2004, Jones, Cayirlioglu et al. 2007, Kwon, Dahanukar et al. 2007). The heterotrimeric G-protein Gαq has been implicated in CO_2 signal transduction (Yao and Carlson 2010). And effectors of Gαq in downstream of CO_2 transduction is possible be members of canonical TRP channels (Badsha, Kain et al. 2012). In AL, CO_2 neurons project into a single glomerulus: the V-glomerulus, which is specialized for CO_2 processing (figure 41B). According to different concentrations of CO_2, the information is conveyed by distinct PNs from AL to higher brain center (Bracker, Siju et al. 2013,

Lin, Chu et al. 2013). More studies show that alternative neural pathways of CO_2 processing from AL to higher brain center are context dependent (Bracker, Siju et al. 2013, Siju, Bracker et al. 2014).

To activate its receptors, CO_2 must be able to cross the biological membranes. It is still unknown whether CO_2 reaches its receptors by passive diffusion or by activate transportation. If CO_2 passes into the liquid lymph surrounding the ORN, it is presumably converted into bicarbonate or H^+. Whether the CO_2 receptors are activated directly by CO_2 or indirectly by converted bicarbonate or H^+ is unknown. Moreover, it is still unclear whether the extracellular domains or intracellular domains of CO_2 receptors are acted on. A single OBP, LUSH, that mediated pheromone (cVA or 11-*cis*-vaccenyl acetate) responses raised the role of OBP in odorant recognition (Ha and Smith 2006, Fan, Francis et al. 2011). Given cVA is hydrophobic molecules, LUSH in lymph, made a function in binding with cVA and active its receptor OR67d (Ha and Smith 2006, Fan, Francis et al. 2011). While another study showed that suppression of one or more OBPs affect the behavioral responses to olfactory odors, it showed the role of OBPs in mediating olfactory perception (Swarup, Williams et al. 2011). These results raise the possibility that OBPs or other small soluble proteins in the lymph may be involved in CO_2 perception. Thus, besides two projects in which I was involved in, I performed additional studies at the molecular and neural levels trying to understand the involvement of potential molecules in CO_2 perception.

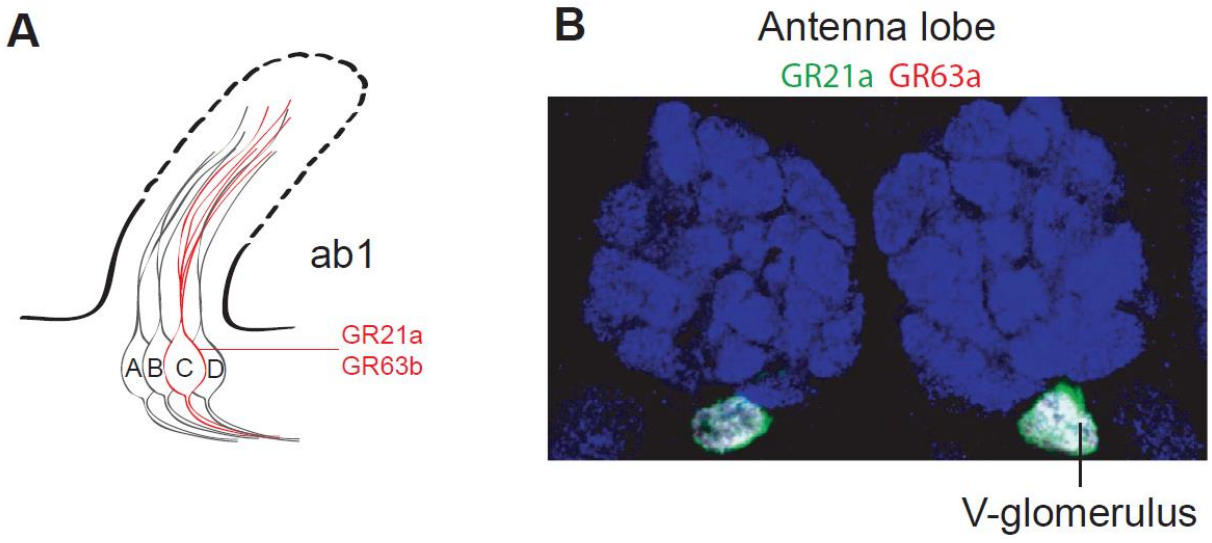

Figure 41 The scheme of CO_2 receptors

(A) Diagram of the antenna basiconic (ab1) sensillum. CO_2 neuron is labelled in red with co-expression of CO_2 receptors. (B) CO_2 receptors co-converge upon V glomerulus in the antenna lobe (GR21a labelled GFP in green and GR63a labelled RFP in magenta). Adapted from Jones et al. 2007.

