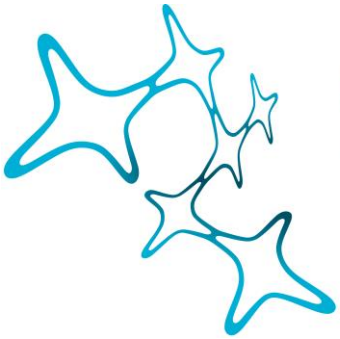

Graduate School of
Systemic Neurosciences
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A NEUROMODULATORY MECHANISM FOR STATE-DEPENDENT NUTRIENT DETECTION IN *DROSOPHILA*

Habibe Üçpunar

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Dissertation der Graduate School of Systemic Neurosciences

der Ludwig-Maximilians-Universität München

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1. List of Figures

2. List of Abbreviations

AL	Antennal Lobe
AMMC	Antennal and Mechanosensory Motor Center
cVA	Cis-Vaccenyl Acetate
DEG/ENaC	Degenerin/Epithelial Na^+ channel
DSO	Dorsal Sense Organ
GPCR	G-protein Coupled Receptor
GPCRs	G-protein Coupled Receptors
GRs	Gustatory Receptors
GSNs	Gustatory Sensory Neurons
iGluRs	Ionotropic Glutamate Receptors
IRs	Ionotropic Receptors
KCs	Kenyon Cells
LH	Lateral Horn
LNs	Local Interneurons
LSO	Labral Sense Organ
MB	Mushroom Body
MBON	Mushroom Body Output Neuron
MIP	Myoinhibitory peptide
OBP	Odorant Binding Protein
OBPs	Odorant Binding Proteins
ODC	Ornithine Decarboxylase
ORs	Olfactory Receptors

OSNs	Olfactory Sensory Neurons
PID	Photoionization Detector
PNs	Projection Neurons
PPK	Pickpocket
Put	Putrescine
SEZ	Subesophageal Zone
sGPNs	Sweet Gustatory Projection Neurons
sNPF	Short Neuropeptide F
sNPFR	Short Neuropeptide - F Receptor
SP	Sex Peptide
Spd	Spermidine
Spm	Spermine
SPR	Sex Peptide Receptor
STG	Stomatogastric Ganglion
TRP	Transient Receptor Potential
VCSO	Ventral and Cibarial Sense Organ

3. Summary

Feeding is an essential routine of an animal to survive. The decision of what to eat is a collective outcome of a sensory evaluation and its nutritional needs. While an animal may decide to eat food just because of its good taste, it is also possible that nutritional needs dictate what to eat through appetite or cravings. Pregnant females often report cravings that potentially arise from a dramatic change in their nutritional needs and physiological and hormonal state.

Food sources attract the attention of many animals primarily by their smell (long range cue) and taste (short range cue). Those sensory cues help these animals to identify a food source and they use them to assess the nutritional value of the food. Due to changing internal needs, animals often make different choices elicited by the same chemosensory cue bouquet. However, little is known how exactly internal needs of an animal modulate its perception and its sensory systems. In this cumulative thesis, I present two published studies addressing this question by using an important class of nutrients, the polyamines. In the first study, I, in collaboration with colleagues, investigated polyamines as chemosensory cues and found that they are detected by both olfactory and gustatory systems of *Drosophila* as signals for beneficial food or egg laying sites. This multimodal polyamine detection is mediated by an ionotropic receptor, IR76b, along with a co-receptor (IR41a) in the olfactory system and by IR76b alone, or with a yet to be identified co-receptor, in the gustatory system. Moreover, female flies significantly increase their reproductive success if they are fed with polyamine enriched food. This finding has provided an entry point to the second paper, in which the role of the mating state in the perception of polyamines has been investigated. Surprisingly, mated female flies exhibit an enhanced attraction towards biologically relevant concentrations of polyamines. This finding is particularly interesting because the modulation takes place right at the sensory neuron level. *Drosophila* is an excellent model to dissect the molecular underpinnings of such a modulation, because of the available genetic toolboxes. By using genetic, behavioral and calcium imaging experiments it was possible to show that a G-protein coupled receptor, sex peptide receptor (SPR), and its ligands, the myoinhibitory peptides (MIPs) regulate this mechanism. Both SPR and MIPs are expressed in the polyamine sensitive OSNs and GSNs, and SPR expression significantly increases upon

mating in sensory neurons thereby modulating their presynaptic output. Interestingly, SPR acts on OSNs' and GSNs' physiology in opposite directions. While increased SPR expression inhibits the presynaptic output of OSNs, GSNs undergo presynaptic facilitation. Altogether, both modulations serve the same behavioral output, to adjust the nutritional preference of the gravid female: enhanced polyamine preference.

From the broader perspective, this study contributes to our understanding of the detection of essential nutrients and neuromodulation of sensory systems according to changing internal states and needs of an animal, including humans.

4. Introduction

Animals are equipped with various sensory modalities to sample and evaluate the environment surrounding them. Among those, chemosensation is thought to be one of the oldest (Philpott et al., 2008). Even single cellular organisms are able to detect and respond to chemical compounds binding to the chemosensory receptors found on their cell membrane (Hazelbauer, 2014). Higher animals, such as amphibians, reptiles, birds and mammals have two main modalities for chemosensation: olfaction and gustation. While olfaction is used to evaluate long range air born cues, gustation is used to evaluate non-volatile substances in close proximity through physical contact with their source. Both modalities collectively serve the survival of the animal through finding good food sources, finding mating partners and avoiding potential dangers.

Importantly, animals move within an invisible bath of chemicals in the air that creates a constantly changing stimulus environment. It leads to continuous computations in the brain to update the latest chemical environment representation and generate the best behavior accordingly. Although there is significant progress in understanding how chemosensory coding takes place, there is still a lot to be answered in the field of chemosensory systems neuroscience: how do chemicals travel through the lymph, the liquid filling the chemosensory sensilla? How do ligands bind to receptors? What are the neuronal networks that extract the relevant information from complex odor mixtures? How is intensity, latency and mixture information encoded in the brain? How is the nervous system modulated to generate different behavioral outputs for the same chemosensory cues at different time points?

To be able to answer all those questions, scientists use model organisms like worms, flies, fishes or mice, which are smaller, easier to manipulate and less complex than humans to solve the basic principles (Figure 1). In addition, model organisms make it possible to develop genetic and experimental tools that make the nervous system accessible for various genetic and experimental manipulations.

4.1.*Drosophila* as a Model Organism

The first systematic and quantitative observation of a single species took place in the middle of 19th century when Gregor Mendel studied the genetics of heredity in peas. Although there is a great variety of life, this early work initiated the idea that the common knowledge of shared principles can be studied on selected few organisms, which are called model organisms today (Müller and Grossniklaus, 2010).

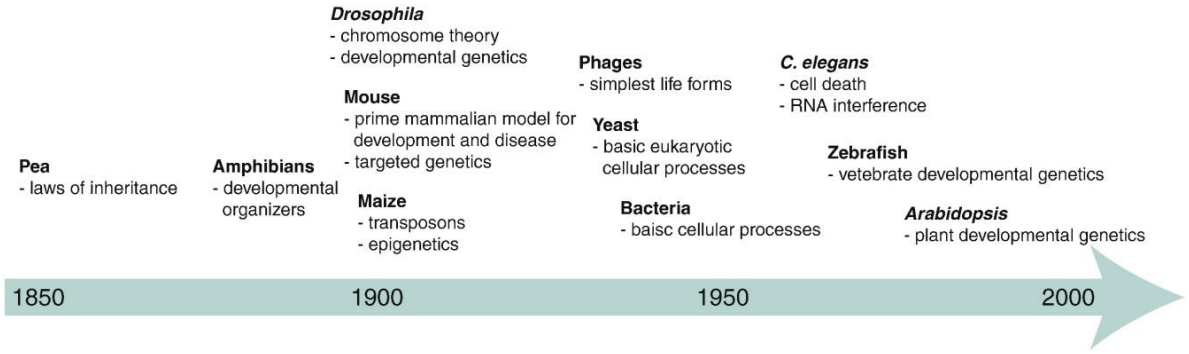

Figure 1: Emergence through time and main contributions of most used organisms.

The timeline showing the main model organisms used in life sciences and key findings obtained through studying them (Müller and Grossniklaus, 2010)**.**

William E. Castle is the first person, who brought *Drosophila* into the laboratory in 1901. He performed breeding studies and tested simple behavioral responses like phototactic, geotactic and mechanosensory responses (Greenspan, 2008). However, *Drosophila* had its golden age with Thomas Hunt Morgan when he started to use it for genetic studies. He found that a physical variation (white eye) shows a Mendelian segregation trait when flies are inbred (Morgan, 1910). This finding was the key for the rise of modern genetics.

Drosophila became one of the prime model organisms for chemosensory research after the identification of its odorant receptor genes (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). Although the *Drosophila* odorant receptor types are structurally different from the

mammalian ones, there is a shared architecture for mammalian and insect olfactory systems (Vosshall and Stocker, 2007). In addition to that, the *Drosophila* olfactory system is comprised of a far lower number of neurons making it an excellent model to investigate the chemosensory system.

4.2.Adult *Drosophila* Chemosensory System

4.2.1. Olfactory System

The *Drosophila* olfactory system is split into two organs found on the rostral side of its head: the antenna and the maxillary palp. The antenna carries most of the olfactory receptors on its third segment that detect olfactory cues and for that reason is counted as the main olfactory organ. The maxillary palp, by contrast, houses a limited number of receptors detecting mainly food odors (Vosshall and Stocker, 2007).

The third segment of the antenna and the maxillary palp are covered with hair-like structures called sensilla. Each sensillum houses one to four olfactory sensory neurons (OSN) that express varying combinations of olfactory receptors. While the shaft of the sensillum covers the dendritic extensions of the OSNs, support cells found next to the cell bodies of the OSNs secrete odorant binding proteins (OBPs) into the sensillum lymph (Shanbhag et al., 2001). Each OSN in a sensillum has a unique spike amplitude and spontaneous activity pattern making it possible to differentiate the activity of a single OSN from others found in the same sensillum during an electrophysiological recording (Joseph and Carlson, 2015).

There are three main types of sensilla on the antenna, each of which differ in size and morphology: small and large basiconic, trichoid and coeloconic sensilla (Shanbhag et al., 2000). The distribution pattern of the sensilla types does not change between the two antennae and in different flies. The only difference exists between male and female flies in the number of large basiconic and trichoid sensilla. While females carry 20% more large basiconic sensilla than males, males have 30% more trichoid sensilla (Stocker, 2001).

Compared to the antenna, the maxillary palp is a simpler organ and contains only one class of sensilla. There are around 120 OSNs housed in basiconic sensilla in pairs (de Bruyne et al., 1999). Unlike antennal OSNs, axons of maxillary palp OSNs follow an indirect path through the taste center, subesophageal zone (SEZ), to reach the antennal lobe (AL). The proximity to the taste detection organs correlates with the role of the MP in detecting food odors.

4.2.1.1. Odorant Receptors

There are three receptor types responsible for odorant detection in *Drosophila*: olfactory receptors (ORs), ionotropic receptors (IRs) and a few gustatory receptors (GRs) that are expressed on the antenna.

After the discovery of mammalian ORs (Buck and Axel, 1991), which are a multigene family of seven transmembrane G-protein coupled receptors (GPCRs), scientists had tried to find the corresponding receptors in flies through a homology-based approach by searching for mammalian-like GPCR families in flies. However, they could not find any homologous ORs in flies and this endeavor failed (Vosshall and Stocker, 2007). Then, instead of homologous GPCRs, they looked for a family of seven transmembrane domain proteins exclusively expressed in fly OSNs and discovered the fly specific ORs (Vosshall et al., 1999). Although the fly ORs have seven transmembrane domains, they do not have a homology with GPCRs or mammalian ORs. Fly ORs have an inverted membrane topology compared to the GPCRs and evolved independently of mammalian ORs (Benton et al., 2006; Vosshall et al., 1999; Wistrand M, Kall L, 2006). In total, there are 60 OR genes and two of them produce two alternative RNA products to give rise to 62 ORs in total (Robertson et al., 2003).

In the vertebrate olfactory system, there is a strict “one neuron to one receptor rule”, meaning that each olfactory sensory neuron expresses only one odorant receptor gene and therefore one type of receptor (Malnic et al., 1999). However, unlike mammals, *Drosophila* olfactory receptors are expressed together with an obligate co-receptor ORCO (previously known as Or83b) (Benton et al., 2006; Vosshall et al., 2000). ORCO expression together with a specific ligand-binding OR is

fundamental and sufficient for the trafficking of the heteromeric OR-X/ORCO receptor complex to the ciliated dendrite of a neuron and their functioning as olfactory receptors. Indeed, almost all basiconic and trichoid ORNs require the expression of ORCO for proper functioning (Benton et al., 2006). The only basiconic ORN that does not require ORCO expression for functioning is the one expressing atypical GRs (GR21a and GR63a) for CO_2 detection (Jones et al., 2007; Kwon et al., 2007).

In addition to having an obligate co-receptor, several *Drosophila* ORNs express two or three ORs along with ORCO introducing a second contrast between the insect and vertebrate olfactory system organization. However, the functional role of this multiple OR co-expression is not known (Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldman et al., 2005).

There is to date no obvious functional or evolutionary reasoning behind which OR is expressed in which olfactory organ, or even in which sensillum type. However, almost all ORs are expressed in either basiconic or trichoid sensilla, and not in coeloconic sensilla (except broadly tuned OR35a). Through electrophysiological recordings it has been shown that coeloconic sensilla are tuned to various ligands including amines, ammonia, water vapor and putrescine, suggesting that additional chemoreceptor families may exist (Yao, 2005). This educated guess had led to the discovery of a second family of olfactory receptors known as ionotropic receptors (IRs) (Benton et al., 2009). IRs are members of the ionotropic glutamate receptor (iGluR) gene family but do not belong to the kainate, AMPA, or NMDA classes of iGluRs and exist as a new class of chemoreceptors. Unlike other iGluRs, IRs contain a divergent ligand binding domain instead of a glutamate binding residue (Benton et al., 2009). IRs are evolutionarily conserved in bacteria, plants and animals suggesting that they are more ancient compared to ORs (Benton et al., 2009). Each IR is expressed together with one or two broadly expressed co-receptors (IR8a, IR25a) acting in different subsets of odor-specific IRs (Abuin et al., 2011). IR8a and IR25a expressing neurons are tuned to complementary chemical classes of odors. While IR8a appears to be the co-receptor for acid-sensing IRs, IR25 seems to be the co-receptor for amine-sensing ones, potentially acting together with IR76b (Silbering et al., 2011). These two groups of IR expressing

OSNs differ from each other not only by the odor repertoire, but also by their developmental pathway through the differential expression of Notch (Silbering et al., 2011).

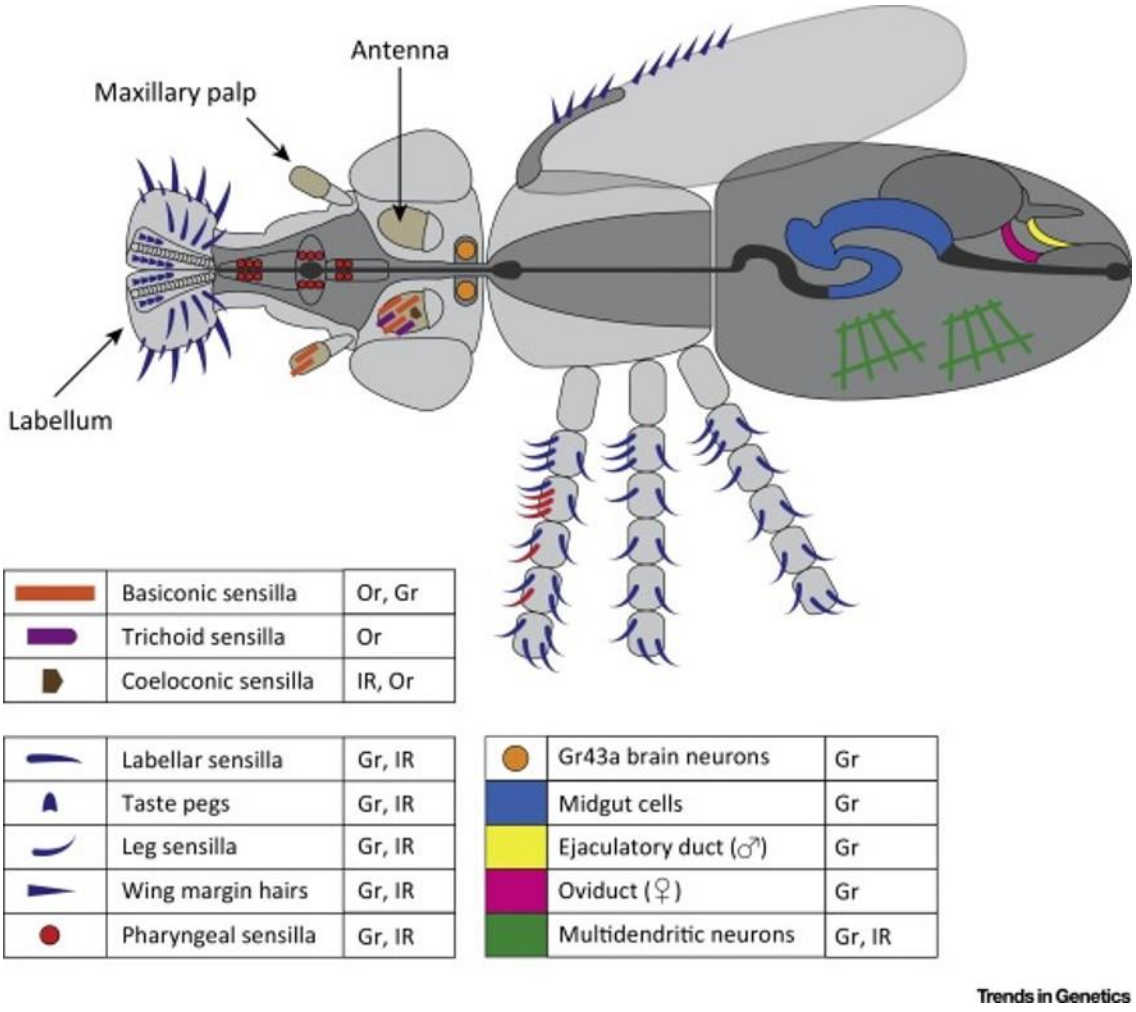

Figure 2: Overview of the OR, GR and IR expression in *Drosophila*

Main chemoreceptor classes, ORs, GRs and IRs are distributed throughout the body of *Drosophila*. Some GRs and IRs are expressed in the internal organs of the fly (dark grey area). On the legs, males carry male specific extra sensilla (the red ones in the picture). Figure is adapted from (Joseph and Carlson, 2015).

Unlike ORs, IRs are not restricted to the olfactory organs (antenna & maxillary palp) and expressed on various anatomical regions in *Drosophila*. In addition to the antenna, they are expressed on legs, labellum, pharynx, anterior wing margins, and internal organs of adult flies

(Figure 2) suggesting other roles than olfaction (Benton et al. 2009; Croset et al. 2010; Grosjean et al. 2011; Zhang et al. 2013; Koh et al. 2014). An interesting example for the gustatory function of IRs is the mediation of low-dose salt attraction through the IR76b receptor expressed on the labellum (Zhang et al. 2013). Moreover, IR52a and IR52c receptors show a sexually dimorphic expression pattern in the forelegs of males and females suggesting a potential pheromone detection role for those IRs (Koh et al., 2014). Being an ancestral chemoreceptor family, IRs seem to be specialized for various chemosensory tasks, most of which still wait to be discovered.

4.2.1.2. Neural Pathway of Olfactory Information

Neuronal signaling of olfactory information gets triggered by the binding of odor molecules to odorant receptors expressed on the ciliated dendrites of olfactory sensory neurons (OSNs), which are housed in the antennal or maxillary palp sensilla. In general, each OSN expresses one specific type of receptor and all OSNs expressing the same type of receptor innervate discrete glomeruli in the AL, which is the first relay center for olfactory information processing (Hallem et al., 2004; Vosshall and Stocker, 2007). This OR-specific targeting of OSNs establishes a stereotypical topographic map in the AL (Couto et al., 2005; Fishilevich and Vosshall, 2005). Here, the axons of OSNs form synapses with two different types of neurons: local interneurons (LNs) and projection neurons (PNs). While LNs establish horizontal connections among glomeruli and carry on inter-glomerular computations, PNs connect each glomerulus with at least two higher olfactory centers, mushroom body (MB) and lateral horn (LH) (Stocker, 1994) (Figure 3).

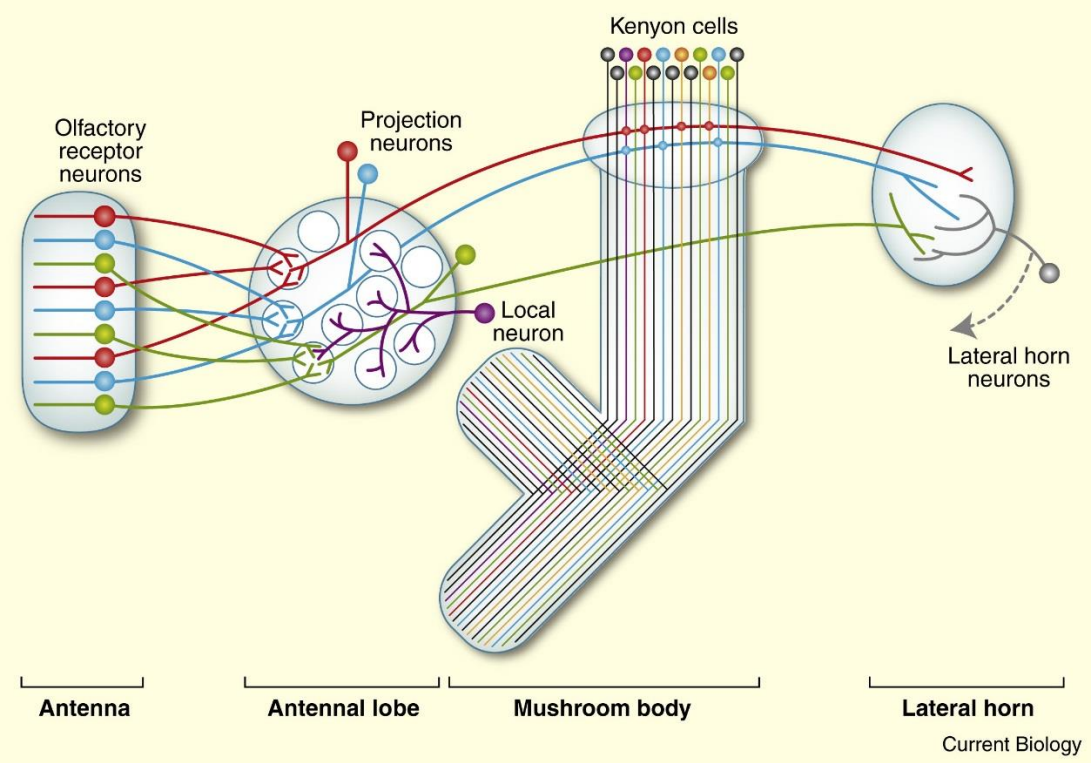

Figure 3: Overview of *Drosophila* olfactory pathway

Olfactory receptor neurons expressing the same type of receptor innervate the same glomerulus in the AL. There, they make connections with LNs and PNs. PNs carry the information to the higher brain centers and target the LH either directly (green line) or through the MB calyx (red and blue lines). Figure is taken from (Masse et al., 2009).

Olfactory receptors can detect more than one odorant and each odorant can activate more than one OSN. Thus, each odorant generates a combinatorial activation code of OSNs providing a broad window of odor detection capability to the animal although flies have a limited number of odorant receptors compared to the panel of odorants that they can detect (Galizia and Szyszka, 2008; Masse et al., 2009). Concentration of an odorant also changes the activation pattern of one or multiple glomeruli and thus the odor experience. The higher the concentration of an odorant gets, the more glomeruli are recruited since the odorant will activate low affinity receptors as well. This change in the combinatorial code of activated OSNs may generate different behavioral outputs as the concentration of an odorant increases. On the other hand, there are some odors, which are highly relevant for the survival of the animal, that activate single dedicated receptors (Jones et al., 2007; Stensmyr et al., 2012; Suh et al., 2004).

From the periphery to the higher brain centers, olfactory information undergoes a series of filtering and modifications. The majority of LNs are GABAergic and form inhibitory synapses with both OSNs and PNs establishing a lateral inhibitory network (Wilson and Laurent, 2005). There are also identified cholinergic LNs forming excitatory synapses with PNs and excite them interglomerularly (Shang et al., 2007). LNs differ with their network area as well, such that there are different types of LNs innervating single, multiple, or all glomeruli and modifying their output accordingly (Chou et al., 2010).

Most PNs form excitatory connections between the AL and the higher brain centers and transmit the odor information to the MB and the LH. Initially, it has been shown that MBs are the center for olfactory learning and memory (Heisenberg, 2003; Keene and Waddell, 2007), but additional findings also show that it is not only the center for learning and memory formation, but also processes context-dependent information such as internal or behavioral state to update and adapt behavior instantanenously (Bräcker et al., 2013; Cohn et al., 2015; Lewis et al., 2015; Owald et al., 2015). Olfactory information is conveyed by PNs to the MB calyx, the main input region of the MBs. MBs contain ~2500 intrinsic neurons, called Kenyon cells (KCs) (Ito et al., 1997; Lee et al., 1999; Strausfeld et al., 2003; Tanaka et al., 2008; Yasuyama et al., 2002). At the calyx, PNs synapse onto a unique and mostly random pattern of KCs and maintain the 'odor image' generated in the AL at higher brain centers level, although the recruited set of PNs differ between individuals. Different PNs innervating the same glomerulus at the AL show an overlapping but unique projection patterns in the higher brain centers. Moreover, the projection pattern of PNs tend to preserve the glomerular neighborhood map of the AL (Marin et al., 2002; Wong et al., 2002). KCs form three main axonal projection area and divide the MBs into at least three different MB lobes: αβ, α'β' and γ (Crittenden et al., 1998; Lee et al., 1999; Tanaka et al., 2008). By looking at the connectivity between PNs and KCs, it has been thought that KCs may act as coincidence detectors since specific Kenyon cell types cover the concentric PN zones and may encode combined PN activity (Perez-Orive 2002; Heisenberg 2003; Wang et al. 2004).

Lateral horns are the second higher brain center in olfactory information processing where PNs target. Unlike KCs' dendritic arborizations, LH third-order neurons are limited to a particular PN

target area and found to be isolated from other areas suggesting little integration between different subsets of the olfactory signal repertoire. Therefore, LHs are thought to be involved in experience-independent (innate) odor responses (de Belle & Heisenberg 1994; Heimbeck et al. 2001; Tanaka et al. 2004). Recent studies show that LHs have functional subdivisions to encode different features of odors like intensity or hedonic valence (Soohong Min et al., 2013; Strutz et al., 2014).

From the periphery to the behavioral output, the odor information goes through several steps of convergence and divergence when it is transmitted from one relay center to another. Activation of a unique combination of numerous OSNs triggers the olfactory signaling and this signal reaches to the limited number of glomeruli with a high ratio of convergence. The signal is sparsened as it steers through the higher brain centers separating different odor representations from each other and forming a clear identity of an odor for decision making (Turner et al., 2008).

4.2.2. Gustatory System

Flies use their gustatory system to find proper food sources, to decide where to lay their eggs, and to stay away from toxic materials. In addition, flies also use their gustatory system for social communication and to initiate courtship and mating (Bray and Amrein, 2003). Unlike the ORs, GRs are not confined to the head but spread throughout the body of the fly (Figure 2). Flies can taste with their mouth (labellum), legs, wings, pharynx, and likely even with their ovipositor in the case of a female (Montell, 2009).

Flies detect tastants through hair like projections called taste bristles. There is a large terminal pore at the tip of a bristle where the taste molecules diffuses into and binds to the receptors expressed on the dendrite. Each gustatory neuron housed in bristles is a bipolar neuron with a dendrite extending to the tip of the sensilla and an axon projecting to the SEZ in the brain. There are three different types of bristles found on the labellum defined according to their size: long (l-type), intermediate (i-type) and short (s-type) (Freeman and Dahanukar, 2015). Each bristle on the labellum contains two to four gustatory sensory neurons (GSNs) responding to a different

tastant including sugar, water, low salt or bitter (including high salt concentrations), respectively (Meunier et al., 2003; Siddiqi and Rodriguez, 1980; Thorne et al., 2004). Bristles containing only two GSNs respond to attractive tastants (sugar and low salt) or aversive tastants (bitter and high salt) (Hiroi et al., 2004). In addition to taste bristles, each labial palp possesses approximately 30 taste pegs on their inner surfaces housing a single GSN (Joseph and Carlson, 2015). Additional GSNs are found on the internal taste organs which are the labral, ventral and cibarial, and dorsal sense organs (LSO, VCSO, DCSO) lining along the pharynx. Those sensilla house up to eight GSNs (Stocker, 1994).

There are other taste receptors spread over the body of the fly. Fly legs contain different types of GSNs responding to sugar, bitter, or neither of the two leaving many 'orphan neurons' to be characterized (Joseph and Carlson, 2015). The chemosensory sensilla found on the anterior margin of the wings respond to tastants triggering grooming behavior (Yanagawa et al., 2014). The ovipositor of the female flies contains bristles that exhibit morphology and innervation patterns to have a potential taste function, however there is not a clearly characterized ovipositor GSN identified so far (Joseph and Carlson, 2015).

4.2.2.1. Gustatory Receptors

So far four different chemoreceptor classes or channels have been shown to mediate responses to various categories of tastants in *Drosophila*: GRs, IRs, TRP and pickpocket (PPK) receptors. Of those, GRs were the first to be characterized and were identified through the same bioinformatics algorithm that led to the discovery of ORs (Clyne et al., 2000). GRs and ORs are distantly related and together form a superfamily of insect chemoreceptor genes (Robertson et al., 2003).

Flies can taste sugar, bitter, water, carbonation, salt and non-volatile pheromones. Sugar sensitive neurons - except the fructose detecting ones – express three main GRs: Gr5a, GR64a and GR64f. An additional five GRs are co-expressed along these three 'core receptors' in sugar sensitive GSNs. While GR5a is responsible for physiological and behavioral responses to

trehalose, GR64a is responsible for the detection of complementary sugar classes, sucrose, maltose and glucose (Jiao et al. 2007, Dahanukar et al. 2007). In both neuronal subsets GR64f is necessary as the co-receptor for the proper functioning of the GRs and detection of the relevant sugar subsets. However, misexpression of GR64f together with either of GR5a or GR64a receptors was not sufficient to generate a sugar response, indicating that additional co-receptors might exist (Jiao et al., 2008). Unlike other sugars, fructose is detected through a single GR, GR43a, although its substrate specificity has not been established yet (Sato et al., 2011). Interestingly, GR43a is expressed also in the central brain monitoring the rise of fructose in the haemolymph after feeding (Miyamoto et al., 2012).

Bitter sensitive neurons are rather heterogeneous compared to the sweet sensitive neurons. Bitter neurons express multiple GRs in partially overlapping subdomains generating distinct classes of response profiles and molecular signatures (Ling et al., 2014; Weiss et al., 2011). Five of those GRs (GR66a, GR32a, GR33a, GR89a and GR39a) are expressed in all bitter sensitive GSNs forming a collection of 'core-bitter GRs'. This suggests that those five receptors could be the obligatory co-receptors for bitter taste detection (Weiss et al., 2011). Consistent with this proposal, misexpression of three broadly expressed GRs (GR66a, GR93a and Gr33a) in a sugar-responsive neuron was insufficient to produce any bitter response in those neurons suggesting that a functional bitter taste receptor complex might be comprised of four or more different subunits (Moon et al., 2009).

In addition to GRs there are different types of receptors that function as gustatory receptors and detect other canonical and non-canonical tastants. Some bitter sensitive neurons express receptors belonging to the Transient receptor potential (TRP) family (S. H. Kim et al., 2010; Zhang et al., 2013b). Moreover, a member of the degenerin/epithelial Na^+ channel (DEG/ENaC) family of PPKs, PPK28, is responsible for water sensation functioning as an osmolarity sensor (Cameron et al., 2010; Inoshita and Tanimura, 2006). An ionotrophic receptor (IR76b), expressed on the labellum, mediates the appetite for low salt (Zhang et al., 2013a). Interestingly, IR76b receptors are also expressed in salt-insensitive GSNs as well as in multiple OSNs targeting different glomeruli in the AL indicating additional roles for this receptor (Benton et al., 2009). It has been

shown that flies can also taste carbonation and fatty acids as appetitive cues, however, receptors detecting these two modalities are still unknown (Fischler et al., 2007; Masek and Keene, 2013).

Flies detect cuticular hydrocarbons as pheromones with their taste receptors. GR68a, the first GR shown to participate in mating behavior, is differentially expressed in the first leg of males (Bray and Amrein, 2003) and detects an anti-aphrodisiac pheromone (3*R*,11*Z*,19*Z*)-3-acetoxy-11,19-octacosadien-1-ol (CH503). It is a cuticular hydrocarbon transferred from males to females during mating and inhibits courtship from other males. It has been shown that GR68a expressing GSNs are necessary for the normal progression of courtship behavior in males (Yew et al. 2009; Shankar et al. 2015). Notably, two receptors belonging to PPK family, PPK23 and PPK25, and an IR receptor, IR52c, also play role as contact chemoreceptors and contribute to courtship behavior (Koh et al., 2014; Lu et al., 2012; Thistle et al., 2012).

4.2.2.2. Neural Pathway of Gustatory Information

The subesophageal zone (SEZ) is the primary relay center of the gustatory information where most of the GSNs innervate the central fly brain. Unlike the glomeruli of AL, the SEZ does not have morphologically distinct anatomical compartments. Initial studies had shown that sweet and bitter sensitive neurons have partially overlapping but distinct patterns of innervations in the SEZ indicating the presence of a spatial map for attractive versus repulsive tastes already at the first stage of taste processing (Thorne et al., 2004; Wang et al., 2004). However, recent efforts to unravel projection patterns by using 67 GAL4 drivers representing the entire repertoire of GR taste receptors revealed ten distinct categories of projection patterns in the SEZ (Kwon et al., 2014). Projection patterns group according to three different criteria: the source organ of the stimulation, the sensillar type the signal comes from, and the taste modality that is represented (Kwon et al., 2014). However, whether each pattern is correlated with a specific behavioral output still needs to be investigated.

Some gustatory neurons do not follow the path of projecting to higher brain centers and rather terminate at the thoracic ganglia generating reflex-like behaviors such as proboscis extension. A

group of tarsal gustatory neurons are among this type of sensory neurons (Kwon et al., 2014). This group of neurons seem to be linking the relevant taste information directly to a motor output without further processing.

Gustatory information is relayed from the SEZ to higher brain centers via second order neurons. Two anatomical structures, MBs and antennal and mechanosensory motor center (AMMC), have been identified as areas receiving gustatory information so far (Kain and Dahanukar, 2015). MBs receive direct inputs from the SEZ as well as indirect inputs through the AMMC. The taste representation in the MB looks to be segregated according to the type of the taste and the source organ of the stimulation. Sweet and bitter tastes, or signals from different organs activate partially overlapping but distinct areas in the MBs (Kirkhart and Scott, 2015). Knowing that MBs are also the processing center for the olfactory information, recent findings are consistent with their previously proposed roles as the site of integration center of multimodal inputs and associative learning (Davis, 2005; Heisenberg, 2003).

The second higher brain center responding to taste information, the AMMC, is connected to the sweet tasting sensory neurons of the labellum via second-order sweet gustatory projection neurons (sGPNs) (Kain and Dahanukar, 2015). Previously, AMMC was shown to be innervated by the sensory axons of the basal antennal segments containing sound, wind and gravity responsive neurons (Homberg et al., 1989; Kamikouchi et al., 2009, 2006; Yorozu et al., 2009). It is an interesting question as to whether there is any integration among these modalities taking place in the AMMC.

The recent findings regarding taste processing in the central brain are exciting. However, so far mostly the GR expressing sensory neurons have been investigated. Today we know that there are multiple classes of receptors involved in detection of canonical and non-canonical tastes beyond the GR expressing GSNs. It will be interesting to understand how and where the taste information is relayed and processed through non-GR-expressing GSNs as the tools are developed allowing to investigate them.

It is possible that orphan gustatory receptors that have not been characterized so far may open the door for unusual tastes and odors. Of those, polyamines stand out as an interesting chemical class, because of their essential role in cellular processes and at the same time their enrichment in some foods as a potential chemosensory cue.

4.3.Polyamines in Health and Disease

Polyamines are small aliphatic polycations found in both prokaryotes and eukaryotes. Polyamines were first described by Antonie van Leeuwenhoek in the 17th century in a human semen sample and thus two of its members are named as spermine (Spm) and spermidine (Spd), although they are found in many other cell types (reviewed in: (Bachrach, 2010)). Putrescine (Put), Spm and Spd are the most prominent members of polyamines. As being polycations, polyamines have the ability of binding to negatively charged molecules (i.e. DNA and RNA) in the cell and directly affect the gene expression and protein synthesis. For this reason, polyamines are essential for survival of organisms and have profound effects on most – if not all – basic cellular functions including cell proliferaton, gene expression and stress response (Miller-Fleming et al., 2015). Ornithine decarboxylase (ODC) is the enzyme catalyzing the formation of putrescine in the cell. Its half-life rapidly changes depending on whether the tissue is growing or not, indicating a strict control of polyamine synthesis and its amount within the organism (Russell, 1970).

Reproduction is a unique event where rapid cell proliferation takes place and polyamine levels need to peak in an animal. For example, it has been shown that spermidine, plays a critical role in mating and egg fertilization efficacy in *C.elegans* as well as in yeast indicating an evolutionarily conserved role in reproduction (Bauer et al., 2013). Animals obtain the necessary polyamines by both *in vivo* synthesis and through nutrition. In addition, guts of many animals contain bacteria that produce polyamines (Minois et al., 2011). *In vivo* synthesis is high in rapidly developing organisms/tissues and significantly drops during aging. Therefore, nutritional supply becomes more and more critical with age (Scalabrino and Feriol, 1984). When the external supply is not sufficient, polyamine deficiency can have severe effects on fertilization and on embryo/fetal

development, contributing to the age-dependent sterility problem (Lefevre et al., 2011). On the other hand, it has been shown that nutritional supply has the potential of reversing these negative outcomes in various model organisms as well as in humans (Eisenberg et al., 2009; Gupta et al., 2013; Kalac, 2014; Minois, 2014).

Although the deficiency of polyamines is associated with important problems mentioned above, in certain diseases, polyamine amounts are found to be significantly higher than in healthy people suggesting that the excess amounts of polyamines may also be detrimental (Pegg, 2013; Ramani et al., 2014). For instance, polyamine concentrations are found to be significantly elevated in urine samples of cancer patients, therefore it is used as a diagnostic marker for cancer progression (Miller-Fleming et al., 2015). Trials to develop inhibitors of polyamine synthesis are an ongoing effort to target tumor tissues to stop cancer progression (Seiler, 2003; Seiler and Raul, 2007). However, it is still not known whether the elevation in polyamine levels is the cause for uncontrollable cell proliferation or the result of it. It could be a sort of a protective stress response of the organism against cancer invasion (Miller-Fleming et al., 2015).

4.3.1. Polyamines as Chemosensory Cues

Although polyamines are fundamental components of the most basic cellular processes and have to be obtained through food, in particular when *in vivo* production is not sufficient, it is currently not known whether animals can actively detect and evaluate them in their environment as chemosensory cues. A recent study has shown that one of the polyamines, cadaverine, is a repulsive cue for zebrafish and they possess an olfactory receptor, trace amine-associated receptor 13c (TAAR13c), to detect it (Hussain et al., 2013). Polyamines have a strongly pungent smell and are unpleasant to humans in pure form or in high concentrations. However, a considerable amount of polyamine containing foods like oranges and cheese possess an appetitive smell to humans showing that the concentration and the composition of the scent are key factors in decision making.

There are several studies and observations providing supportive information on polyamines as positive chemosensory cues for insects. Polyamines, like putrescine and cadaverine, are released from fermenting organic materials, and *Drosophila* are highly attracted to overripe and fermenting fruits (Dweck et al., 2013; Takeda et al., 1997). Various plants use this strong attraction to trap flies and transfer their pollens via mimicking the carrion odor of fermenting food smell (Stensmyr et al., 2002; Stökl et al., 2010), indicating that polyamines could be attractive food cues for *Drosophila*. Another example is that *Calliphora*, blowflies show a selective attraction to decaying flesh and lay their eggs into them, indicating that cadaverine, one of the signature odors of carrion smell could be the cue for egg laying and feeding sites for these flies (Johansen et al., 2014; Paczkowski et al., 2012). Mosquitoes also prefer to lay their eggs into polyamine rich areas like standing waters with a lot of decaying organic materials in them (Ponnusamy et al., 2008; Wong et al., 2011). Although all these examples indicate a high correlation between the favorite food sources and egg laying sites of certain insects, and the high amount of polyamines in those sites, so far the role of polyamines in their choice behavior and chemosensory receptors for polyamines remain uncharacterized in insects.

4.4.Modulation of Neural Networks

Animals have constantly fluctuating internal states and so do their sensory input priorities. Sensory information collected from the environment has to be actively filtered and prioritized according to the changing needs of an animal. For instance, if an animal is hungry and eating is at the top of the list of needs, the most attended stimuli will be food cues in all modalities at that time point, and all other cues irrelevant to the immediate need will be filtered out as the secondary-level important stimuli (Sternson, 2013). Intriguingly, we and all other animals have the capability of re-evaluating a negative stimulus in different contexts and can make different decisions. For instance, a noxious olfactory cue, or a bitter taste generates an aversive behavior under neutral circumstances. However, if there is a challenging internal state, i.e. hunger, the repulsive chemosensory inputs might be “ignorable” even if those noxious stimuli are in the same environment with the food source (Bräcker et al., 2013; Cohn et al., 2015; Lewis et al., 2015; Owald et al., 2015). Once the need is satiated, the choice behavior is dominated by noxious

stimuli again. The question here is, how the nervous system integrates fluctuating internal physiological states with the external stimuli and generates appropriate behavioral responses? And how is such a dynamic and reversible modulation orchestrated in a neural network with a great number of anatomically-specified possibilities?

Neuromodulation is the physiological process generating multiple functional circuits from a single anatomically defined network via small peptides or other molecules (reviewed in: (Bargmann, 2012; Harris-Warrick, 1991)). Neuromodulators are small molecules released by neurons and diffuse to larger areas affecting short and long distance elements of a circuit. They can reshape neuronal circuits' anatomy by excluding an existing neuron or recruiting additional neurons to the circuit to compose different set of neurons to generate different behaviors under different context and internal state conditions (Bargmann, 2012). Moreover, they can also generate different behaviors by changing intrinsic properties of neurons in a network and thereby changing synaptic efficacy in a reversible manner (Harris-Warrick, 1991).

The first systematically analyzed example of neuromodulatory events was the modulation of the crustacean stomatogastric ganglion (STG) (Marder and Bucher, 2007). Possibility of an *ex-vivo* preparation allowed scientists to identify circuit elements and their properties under different conditions. Basically, in the ganglion there are two neuronal networks generating phyloric and gastric rhythms oscillating in different phases. Each rhythm is generated through different intrinsic properties of composing neurons in two circuits. On top of that, the ganglion is innervated with neurons that provide neuromodulatory inputs in the form of neuropeptides and biogenic amines (Johnson et al., 2005; Marder and Bucher, 2007) that can change both circuit dynamics (Eisen and Marder, 1984) and circuit composition (Weimann and Marder, 1994). Another well-known example of neuromodulation is the mammalian retina in the context of high/low light intensity environments (Baldridge et al., 1998; Mills and Massey, 1995; Xia and Mills, 2004). At the low vs high light levels, retinal processes are dominated by rod or cone cells, respectively. Modulation takes place at the AII amacrine cell-cone bipolar cell connection where cone bipolar cell receives direct input from cones and indirect input from rods through rod bipolar cells and AII amacrine cells. When the animal moves from low light to high light

environment, the rod pathway is dissociated from the network via uncoupling the gap junctions between AII amacrine cells and cone bipolar cells. This connection is open to modulations if different light levels, circadian rhythm and neuromodulators like dopamine applied (Baldridge et al., 1998; Mills and Massey, 1995; Xia and Mills, 2004).

Neuromodulators mostly act by binding to G-protein coupled receptors (GPCRs) (Bargmann, 2012). GPCRs are receptors anchored to the cell membrane through seven transmembrane domains and can "sense" the outer environment of a cell through binding ligands (Pierce et al., 2002). Ligands activating GPCRs span a vast area of molecules, including but not limited to photons, odors, tastes, hormones, neuropeptides and neurotransmitters (Rosenbaum et al., 2009; Syrovatkina et al., 2016). GPCRs are one of the biggest receptor superfamilies regulating many different functions at various sites in the body (Syrovatkina et al., 2016). Indeed, G-protein-mediated signal transduction is the most widely used transmembrane signaling system in higher organisms (Wettschureck and Offermanns, 2005). As the name indicates, GPCRs function together with G-proteins (Gilman, 1987; Simon et al., 1991). G-proteins function as intermediaries in the signaling cascade between the GPCRs and the second messanger systems. Upon binding of a ligand, G-proteins relay the signal into the cell via its subunits, α, β, and γ. β- and γ-subunits act together by forming an undissociable complex. When a ligand binds and changes the receptor's conformation to the active state, α and βγ subunits dissociate from the receptor complex and initiate series of reactions that at the end generates the cellular response to the ligand (Gilman, 1987; Simon et al., 1991).

Among the ligand family of GPCRs with neuromodulatory function, neuropeptides are crucial factors playing role in feeding, courtship, sleep, learning and memory, stress, addiction, and social behaviors (Schoofs et al., 2017). Neuropeptides are small proteins that are released by neurons or neurosecretory cells. They have essential role in regulating a myriad of physiological processes as neurotransmitters, hormones, neuromodulators, and growth factors. In *Drosophila*, bioinformatics analysis resulted with 119 neuropeptide precursor genes, out of which 46 neuropeptides derived from 19 genes were biochemically characterized (Clynen et al., 2010).

Mating in *Drosophila* is a very good example of how neuropeptides play role in translating an internal state into a behavioral switch. Mating causes a series of changes in physiology and behavior of female insects (Ringo, 1996; Saveer et al., 2012; Schoofs et al., 2017). Following mating, female flies' egg production rate substantially increases and they switch to egg laying behavior. Moreover, they become unreceptive to any re-mating attempts for a period of time and reject males by either kicking them or extruding their ovipositor. All these changes are collectively called as post-mating switch and triggered through sex peptide (SP), a seminal fluid protein that is transferred from the male during copulation (Ringo, 1996; Saveer et al., 2012; Schoofs et al., 2017). Females mated to SP mutant males do not develop the rejection behavior and continue being receptive (Liu and Kubli, 2003), and it is sufficient for virgin females to be injected with extracted SP to stop mating and reject any further copulation attempt (Chen, 1988). Mechanistically, SP achieves its role via binding to a GPCR, sex peptide receptor (SPR), initiating a cascade of changes in reproductive tissues and behavior (Yapici et al., 2008). SPR is widely expressed in the CNS. However, it has been shown that only a small subset of those neurons, co-expressing *doublesex* and *fruitless,* are necessary and sufficient for the post mating switch behavior (Rezával et al., 2017), leaving the door open for the possibility of alternative functions for SPR^{+} neurons.

Neuromodulation occurs basically at all levels of the nervous system (Harris-Warrick, 1991). In the context of chemosensory systems, it can be categorized according to where the neuromodulatory event takes place as: 1- Peripheral modulation and 2- Central modulation.

4.4.1. Modulation of Peripheral Sensory Neurons in Chemosensory Processing

Synapses can form on axon terminals, cell body, or dendrites of a neuron (Kandel et al., 2000). While axodendritic and axosomatic inputs have direct effect on the trigger zone of the postsynaptic neuron, axo-axonic contacts selectively control individual axonal branches of the postsynaptic neuron. These axo-axonic synapses can inhibit or enhance transmitter release via affecting the Ca^{+2} influx into presynaptic terminals of the postsynaptic neuron. It is called

"presynaptic inhibition", if the neuronal contact reduces transmitter release onto the postsynaptic neuron. Whereas it is called "presynaptic facilitation", if the presynaptic contact of the modulatory neuron enhances transmitter release onto the postsynaptic neuron (Figure 4). Presynaptic modulations occur at early steps of the sensory pathways (Kandel et al., 2000) and chemosensory systems are among those.

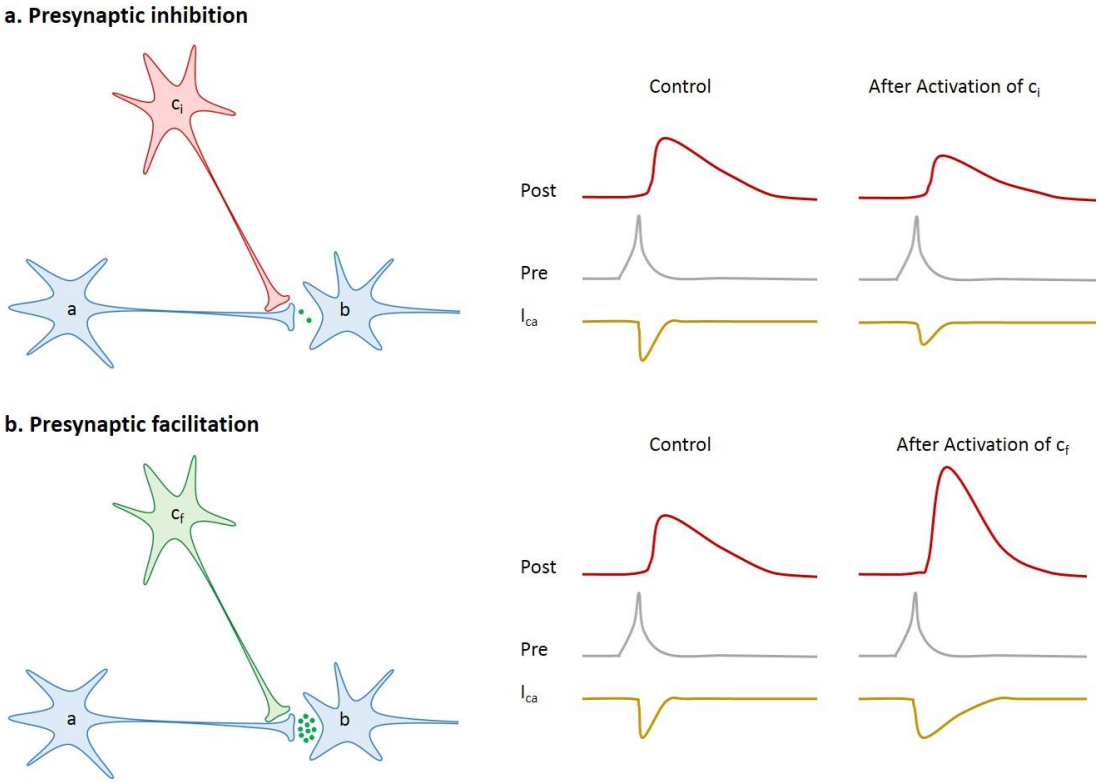

Figure 4: Presynaptic modulation of neuronal response.

Axo-axonic synaptic actions on presynaptic terminals can decrease or increase the amount of neurotransmitter released from the postsynaptic cell. **(a)** An inhibitory neuron (c_i) synapses onto the the axon terminal of neuron a. This depresses the calcium current in neuron a and reduces the amount of transmitters released. Thus, the postsynaptic potential is depressed in cell b. **(b)** A facilitating neuron (c_f) synapses onto the axon terminal of neuron a. c_f depresses K^+ current in cell a and increases Ca^{+2} influx through voltage-gated Ca^{+2} channels. Thus, the postsynaptic potential increases in cell b.

The olfactory system is comprised of several layers of processing centers through which olfactory information is translated into a behavior (see 4.2.1.2). Ascending from the periphery to the central brain, not only the identity and intensity of complex odorants, but also the internal

physiological states of the animal are integrated to drive a certain behavior. Recent years of work has shown that the peripheral sensory neurons are not the passive conductors of odorant detection. Indeed, they are subjected to various modulations correlated with different internal states changing their odor sensitivity interval and adaptation properties (Chalasani et al., 2010; Dey et al., 2015; Ignell et al., 2009; Root et al., 2011).

Studies carried out in worms, flies, and mice demonstrated that certain neuropeptides, which are released either from sensory neurons or from interneurons in the olfactory system are able to inhibit or enhance sensory neuron sensitivity or synaptic output (Bargmann, 2012; Harris-Warrick, 1991; Leinwand and Chalasani, 2011). In addition to classical neurotransmitters, neuropeptides are molecules used to regulate physiological processes and adaptive animal behaviors like feeding, reproduction, circadian rhythm and developmental processes (Nässel and Winther, 2010). For instance, in worms the neuropeptide NLP-1 is released from the olfactory sensory neuron AWC and binds to its post-synaptic G-protein coupled receptor NPR-11, thereby suppressing the sensory neuron's activity by generating a neuropeptide feedback loop (Chalasani et al., 2010). There is a comparable mechanism found in flies regulating olfactory sensitivity to food odors by enhancing synaptic output of the OSN in a hunger-dependent manner (Root et al., 2011). Global increase of insulin in the circulatory system affects the synaptic output of food odor responding OR42b OSNs via increased expression of short neuropeptide F receptor (sNPFR) in sensory neurons. Both short neuropeptide F (sNPF) and sNPFR are expressed in OR42b sensory neurons and hunger facilitates the activity of these OSNs by changing sNPFR expression presynaptically (Root et al., 2011). Furthermore, very recent work in mice has shown that OSNs in vomeronasal organs of female mice are also subject to modulation (Dey et al., 2015). The study demonstrated that females show changing sensitivity to male pheromones in an estrus cycle-dependent manner. Increased progesterone expression in the non-ovulating period acts directly on sensory neurons and selectively silences male pheromone responding OSNs in vomeronasal organs of female mice. This "blinds" the females to male pheromones (Dey et al., 2015). Collectively these examples show that sensory neuron modulation in olfactory systems is a conserved mechanism from simple to complex organisms. Through this mechanism the nervous system is presumably prevented from processing

irrelevant information, or the input, relevant for a specific internal state, is enhanced from the first relay on.