6.2.1 A small screen of potential molecules involved in CO_2 detection

In addition to my main projects, which I collaborated in, I performed a side project to study the molecular mechanism of CO_2 receptor binding and activation in *Drosophila*. During CO_2 detection, gaseous CO_2 has to travel through a liquid environment to reach its receptors GR21a and GR63a (Jones, Cayirlioglu et al. 2007). Besides the olfactory detection of CO_2, the gustatory system also detects CO_2 in solution as bicarbonate (Fischler, Kong et al. 2007). How the odorant CO_2 passes the liquid and activates the lymph surrounded ORNs is unknown. Some soluble carrier proteins such as odorant binding proteins (OBPs) in *Drosophila* antenna are thought to be involved in carrying odors (Anholt and Williams 2010). To better understand the process of CO_2 detection and the related physiological effects, I performed a small screen testing CO_2 avoidance behavior of RNAi flies in T-maze assay looking for potential molecules involved in CO_2 detection. Candidate molecules were downregulated using *in vivo* RNAi. And the mutants of potential candidate molecules were tested in parallel. In addition to OBPs, the candidate molecules also included guanylate cyclase (GC; a receptor expressed on CO_2 ORNs in mammals), cGMP dependent protein kinase (cGK; promoting GC signaling), cyclic-nucleotide-gated ion channel (CNG; downstream channel activated by GC produced cGMP signaling), carbonic anhydrase (CAH; enzyme catalyzes the conversion of CO_2 into bicarbonate and proton), transient receptor potential channels (TRPs; family of ion channels mediates vision, olfaction and taste function of flies) and hemoglobin (globin) (Luo, Sun et al. 2009, Scott 2011, Fowler and Montell 2013). 1% CO_2 was used to test flies. RNAi lines of candidate genes were driven by actin-Gal4 across the development period and the adulthood. The flies were test in adulthood around 5-7days after hatched. RNAi flies of GR21a, the CO_2 receptor, were used as positive control in the same condition. I did not find any CO_2 avoidance phenotypes in flies with downregulated OBPs, GC, CNG and CAHs (figure 42A and B). Whereas, in the mutants, the CO_2 avoidance was largely reduced in *TRPs* and *globin1* mutant flies (figure 42C and D).