In addition to OSNs, gustatory sensory neurons are also subject to modulation. So far several mechanisms have been identified mediating this purpose in different ways. Activation of bitter sensitive GSNs can directly suppress the sugar GSN responses either via an odorant binding protein (OBP), OBP49a, present in the sensillar lymph (Jeong et al., 2013) or through GABAergic interneurons connecting bitter and sweet neurons presynaptically (Chu et al., 2014). Notably, previous experiences can also change taste neuron sensitivity. Bitter sensitive neurons respond less to camphor, a non-toxic bitter compound, thereby increasing its acceptance by the animal when flies are exposed to the compound for a certain time (Zhang et al., 2013b). Moreover, feeding state can also change peripheral taste sensitivity for sweet and bitter modalities through two independent neuromodulatory pathways. On the one hand, starvation makes sweet sensing neurons more sensitive by the release of dopamine onto primary GSNs and increases their excitability (Inagaki et al., 2012). On the other hand, the sensitivity of bitter neurons decreases with extended starvation in an sNPF dependent manner via GABAergic interneurons acting on bitter neurons leading to a less selective feeding behavior (Inagaki et al., 2014). All in all, neuromodulation of peripheral chemosensory neurons is a strategy that is used through multiple molecular and physiological mechanisms which lead to the ultimate goal: fine tuning of sensory stimuli according to the organism's immediate or longer-term needs.

4.4.2. Central Modulation of Chemosensory Processing

Odors are the main sensory cues for food foraging behavior of *Drosophila*. However, food sources do not emit a single type of molecule, but rather a bouquet of ligands that activate multiple glomeruli in the AL. Moreover, a food source may not always contain only the attractive cues, but can be spoiled or emit a mixture of cues that are attractive or repulsive on their own. Hunger is the key internal state that changes how and how much the odors emitted from those sources will be perceived via modulating the olfactory system. There is substantial work carried out in recent years to find out how odor information is modulated at the central nervous system

in hungry vs. fed flies. How other internal states impact on chemosensory processing is much less well understood.

As described in 4.2.1.2, olfactory information is computed and relayed through several layers of neurons from the periphery to central brain areas. Projection neurons carrying the olfactory signal to higher brain centers can be either excitatory or inhibitory and innervate distinct regions of the higher brain centers (Liang et al., 2013). Excitatory PNs form uniglomerular connections and innervate both the LH and the MBs, however, inhibitory PNs can integrate multiple glomeruli and only innervate the LH (Jefferis et al., 2007; Wang et al., 2014). In the LH excitatory and inhibitory PNs collectively computes the features of innate odors (Jefferis et al., 2007) (Strutz et al., 2014). Moreover, sNPF-positive higher brain neurons innervating the LH (Wang et al., 2013) show increased sensitivity by hunger, thereby modulating the food odor induced foraging behavior in a hunger-dependent manner (Beshel and Zhong, 2013). Thus, the idea that the LH only responds to hard-wired odors with innate valence is changing with the findings that even these odors are subjected to modulation at different levels of the olfactory system via changing internal state of the animal.

In contrast to the LH, MBs are known as the center where odor experiences are encoded (Davis, 2005; Guven-ozkan and Davis, 2014; Heisenberg, 2003; Kirkhart and Scott, 2015). Mushroom body circuits are formed by mainly three types of neurons: 1- KCs, which are around 2000 intrinsic neurons of MBs, 2- dopaminergic neurons that innervate KC axons and 3- Mushroom body output neurons (MBON) which are merely 34 neurons that KCs synapse onto (Aso et al., 2014). As it is seen in the numbers of KCs and the MBONs, a huge convergence takes place when the signal travels from KCs to MBONs. While KCs show stimulus-specific odor responses (Campbell et al., 2013), MBONs are tuned differently in every animal and their tuning parameters are shaped by experience (Campbell et al., 2013; Hige et al., 2015). On the other hand, dopaminergic neurons provide neuromodulatory axonal inputs to KC-MBON synapses and mostly depress neurotransmission between specific KCs and MBONs (Cohn et al., 2015; Musso et al., 2015; Owald et al., 2015).

In addition to learning and encoding experience dependent odor information, recent studies show that MBs may have additional roles. The work carried out in our lab demonstrated that MBs are not only the center of odor learning, but also mediate the computations of immediate responses to conflicting innate odors – innately repulsive CO_2 and innately attractive vinegar - in a satiety dependent-manner by dopaminergic modulation of KC-MBON synapses (Bräcker et al., 2013; Lewis et al., 2015). Moreover, MBs are also involved in the modulation of responses to other innate stimuli like the humidity in the air (Lin et al., 2014) and hot/cold stimuli (Frank et al., 2015; Tomchik, 2013). Together, it has been demonstrated that MBs modulate the response to odor stimuli in two different ways so far: 1- As a coincidence detector associating neutral odors with unconditioned stimuli thereby establishing a learned memory of a given odor, and 2- Modulating instantaneous behaviors according the external and internal circumstances. It has been shown that the precise behavioral state of the animal plays a role on the saliency of the stimulus to drive a certain behavior (Cohn et al., 2015). Dopaminergic neurons reroute the same olfactory signal to a different set of circuits to drive different behaviors depending on the internal and external context of the animal (Cohn et al., 2015).

Mushroom bodies are involved in modulation of taste responses as well (Masek and Scott, 2010). Notably, the gamma lobes of the mushroom bodies are necessary for taste associative learning together with a subset of dopaminergic neurons (Kirkhart and Scott, 2015). Interestingly, taste neurons in legs, proboscis or wings activate partially overlapping but distinct subsets of KCs in the MBs, suggesting that organotopic SEZ innervation (Stocker, 1994; Wang et al., 2004) of taste neurons seems to be maintained in the higher brain. Therefore, the same sweet input, for instance, can potentially form taste organ specific associations, if used to condition different taste organs (Kirkhart and Scott, 2015).

Mating is an internal state that appears to modulate olfactory driven behaviors, too. Cis vaccenyl acetate (cVA) is a well studied male pheromone that is transferred to the female during the insemination, triggering sex-specific courtship behaviors (Keleman et al., 2012; Kurtovic et al., 2007) and playing role in social behaviors (Bartelt et al., 1985; Wang and Anderson, 2010). Female flies change their behavioral response to cVA right after mating and stop finding it

attractive (Lebreton et al., 2014). This modulation is achieved through lateral interactions between the two cVA sensitive olfactory channels, OR67d and OR65a, at the PN level. OR65a channel activity inhibits the OR67d channel activity at the PN level, thereby inhibits the attraction behavior (Lebreton et al., 2014).

Pathways from the SEZ to higher brain regions are not as well identified as AL pathways in *Drosophila*. However, a recent study demonstrated a novel anatomical region, AMMC, that receives input from the SEZ. It also receives mechanosensory and olfactory inputs along with the gustatory ones that potentially interact to form multi-modality associations (Kain and Dahanukar, 2015). Moreover, the sweet gustatory projection neurons (sGPNs) connecting the SEZ to the AMMC show increased sucrose sensitivity upon starvation (Kain and Dahanukar, 2015) suggesting that internal nutrient state has a modulatory effect on the higher order neurons innervating this brain region.

Two other recent studies identified different types of neurons whose activity is modulated in a satiety dependent manner. (Marella et al., 2012) showed that a subset of dopaminergic neurons modulate proboscis extension behavior to sucrose. Another study identified a pair of interneurons that integrates satiety information with the food presence and triggers the motor program of feeding (Flood et al., 2013). However, both studies did not show a direct connection between those neurons and the GSNs.

4.5.Thesis Aims

Flies are highly attracted to overripe and rotting fruits which emit high amounts of polyamines. Polyamines are essential nutrients both produced *in vivo* and obtained through feeding. As the *in vivo* production declines by age, nutritional supply becomes highly important especially for cell proliferation and reproduction. It is not known whether flies use these essential nutrient as chemosensory cues to assess the food quality. Therefore, the first aim of this study was to identify the behavioral response of flies to polyamines and find receptors detecting polyamine smell and taste through behavioral analysis, calcium imaging and electrophysiological recordings.

After characterizing the relevant receptors and seeing that polyamines increase the reproductive success of female flies, the second aim of this thesis was to characterize the role of reproductive state on the perception of polyamines. Finding that mated females have increased preference for polyamine taste and smell carried us to the third aim of this study: using the reproductive state dependent polyamine detection as a model to find out neuronal and molecular mechanisms of neuromodulation of sensory systems in an internal state dependent-manner through a number of techniques, including behavioral analysis and calcium imaging.

5. Results

5.1. First paper: Hussain A*, Zhang M*, Üçpunar HK, Svensson T, Quillery E, Gompel N, Ignell R, Grunwald Kadow IC. (2016) Ionotropic chemosensory receptors mediate the taste and smell of polyamines. PLoS Biol 14(5): e1002454

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RESEARCH ARTICLE

Ionotropic Chemosensory Receptors Mediate the Taste and Smell of Polyamines

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Abstract

The ability to find and consume nutrient-rich diets for successful reproduction and survival is fundamental to animal life. Among the nutrients important for all animals are polyamines, a class of pungent smelling compounds required in numerous cellular and organismic processes. Polyamine deficiency or excess has detrimental effects on health, cognitive function, reproduction, and lifespan. Here, we show that a diet high in polyamine is beneficial and increases reproductive success of flies, and we unravel the sensory mechanisms that attract *Drosophila* to polyamine-rich food and egg-laying substrates. Using a combination of behavioral genetics and in vivo calcium imaging, we demonstrate that *Drosophila* uses multisensory detection to find and evaluate polyamines present in overripe and fermenting fruit, their favored feeding and egg-laying substrate. In the olfactory system, two coexpressed ionotropic receptors (IRs), IR76b and IR41a, mediate the long-range attraction to the odor. In the gustatory system, multimodal taste sensation by IR76b receptor and GR66a bitter receptor neurons is used to evaluate quality and valence of the polyamine providing a mechanism for the fly's high attraction to polyamine-rich and sweet decaying fruit. Given their universal and highly conserved biological roles, we propose that the ability to evaluate food for polyamine content may impact health and reproductive success also of other animals including humans.

Author Summary

Animals, including humans, evaluate food by its smell and taste. Odors and tastes not only signal the presence of food, they also reveal details about the type and amount of nutrients contained in it. A preference for certain foods frequently reflects the specific metabolic needs of an animal. Among the important but less known compounds that animals consume with their diet are polyamines. These pungent smelling molecules are essential for reproduction, development, and cognition. Interestingly, they are also produced by the cell and body, but their levels decline with age. A diet high in polyamines can improve age-related memory deficits and loss of fertility. We have used the model fly *Drosophila melanogaster* to unravel if and how animals detect polyamines in their food and environment,

data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abbreviations: AL, antennal lobe; *ato*, *atonal*; GR, gustatory receptor; GRN, gustatory receptor neuron; iGluRs, ionotropic glutamate receptors; IR, ionotropic receptor; MFC, mass flow controller; NMDA, N-methyl-D-aspartate receptor; OR, olfactory receptor; Orco, OR coreceptor; OSN, olfactory sensory neuron; PI, preference index; PID, photoionization detector; PN, projection neuron; ROI, region of interest; SEM, standard error of the mean; SEZ, subesophageal zone; SSR, single sensillum recording; TAAR13c, trace amine-associated receptor 13c; TCC, tricholine citrate.

and which role this detection plays in their food choice behavior. Polyamine levels are particularly high in the fly's favorite food and egg-laying substrate, overripe and decaying fruit. We found that food supplemented with polyamines indeed improves the reproductive success of a fly couple. We show that *Drosophila* is highly attracted to polyamines and uses them to identify promising egg-laying and feeding sites. It detects them through an ancient clade of receptor proteins on its olfactory and taste organs. We speculate that other animals can also detect polyamines and use their smell and taste to identify sources of these beneficial nutrients.

Introduction

Animals make use of all of their senses while searching and evaluating food. For most, smell and taste are the major modalities to assess the quality and nutritional value of food. While odors help the animal to track down food over long distances, short-range evaluation using the sense of taste is ultimately crucial for the decision whether to feed or to lay an egg [1,2]. In general, animals prefer calorie-dense and sweet foods to bitter foods (see for instance [3]). In addition, animals need to consume food containing other important nutrients. Among the vital dietary constituents are polyamines [4]. Polyamines, most notably putrescine, spermine, and spermidine, are essential for basic cellular processes such as cell growth and proliferation, and are of specific importance during reproduction [5,6]. While polyamines can be generated by endogenous biosynthesis or microbes in the gut, a significant fraction comes from the diet [4]. Animal products, soybeans, or certain fruits are rich sources of polyamines [4]. In cells, these polycations are bound to nucleic acids, proteins, and phospholipids, where they participate in fundamental cellular processes such as DNA replication, RNA translation, and mitosis [6,7]. Polyamine deficiency can have fatal consequences on reproductive success [5]; and low polyamine levels have been linked to neurodegenerative diseases and ageing [8]. Yet, high polyamine concentrations are found in cancer cells suggesting that their excess could be unhealthy [6]. Notably, the enzymes that generate endogenous polyamines decline with ageing [5–7]. And the exogenous supply through high polyamine diets can have beneficial effects on ageing, memory loss, and reproduction in a variety of model species and humans [4,8–11]. Given their beneficial but also detrimental roles, food industry has consequently measured the amount of polyamines in many food items [12]. Whether animals can directly evaluate polyamine content for instance through their taste organ is not known. The smell of polyamines, however, can be detected by animals, including humans; it is strongly pungent and at higher concentrations unpleasant to humans. There is circumstantial evidence indicating that insects may detect the smell and taste of polyamines. For instance, *Calliphora* blowflies are attracted to decaying flesh and lay their eggs exclusively into corpses. This attraction could be due to the carrion smell of cadaverine [13]. *Drosophila* flies are notorious for their attraction to overripe and fermenting fruit [14,15]. The amount of polyamines increases dramatically in fruit within days after harvest [16] and during fermentation [17]. Therefore, for these flies, polyamines are candidate molecules for the detection of beneficial feeding and egg-laying sites. This may also hold true for females of other insects. For instance, females of the dengue fever vector, the mosquito *Aedes aegypti* typically lay their eggs in batches in standing waters such as flowerpot plates with decaying organic and polyamine-rich materials [18,19]. Although an olfactory receptor, trace amine-associated receptor 13c (TAAR13c), for one polyamine, cadaverine, was recently described in zebrafish [20], no taste receptor has been identified so far. Chemosensory receptors for the detection of polyamines remain uncharacterized in insects.

To detect chemosensory stimuli animals use highly specialized families of receptor proteins that are present in sensory neurons on peripheral taste or smell organs [21]. Given their importance in animal life, a large effort goes into identification of these receptors and their putative odors or tastes. *D. melanogaster* has proven to be a useful model in matching olfactory and gustatory receptors (GRs) to their ligands and has contributed much to our understanding of chemosensory coding in the nervous system [22].

Insects possess three classes of olfactory receptors (ORs): the classical ORs, the more recently described but evolutionarily older family of ionotropic receptors (IRs), and a few GRs [23–26]. Each olfactory sensory neuron (OSN) is located in a sensillum on either antenna or maxillary palp and expresses a specific type or very small combination of receptors, which are tuned to a narrow group of molecules. All OSNs that express the same receptor project their axons to one of ~50 glomeruli in the antennal lobe (AL) in the central brain [27]. This highly conserved architecture allows the translation of a nonspatial sensory cue into a highly organized spatial map and provides the logic for odor coding [21]. Upon additional local processing at the level of the AL, the odor information is sent via projection neurons (PNs) to two main higher brain centers, the mushroom body and the lateral horn [28]. While many of the ~45 ORs have been deorphanized, ligands for a number of IRs remain uncharacterized [22,29]. Previous work showed that most of the IR OSNs express one of the putative coreceptors IR8a or IR25a [30,31]. Among the deorphanized IRs is IR92a, the receptor for ammonia and small amines [32]. The behavioral role of most IRs, however, remains elusive with few exceptions such as IR84a and IR64a [29,33,34].

Gustatory receptor neurons (GRNs), in contrast to OSNs, are found on many peripheral as well as internal organs [35]. On the external sensory organs, GRN-containing sensilla are mainly found on the labellum, the legs, and the wing margins [35]. The labellum carries ~60 morphologically distinct sensilla with four GRNs each that are tuned to distinct flavors such as sweet, salty, water (appetitive) or bitter, and acidic (noxious). While GRs form the best-characterized family of taste receptors to date [22,36], more recent members of the IRs have been implicated in the sensation of tastants [37–39]. For instance, IR76b was shown to be essential for the detection of appetitive concentrations of salt [40]. Interestingly, IR76b is also expressed in GRNs that do not detect salt, but a role for these neurons has not been assigned yet. Finally, increasing evidence suggests that dietary amines can be tasted, but receptors have not been identified yet [37]. Peripheral GRNs project to the central brain or in the case of GRNs on tarsae or wings, also to the ventral nerve cord [35]. In the central brain, the distinct patterns of innervation in an area called the subesophageal zone (SEZ) by bitter and sweet neurons indicated a taste map similar to but less structured than the map found in the AL of the olfactory system [41]. In contrast to the olfactory system, however, higher order processing of tastes is still not well understood [42].

We have analyzed at the sensory, molecular, and behavioral levels how polyamines guide insect preference behavior. First, we demonstrate that a polyamine-rich diet significantly increases the number of progeny of flies. Second, we show that flies can find and evaluate polyamine-rich feeding and egg-laying sites using their senses of smell and taste. We characterize the polyamine receptors and demonstrate an essential role for specific IRs in olfactory and gustatory organs. Altogether, our data characterize sensory receptors for polyamines and their behavioral role in insects and indicate that the ability to sense polyamines promotes reproductive success and survival.

Results

Polyamine-Rich Diet Increases Reproductive Success of *Drosophila*

Given the evidence that polyamines are vital molecules during reproduction, we asked whether males and females feeding on polyamine-rich food would produce more offspring compared to

flies on standard fly food (see Materials and Methods). Therefore, we crossed single males to single females in two different conditions—on standard fly food and fly food that had been supplemented with putrescine or cadaverine solution (~2.5 mmol polyamine/l of food). After 4 d, the parental generation was discarded, and the number of eggs laid was quantified. In addition, once these eggs had developed into flies, these were counted again. Females on polyamine-enriched food laid ~3 times the amount of eggs compared to females on standard food. Similarly, fly pairs fed a high-polyamine diet had ~3 times more offspring than the couples on standard fly food (Fig 1A). Thus, it indeed appeared that polyamine-enriched food was beneficial for reproduction of flies similarly as what has been suggested for other species such as humans.

Drosophila Is Strongly Attracted to Volatile Polyamines

D. melanogaster flies are notorious for being attracted to, and for laying their eggs into, decaying fruits [14]. We asked whether this preference was rooted in the need to consume polyamines, present in fermenting fruit. First, we quantified the attraction of male and female flies to fruits at different stages of maturity with a laboratory choice assay, the T-maze. We found that flies show a strong aversion to green bananas, are indifferent to yellow bananas, but are highly attracted to the same batch of overripe bananas 5–7 d later (Fig 1B) [43]. Next, we tested whether *Drosophila*'s attraction to decaying fruit could, in part, be attributed to increased concentrations of polyamines produced during ripening and decay [16]. Running the same T-maze assays with different polyamines, we found that female and male flies were strongly attracted to the odor of spermine (sperm.), spermidine (sperd.), diaminopropane (prop.), putrescine (put.), cadaverine (cad.), diaminohexane (hex.), diaminoheptane (hept.), diaminooctane (oct.), and diaminodecane (dec.) (Fig 1C). The responses were dose-dependent with 1 mM (~10 ppm) eliciting the strongest attraction compared to lower as well as higher concentrations (S1A Fig; 1 μM–1 M). This concentration roughly corresponded to the amount of putrescine found in fermented banana (~0.9 mmol/kg) or fresh oranges (~1.3 mmol/kg) [12]. We observed the same attraction when single female flies were assayed in the T-maze, suggesting that individual flies perceive and are attracted to the odor (S1B Fig).

To uncover the neural basis of the fly's attraction to this class of important nutrients, we sought to identify the receptor for polyamine sensation. Preference to a chemical in the T-maze is typically mediated by the olfactory system. Indeed, flies with surgically removed antennae, the main olfactory organ, lost their T-maze preference for polyamines (S1C Fig). Previous reports indicated that OR- and IR-expressing OSNs respond to putrescine in single sensillum recordings (SSR) [23,44–46]. From these two receptor classes, the entire OR system can be impaired at once by mutating the obligatory OR coreceptor (Orco) [47]. *Orco* mutant flies maintained normal attraction to putrescine (S1D Fig), excluding this family of receptors from our search as suggested before [46]. We then analyzed the requirement of IRs. To suppress all IR-mediated chemosensation, we relied on *atonal* (*ato*) mutants, as they fail to develop IR-expressing coeloconic sensilla [23,48,49]. *ato* mutant flies did not show any preference for putrescine or cadaverine in the T-maze (S1E Fig). We concluded that IRs mediate attraction to volatile polyamines. To identify specific IRs, we carried out a small genetic screen using loss of function of single IRs. From all IR mutants tested, including the two putative coreceptors *IR8a* and *IR25a*, only *IR76b* mutants showed a significant reduction in polyamine attraction in the T-maze (Fig 1D, S1F Fig). To confirm a requirement for IR76b, we silenced the activity of IR76b neurons by expressing the inward-rectifier potassium channel Kir2.1 [50]. Flies of the genotype *IR76b-Gal4;UAS-Kir2.1* showed a strong impairment in attraction to putrescine or cadaverine (Fig 1E).

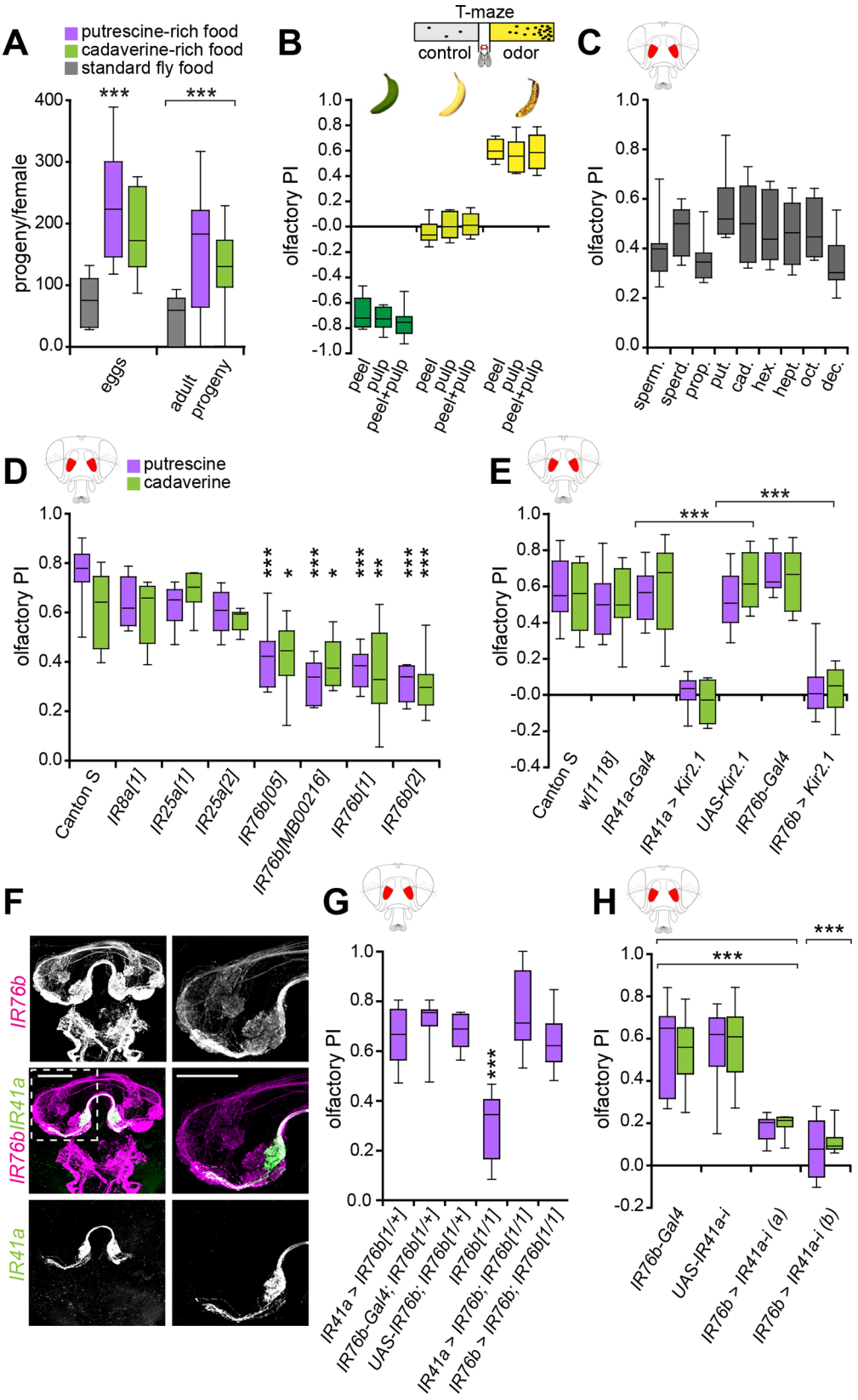
A
putrescine-rich food
cadaverine-rich food
standard fly food
progeny/female
eggs
adult progeny
B
T-maze
control
odor
olfactory PI
peel
pulp
peel+pulp
C
olfactory PI
sperm.
sperd.
prop.
put.
cad.
hex.
hept.
oct.
dec.
D
putrescine
cadaverine
olfactory PI
Canton S
IR8a[1]
IR25a[1]
IR25a[2]
IR76b[05]
IR76b[MB00216]
IR76b[1]
IR76b[2]
E
olfactory PI
Canton S
w[1118]
IR41a-Gal4
IR41a > Kir2.1
UAS-Kir2.1
IR76b-Gal4
IR76b > Kir2.1
F
IR76b
IR76b/IR41a
IR41a
G
olfactory PI
IR41a > IR76b[1/+]
IR76b-Gal4; IR76b[1/+]
UAS-IR76b; IR76b[1/+]
IR76b[1/1]
IR41a > IR76b; IR76b[1/1]
IR76b > IR76b; IR76b[1/1]
H
olfactory PI
IR76b-Gal4
UAS-IR41a-i
IR76b > IR41a-i (a)
IR76b > IR41a-i (b)

Fig 1. IR41a and IR76b mediate olfactory attraction to polyamines. (A) A diet high in polyamines increases reproductive success. Single males were crossed with single females in two different conditions for 4 d. Standard fly food and fly food with additional putrescine or cadaverine solution (~2.5 mmol polyamine/l of food). The number of eggs laid per single female and the number of eclosed flies per single female was quantified. Box plot show median and upper/lower quartiles (n = 8, 2 flies/trial 1 ♀ and 1 ♂). (B) Schematic illustration of the T-maze assay (top). *Drosophila* is attracted to the smell of overripe banana. Bars show olfactory preference index (PI) of wild type Canton S flies to peel, pulp, and peel + pulp of green banana, just yellow banana and brown-speckled banana, respectively. y-axis value of 0 indicates indifference while positive values indicate the degree of attraction and negative values indicate aversion. Box plots show median and upper/lower quartiles (n = 8, 60 flies/trial, 30 ♀ and 30 ♂). (C) Flies are attracted to several polyamines (of chain lengths C3-C10) with different sensitivities. Graph shows olfactory PI of wild type flies to 1 mM spermine (sperm.), spermidine (sperd.), diaminopropane (prop.), putrescine (put.), cadaverine (cad.), diaminohexane (hex.), diaminoheptane (hept.), diaminooctane (oct.), and diaminodecane (dec.), respectively. Box plots show median and upper/lower quartiles (n = 8, 60 flies/trial, 30 ♀ and 30 ♂). (D) Olfactory PI of Canton S (control) and IR mutant flies to putrescine and cadaverine in the T-maze assay. Box plots show median and upper/lower quartiles (n = 8, 60 flies/trial, 30 ♀ and 30 ♂). Asterisks denote significant reduction in olfactory preference to putrescine and cadaverine. (E) Bar graph shows olfactory PI of *IR41a-Gal4;UAS-Kir2.1* and *IR76b-Gal4;UAS-Kir2.1* with Canton S and genetic controls to putrescine and cadaverine in the T-maze assay. Asterisks denote a significant reduction in olfactory preference to putrescine and cadaverine. Box plots show median and upper/lower quartiles (n = 8, 60 flies/trial, 30 ♀ and 30 ♂). (F) Costaining of *IR41a-Gal4;UAS-mCD8GFP* (green) and *IR76b-QF;QUAS-mdtomato* (magenta) in the fly brain. The boxed region is centered on the AL, which is shown enlarged in the right panels. IR41a and IR76b expressing axons coinnervate a single glomerulus (VC5) in the AL. (G) IR76b is necessary in IR41a neurons to mediate the behavioral response to polyamine odor. IR76b was re-expressed in the *IR76b* mutant background using *IR76b-Gal4* or *IR41a-Gal4*. While *IR76b* mutants show a significantly reduced response to putrescine, re-expression of IR76b in either IR76b or IR41a neurons fully rescued this defect. (H) IR41a receptor is essential for polyamine attraction. Bar graphs show olfactory PIs of flies carrying *IR76b-Gal4; UAS-IR41a-RNAi* of two different RNAi transgenes flies and their genetic controls to putrescine and cadaverine in the T-maze assay. Box plots show median and upper/lower quartiles (n = 8, 60 flies/trial, 30 ♀ and 30 ♂). All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison posthoc test (ns > 0.05, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$). In all figures, asterisks above a single bar refer to p-values of comparison to the control (first bar of the panel). Lines joining two bars or groups of bars denote all other comparisons.

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It has been suggested that IRs may form functional heteromers in olfactory neurons, similarly to ORs [30]. Furthermore, IR76b as judged by reporter expression using a previously characterized *IR76b-Gal4* transgene [40] was expressed in multiple types of OSNs with axons innervating four glomeruli strongly and three weakly in the AL (Fig 1F; [46]). This strengthened the notion that another receptor might be used in conjunction with IR76b as previously hypothesized [46]. Among the OSNs previously shown to respond to putrescine [46] is a subset of IR41a-expressing neurons, housed in ac2 sensilla [31,46]. We blocked the activity of IR41a neurons with Kir2.1 *(IR41a-Gal4;UAS-Kir2.1)* and found that similar to IR76b neuron silencing, these flies showed no attraction to polyamines (Fig 1E). Using double labeling with *IR41a-Gal4* and *IR76b-QF*, which labels the same neurons as *IR76b-Gal4* (S2 Fig and see also Silbering et al. [46]), we found that a small subset of OSNs innervating a ventral and central (VC5) glomerulus coexpresses IR41a and IR76b (Fig 1F).

To obtain more direct evidence of a requirement of IR76b in IR41a neurons, we re-expressed IR76b in the *IR76b* mutant background selectively in IR41a or in all IR76b neurons (Fig 1G). As expected, re-expression of IR76b in IR76b neurons fully rescued the flies' attraction to polyamine odor (*IR76b-Gal4;UAS-IR76b;IR76b*^{1}, Fig 1G). Importantly, the same rescue was observed when we re-expressed IR76b selectively in IR41a neurons (*IR41a-Gal4; UAS-IR76b;IR76b*^{1}, Fig 1G). In a reciprocal experiment to test the role of IR41a in these neurons, we used RNAi to knockdown IR41a in IR76b-expressing neurons (*IR76b-Gal4; UAS-IR41a-i*) and assayed the effect in the T-maze. Knockdown of IR41a using two different RNAi transgenes reduced attraction to polyamines significantly as compared to control flies (Fig 1H).

These data, taken together, provide strong evidence that IR41a/IR76b coexpressing neurons are necessary and sufficient to mediate polyamine attraction.

IR76b Is Required for the Polyamine Odor Response of IR41a OSNs

To strengthen the evidence for a role of IR41a/IR76b OSNs in the detection of polyamine odor, we used in vivo calcium imaging as a proxy of neuronal activity of these OSNs. To this end, we expressed the genetically encoded calcium indicator GCaMP6f [51] under the control of *IR76b-Gal4* or *IR41a-Gal4* (*IR-Gal4;UAS-GCaMP6f*) and recorded increases in intracellular calcium levels in response to the polyamine odor stimulus at the level of the axon terminals in the AL in the brain (Fig 2A). When GCaMP was expressed exclusively in IR41a neurons, the innervated glomerulus strongly responded to putrescine in a concentration-dependent manner (Fig 2B–2D; see also [46]). Similarly, only a single glomerulus, the one innervated by IR41a/IR76b neurons, responded strongly to polyamine odor when GCaMP was expressed in all IR76b neurons (Fig 2E–2H). An independent IR76b, but not IR41a, neuron-innervated glomerulus, by contrast, did not respond significantly to polyamines (S3A–S3C Fig). Notably, the response was very long lasting (> 15 s, Fig 2D and 2H). Photoionization detector (PID) measurements suggested that odor might have been released into the airstream for up to 4 s with a 500 ms stimulus, because polyamine leaves a trace in the delivery line, which is cleaned out by air. This could, in part, explain this long-lasting response. Alternatively, this prolonged response could be a feature of some IR neurons as has been observed for other odors [32].

To test a requirement for IR76b in the observed odor response, we analyzed $IR76b^{1}$ mutant flies. The response of the IR41a glomerulus was strongly reduced in *IR76b* loss of function mutants (*IR76b-Gal4;UAS-GCaMP6f;*$IR76b^{1}$) confirming the essential role of IR76b for polyamine odor detection (Fig 2I–2K).

These experiments suggest that IR41a and IR76b function in the same neurons as polyamine receptors used by flies to detect polyamine-rich food sources such as overripe fruit.

Taste Neurons Mediate Short-Range Egg-Laying Choices on Polyamines

Our data indicated that flies are attracted to a source of volatile polyamine such as overripe fruit through specific olfactory neurons. Furthermore, we showed that females on polyamine-enriched food laid more eggs and had more offspring than females on standard food (see Fig 1A). We therefore asked if females use polyamines as a hallmark of beneficial egg-laying sites. To analyze this, we quantified the number of eggs laid on a polyamine-rich but otherwise plain, sugar-free substrate (polyamine and 1% agarose) versus a control substrate (1% agarose) in an oviposition assay (Fig 3A and 3B). Surprisingly, and in contrast to their olfactory preference, female flies avoided the polyamine-rich substrate and laid the majority of eggs on the polyamine-free site (Fig 3B, S4A–S4C Fig). Single females made the same choice as groups of females and laid their eggs away from polyamines showing that this aversion was not a consequence of overcrowding (Fig 3C, S4D Fig). While it was previously suggested that female flies avoid laying their eggs directly into feeding substrates [52], the apparent dislike of the beneficial polyamine-rich substrates as oviposition sites was surprising. This behavior could reflect the rather artificial assay conditions, where flies chose between polyamine-rich and polyamine-free but an otherwise taste- or odorless substrate. Because in a decaying fruit, polyamines appear together with other food odors and tastes, we reasoned that females might find them more appealing for oviposition when combined to other chemosensory cues. We tested this by mixing either of two polyamines, putrescine or cadaverine, with apple juice and gave the flies the choice to lay their eggs either on apple juice alone or on the mixture. While flies prefer to

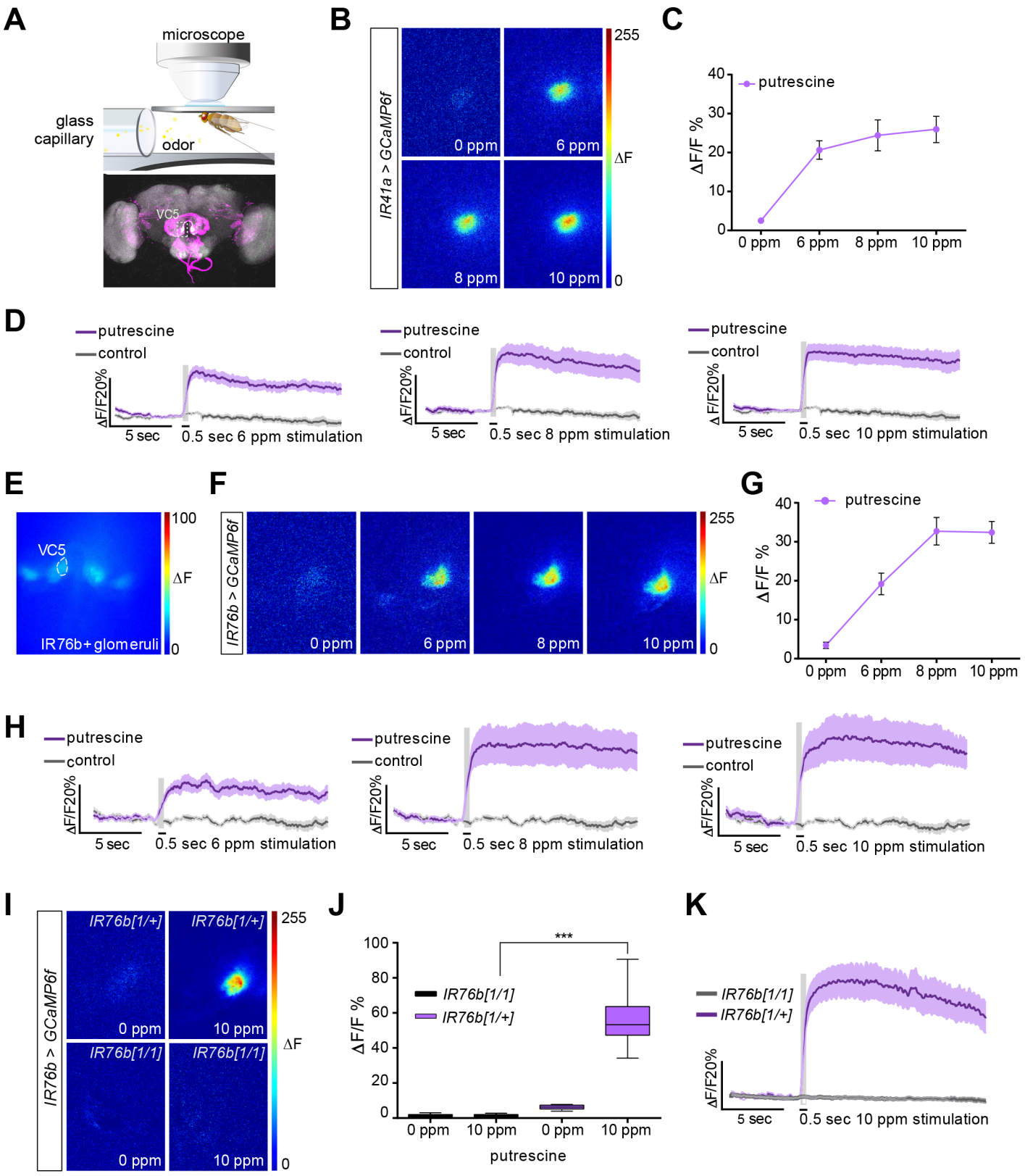

Fig 2. IR76b is required for the polyamine odor response of IR41a OSNs. (A) In vivo imaging setup illustration (top). Illustrative confocal image showing the IR41a and IR76b OSN innervating glomeruli pattern (bottom). VC5 is the glomerulus innervated by the polyamine-responding IR41a sensory

neurons. (B–D) In vivo calcium imaging of *IR41-Gal4;UAS-GCaMP6f* flies stimulated with water and 6, 8, and 10 ppm putrescine, respectively. Please note that that based on PID measurements, 10 ppm most closely represented the 1 mM concentration used in behavioral experiments due to the technical differences of odor application. (B) Representative pseudocolor images showing the response to water and increasing doses of putrescine, respectively. (C) Quantification of peak ΔF responses of the VC5 glomerulus ($n = 9$). (D) Average activity trace of VC5 glomerulus upon polyamine stimulation in %ΔF/F. (E) Prestimulation fluorescence micrograph showing IR76b positive glomeruli. (F–H) In vivo calcium imaging of *IR76b-Gal4; UAS-GCaMP6f* flies stimulated with water and 6, 8, and 10 ppm putrescine, respectively. (F) Representative pseudocolor images showing the response to increasing doses of putrescine. (G) Quantification of peak ΔF responses of the VC5 glomerulus ($n = 6$). (H) Average activity trace of VC5 glomerulus in %ΔF/F. (I–K) In vivo calcium imaging of *IR76b-Gal4,UAS-GCaMP6f;IR76b*^{1} and heterozygous control flies stimulated with water and 10 ppm putrescine, respectively. (I) Representative pseudocolor images showing the response in the VC5 glomerulus. (J) Quantification of peak ΔF responses in mutant and control flies. Boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. ***$p < 0.001$ by unpaired *t* test with Welch correction ($n = 6$). (K) Average activity trace of VC5 glomerulus. (D, H, K) The gray column represents the 0.5 second stimulation period. Dark colored line is the average response and the light shade is the standard error of the mean (SEM).

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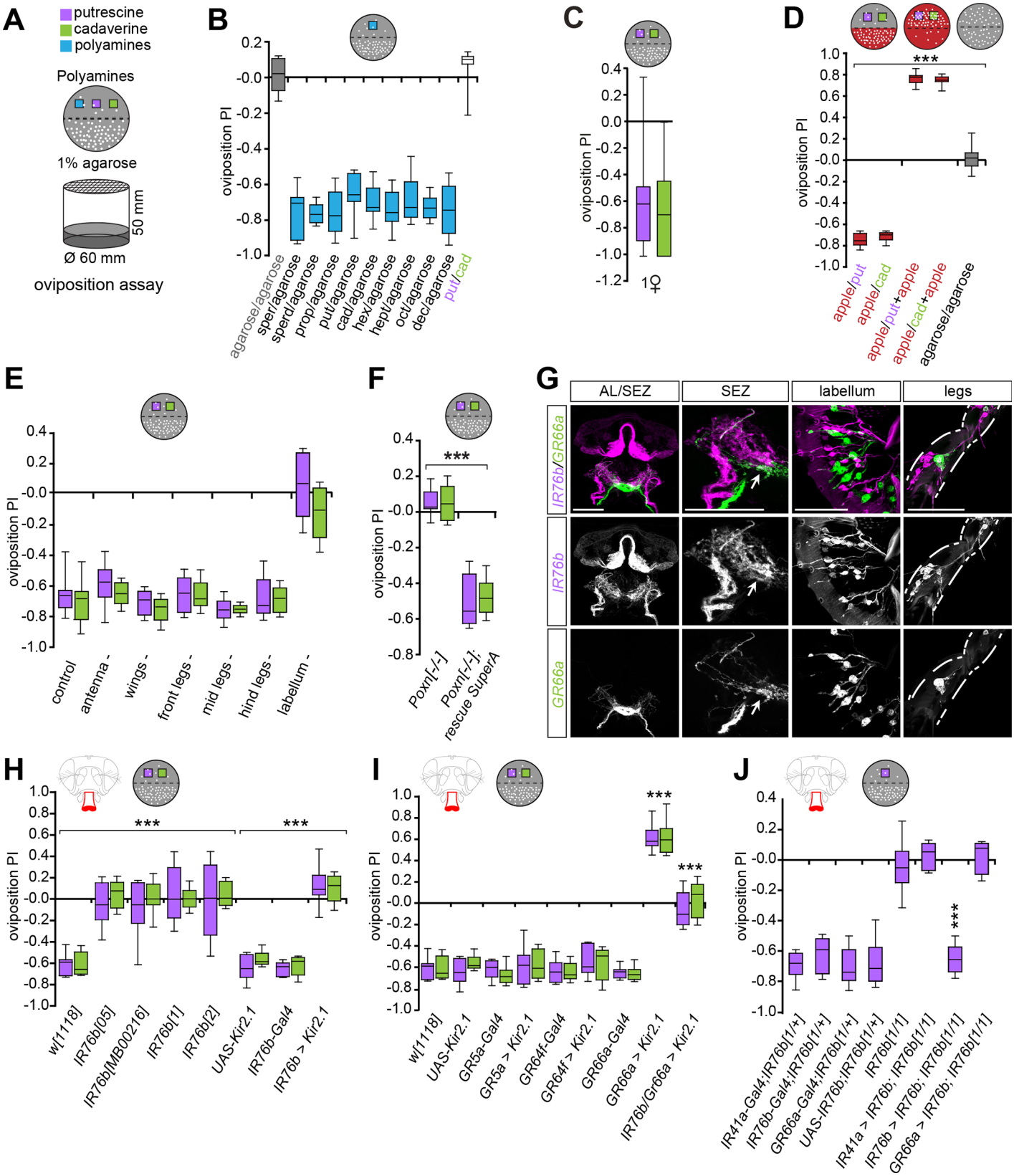
lay their eggs on apple juice compared to pure polyamine, they strongly preferred the mixture of apple juice and polyamines to apple juice alone (Fig 3D, S4E Fig). Similarly, flies laid significantly more eggs onto a substrate that contained sugar and putrescine than on sugar alone (S4F Fig). Thus, polyamines are not only beneficial for egg-laying and increase the number of progeny, they also provide and enhance appealing landmarks for oviposition.

The data highlighted that females use combinatorial sensory cues to decide where to lay an egg. We therefore examined which sensory modalities contributed to this short-range decision during oviposition. To facilitate the dissection of sensory mechanisms, we returned to assays using polyamine alone (agarose + polyamine versus agarose) and first determined the role of different sensory organs in polyamine choice during egg-laying. We found that ablation of antennae had no effect on oviposition avoidance to putrescine showing that IR41a/IR76b OSNs were dispensable once the female had closed in on an egg-laying substrate (Fig 3E, S4G Fig). We therefore turned to the other chemosensory modality, gustation. Taste organs are specified during development by the transcription factor Poxn. In *Poxn* mutants, most taste organs with the exception of some taste organs in the pharynx [53] are transformed into mechanosensory organs, including those found on the labellum, the legs, and the wings [54,55]. Compared to wild type females, the taste-impaired *Poxn* mutant females completely lost their aversion to oviposit onto polyamine-rich substrate and laid their eggs in equal numbers of both sites of the assay (Fig 3F, S4H Fig). This phenotype was rescued to wild type levels when *Poxn* was re-expressed using a full-genomic *Poxn* construct that rescued all taste neurons (Fig 3F, S4H Fig) [55]. Therefore, we concluded that the ultimate choice for egg-laying choice depends on GRNs, but does not involve pharyngeal taste neurons. We next sought to determine the external taste organs most critical for the female's egg-laying decision. To this end, we ablated single taste organs and assayed oviposition behavior. Ablation of the lower segments of front, middle, or hind legs had no effect on the oviposition choice of wildtype females and the majority of eggs were laid on the polyamine-free site (Fig 3E, S4G Fig). Similarly, females with clipped wings behaved normally to polyamines (Fig 3E, S4G Fig). By contrast, ablation of the labellum resulted in equal egg-laying on both sides of the plate and complete loss of oviposition preference, strongly suggesting, together with the *Poxn* mutant data, that polyamine-based oviposition choice requires taste neurons on the fly's labellum (Fig 3E, S4G Fig). As we cannot ablate all legs simultaneously, it remains possible that tarsal IR76b neurons also contribute to some extent to the choice. Nevertheless, these other IR76b neurons cannot compensate for the lack of labellar neurons. Thus, gustatory neurons on the labellum are essential to mediate the short-range polyamine choice behavior during egg-laying.

Taste sensilla contain several types of neurons including bitter-, salt-, and sugar-sensitive neurons [37]. How flies taste any amine including polyamines is unknown [37]. We thus sought to determine which taste receptors mediate the gustatory perception of polyamines. Some GRNs express IRs [37,56]. In contrast to the odor-detecting IR41a, IR76b is expressed in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IR41a > IR76b; IR76b[1/1]
IR76b > IR76b; IR76b[1/1]
GR66a > IR76b; IR76b[1/1]

Fig 3. Oviposition site choice requires IR76b and bitter taste neurons. (A) Schematic drawing of the oviposition assay setup (bottom) and a sample plate used to calculate the oviposition preference (top). *D*. *melanogaster* evaluates polyamine levels during egg-laying choices. The egg-laying plate halves contain 1% low melting agarose alone or agarose supplemented with a specific polyamine (purple/green boxes) in all cases with the exception of Fig 3D. (B–J) Box plots show oviposition PI of flies. *y*-axis value of 0 indicates indifference, while positive values indicate the degree of attraction and negative values indicate aversion. (B) Oviposition assay using plain agarose versus agarose + different polyamines at 1 mM. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀/trial). (C) Same assay as in B with single females showing group-independent decision-making to polyamines. ($n = 30$, 1 ♀ flies/trial). (D) Polyamines increase attractiveness of fruit. Oviposition PI of females for putrescine or cadaverine (grey plate) versus apple juice (red plate). While apple juice is more attractive than plain putrescine or cadaverine, apple juice supplemented with polyamine is more attractive than apple juice alone. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀/trial). (E) Oviposition assay (agarose versus putrescine or cadaverine) with females missing either antennae, wings, different tarsae (legs), or labellum compared to intact flies (control). Flies missing the labellum show no preference, while all other ablations had no effect on the PI. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀/trial). (F) Oviposition PI of loss of function *Poxn* females ($Poxn^{-/-}$) and *Poxn* rescue construct (SuperA-158 (53)) for putrescine and cadaverine. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀/trial). (G) Expression of GR66a (*GR66a-Gal4;UAS-mCD8GFP*, green) and IR76b (*IR76b-QF;QUAS-mtdTomato-3xHA*, magenta) in the AL, SEZ, labellum and legs. GR66a and IR76b are not expressed in the same taste neurons but innervate neighboring areas in the SEZ (arrow). (H) *IR76b* mutants lose their preference behavior to polyamine taste ($IR76b^{05}$, $IR76b^{MB00216}$, $IR76b^{1}$, $IR76b^{2}$), *IR76b-Gal4;UAS-Kir2.1*, and appropriate genetic controls. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀/trial). (I) Two taste receptors mediate oviposition preference. Oviposition PI of silenced sweet tasting GRs (*GR5a-Gal4;UAS-Kir2.1* and *GR64f-Gal4;UAS-Kir2.1*), and inactivated bitter tasting receptor neurons (*GR66a-Gal4;UAS-Kir2.1*) and appropriate controls. Silencing of bitter neurons makes polyamines attractive, while silencing sweet neurons has no effect. This attractiveness is dependent on the activity of IR76b neurons as *GR66a-Gal4*,*IR76b-Gal4;UAS-Kir2.1* flies show no preference behavior. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀/trial). (J) IR76b is required to mediate the behavioral response to polyamine odor. IR76b was re-expressed in the *IR76b* mutant background using *IR76b-Gal4*, *IR41a-Gal4 or GR66a-Gal4*. While *IR76b* mutants show no preference behavior to putrescine, re-expression of IR76b in IR76b but not in IR41a or GR66a neurons fully rescued this defect. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀/trial). All *p*-values were calculated via two-way ANOVA with the Bonferroni multiple comparison posthoc test (ns > 0.05, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$). In all figures, asterisks above bars refer to *p*-values of comparison to wild type control (first bar of the panel). Lines joining two bars or groups of bars denote all other comparisons.

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the gustatory system including GRNs of the labellum. These IR76b neurons project to the SEZ [40] (Fig 3G). Given the requirement of IR76b olfactory neurons in attraction to polyamines, we tested the involvement of their gustatory counterparts in oviposition behavior. Four loss of function mutants of IR76b (e.g., $IR76b^{1}$), as well as the Gal4-mediated neuronal silencing of IR76b neurons (*IR76b-Gal4;UAS-Kir2.1*) completely abolished polyamine avoidance during egg-laying (Fig 3H, S4I Fig). By contrast, mutants for other IRs (e.g., *IR8a and IR25a*) or *ORCO* or silencing of sugar-sensitive neurons (GR5a and GR64f) by Kir2.1 had no effect on polyamine avoidance of pure polyamine-agar substrate during oviposition (Fig 3I, S4I–S4K Fig). These data implicate labellar IR76b taste neurons into the detection of polyamine taste.

Polyamines are strongly bitter-tasting compounds to humans [57]. We tested whether this sensation could trigger the aversion of polyamines in the absence of fruit or sugar (see above). When we silenced bitter-sensing GR66a taste neurons [58] during egg-laying, the aversive (pure) polyamine became attractive to these females (*GR66a-Gal4;UAS-Kir2.1*), which then strongly preferred to lay their eggs on the polyamine-rich otherwise plain substrate (Fig 3I, S4K Fig). These results confirm that polyamines are highly attractive egg-laying substrates and suggest that GR66a neurons may inhibit or counteract such attractiveness. This result also provides a mechanistic explanation for why polyamines in the context of fruit or sugar are highly attractive to flies, because sweet sensation can quench the perception of bitter ([59] see Fig 3D, S4E and S4F Fig). Silencing of both IR76b and GR66a neurons simultaneously (*GR66a-Gal4*, *IR76b-Gal4;UAS-Kir2.1*) abolished this preference for the polyamine-rich substrate. This further demonstrates that the attraction to polyamine indeed depends on IR76b (Fig 3I, S4K Fig).

Given that IR76b and GR66a are both expressed in taste organs, we carried out double-labeling experiments using genetic reporters to analyze their relative position on the labellum and their axonal projections into the SEZ (Fig 3G, S5 Fig). We first confirmed that *IR76b-QF* and *IR76b-Gal4* used in the behavioral assays were coexpressed in gustatory neurons. Although the relative expression levels of the two reporters showed some variation, they were by and large coexpressed (S2 Fig). We therefore used the *IR76b-QF* reporter to analyze potential expression in GR bitter neurons (*GR66a-Gal4*). Our reporter expression data suggested that

these receptors are not expressed in the same neurons in the labellum as the respective axon populations innervated distinct regions of the SEZ (Fig 3G, S5 Fig, e.g., SEZ panel) [40]. These results suggest that two different taste neuron populations on the labellum are required to taste and evaluate polyamine-rich egg-laying substrates.