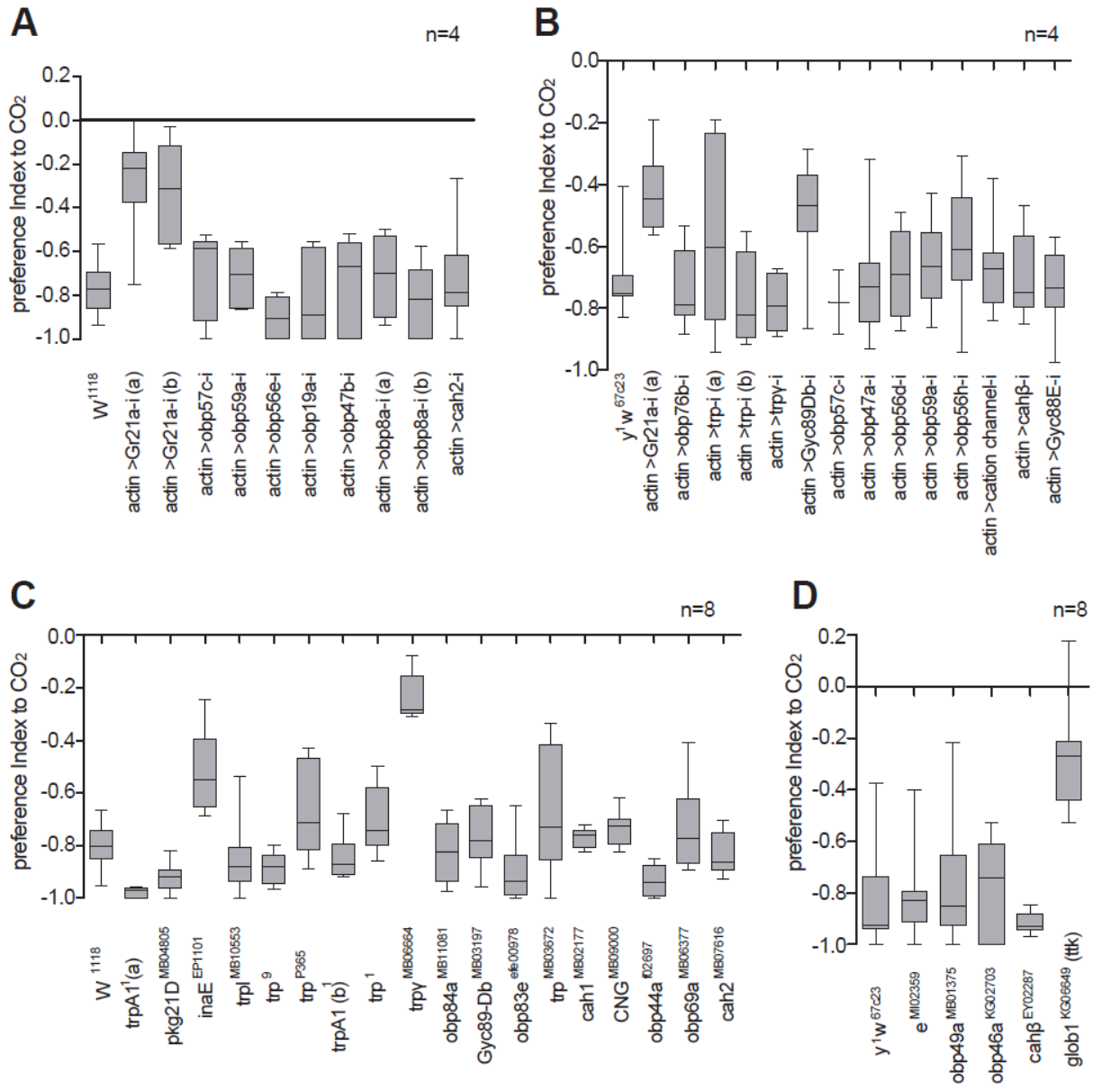

Figure 42 Preliminary results of screening on potential molecules involved in CO_2 detection

(A) and (B) RNAi suppression of OBPs, CAHs and other potential molecules did not affect the avoidance preference of flies to CO_2 (n=4 ± SEM). (C) and (D) Mutants of Trpγ, inaE and globin1 showed reduced CO_2 avoidance in behavior (n=8 ± SEM).

The family of TRP members are a class of cationic channels functioning downstream of G-proteins in the photoreceptors of the flies' visual system (Fowler and Montell 2013). I got two positive hits, the transient receptor potential cation channel γ (Trpγ) and an enzyme inaE which produce excitatory message linked with the opening of TRP channels (Leung, Tseng-Crank et al.

2008). Both mutants of *Trpγ* and *inaE* showed reduced CO_2 avoidance behaviors. The result indicated that the gene of *Trpγ* and *inaE* function as downstream factors of CO_2 receptor in CO_2 processing. However, another group published similar work on Trpγ before I was able to finish my work (Badsha, Kain et al. 2012). They showed that down-regulation of Trpγ and TrpL channels reduced the response to CO_2 in both behavioral and electroantennogram (EAG) responses. Contradicting their electrophysiological results, I did not observe reduced responses in CO_2 receptor neurons via single sensillum recording (SSR) (figure 43). However, the method that I used recorded the responses of individual CO_2 receptors, compared to the converged responses of all CO_2 ORNs recorded by EAG. This difference in methods used by these two works might indicate the changes between the converged information of nerves and the character of individual neurons.