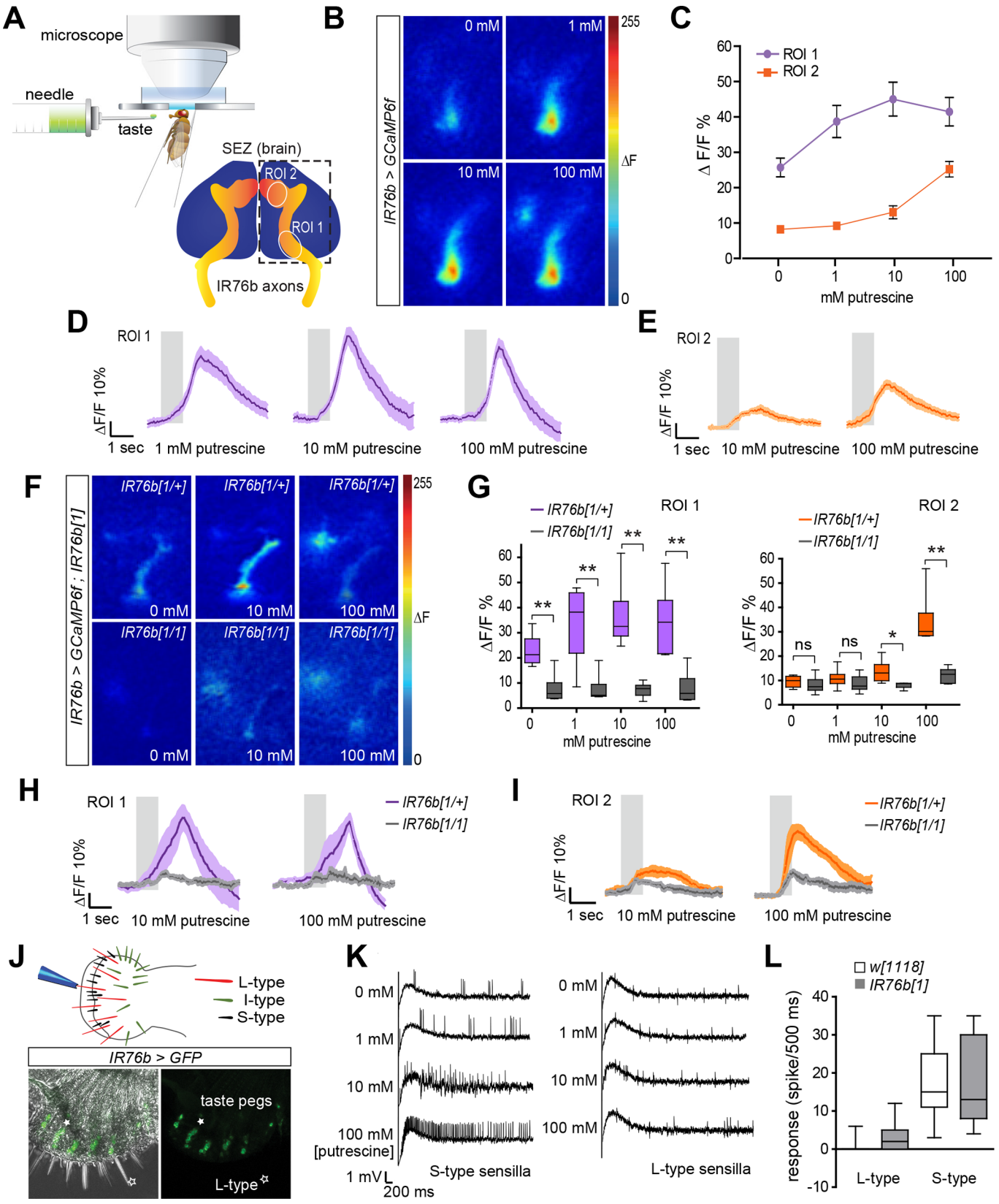
To strengthen this evidence and to confirm that IR76b receptors were indeed required to taste polyamines, we carried out rescue experiments in *IR76b*^{1} mutant females. Re-expression of IR76b in IR76b receptor neurons (*IR76b-Gal4,UAS-IR76b;IR76b*^{1}) resulted in a full rescue of oviposition behavior (Fig 3J, S4L Fig). By contrast, re-expression of IR76b selectively in IR41a OSNs (*IR41a-Gal4,UAS-IR76b;IR76b*^{1}) did not rescue oviposition choice confirming that this behavior depended on taste neurons (Fig 3J, S4L Fig). Finally, we re-expressed IR76b in GR66a neurons (*GR66a-Gal4,UAS-IR76b;IR76b*^{1}) and observed no rescue of the choice behavior confirming that IR76b receptors critical for polyamine taste do not reside in GR66a bitter neurons (Fig 3J, S4L Fig). Hence, the fly receives and integrates two types of information, quality and valence, from one molecule, using two types of taste neurons. Given our data on polyamine and apple juice/sugar, such integration could potentially follow a mechanism that was recently demonstrated for sugar neurons, which are indirectly inhibited by bitter neurons via GABAergic inhibitory neurons of the SEZ [59]. This type of multimodal taste integration would allow the fly to measure the relative levels of polyamines and sugars during the evaluation of food.

Taste Neurons Respond to Polyamines

Our behavioral data provides strong evidence that polyamine sensing in the context of oviposition requires IR76b receptor in taste neurons on the female's labellum. To test more directly whether taste neurons responded to polyamines, we monitored the response of IR76b neurons to putrescine using in vivo calcium imaging with GCaMP6f (*IR76b-Gal4; UAS-GCaMP6f*; Fig 4A). Labellar stimulation with a putrescine solution led to a significant increase in GCaMP-fluorescence in primarily two regions of the SEZ innervated by at least two distinct sets of IR76b taste neurons (Fig 4A–4E). One of these regions (region of interest [ROI] 1) responded more strongly at for egg-laying behavior relevant concentrations (1 mM) (Fig 4C and 4D). By contrast, ROI 2 responded to higher concentrations of polyamine (100 mM; Fig 4C and 4E). We also observed calcium increases in ROI 1 in IR76b neuron axon terminals upon stimulation with salt consistent with a previous report implicating IR76b in the detection of low salt concentrations (S6A Fig, 50 mM NaCl, [40]). Given that IR76b neurons are also found on the legs, we tested their responses to polyamines. To this end, we recorded GCaMP-fluorescence directly from the neurons on the tarsae (S6B–S6D Fig). Our results suggest that polyamine-sensitive tarsal taste neurons exist on all legs and therefore could potentially contribute—although probably to a lesser extent—to the egg-laying choice. Of note, we only observed a significant response to high (100 mM) but not to lower polyamine concentrations (1 mM).

To gain more evidence for a role of IR76b as polyamine receptor, we recorded changes in GCaMP fluorescence in *IR76b*^{1} mutant flies and found that the response to polyamine was absent in both the ROI 1 and ROI 2 areas (Fig 4F–4I). Similarly, the response to salt was also significantly reduced (S6E Fig). These results demonstrate that IR76b receptor is required for polyamine detection by GRNs. Together with our behavioral data, these results show that IR76b, in addition to its function as salt receptor [40], is a taste receptor for polyamines.

We wondered which type of IR76b-expressing labellar GRN detects polyamines. Labellar taste neurons are housed in three types of taste bristles, short (S), intermediate (I), and long (L), as well as in taste pegs [35] (Fig 4J). L-type GRNs expressing IR76b promote the attraction to low levels of salt [40]; and IR76b expression as judged by the Gal4 reporter (*IR76b-Gal4; UAS-mCD8GFP*) can be observed in L-type sensilla [40]. In addition, we found that the


A
microscope
needle
taste
SEZ (brain)
ROI 2
ROI 1
IR76b axons
B
IR76b > GCaMP6f
0 mM
1 mM
10 mM
100 mM
255
ΔF
0
C
ROI 1
ROI 2
Δ F/F %
mM putrescine
D
ROI 1
ΔF/F 10%
1 sec
1 mM putrescine
10 mM putrescine
100 mM putrescine
E
ROI 2
ΔF/F 10%
1 sec
10 mM putrescine
100 mM putrescine
F
IR76b > GCaMP6f ; IR76b[1/1]
IR76b[1/+]
IR76b[1/1]
0 mM
10 mM
100 mM
255
ΔF
0
G
IR76b[1/+]
IR76b[1/1]
ROI 1
ROI 2
ΔF/F %
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*
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ROI 1
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IR76b[1/1]
ΔF/F 10%
1 sec
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100 mM putrescine
I
ROI 2
IR76b[1/+]
IR76b[1/1]
ΔF/F 10%
1 sec
10 mM putrescine
100 mM putrescine
J
L-type
I-type
S-type
IR76b > GFP
taste pegs
L-type
K
0 mM
1 mM
10 mM
100 mM [putrescine]
1 mV
200 ms
S-type sensilla
L-type sensilla
L
w[1118]
IR76b[1]
response (spike/500 ms)
L-type
S-type

Fig 4. IR76b neurons respond to polyamines in gustatory processing. (A) Schematic drawing of SEZ calcium imaging setup and position of ROIs within the SEZ, which were used for quantification of relative changes in GCaMP-fluorescence (%ΔF/F). (B) Representative images of SEZ imaging of *IR76b-Gal4;UAS-GCaMP6f* female flies stimulated with distilled water or increasing concentrations of putrescine. (C) Quantification of GCaMP6f-fluorescence peak responses (in %ΔF/F) in the ROI 1 and ROI 2 area, respectively, when female flies were stimulated with increasing concentrations of putrescine ($n = 11 \pm$ SEM). (D) Average response trace of ROI 1 area to putrescine ($n = 11 \pm$ SEM). The gray bar shows the stimulation period. Dark colored line in the middle presents the average value, and the light shade presents the SEM. (E) Average response trace of ROI 2 to putrescine ($n = 11 \pm$ SEM). (F) Representative images of SEZ of *IR76b-Gal4,UAS-GCaMP6f; IR76b*^{1} and heterozygous control female flies stimulated with distilled water or increasing concentrations of putrescine. (G) Quantification of peak responses (in %ΔF/F) in *IR76b* mutant and control. Responses in the ROI 1 and ROI 2 are calculated separately with increasing concentrations of putrescine ($n = 6 \pm$ SEM). Boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. (H) Average response trace of ROI 1 in *IR76b* mutant and control ($n = 6 \pm$ SEM). (I) Average response trace of ROI 2 in *IR76b* mutant and control ($n = 6 \pm$ SEM). (J) Scheme of the *Drosophila* labellum with different types of sensilla. IR76b *(IR76b-Gal4;UASmCD8GFP)* is expressed in peg taste sensilla on the labellum. Filled star indicates peg taste neuron, open star indicates L-type sensillum. (K) Electrophysiological recording of S-type and L-type sensilla to putrescine at different concentrations (0 mM–100 mM, $n = 8 \pm$ SEM). (L) Quantification of the response of S-type and L-type sensilla to putrescine in *IR76b* mutant and wild-type control. ($n = 8 \pm$ SEM). All *p*-values were calculated via Student's *t* test (ns > 0.05, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

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reporter was strongly expressed in taste pegs (Fig 4J). Consistent with this, the innervation of the ROI 1 area in the SEZ by IR76b GRN axons resembled the innervation previously observed for GRNs in taste pegs (e.g., GRNs responding to carbonation, [60]). As the ROI 1 area responded most strongly to behaviorally relevant concentrations of polyamines, it is conceivable that IR76b peg neurons are required for oviposition choice behavior. To test which neurons responded to the taste, we used tip recordings and recorded putative responses of the taste neurons housed in sensilla. In particular, we asked whether L-type sensilla were activated by polyamines. We found that none of the recorded L-type sensilla showed a response to putrescine consistent with our hypothesis that a different GRN type detects polyamines such as the peg neurons (Fig 4K and 4L). We next tested the responses of S-type sensilla. These sensilla responded significantly to polyamines (Fig 4K and 4L), but IR76b did not mediate these responses, as tip recordings on *IR76b*^{1} mutants still showed strong responses of S-type sensilla to putrescine (Fig 4L). Given that bitter receptors are prominent in these sensilla, we hypothesize that bitter receptors such as GR66a might instead mediate these responses consistent with their involvement in oviposition behavior.

We conclude that the fly's taste sensation of polyamines requires IR76b in labellar GRNs, most likely in peg neurons but not in L-type sensilla that mediate the response to salt [40]. IR76b is also not required in S-type sensilla, which presumably mediate the bitter taste of these compounds. Finally, it appears that amongst IR76b taste neurons, some are involved in the sensation of polyamines while others sense salt, indicating the involvement of up-to-now unidentified partner receptors for salt and polyamines, respectively.

Multisensory Detection of Polyamines Explain Egg-Laying Behavior

Our data suggest that female flies sense and interpret polyamine odor and taste. To address how flies use and integrate this multisensory input elicited by a single compound during egg-laying site selection, we video-monitored the time flies spend on polyamine-rich compared to control substrate in the setup of the oviposition assay (Fig 5A). To simplify the assay and the interpretation, we carried out the experiments on polyamine-rich substrates devoid of additional odors or tastes. We found that wild type female flies spent significantly more time on the polyamine site (positive position index; solid lines), although they preferred to lay their eggs on the control site (negative oviposition index; dashed lines) (Fig 5B, S7A Fig). Notably, flies showed the strongest positional preference for polyamine at the beginning of the experiment, but this preference steadily declined in parallel to an increase of eggs laid (Fig 5B). We investigated which of the identified receptor neurons and thus sensory modalities are used for position and oviposition, respectively. Taste neuron-deficient

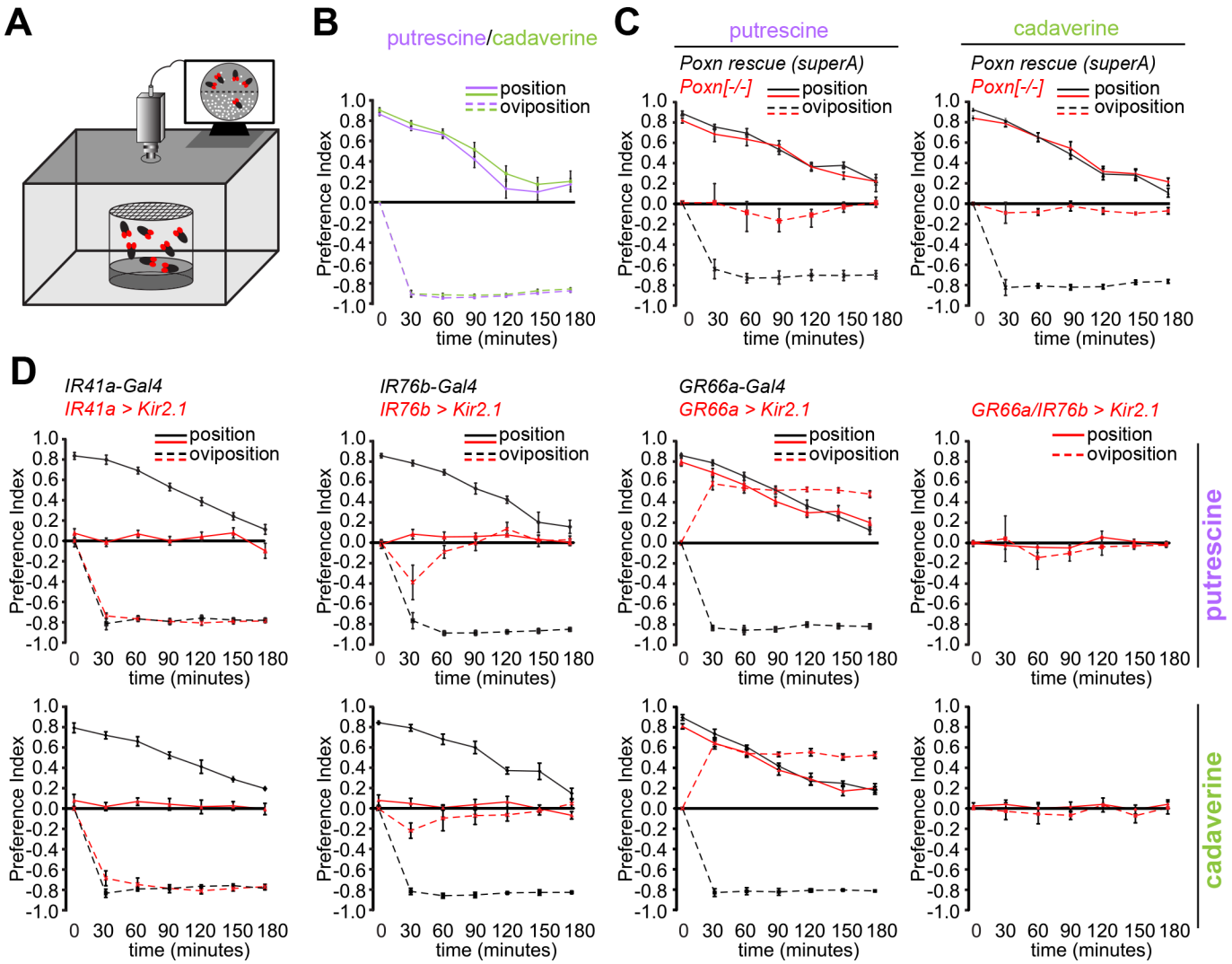

Fig 5. Females detect odor and taste of polyamines during oviposition site selection. (A) Scheme of the video-tracking position-oviposition assay. (B) Females spend more time on the polyamine-rich site but avoid pure polyamines for egg laying. Position (solid line) and oviposition (dashed line) preference of Canton S to putrescine and cadaverine over time in position–oviposition assay. *y*-axis shows position and oviposition PI. *x*-axis shows total time (min) of the assay. The female's position–oviposition behavior was quantified in 30 min intervals. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀ flies/trial, total time of assay of 3 h. (C) Position and oviposition preference of loss of function mutant of *Poxn* (*Poxn*$^{-/-}$) and *Poxn* full rescue (rescue superA) to putrescine in position–oviposition assay. Red lines indicate behavior of *Poxn*$^{-/-}$ females, while black lines designate behavior of *Poxn* rescue SuperA. Loss of sense of taste does affect oviposition but not position preference. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀ flies/trial). (D) Position and oviposition preference of *IR41a-Gal4;UAS-Kir2.1*, *IR76b-Gal4;UAS-Kir2.1*, *Gr66a-Gal4;UAS-Kir2.1*, and *Gr66a-Gal4*,*IR76b-Gal4;UAS-Kir2.1* and their respective genetic controls to putrescine over time in position–oviposition assay. Position preference is mediated by IR41a olfactory neurons, while taste neurons trigger oviposition avoidance. Red lines indicate *receptor-Gal4;UAS-Kir2.1*, while black lines indicate controls. Box plots show median and upper/lower quartiles ($n = 8$, 60 ♀ flies/trial). All *p*-values were calculated via two-way ANOVA with the Bonferroni multiple comparison posthoc test (ns > 0.05, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

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Poxn mutants confirmed that oviposition aversion was dependent on taste; *Poxn* mutants showed no aversion and laid their eggs on both sides of the assay (Fig 5C, S7B and S7C Fig). By contrast, positional attraction to polyamines remained intact in these flies (Fig 5C). Of note, *Poxn* mutant females laid significantly less eggs in the first three hours that were observed in this assay (S7B Fig). However, in the longer assay as shown above (S4H Fig), the total number of eggs was comparable to controls suggesting that a lack of the sense of taste may initially inhibit the female's willingness to deposit her eggs.

We found that silencing IR41a OSNs (*IR41a-Gal4;UAS-Kir2.1*) selectively affected position preference and not oviposition avoidance, suggesting that positional preference required olfactory detection of polyamines (Fig 5D, S7D Fig). Furthermore, females with silenced IR76b neurons (*IR76b-Gal4;UAS-Kir2.1*), which included the IR41a/IR76b OSN population as well as the IR76b taste neurons, were completely indifferent to polyamines in position and oviposition (Fig 5D, S7D Fig). They also showed a similar phenotype as *Poxn* mutant females and appeared to hold their eggs longer before deciding to lay them consistent with a deficient taste system (S7D Fig and S4K Fig). Silencing of GR66a bitter taste neurons (*GR66a-Gal4;UAS-Kir2.1*) had no effect on position behavior, but as reported above, reversed oviposition avoidance to preference (Fig 5D, S7D Fig). Egg numbers were comparable to controls (S7D Fig). Furthermore, concurrent inactivation of IR76b and bitter tasting neurons (*Gr66a-Gal4,IR76b-Gal4;UAS--Kir2.1*) completely abolished positional and oviposition preference (Fig 5D, S7D Fig).

In summary, these data show that odor- and taste-induced behaviors do not depend on each other but rather happen in a parallel or sequential manner. Polyamine odors could be long-range attractive cues for female flies to navigate to putative oviposition sites. Upon arrival on or in close proximity of such sites (a decaying fruit or the confined space of our oviposition assay), the sense of smell is dispensable, and females refine their choice for oviposition with their taste organs, likely integrating polyamine inputs with other tastes (e.g., sugar).

Polyamines Attract *Ae*. *aegypti* Mosquitoes to Egg-Laying Sites

Having shown that *Drosophila* flies use polyamines to identify egg-laying sites, we next investigated a more general role of these compounds beyond the vinegar fly. *Ae*. *aegypti* mosquitoes transmit the dangerous disease dengue fever and cause about 25,000 deaths per year [61]. ORs are among the potential targets of measures for pest control.

Because *Aedes* mosquito adults live in human households and are often found attached to sheets, curtains, etc. [62], insecticide treatments of adults are often limited by their proximity to humans. Targeting breeding sites might therefore be more practical and efficient [63]. Using laboratory oviposition assays, we asked whether *Aedes* females are attracted to the odor of polyamines for egg-laying (Fig 6A). Single females were released into a caged area and given the choice between laying into a cup that smelled of polyamines and an odorless cup. They were prevented from tasting the compounds in this assay and forced to use their sense of smell. We found that egg-laying female mosquitoes deposit significantly more eggs into water that smelled of putrescine or cadaverine compared to control (Fig 6B and 6C). This attraction was concentration-dependent, and mosquitoes were most attracted at 1μM and 10 μM of putrescine or cadaverine (Fig 6B and 6C). By contrast, concentrations as of 100 μM started to become repulsive, and females preferred to lay their eggs into the nonsmelling cup (Fig 6B and 6C).

These experiments suggest that also other insects such as mosquitoes use polyamines to find feeding and egg-laying sites. These polyamine-based behaviors and possibly the detection mechanisms might be conserved in other species.

Discussion

Here, we show that polyamines, important biogenic amines, are beneficial components of a diet that increases the reproductive success of *Drosophila* flies. In line with this, flies are highly attracted to polyamines and use them to identify food sites for instance for egg-laying. The decision to approach or lay eggs on polyamine-rich substrates requires multisensory integration of two distinct sensory modalities, olfaction and taste. We have identified the sensory and molecular mechanisms involved in polyamine-guided behavior in both modalities and show that they share the requirement of the IR, IR76b.

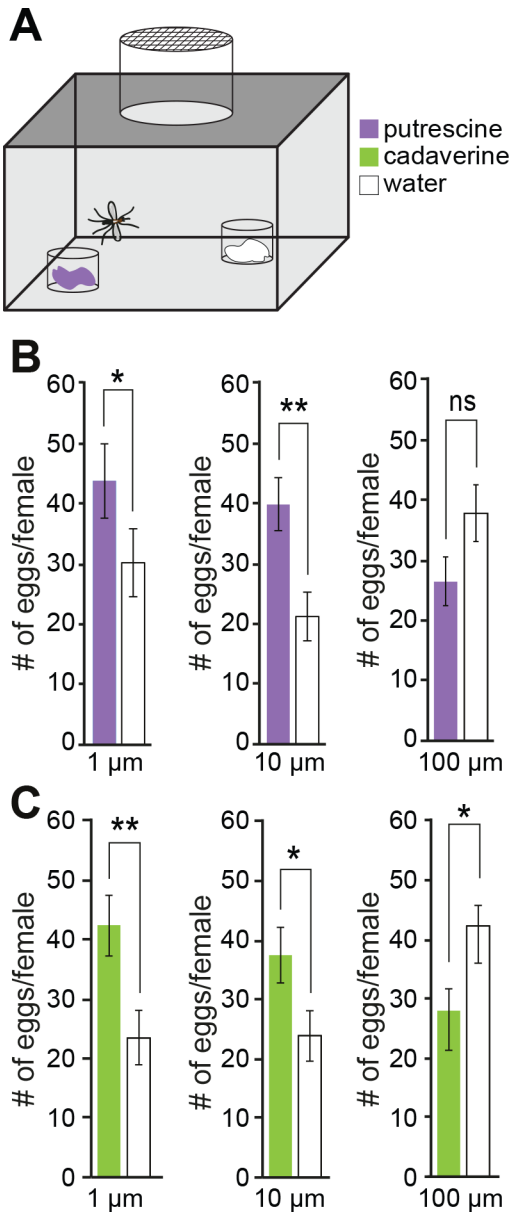

Fig 6. *Ae. aegypti* mosquitoes are attracted to polyamines for egg laying. (A) Schema of single female mosquito egg-laying assay. Mated, gravid females were given the choice between two small cups containing either pure water or water enriched with different concentrations of polyamines (1 μM to 100 μM). Females were exposed only to the odor of polyamines, and direct contact to polyamines was prevented (see materials and methods). (B–C) Females were most attracted to 1 and 10 μM of putrescine (purple, upper panels) or cadaverine (green, lower panels). 100 μM polyamine repelled females from laying eggs into the cup. PIs are averaged ($n = 8 \pm$ SEM, 1 female/trial). All p-values were calculated via Student's t test (ns > 0.05, *$p \leq 0.05$, **$p \leq 0.01$).

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Polyamines Are Beneficial Nutrients Involved in Reproductive Success

Polyamines represent an important component of animal nutrition [6,12]. Deficiency as well as excess of polyamines can be detrimental to health and reproduction [6]. Therefore, species might have undergone a selection to choose food with levels of polyamines that meet their

physiological needs. Our results provide important biological significance for the preference of *D. melanogaster* for polyamine-rich food such as fermented fruit or fresh oranges (see also [14,15]). In particular, we show that a polyamine-enriched diet increases the number of offspring produced by a fly couple. Females feeding on polyamine-supplemented food lay significantly more eggs. Mechanistically, we speculate that the diverse and conserved roles of polyamines during cell cycle progression, differentiation, and autophagy among others are responsible for the beneficial effect [5]. Interestingly, in addition to uptake through the diet, polyamine-synthesis enzymes (i.e., ornithine decarboxylase) are selectively up-regulated after mating in the spermatheca of female mosquitoes, a tissue involved in sperm storage, egg-production, and laying in mosquito and *Drosophila*, consistent with a role in fertility and reproduction [64].

Conception and proper embryonic development depend on polyamines also in humans [5]. Polyamines also form a substantial part of the male ejaculate (i.e., spermine and spermidine) and infertile men show lower levels of spermine, spermidine, and putrescine in their semen [5]. Interestingly, polyamine-synthesizing enzymes in the cell decay with age, spurring studies on the beneficial effects of polyamines in the diet. Polyamine supplementation might counteract age-related loss of fertility in men and women, but also other ageing-induced deficits such as loss of memory or even lifespan [4,8,10,11]. On the other hand, an excess of these compounds in the cell, and therefore possibly in the diet has been linked with the occurrence and progression of cancer and other diseases suggesting that polyamine intake should be carefully regulated [6]. With the characterization of sensory mechanisms underpinning the attraction to polyamines, we can begin to analyze how fundamental physiological needs shape sensory processing and ultimately impinge on feeding and reproductive behavior.

Multisensory Detection of Polyamines

Flies detect the odor and taste of polyamines. In addition, our data suggests that two types of gustatory neurons evaluate quality and valence of polyamines separately. The female to identify egg-laying sites uses these two taste modalities, polyamine taste and bitter taste. In this context and presumably during feeding, sugar in the fruit appear to override the bitter taste of polyamines, which translates at the behavioral level to a preference for polyamine-rich compared to polyamine-poor fruity substrates. This claim is supported by data showing that female flies strongly prefer to lay their eggs into pure polyamine substrates when their bitter sense is silenced genetically. Therefore, several modalities contribute to polyamine choice behavior. Such multimodal detection of polyamines might ensure that the animal only consumes polyamines in the context of a suitable food source and at beneficial concentrations. A similar interaction was suggested between sugar and bitter taste sensation in the fly, indicating that concentrations might be generally estimated by assessing the relative amounts of tastants [59,65,66]. Multimodal taste experiences are essential to judge food quality also for humans. Therefore, sugar is frequently used to quench bitter tastes in food and to make medication more palatable [67].

In addition to integrating two types of taste modalities, flies also appear to use their sense of smell to find polyamine-rich foods. Our tracking data shows that these two kinds of information, smell and taste, are used sequentially consistent with odor being a long-range and taste a short-range signal. Flies that have found the source of the polyamine using their sense of smell do no longer require it to make the decision on where to lay eggs. However, whether odor and tastes are integrated in a more complex odor/taste environment of polyamines and other cues remains to be investigated.

IR76b Receptors Are Required to Detect the Smell and Taste of Polyamines

Our behavioral data shows that flies are attracted to all polyamines tested including putrescine, spermine, and cadaverine. Notably, they appear to prefer concentrations that are typically found in fermented foods, overripe fruit, or oranges, also a favorite of flies [15]. The receptor IR76b is required for the detection of the odor as well as the taste. Mutants for *IR76b* show significantly reduced attraction to polyamines. In this context, IR76b seems to work with another receptor, IR41a, which is specific to a very small subset (~7) of antennal OSNs. Although IR76b is more broadly expressed than IR41a in the olfactory system, it is unclear whether it plays the role of a coreceptor like IR8a or IR25a [31]. IR25a, which appears to be coexpressed with IR76b and IR41a, could play this role in polyamine detection. However, *IR25a* mutant flies show no decrease in polyamine attraction compared to controls. Thus, it is possible that IR76b and IR41a work as functional receptor heteromers, a configuration that is necessary and sufficient to form functional chemoreceptors [30,31].

Notably, the effect of the *IR76b* mutation in calcium imaging of OSNs appeared significantly stronger than the effect of the mutation in odor-guided behavior (see Fig 1 and Fig 2), although IR76b and the IR41a glomerulus appeared to be necessary and sufficient to mediate polyamine odor attraction (see Fig 1). This difference could be due to the slightly different conditions in behavior and imaging. On the one hand, animals are freely moving during behavior and might experience odor plumes rather than constant streams. On the other hand, animals are exposed for longer periods of times to the odor in the T-maze as compared to the imaging experiments. These effects might also explain a discrepancy between our behavioral data and a previous study implicating the IR coreceptor IR8a in the detection of putrescine using single sensillum electrophysiology [31]. As mentioned above the same *IR8a* loss of function mutants as used by Abuin et al. [31] did not show any significant reduction in polyamine odor attraction in behavioral assays.

In the gustatory system, IR76b expressed in labellar GRNs is necessary and sufficient to mediate polyamine choice behavior. In contrast to the olfactory system, a mutation in *IR76b* results in complete loss of preference behavior to polyamines as well as a loss of calcium responses in GRNs. Calcium imaging and tip recording along with expression data indicate that polyamines are not recognized by IR76b expressed in L-type or S-type sensilla on the labellum, but instead might be detected by peg taste neurons that express IR76b. Furthermore, IR76b taste neurons on the leg, although not essential for egg-laying decisions, responded to polyamines. Based on our data, we can exclude and infer the involvement of certain types of IR76b taste neurons for polyamine detection, but further experiments will be required to reveal their exact identity and position.

A previous study showed that IR76b in L-type neurons was required for the fly's attraction to low levels of salt [40]. How can the same receptor mediate two or more different taste modalities? The easiest explanation might be the involvement of at least one coreceptor for either salt or polyamine. Given that IR76b expressing L-type sensilla do not respond to polyamines, this appears to be a likely scenario at least for the detection of polyamines. Up to now, our candidate approaches, however, have not identified such a coreceptor. The other possibility is that the same receptor has putative binding sites for both of these ligands. In fact, ORs with few exceptions such as the CO_2 receptors [24,26] detect multiple odorants. Nevertheless, salt and polyamines appear to be very different types of ligands. The activation of IR76b receptor by salt seems to depend on a particular amino acid located in the transmembrane domain, which is required for ion conductance in ionotropic glutamate receptors (iGluRs) [40]. The

authors propose that this amino acid will keep the channel in a constitutively open or partly open position, which allows sodium entry when GRNs contact salt.

The structural similarity of IRs and iGluRs might provide hints for how polyamines could activate IRs. Polyamines are released from presynaptic terminals and can thus interact with the extracellular domains of synaptic iGluRs [68]. Such interactions appear to modulate the activity of some iGluRs (e.g., [69]). For instance, spermine can potentiate the activity of NMDA (N-methyl-D-aspartate) receptor by binding at a site within the extracellular domains of one of the subunits, the NR1 subunit [69–71]. Comparison of the putative structures of IR41a and IR76b and the structure of the NR1 subunit shows that these receptors share a high structural similarity (S8 Fig). It is thus conceivable that polyamine activation of IR41a and IR76b follows a similar mechanism as polyamine potentiation of NMDAR [72]. Structure–function analysis guided by studies on the NMDAR will help to test this model. It is certainly exciting to speculate that the binding and modulation by polyamines has been acquired early on in the evolution of iGluRs and further optimized in specific IRs.

Materials and Methods

Fly Rearing and Lines

D. melanogaster stocks were raised on conventional cornmeal-agar medium at 25°C temperature and 60% humidity and a 12 hr light:12 hr dark cycle. Following fly lines were used to obtain experimental groups of flies in the different experiments:

(1) Canton S

(2) w^{1118}

(3) $orco^{1}$

(4) *eyflp; FRT82B CL / FRT82B*

(5) *eyflp; FRT82B CL / FRT82B* ato^{1}

(6) $IR8a^{1}$;Bl^{1} L^{2}/*CyO*

(7) *w*;* $IR25a^{1}$/*CyO*

(8) *w*;* $IR25a^{2}$/*CyO*

(9) $y^{1}w^{*}$;*P(w[+mC] = UAST-YFP.Rab39.S23N]IR76b*^{05}

(10) y^{1}*w[67c23];Mi[ET1]IR76b [MB00216]*

(11) *w*;*$IR76b^{1}$

(12) *w*;*$IR76b^{2}$

(13) *w*;+; UAS-Kir2.1::eGFP*

(14) *w*;P[IR41a-GAL4.2474]attP40;TM2/TM6B,Tb*

(15) *w*; P[IR76b-GAL4.916]226.8;TM2/TM6B,Tb*

(16) *w*;P[IR76b-QF.1.5]2*

(17) w^{1118}*;UAS-mCD8GFP,QUAS-mtd-tomato-3xHA*

(18) w^{1118}*;UAS-IR41a-RNAi:P[KK104134]VIE-260B (a)* and

$y^{1}v^{1}$*; P[y[+t7.7] v[+t1.8] = TRiP.HMJ21838]attP40 (b)*

(19) *w^{1118};Poxn[ΔM22-B5]/CyO*, (a genomic deletion of the *Poxn* locus; a detailed description of the allele can be found in [73])

(20) *w^{1118};Poxn[ΔM22-B5];P[w6 Poxn_resc]superA158-119*, (the same genomic deletion of the *Poxn* locus in combination with a genomic rescue construct that rescues all aspects of *Poxn* loss of function; a detailed description of the allele can be found in [73])

(21) *w^{*};GR5a-Gal4-6/CyO*

(22) *w^{*};GR64f-Gal4/CyO*

(23) *w^{*};GR66a-Gal4/Cyo;TM2/TM6B, Tb*

(24) *w^{1118}; PBac[w[+mC] = WH]IR31a[f06333]*

(25) *y^{1} w[67c23]; Mi[ET1]IR75a[MB00253]*

(26) *$y^{1}w^{*}$; Mi[y[+mDint2] = MIC]IR75a[MI00303]*

(27) *w^{1118};Mi[ET1]IR75d[MB04616]*

(28) *$y^{1}w^{*}$;Mi[y [+mDint2] = MIC]IR84a[MI00501]*

(29) *w^{1118}; Mi[ET1]IR92a[MB03705]*

(30) *w^{*};UAS-mCD8GFP*

(31) *w^{*};UAS-GCaMP6f*

(32) *w^{*}; Bl^{1}/CyO; P[w[+mC] = UAS-Ir76b.A]298.7* and

w^{*}; P[w[+mC] = UAS-Ir76b.Z]2/CyO; TM2/TM6B, Tb

The majority of the lines were obtained from Bloomington (http://flystocks.bio.indiana.edu/) or the VDRC stock center (http://stockcenter.vdrc.at). The Poxn lines were a gift by Werner Boll and IR76b-QF was a gift by Craig Montell.

Behavioral Assays for *Drosophila melanogaster*

T-maze assay. The use of the T-maze assay is indicated in all figures with a fly head schematic with red colored antennae to show that polyamine preference depends on OSNs on the antenna. Five to seven days old flies raised at 25°C were used for all experiments with the exception of experiments where RNAi was used. For these experiments, flies were raised at 30°C to increase the efficacy of RNAi. Flies were tested in groups of ~60 (30 females and 30 males or 60 females) in a T-maze and were allowed 1 min to respond to stimuli. Experimentation was carried out within climate controlled boxes at 25°C and 60% rH in the dark. 50 μl of fresh odor solution at different concentrations diluted in distilled water applied on Whatman chromatography paper was provided in the odor tube while 50 μl of distilled water (polyamine solvent) applied on Whatman chromatography paper was placed into the control tube. Unless otherwise indicated 1 mM (according to photo-ionization detector (PID) measurements corresponds to ~10 ppm) of either putrescine or cadaverine were used. After experimentation, the number of flies in each tube was counted. An olfactory 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 by the total number of flies. Statistical analysis was performed using two-way ANOVA and the Bonferroni multiple comparisons post-hoc test using Prism GraphPad 6.

Oviposition assay. The oviposition assay is indicated in all figures by an illustration of the fly head with a red-labeled proboscis showing that oviposition preference depends on labellar

taste neurons. In addition, oviposition assays are shown in simple schemes in most figures. Here, the gray circle shows the oviposition plate filled with 1% agarose and the colored squares indicate the addition of putrescine or cadaverine on one half of the plate. Unless otherwise stated 1 mM of polyamine was used in all assays. Five to seven day old flies raised at 25°C were used for all experiments with the exception of experiments where RNAi was used. For these experiments, flies were raised at 30°C to increase the efficacy of RNAi. Mated female flies, reared on standard cornmeal medium at 25°C and 60% rH, were separated on ice from male flies at day 4 d posteclosion. Female flies were kept for two more days on fly food and used on day 6 for the oviposition assays. 1% low melting agarose was poured in 60 x 15 mm petri dish, and two halves were marked with a permanent marker on the bottom of the dish. 50 μl of polyamine solution at different concentrations was applied on one site (test) of the dish. In initial experiments, we also tested odor mixed into 1% low melting agarose compared to agarose only and obtained the same results as with applying the polyamine solution onto the hardened agarose. 60 female flies were put in a gauzed top round cage and the cage was closed with the test petri dish. Flies were kept for exactly 16 h in a light:dark cycle at controlled temperature and humidity conditions. An oviposition PI was calculated by subtracting the number of eggs on the test site from the number of eggs on the control site and normalized by the total number of eggs. Statistical analysis was performed using two-way ANOVA and the Bonferroni multiple comparisons posthoc test using Prism GraphPad 6.

Position-oviposition assay. The same experimental set up as used for the regular oviposition assays was placed in 75 x 45 x 47 cm black box with infrared light at 25°C and 60% rH for 3 h. Behavior of flies was video-tracked, and position preference of flies was quantified in 30 min time intervals. The number of eggs was quantified also every 30 min for 3 h. Position and oviposition preference indices were calculated as described above for individual time points. Statistical analysis was performed using standard two-way ANOVA and the Bonferroni multiple comparisons post-hoc test using Prism GraphPad 6.

Behavioral Assays for *Ae*. *aegypti*

Animal rearing. *Ae*. *aegypti* (Rockefeller strain) were reared at 27 ± 2°C, 70 ± 2% RH under a 12 h:12 h light:dark period. Larvae were reared in plastic containers (20 x 18 x 7 cm) and fed Superwhite fishfood (Tropical, Poland). Pupae were transferred into 20 ml plastic cups and placed into Bugdorm-1 cages (Megaview, Taiwan; 30 x 30 x 30 cm). Adults had ad libitum access to 10% sucrose presented on a filter paper. Four to six days post emergence, females were starved for 6 h prior being offered defibrillated sheep blood (Håtuna lab, Sweden) through a membrane feeding system (Hemotek Ltd, UK). After taking a full blood meal, the females were transferred to the experimental chamber for oviposition experiments. Females had ad libitum access to the sucrose solution.

Oviposition assay. Oviposition assays were performed in a climate chamber maintained under the same conditions as the rearing chamber. The assays consisted of a Bugdorm-1 cage with two oviposition cups diagonally oriented at a distance of 20 cm from each other. Four separate cups made up an oviposition cup. In the bottom, a transparent 250 ml cup (Houseware Nordic Business Association AB, Sweden), filled with 1 ml of the test compound or control (water). Two paper cups (230 ml; Clas Ohlson, Sweden), the first with a 10 mm centre hole and the second with eight 1 mm piercings, were stacked into the plastic cup. In the second paper cup, a 30 ml cup (Nolato Hertila, Sweden), with a folded filter paper (Munktell Filter AB, Sweden) and 5 ml dH2O, provided the oviposition substrate. Four days post blood meal, female mosquitoes were transferred from the rearing cage to individual test cages where they were deprived of the sucrose solution. Female *Ae*. *aegypti* were then allowed to oviposit for three

consecutive L:D cycles after which the number of eggs laid in the test cup and control cup were counted. The test compounds, putrescine and cadaverine, tested at 1, 10, and 100 μM, and the control, were refreshed on a daily basis. Statistical analysis was performed using Minitab (Minitab Inc, State College, Pennsylvania, USA).

Anatomy. Adult fly brains were dissected, fixed and stained as described previously [74]. Briefly, brains were dissected in cold PBS, fixed with paraformaldehyde (2%, overnight at 4°C or for 2 h at RT), washed in PBS, 0.1% Triton X-100, 10% donkey serum, and stained overnight at 4°C or for 2 h at RT with the primary and after washes in PBS, 0.1% Triton X-100 with the secondary antibody using the same conditions. All microscopic observations were made at an Olympus FV-1000 confocal microscope. Images were processed using ImageJ and Photoshop. The following antibodies were used: chicken anti-GFP (molecular probes, 1:100), rabbit anti-Dsred (Clontech, Living colors DsRed polyclonal AB, 1:200), and rat anti-N-cadherin (anti-N-cad DN-Ex #8, Developmental Studies Hybridoma Bank, 1:100). Secondary antibodies used were: anti-chicken Alexa 488 (molecular probes, 1:250) and anti-rabbit Alexa 549 (molecular probes, 1:250), respectively.

In vivo calcium imaging. For calcium imaging experiments, GCaMP6f was expressed under the control of IR41a-Gal4 or IR76b-Gal4. In vivo preparations of flies were prepared according to a method previously described [74]. In vivo preparations were imaged using a Leica DM6000FS fluorescent microscope equipped with a 40x water immersion objective and 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 s with 4 x 4 binning mode. During all measurements the exposure time was kept constant at 20 ms. For all experiments with odor stimulation, the stimulus was applied 5 s after the start of each measurement. A continuous and humidified airstream (2000 ml/min) was delivered to the fly throughout the experiment via an 8 mm diameter glass tube positioned 10 mm away from the preparation. A custom-made odor delivery system (Smartec, Martinsried), consisting of mass flow controllers (MFCs) and solenoid valves, was used for delivering a continuous airstream and stimuli in all experiments. In all experiments stimuli were delivered for 500 ms and during stimulations the continuous flow was maintained at 2,000 ml/min. A continuous and humidified airstream (2,000 ml/min) was delivered to the fly head throughout the experiment via an 8 mm diameter glass tube positioned 10 mm away from the preparation. For putrescine stimulation, 1 ml of odor solution (0 mM (air stimulus), 1 mM, 10 mM, 100 mM diluted in pure water) was filled in the odor delivery cup and the collected airspace odor was injected into the main airstream for 500 ms without changing airstream strength. PID measurements suggested that 100 mM corresponded to ~10 ppm of odor and therefore compared best to the optimal concentration used in behavioral experiments. To measure the fluorescent intensity change, the ROI 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 basal 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 softwares, respectively.

Imaging with taste stimuli was performed in a similar setup as described above with some modifications. The flies expressing GCaMP-fluorescence under IR76b-Gal4 were prepared according to a method previously described [75]. The proboscis of the fly was pulled out by suction and fixed by gluing to prevent it from going back into the head capsule. 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 a stereomicroscope. A drop of taste was delivered to touch the labellum. The stimulus was applied for 1 s after the start of each measurement. Imaging of leg IR76b taste neurons was carried out as previously described [76]. Briefly, legs were detached from the body of the fly and glued to double-sided tape on a slide. 100 μl of water were added to the free lower segments of the leg to help focus the preparation on the IR76b neurons. For stimulation, different concentrations of 100 μl polyamine solution were added as 2 x to the water. All analysis and statistical tests were done using Excel and GraphPad6 Prism softwares as described above.

Electrophysiology. Tip recordings were carried out as previously described tip [77] with minor modifications. Female flies at 5–7 d after eclosion were used for all experiments. Legs and wings were removed to reduce movement. For recording, the flies were wedged into the narrow neck of a 200 μl pipette tip. 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 the 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 (10–2,800 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 [74].

The numerical data used in all main and supplementary figures are included in S1 Data.

Supporting Information

S1 Data. The excel spreadsheet contains, in separate sheets, the underlying numerical data and statistical analysis for the following figures with their relative panels: Fig 1, Fig 2, Fig 3, Fig 4, Fig 5, Fig 6, S1 Fig, S2 Fig, S3 Fig, S4 Fig, S5 Fig, S6 Fig, S7 Fig and S8 Fig.
(XLSX)

S1 Fig. Olfactory attraction to polyamines. (A) Dose-dependent (0.001–1,000 mM) olfactory preference of Canton S flies to putrescine and cadaverine. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (B) Analogous to group olfactory behavior, single flies chose polyamine side over non-polyamine side in T-maze assay. Graphs show preference of polyamine over non-polyamine side by single fly in 30 T-maze trials. (C) Polyamine-associated attraction of *Drosophila* is dependent on the main olfactory organ, the antenna. Bars show olfactory PI of wild type flies with or without antenna to 1 mM putrescine and cadaverine in the T-maze assay. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (D) ORs are not required for polyamine attraction. Bars show olfactory PI of *Orco-/-* flies to putrescine and cadaverine. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (E) IRs mediate olfactory attraction to polyamines. Bars show olfactory PI of control (*wt*: *eyflp; FRT82B/FRT82B cell lethal*) and mosaic atonal mutant (*ato-/-: eyflp; FRT82B ato[1]/FRT82B cell lethal*) flies to putrescine and cadaverine in the T-maze assay.

Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (F) Olfactory PI of putative candidate receptor mutants (*IR31a-/-*, *IR75a-/-*, *IR75d-/-*, *IR84a-/-* and *IR92a-/-*) for polyamine detection in the T-maze assay. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). All p-values were calculated via standard t test (ns > 0.05, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).
(TIF)

S2 Fig. IR76b-Gal4 and IR76b-QF show highly overlapping expression pattern. Comparison of GFP/RFP signals in *IR76b-QF;QUAS-mdTomato-3xHa* and *IR76b-Gal4;UASmCD8GFP* flies. Neurons in legs, antenna, labellum, and wings always show both GFP and RFP staining. The expression is by and large overlapping with few exceptions where green cells appear to be stained more strongly than red cells. Furthermore, the distribution of the fluorescence within the cells is different because of the nature of the respective reporter protein. Confocal images were taken at an Olympus Confocal microscope. Step size 0.5 μM. Single sections or small stacks are shown.
(TIF)

S3 Fig. Non-Ir41a IR76b-expressing OSNs do not respond to putrescine. (A) Prestimulation fluorescence micrograph showing IR76b OSN axon-innervated glomeruli. VC5 is innervated by IR41a OSNs, which are polyamine responsive. The indicated ROI marks another IR76b innervated glomerulus that was analyzed for a putative response to putrescine. (B) Quantification of peak ΔF responses in mutant ($IR76b^{1/1}$) and control flies that express *UAS-GCaMP6f* under the control of *IR76b-Gal4*. Boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. $p > 0.05$ by unpaired t test with Welch correction (n = 6). (C) Average activity trace of non-VC5 glomerulus. The gray bar represents the 0.5 second stimulation period. Dark colored line is the average response, and the light shade is the SEM.
(TIF)

S4 Fig. IR76b receptor is required for oviposition preference. (A) Average total number of eggs after 16 h in oviposition assay of Canton S females. Female Canton S flies prefer to lay eggs on the control side (1% low melting agarose only, shown in gray) compared to polyamine side (shown in orange). The number of eggs is averaged for each stimulus (nv = v8 ± SEM, 60 ♀ flies/trial). (B) Dose-dependent (0.001–1,000 mM) oviposition preference of Canton S to putrescine and cadaverine (magenta: putrescine, green: cadaverine, gray: agarose). Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial). (C) Average number of eggs on stimulus and nonstimulus (1% low melting agarose, shown in gray bar) sites at different concentrations in oviposition assay. (D) Average total number of eggs in oviposition assay of single Canton S female fly (n = 30 ± SEM, 1 ♀ flies/trial). (E) Average total number of eggs in oviposition assay of Canton S female fly (n = 8 ± SEM, 60 ♀ flies/trial). (F) Addition of polyamines significantly increases the attractiveness of sugar as egg-laying substrate compared to sugar alone. Graphs show number of eggs in the presence and absence of polyamines (putrescine) after 16 h, number of eggs are averaged for each stimulus (n = 8 ± SEM, 10 ♀ flies/trial). (G) Polyamine-triggered oviposition choice behavior depends on the sense of taste (labellum). Bars show average total number of eggs for antenna, legs, wings, and labellum ablated flies (− depicts ablation, + shows non ablation). (n = 8 ± SEM, 60 ♀ flies/trial). (H) Egg numbers of 16 h oviposition assay of *Poxn* mutants ($Poxn^{-/-}$) and $Poxn^{-/-}$ rescues SuperA. (I) Oviposition PI of odorant coreceptor mutant ($Orco^{-/-}$) and mutants of putative ionotropic coreceptor mutants ($IR8a^{-/-}$, $IR25a^{-/-}$, and $IR76b^{-/-}$) to polyamines. The gray box serves as a control and shows that females show no side preference on plain agar plates. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial). (J) Egg numbers of S4I Fig. PIs are averaged (n = 8 ± SEM, 60 ♀

flies/trial). (K) Egg numbers of 16 h oviposition assay of different mutants and controls corresponding to Fig 3H–3I. Number of eggs are averaged ($n = 8 \pm$ SEM). (L) Average number of eggs after 16 h oviposition assay corresponding to Fig 3J. Number of eggs is averaged ($n = 8 \pm$ SEM).
(TIF)

S5 Fig. IR76b and Gr66a are not coexpressed in labellar taste neurons. Expression analysis of IR76b (*IR76b-QF;QUAS-mdTomato-3xHa*) and Gr66a (*Gr66a-Gal4;UASmCD8GFP*) in proboscis, brain (SEZ), and legs. No coexpression could be observed in neurons of the labellum and axons projecting from these to the SEZ innervated neighboring regions and did not overlap. Coexpressing cells were occasionally found in the leg. The legs, however, were redundant for fly's taste preference behavior. Confocal images were taken at an Olympus Confocal microscope. Step size 0.5 μM. Single sections or small stacks are shown.
(TIF)

S6 Fig. IR76b neurons mediate responses to low salt concentration. (A) Quantification of peak responses of GCaMP6f-fluorescence (in %ΔF/F) in the ROI 1 and ROI 2 areas, respectively, when *IR76b-Gal4;UAS-GCaMP6f* female flies were stimulated with distilled water or 50 mM $NaCl_2$ ($n = 6 \pm$ SEM). (B) Representative image of calcium responses of a leg expressing GCaMP under the control of IR76b-Gal4 stimulated with polyamine. (C) Average response trace of tarsal IR76b neurons ($n = 8 \pm$ SEM). (D) Tarsal IR76b neurons respond to high concentrations of polyamines ($n = 8 \pm$ SEM). (E) Quantification of peak responses (in %ΔF/F) of *IR76b* mutant and heterozygous controls in the ROI 1 and ROI 2 areas, respectively to 50 mM $NaCl_2$ stimulation ($n = 6 \pm$ SEM). Boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. All *p*-values were calculated via Student's *t* test (ns > 0.05, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$).
(TIF)

S7 Fig. Egg numbers after 3 h of egg laying. (A–D) Graphs display the number of eggs females laid in the first 3 h of a position–oviposition assay on control (1% low melting agarose) or stimulus (agarose plus polyamines) site corresponding to Fig 5. Note that the low number of eggs in some test lines reflects the slow start of oviposition due to genetic or other manipulations. Egg numbers caught up later significantly (see for instance S4 Fig). However, it is important to interpret some of the oviposition preferences with caution due to the low number of eggs. Number of eggs are averaged for each time point ($n = 8 \pm$ SEM, 60 ♀ flies/trial).
(TIF)

S8 Fig. Structural similarity of NMDA receptor subunit NR1 and polyamine receptors. (A) Schematic presentation of putative structure of the NR1 subunit of the NMDA receptor. Acidic amino acids have been marked in red. In particular, several acidic residues in domains S1, S2, R1, and R2 have been implicated in polyamine-mediated potentiation [69–71]. (B) Putative structure of IR76b. (C) Putative structure of IR41a. (D) Structure comparison of NR1 with IR76b, (E) with IR41a, and (F) structure comparison between IR41a and IR76b.
(TIF)

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Conceived and designed the experiments: ICGK AH HKÜ MZ RI. Performed the experiments: AH MZ HKÜ TS EQ. Analyzed the data: AH MZ HKÜ TS EQ RI ICGK. Contributed reagents/materials/analysis tools: AH MZ HKÜ TS EQ RI ICGK NG. Wrote the paper: ICGK NG AH.