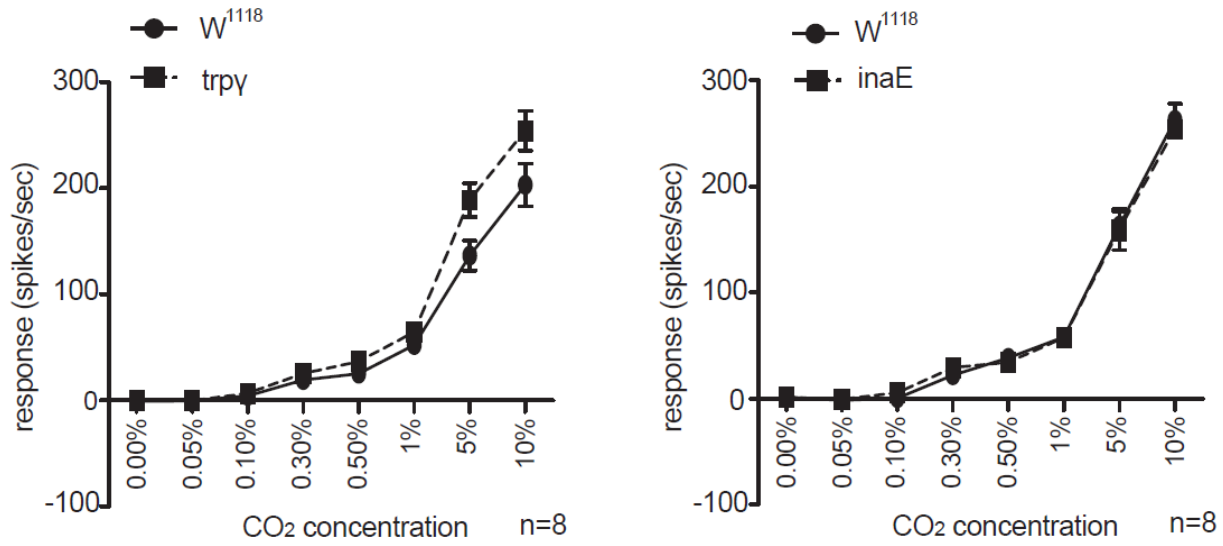

Figure 43 Single sensillum recording results of Trpγ and inaE mutants in CO_2 detection

No difference of neural responses in mutants of *Trpγ* and *inaE* compared with controls separately (n=8± SEM).

6.2.2 A potential candidate molecule involved in CO_2 detection

Another candidate gene *globin1* codes for an oxygen binding protein (Burmester, Storf et al. 2006). To test whether the mutant hit was truly positive, I tested additional mutant alleles of *globin*. However, none of the other alleles of g*lobin1* showed a similar phenotype in my following tests. We hypothesized that perhaps the P-element of the mutant allele that showed reduced CO_2 avoidance might carry a mutation not only in globin but also potentially in another gene

responsible for the phenotype. To find out if that was the case I carried out inverse polymerase chain reaction (PCR) to check the insertion of the P-element and to verify the mutation in globin.

The affected gene turned out to be *tramtrack (ttk*), because the p-element had inserted into the ttk locus. Ttk is a transcriptional factor and determines neuronal cell fate in the peripheral nervous system (PNS) during development of *Drosophila*. *Ttk* is known as a regulator of cell fate specification, cell proliferation and cell cycle regulation in the visual and tracheal systems of *Drosophila* (Li, Li et al. 1997, Araujo, Cela et al. 2007). This initial *ttk* allele flies (flies were labelled as *globin1* from Bloomington stock center) showed reduced CO_2 avoidance in behavior (figure 42D). Another allele of *ttk* mutant flies that lost expression of Ttk also showed reduced avoidance to CO_2 (figure 44A). However, when I used deletion and other mutants of *ttk* making combination of different *ttk* alleles, part of combinations did not affect the CO_2 avoidance in behavior (figure 44B). When I used one construct *UAS-tramtrack.p69* to rescue the phenotype in initial *ttk* homozygous, the reduced CO_2 avoidance in behavior could be rescued (figure 44C). All these results indicate a complicated mechanism of *ttk* regulating the CO_2 detection. Up-or downstream factors of *ttk* could be involved. To understand the function of *ttk* in CO_2 detection, more studies will be performed at the neural and molecular level.