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Ionotropic Chemosensory Receptors Mediate the Taste and Smell of Polyamines.

Hussain A*, Zhang M*, Üçpunar HK, Svensson T, Quillery E, Gompel N, Ignell R, Grunwald Kadow IC. (2016) PLoS Biol 14(5): e1002454 (* Equal contribution)

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Supplementary Figures

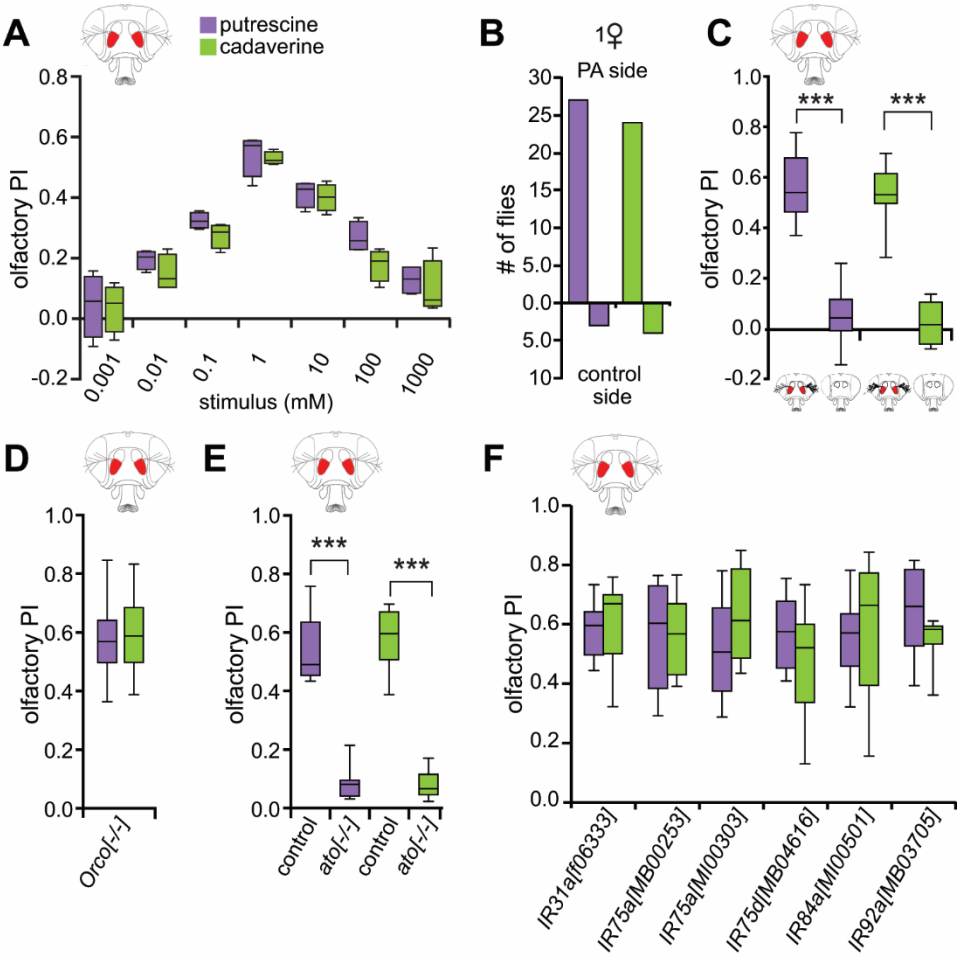

Fig. S1 Olfactory attraction to polyamines.

(A) Dose-dependent (0.001–1,000 mM) olfactory preference of Canton S flies to putrescine and cadaverine. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (B) Analogous to group olfactory behavior, single flies chose polyamine side over non-polyamine side in T-maze assay. Graphs show preference of polyamine over non-polyamine side by single fly in 30 T-maze trials. (C) Polyamine-associated attraction of *Drosophila* is dependent on the main olfactory organ, the antenna. Bars show olfactory PI of wild type flies with or without antenna to 1 mM putrescine and cadaverine in the T-maze assay. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (D) ORs are not required for polyamine attraction. Bars show olfactory PI of *Orco-/-* flies to putrescine and cadaverine. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (E) IRs mediate olfactory attraction to polyamines. Bars show olfactory PI of control (*wt*: *eyflp; FRT82B/FRT82B cell lethal*) and mosaic atonal mutant (*ato-/-: eyflp; FRT82B ato[1]/FRT82B cell lethal*) flies to putrescine and cadaverine in the T-maze assay. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). (F) Olfactory PI of putative candidate receptor mutants (*IR31a-/-*, *IR75a-/-*, *IR75d-/-*, *IR84a-/-* and *IR92a-/-*) for polyamine detection in the T-maze assay. Box plots show median and upper/lower quartiles (n = 8, 60 ♀/trial, 30 ♀ and 30 ♂). All p-values were calculated via standard t test (ns > 0.05, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

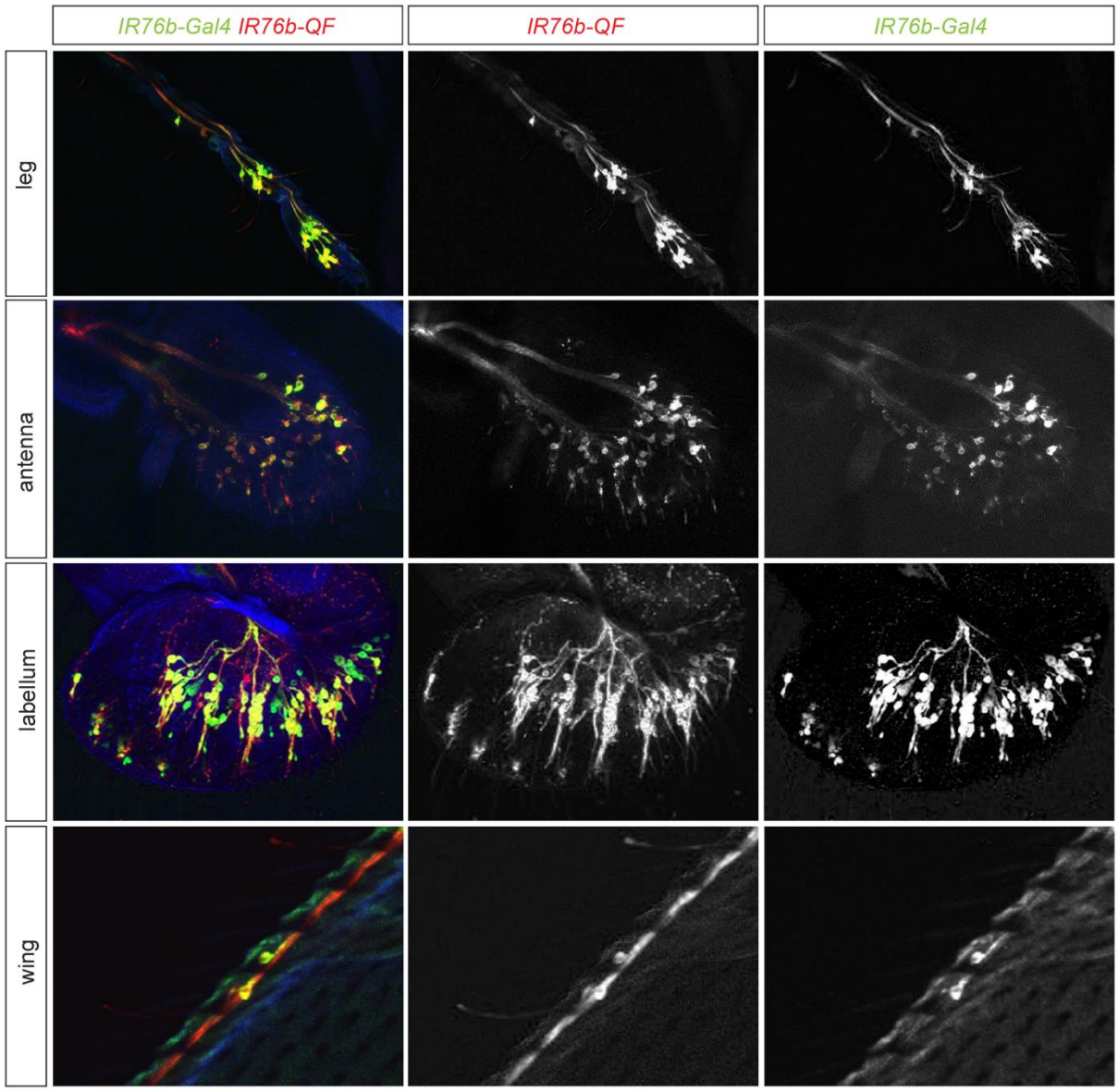

Fig. S2 IR76b-Gal4 and IR76b-QF show highly overlapping expression pattern.

Comparison of GFP/RFP signals in *IR76b-QF;QUAS-mdTomato-3xHa* and *IR76b-Gal4;UASmCD8GFP* flies. Neurons in legs, antenna, labellum, and wings always show both GFP and RFP staining. The expression is by and large overlapping with few exceptions where green cells appear to be stained more strongly than red cells. Furthermore, the distribution of the fluorescence within the cells is different because of the nature of the respective reporter protein. Confocal images were taken at an Olympus Confocal microscope. Step size 0.5 µM. Single sections or small stacks are shown.

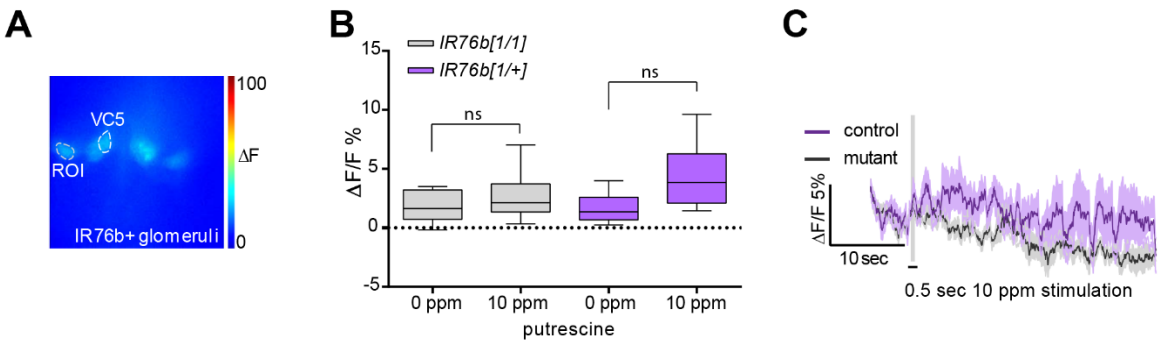

Fig. S3 Non-Ir41a IR76b-expressing OSNs do not respond to putrescine.

(A) Prestimulation fluorescence micrograph showing IR76b OSN axon-innervated glomeruli. VC5 is innervated by IR41a OSNs, which are polyamine responsive. The indicated ROI marks another IR76b innervated glomerulus that was analyzed for a putative response to putrescine. (B) Quantification of peak ΔF responses in mutant (*IR76b*$^{1/1}$) and control flies that express *UAS-GCaMP6f* under the control of *IR76b-Gal4*. Boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. $p > 0.05$ by unpaired *t* test with Welch correction ($n = 6$). (C) Average activity trace of non-VC5 glomerulus. The gray bar represents the 0.5 second stimulation period. Dark colored line is the average response, and the light shade is the SEM.

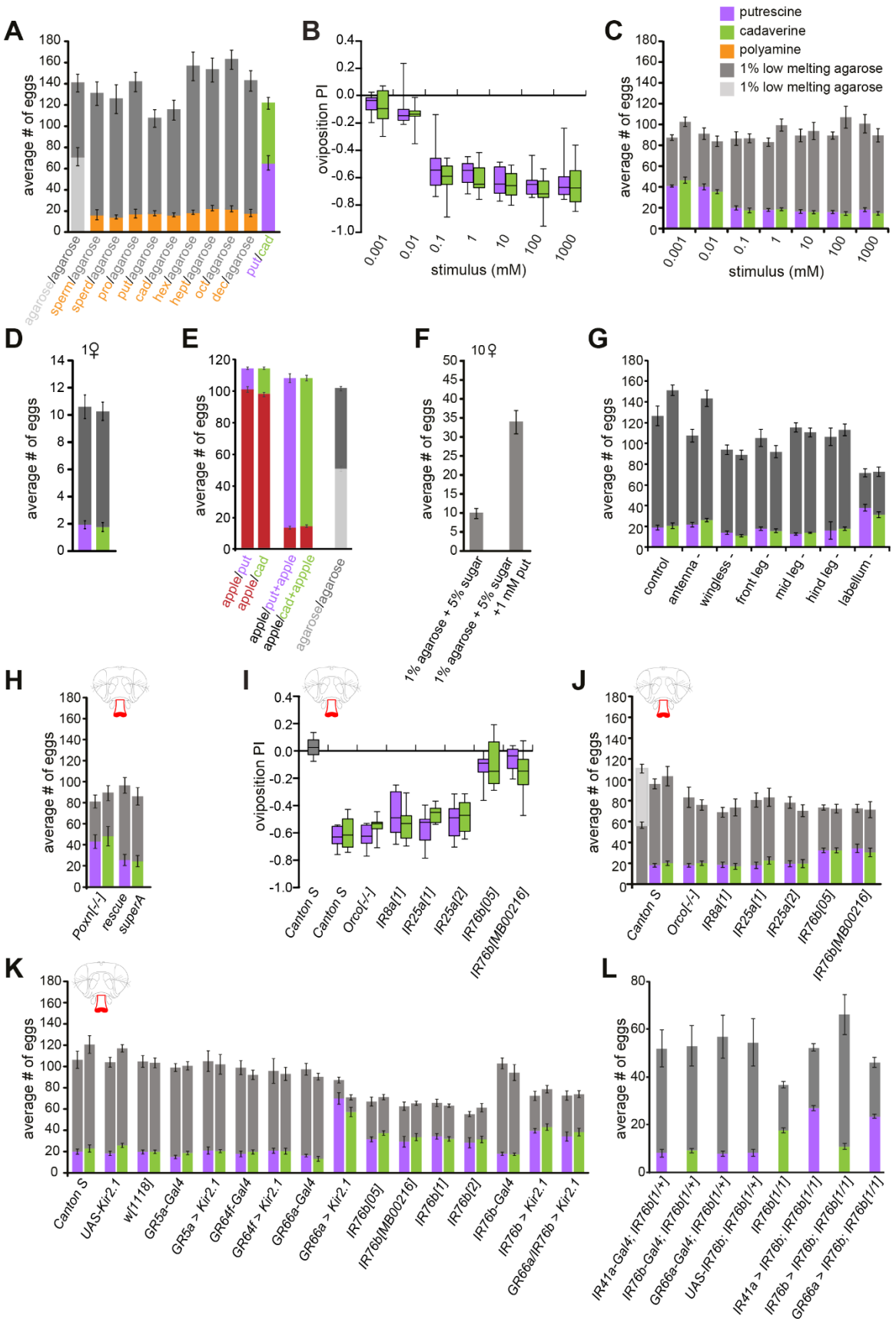
A
average # of eggs
agarose/agarose
sperm/agarose
sperd/agarose
pro/agarose
put/agarose
cad/agarose
hex/agarose
hep/agarose
oct/agarose
ded/agarose
put/cad
B
oviposition PI
stimulus (mM)
0.001
0.01
0.1
1
10
100
1000
C
putrescine
cadaverine
polyamine
1% low melting agarose
1% low melting agarose
average # of eggs
stimulus (mM)
D
1♀
average # of eggs
E
average # of eggs
apple/put
apple/cad
apple/put+apple
apple/cad+appple
agarose/agarose
F
10♀
average # of eggs
1% agarose + 5% sugar
1% agarose + 5% sugar +1 mM put
G
average # of eggs
control
antenna -
wingless -
front leg -
mid leg -
hind leg -
labellum -
H
average # of eggs
Poxn[-/-]
rescue
superA
I
oviposition PI
Canton S
Canton S
Orco[-/-]
IR8a[1]
IR25a[1]
IR25a[2]
IR76b[05]
IR76b[MB00216]
J
average # of eggs
Canton S
Orco[-/-]
IR8a[1]
IR25a[1]
IR25a[2]
IR76b[05]
IR76b[MB00216]
K
average # of eggs
Canton S
UAS-Kir2.1
w[1118]
GR5a-Gal4
GR5a > Kir2.1
GR64f-Gal4
GR64f > Kir2.1
GR66a-Gal4
GR66a > Kir2.1
IR76b[05]
IR76b[MB00216]
IR76b[1]
IR76b[2]
IR76b-Gal4
IR76b > Kir2.1
GR66a/IR76b > Kir2.1
L
average # of eggs
IR41a-Gal4; IR76b[1/+]
IR76b-Gal4; IR76b[1/+]
GR66a-Gal4; IR76b[1/+]
UAS-IR76b; IR76b[1/+]
IR76b[1/1]
IR41a > IR76b; IR76b[1/1]
IR76b > IR76b; IR76b[1/1]
GR66a > IR76b; IR76b[1/1]

Fig. S4 IR76b receptor is required for oviposition preference.

(A) Average total number of eggs after 16 h in oviposition assay of Canton S females. Female Canton S flies prefer to lay eggs on the control side (1% low melting agarose only, shown in gray) compared to polyamine side (shown in orange). The number of eggs is averaged for each stimulus (*n*v = v8 ± SEM, 60 ♀ flies/trial). (B) Dose-dependent (0.001–1,000 mM) oviposition preference of Canton S to putrescine and cadaverine (magenta: putrescine, green: cadaverine, gray: agarose). Box plots show median and upper/lower quartiles (*n* = 8, 60 ♀/trial). (C) Average number of eggs on stimulus and nonstimulus (1% low melting agarose, shown in gray bar) sites at different concentrations in oviposition assay. (D) Average total number of eggs in oviposition assay of single Canton S female fly (*n* = 30 ± SEM, 1 ♀ flies/trial). (E) Average total number of eggs in oviposition assay of Canton S female fly (*n* = 8 ± SEM, 60 ♀ flies/trial). (F) Addition of polyamines significantly increases the attractiveness of sugar as egg-laying substrate compared to sugar alone. Graphs show number of eggs in the presence and absence of polyamines (putrescine) after 16 h, number of eggs are averaged for each stimulus (*n* = 8 ± SEM, 10 ♀ flies/trial). (G) Polyamine-triggered oviposition choice behavior depends on the sense of taste (labellum). Bars show average total number of eggs for antenna, legs, wings, and labellum ablated flies (− depicts ablation, + shows non ablation). (*n* = 8 ± SEM, 60 ♀ flies/trial). (H) Egg numbers of 16 h oviposition assay of *Poxn* mutants ($Poxn^{-/-}$) and $Poxn^{-/-}$ rescues SuperA. (I) Oviposition PI of odorant coreceptor mutant ($Orco^{-/-}$) and mutants of putative ionotropic coreceptor mutants ($IR8a^{-/-}$, $IR25a^{-/-}$, and $IR76b^{-/-}$) to polyamines. The gray box serves as a control and shows that females show no side preference on plain agar plates. Box plots show median and upper/lower quartiles (*n* = 8, 60 ♀/trial). (J) Egg numbers of S4I Fig. PIs are averaged (*n* = 8 ± SEM, 60 ♀ flies/trial). (K) Egg numbers of 16 h oviposition assay of different mutants and controls corresponding to Fig 3H–3I. Number of eggs are averaged (*n* = 8 ± SEM). (L) Average number of eggs after 16 h oviposition assay corresponding to Fig 3J. Number of eggs is averaged (*n* = 8 ± SEM).

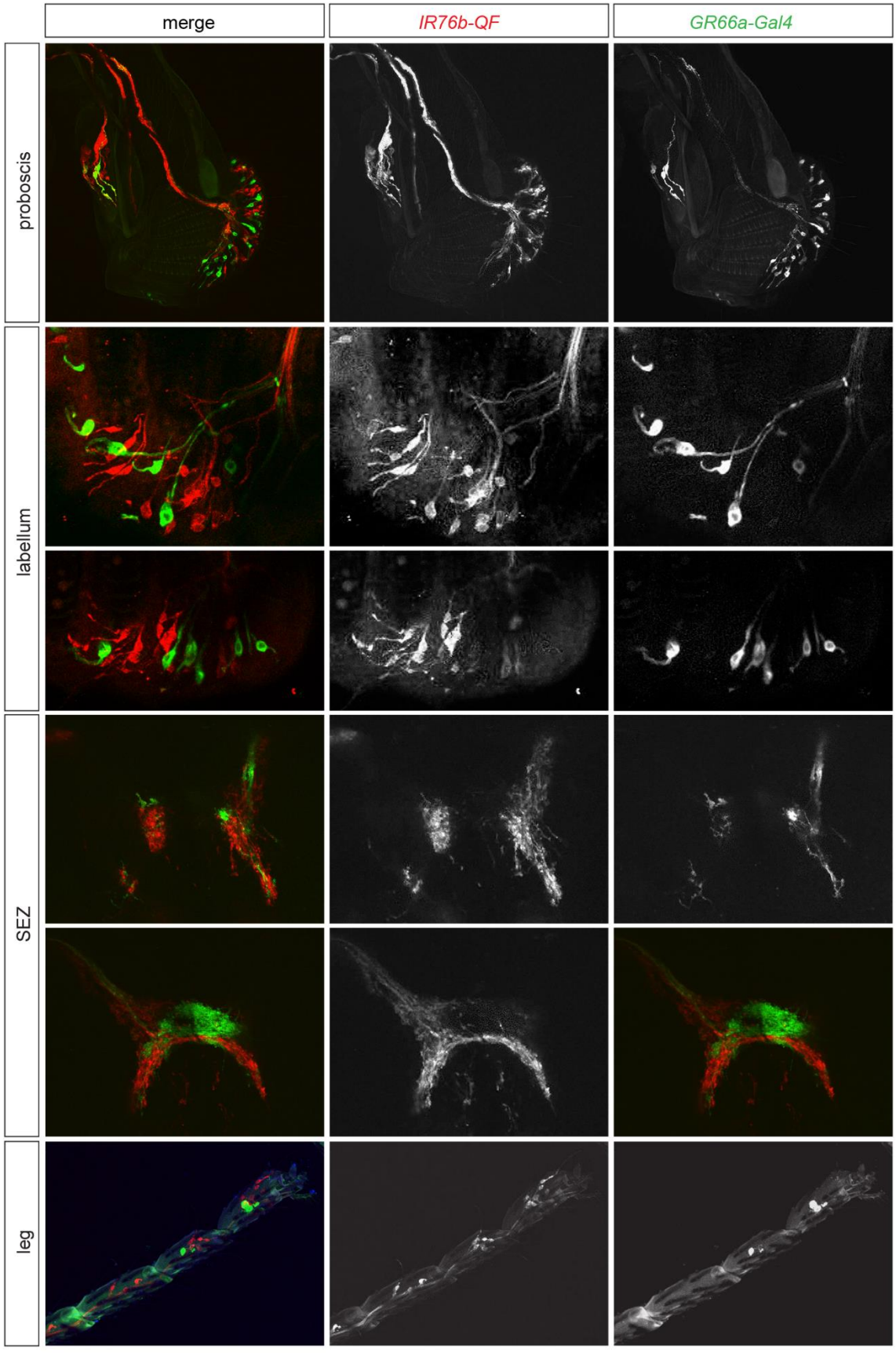
merge
IR76b-QF
GR66a-Gal4
proboscis
labellum
SEZ
leg

Fig. S5 IR76b and Gr66a are not coexpressed in labellar taste neurons.

Expression analysis of IR76b (*IR76b-QF;QUAS-mdTomato-3xHa*) and Gr66a (*Gr66a-Gal4;UASmCD8GFP*) in proboscis, brain (SEZ), and legs. No coexpression could be observed in neurons of the labellum and axons projecting from these to the SEZ innervated neighboring regions and did not overlap. Coexpressing cells were occasionally found in the leg. The legs, however, were redundant for fly's taste preference behavior. Confocal images were taken at an Olympus Confocal microscope. Step size 0.5 µM. Single sections or small stacks are shown.

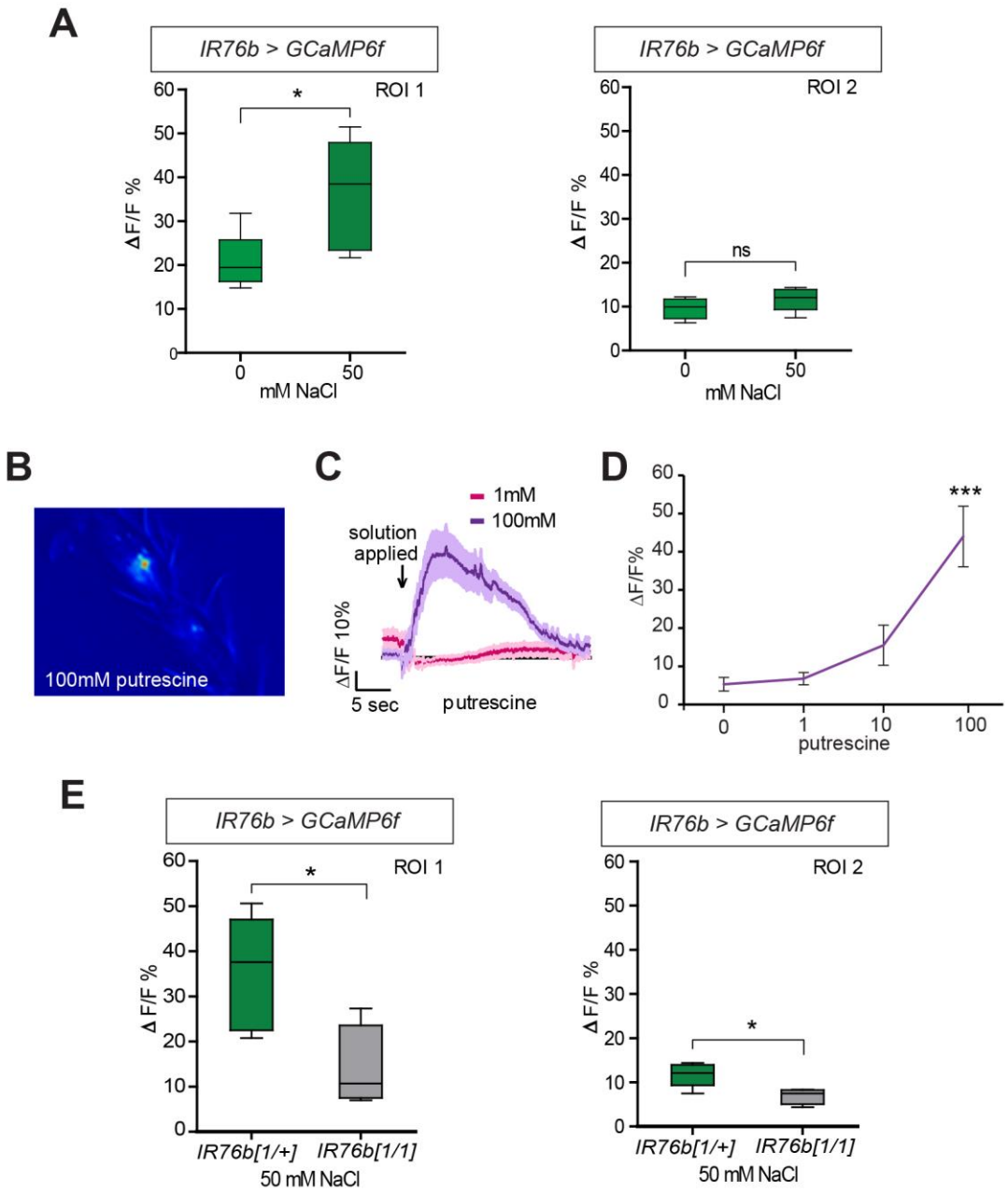

Fig. S6 IR76b neurons mediate responses to low salt concentration.