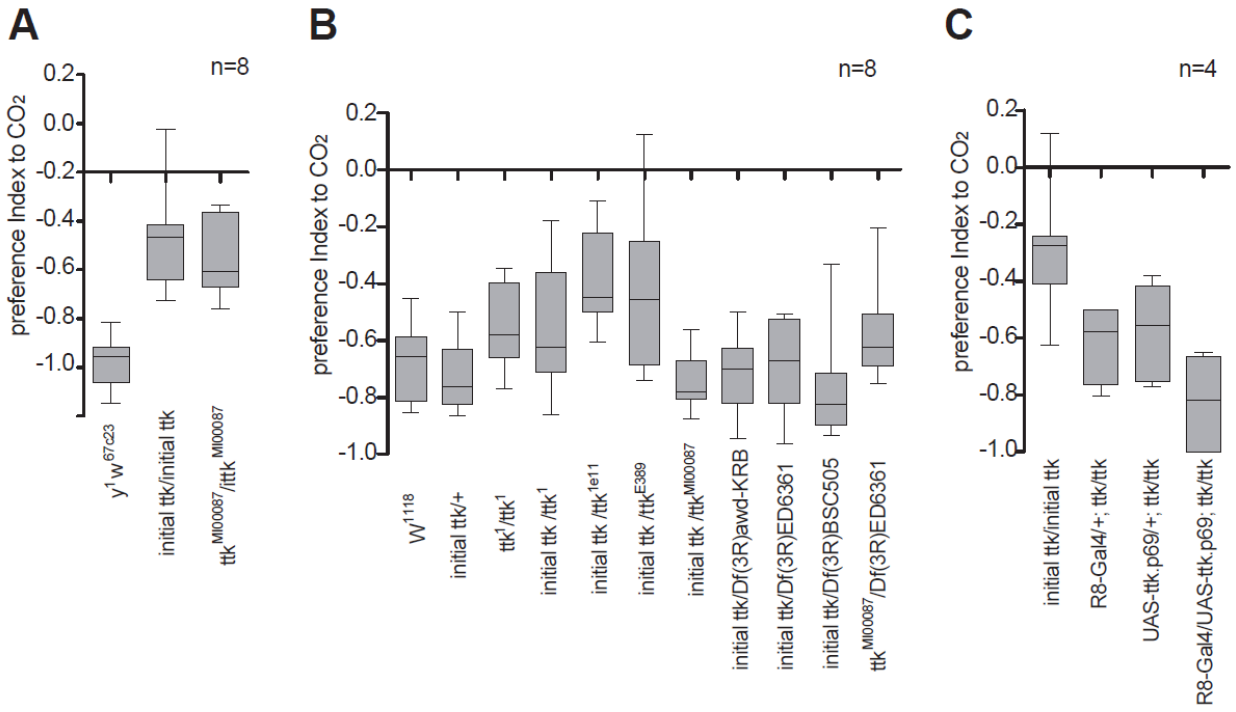

Figure 44 Combination of different ttk alleles shows partly effect on CO_2 detection

(A) The initial *ttk* allele flies and another *ttk* mutants showed reduced CO_2 avoidance in behavior (n=8 ± SEM). (B) Not all of the combination of these two allele with other *ttk* alleles and deletions showed affected CO_2 avoidance in behavior (n=8 ± SEM). (C) Re-expressing *ttk* using a construct *UAS-tramtrack.p69* could rescue the phenotype in the initial *ttk* mutant.

Taken together, I screened small molecules potentially involved in CO_2 detection. I found that the transient receptor potential cation channel *Trpγ* and its excitatory enzyme *inaE* are required in CO_2 detection. However, I didn't continue to work on them. In addition, I found a mutant allele of either the genes *globin* or *tramtrack* (this was not immediately clear as the insertion of the mutation could have been in either gene), which led to a reduced CO_2 avoidance behavior. However, the behavioral results on alternative alleles of *globin* or *tramtrack* were not consistent with this allele. The results indicated a more complicated mechanism of this affected gene in regulating CO_2 avoidance behavior. This allele might be affecting an alternative gene and neither *globin* nor *tramtrack*. The effected gene could also be possibly regulated by transcription factors up-or downstream of tramtrack. Facing too many possibilities, I stopped this project and turned to join more promising projects ongoing in the lab.

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