(A) Quantification of peak responses of GCaMP6f-fluorescence (in %ΔF/F) in the ROI 1 and ROI 2 areas, respectively, when *IR76b-Gal4;UAS-GCaMP6f* female flies were stimulated with distilled water or 50 mM $NaCl_2$ (n = 6 ± SEM). (B) Representative image of calcium responses of a leg expressing GCaMP under the control of IR76b-Gal4 stimulated with polyamine. (C) Average response trace of tarsal IR76b neurons (n = 8 ± SEM). (D) Tarsal IR76b neurons respond to high concentrations of polyamines (n = 8 ± SEM). (E) Quantification of peak responses (in %ΔF/F) of *IR76b* mutant and heterozygous controls in the ROI 1 and ROI 2 areas, respectively to 50 mM $NaCl_2$ stimulation (n = 6 ± SEM). Boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. All p-values were calculated via Student's t test (ns > 0.05, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

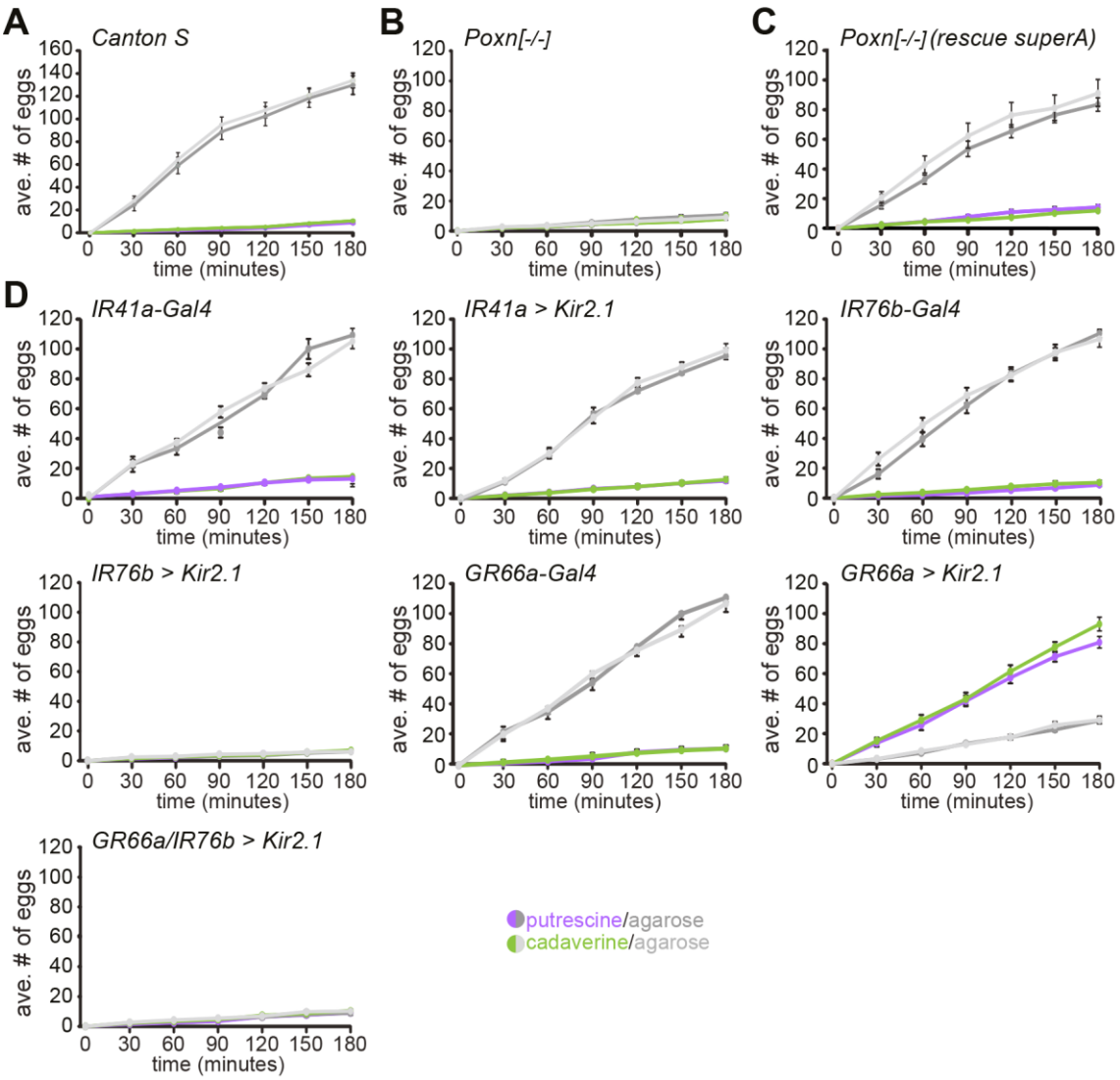

Fig. S7 Egg numbers after 3 h of egg laying.

(A–D) Graphs display the number of eggs females laid in the first 3 h of a position–oviposition assay on control (1% low melting agarose) or stimulus (agarose plus polyamines) site corresponding to Fig 5. Note that the low number of eggs in some test lines reflects the slow start of oviposition due to genetic or other manipulations. Egg numbers caught up later significantly (see for instance S4 Fig). However, it is important to interpret some of the oviposition preferences with caution due to the low number of eggs. Number of eggs are averaged for each time point (n = 8 ± SEM, 60 ♀ flies/trial).

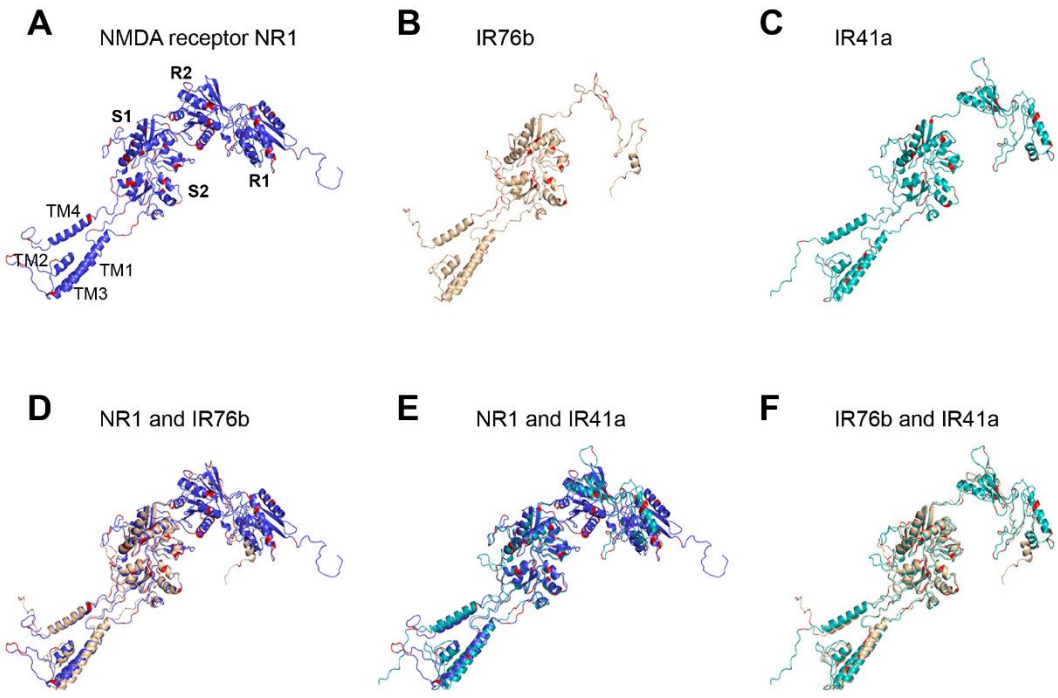

Fig. S8 Structural similarity of NMDA receptor subunit NR1 and polyamine receptors.

(A) Schematic presentation of putative structure of the NR1 subunit of the NMDA receptor. Acidic amino acids have been marked in red. In particular, several acidic residues in domains S1, S2, R1, and R2 have been implicated in polyamine-mediated potentiation [69–71]. (B) Putative structure of IR76b. (C) Putative structure of IR41a. (D) Structure comparison of NR1 with IR76b, (E) with IR41a, and (F) structure comparison between IR41a and IR76b.

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RESEARCH ARTICLE

Neuropeptides Modulate Female Chemosensory Processing upon Mating in *Drosophila*

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Abstract

A female's reproductive state influences her perception of odors and tastes along with her changed behavioral state and physiological needs. The mechanism that modulates chemosensory processing, however, remains largely elusive. Using *Drosophila*, we have identified a behavioral, neuronal, and genetic mechanism that adapts the senses of smell and taste, the major modalities for food quality perception, to the physiological needs of a gravid female. Pungent smelling polyamines, such as putrescine and spermidine, are essential for cell proliferation, reproduction, and embryonic development in all animals. A polyamine-rich diet increases reproductive success in many species, including flies. Using a combination of behavioral analysis and in vivo physiology, we show that polyamine attraction is modulated in gravid females through a G-protein coupled receptor, the sex peptide receptor (SPR), and its neuropeptide ligands, MIPs (myoinhibitory peptides), which act directly in the polyamine-detecting olfactory and taste neurons. This modulation is triggered by an increase of SPR expression in chemosensory neurons, which is sufficient to convert virgin to mated female olfactory choice behavior. Together, our data show that neuropeptide-mediated modulation of peripheral chemosensory neurons increases a gravid female's preference for important nutrients, thereby ensuring optimal conditions for her growing progeny.

Author Summary

Food choices often correlate with nutritional needs or physiological states of an animal. For instance, during pregnancy, women frequently report that their food preferences change—sometimes dramatically. In part, this change in preference is brought about by a change in the perception of smells and tastes. Research has shown that female insects also change their food and egg-laying site preferences depending on their reproductive state. However, the mechanisms that trigger these changes are not understood in either mammals or insects. We have unraveled a mechanism that changes a mated female's perception of odors and tastes and thereby adapts her choices to her reproductive state. Using the

role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Abbreviations: AL, antennal lobe; GPCR, G-protein coupled receptor; GRN, gustatory receptor neuron; IR, ionotropic receptor; MIP, myoinhibitory peptide; OSN, olfactory sensory neuron; PI, preference index; PID, photo-ionization detector; PLC, phospholipase C; PN, projection neuron; RNAi, RNA interference; ROI, region of interest; SEZ, subesophageal zone; sNPF, short neuropeptide F; sNPFR, short neuropeptide F receptor; SP, sex peptide; SPR, sex peptide receptor; VDRC, Vienna Drosophila Resource Center.

model fly *Drosophila melanogaster*, we show that mating increases females' interest in sources of specific beneficial nutrients: polyamines such as spermine and putrescine. Polyamine levels in the body are maintained by diet, microorganisms in the gut, and own synthesis. Increased levels are required during pregnancy and reproduction. Indeed, mated females were more attracted to the taste and smell of polyamines than virgins were. We found that this behavioral modulation is regulated through a secreted peptide and its receptor, whose expression rises markedly in sensory organs upon mating. This signal appears to change the intensity of how polyamine taste or smell information reaches the brain and ultimately elicits a choice. Given that odor and taste processing in mammals and insects are similar, our findings in flies can lead to a better understanding of how dynamic physiological states affect our perception of the environment and lead us to adapt our choices of food and other relevant decisions.

Introduction

The behavior of females in most animal species changes significantly as a consequence of mating. Those changes are interpreted from an evolutionary standpoint as the female's preparation to maximize the fitness of her offspring. In general, they entail a qualitative and quantitative change in her diet, as well as the search for an optimal site where her progeny will develop. In humans, the eating behavior and perception of tastes and odors of a pregnant woman are modulated in concert with altered physiology and the specific needs of the embryo [1–3]. While several neuromodulatory molecules such as noradrenaline are found in the vertebrate olfactory and gustatory systems, little is known about how reproductive state and pregnancy shape a female's odor and taste preferences [4,5]. Very recent work in the mouse showed that olfactory sensory neurons (OSNs) are modulated during the estrus cycle [6]. Progesterone receptor expressed in OSNs decreases the sensitivity of pheromone-detecting OSNs and thereby reduces the non-sexually receptive female's interest in male pheromones. The mechanisms of how mating, pregnancy, and lactation shape the response of the female olfactory and gustatory systems remain poorly understood.

The neuronal underpinnings of mating and its consequences on female behaviors have arguably been best characterized in the fruit fly *Drosophila melanogaster* [7,8]. Shortly after copulation, female flies engage in a series of post-mating behaviors contrasting with those of virgins: their sexual receptivity decreases, and they feed to accumulate essential resources needed for the production of eggs [9–12]; finally, they lay their eggs. This suite of behaviors results from a post-mating trigger located in the female's reproductive tract [12]. Sensory neurons extending their dendrites directly into the oviduct are activated by a component of the male's ejaculate, the sex peptide (SP) [13,14]. Sex peptide receptor (SPR) expressed by these sensory neurons triggers the post-mating switch [15]. Mated females mutant for *SPR* produce and lay fewer eggs while maintaining a high sexual receptivity [13–15]. In addition to SP, male ejaculate contains more than 200 proteins, which are transferred along with SP into the female. These have been implicated in conformational changes of the uterus, induction of ovulation, and sperm storage [7,16–18].

Additional SPR ligands have been identified that are not required for the canonical post-mating switch, opening the possibility that this receptor is involved in the neuromodulation of other processes [19–22]. These alternative ligands, the myoinhibitory peptides (MIPs)/allatostatin-Bs, unlike SP, have been found outside of drosophilids, in many other insect species such as the silkmoth (*Bombyx mori*), several mosquito species, and the red flour beetle (*Tribolium*

castaneum) [19]. They are expressed in the brain of flies and mosquitoes, including in the centers of olfactory and gustatory sensory neuron projections, the antennal lobe (AL), and the subesophageal zone (SEZ), respectively [19,23,24]. Although these high-affinity SPR ligands have recently been implicated in the control of sleep in *Drosophila* males and females [25], nothing thus far suggests a function in reproductive behaviors [19].

To identify optimal food and oviposition sites, female flies rely strongly on their sense of smell and taste [26–29]. *D. melanogaster* females prefer to oviposit in decaying fruit and use byproducts of fermentation such as ethanol and acetic acid to choose oviposition sites [29,30]. Their receptivity to these byproducts is enhanced by their internal state [29,31]. It was shown, for instance, that the presence of an egg about to be laid results in increased attraction to acetic acid [31]. Yet the mechanisms linking reproductive state to the modulation of chemosensory processing remain unknown.

We have examined the causative mechanisms that integrate reproductive state into preference behavior and chemosensory processing. We have focused on the perception of another class of byproducts of fermenting fruits, polyamines. Polyamines such as putrescine, spermine, and spermidine are important nutrients that are associated with reproductive success across animal species [32]. A diet high in polyamines indeed increases the number of offspring of a fly couple, and female flies prefer to lay their eggs on polyamine-rich food [33]. Importantly, we have previously characterized the chemosensory mechanisms flies use to find and evaluate polyamine-rich food sources and oviposition sites. In brief, volatile polyamines are detected by OSNs on the fly's antenna, co-expressing two ionotropic receptors (IRs), IR41a and IR76b [33,34]. Interestingly, the taste of polyamines is also detected by IR76b in labellar gustatory receptor neurons (GRNs) [33].

This beneficial role of polyamines has a well-characterized biological basis: polyamines are essential for basic cellular processes such as cell growth and proliferation, and are of specific importance during reproduction [35]. They enhance the quality of sperm and egg and are critical during embryogenesis and postnatal development [32,36]. While the organism can generate polyamines, a significant part is taken in with the diet [37,38]. Moreover, endogenous synthesis of polyamines declines with ageing and can be compensated for through a polyamine-rich diet [32]. Therefore, these compounds represent a sensory cue as well as an essential component of the diet of a gravid female fly.

Here, we show that the olfactory and gustatory perception of polyamines is modulated by the female's reproductive state and guides her choice behavior accordingly. This sensory and behavioral modulation depends on SPR and its conserved ligands, the MIPs that act directly on the chemosensory neurons themselves. Together, our results suggest that mating-state-dependent neuropeptidergic modulation of chemosensory neurons matches the female fly's decision-making to her physiological needs.

Results

Mating State Modulates the Perception of Polyamines

Males and female flies are strongly attracted to polyamines [33]. The perception of sensory stimuli, however, can be modulated and depends on behavioral context [39]. Given that polyamine-rich foods increase the number of progeny [33], we wondered whether mating state influences the perception of these important molecules. To test this, we compared olfactory and oviposition behaviors of mated to virgin female flies. In an olfactory choice assay, the T-maze, mated females showed a strong attraction to volatile polyamines, which requires their sense of smell, as we have shown in the companion paper and as previously suggested by Silbering et al. [33,34]. Virgin flies displayed a significantly altered preference for the polyamines

putrescine and cadaverine compared to mated flies (Fig 1A). While mated females preferred relatively high concentrations of polyamines typically present in fermenting fruit (1 mM or 10 ppm, [36,37]), virgin females showed strong attraction to only the lowest levels and increasing avoidance of higher levels of these odors (Fig 1B).

We next analyzed whether virgin flies would make different egg-laying choices compared to mated flies. Mated females taste polyamines with taste sensilla on their labellum and use this information during egg-laying decisions [33]. Although egg-laying substrates containing just polyamines are avoided as egg-laying substrates because of their bitter taste, polyamine-rich sugary substrates such as decaying fruit are strongly preferred over fresh fruit [33]. To assay the egg-laying preferences, we used a simple oviposition assay consisting of a plate with a plain agarose substrate (1%) that was on one-half of the egg-laying plate supplemented with the polyamines, putrescine or cadaverine (1 mM, Fig 1C, see Materials and Methods). Consistent with our dissection of polyamine perception [33], mated flies displayed a strong preference and laid the majority of their eggs on plain agarose (Fig 1C). By contrast, virgin females, albeit laying very few (and unfertilized) eggs, distributed their eggs equally between polyamine and control sides (Figs 1C and S1A). Therefore, we concluded that, while mated females actively develop a choice behavior, virgin females are indifferent to polyamines as an egg-laying substrate. Taken together, odor as well as taste perception of polyamines strongly depends on the female fly's mating state.

We have shown that a polyamine-rich diet increases the number of offspring of a fly couple [33]. These data could potentially indicate that needs arising through egg production and laying, and not exclusively or primarily through mating, drive a female to seek polyamines. We therefore first tested whether polyamine choice behavior correlated with the female's egg-laying activity and time after mating. This appeared to be the case, because mated females that had ceased to lay eggs at 14 d after mating returned to their pre-mating preference behavior and made choices that resembled the choices of virgin flies (Fig 1D). This return to virgin behavior could be due to the time elapsed after mating or to a reduction in egg-laying. To dissect the relative contribution of egg-laying activity and mating, we analyzed the preference behavior of mated *ovoD1* mutant females [40]. These females are sterile due to an atrophy of the ovaries. Mated *ovoD1* mutant females showed the same preference to polyamines in the T-maze compared to control mated females (Fig 1E). From these data, it appears that mating itself provides a key signal that changes the female's perception and stimulates her to seek polyamines.

While previous research has shown that mating state and egg-laying activity influence the choice behavior of female flies when selecting food or oviposition substrates [9,29,31], how mating state modulates neural sensitivity and processing of sensory information remains not understood. Having defined the gustatory and olfactory receptors and sensory neurons for the detection of polyamines [33], we sought to identify the mechanism that modulates this detection and processing in a mating state-dependent manner. SPR and SP are required for the classical post-mating switch (see Introduction) and changes in feeding behavior [9,10,41]. To test the role of SPR in mating-state-dependent polyamine choice behavior, we initially examined the olfactory preference and oviposition behavior of *SPR* mutant females (*Df(1)Exel6234*) [15]. Mated *SPR* mutant females showed a significantly reduced preference behavior in the T-maze (odor) as well as in oviposition assays (taste) compared to that of mated control females (Fig 1F and 1G). Importantly, *SPR* mutant males maintained the same level of attraction as wildtype control males, possibly representing the constant need of polyamines such as spermine and spermidine for sperm production (Fig 1H). These results indicated that the SPR pathway is part of the mechanism that controls mating-induced changes in the perception of the smell and taste of polyamines.

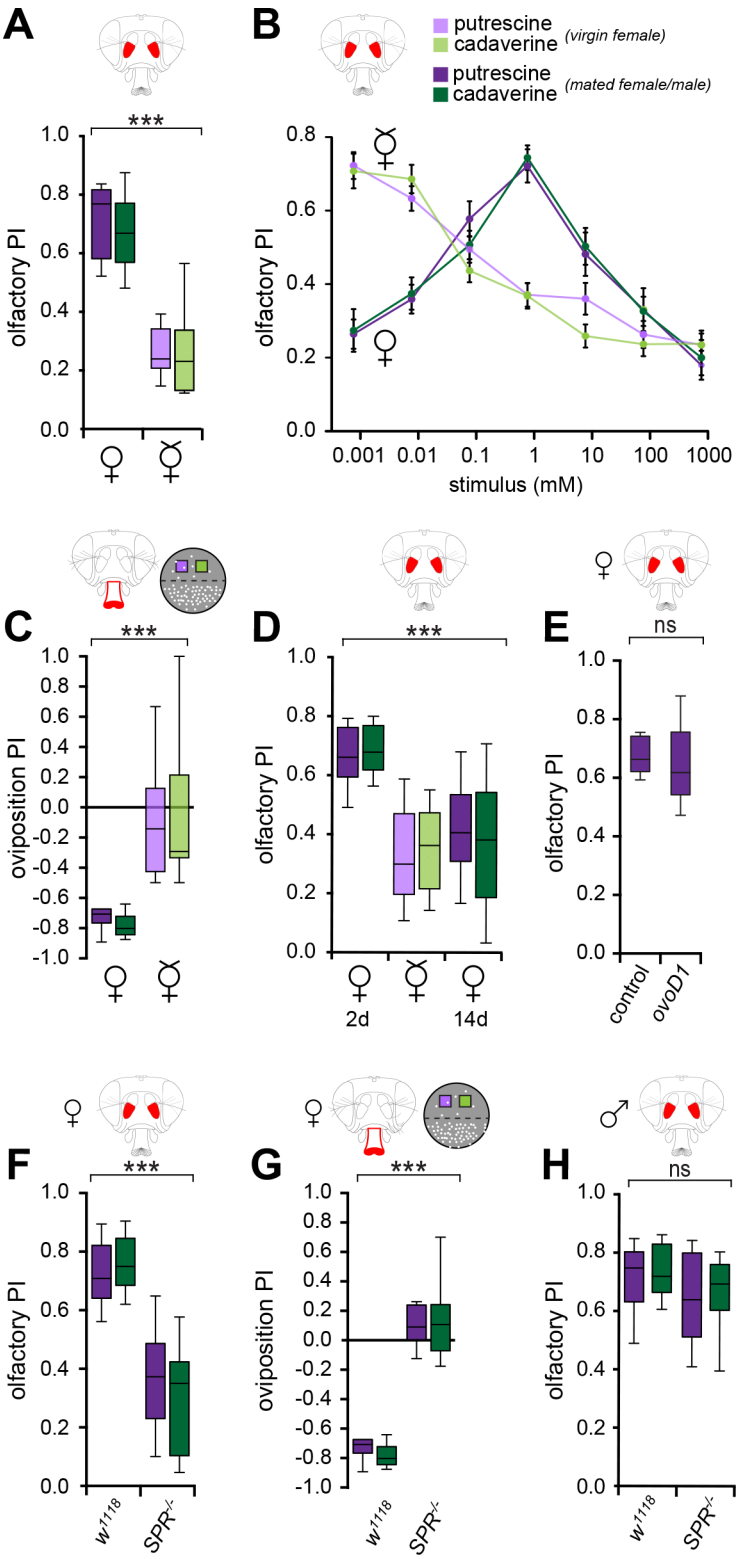

Fig 1. Mating state modulates the perception of polyamines. (A) Virgin flies are less attracted to a high polyamine concentration of 1 mM (10 ppm) as compared to mated flies. Olfactory preference index of Canton S mated (♀) and Canton S virgin (☿) females in the T-maze assay. Violet and green bars represent putrescine and cadaverine, respectively. (n = 8, 60 mated (♀) or virgin (☿) flies/trial). (B) Mated females, unlike virgins, preferred relatively high concentrations of polyamines, naturally present in fermenting fruit (1 mM or 10ppm). By contrast, virgin females were most attracted to very low concentrations of polyamine. Line graph shows

dose-dependent olfactory preference index of mated (♀) and virgin (☿) flies to polyamines. (n = 8 ± SEM, 60 mated (♀) or virgin (☿) flies/trial). (C) Virgin flies show no preference between polyamines (putrescine or cadaverine, 1 mM) and 1% low melting agarose, and deposited their low number of eggs on either site of the assay. (n = 8, 60 mated (♀) or virgin (☿) flies/trial). (D) Polyamine preference appears to correlate with the female's egg-laying activity. Graphs show olfactory preference index of females 2 d post-mating (♀, 2 d), virgin females (☿), and females 14 d post-mating (♀, 14 d) to 10 ppm of polyamine. (n = 8, 60 mated (♀, 2 d), virgin (☿) and mated (♀, 14 d) female flies/trial). (E) Mated but sterile *ovoD1* mutant females (*ovoD1*/+, Canton S) show similar attraction to polyamine odor compared to wildtype controls (+/+, Canton S). (F) Mated sex peptide receptor mutant ($SPR^{-/-}$) female flies display a significantly reduced attraction to polyamine odor (n = 8, 60 flies/trial). (G) Oviposition preference index of mated sex peptide receptor mutant ($SPR^{-/-}$) females. Mated *SPR* mutant females show indifference to polyamines. (n = 8, 60 mated (♀) flies/ trial). (H) Olfactory preference for 10 ppm polyamine of $SPR^{-/-}$ male flies is comparable to control males. Box plots show median and upper/lower quartiles (n = 8, 60 flies/trial). All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test, with the exception of (E), where p-values were calculated with an unpaired t-test (ns > 0.05, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

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G-Protein Coupled Receptor (GPCR) Signaling in Chemosensory Neurons Modulates Female Perception

Increasing evidence in different model organisms indicates that chemosensory neurons themselves are potent targets for neuromodulation [6,42–44]. Although SPR is required in specific internal sensory neurons in the female reproductive tract for the canonical post-mating switch, its rather broad expression in the nervous system, including chemosensory organs and their projection zones in the brain [15,45], prompted us to ask whether SPR signaling was acting directly in peripheral chemosensory neurons. Previous work successfully employed RNA interference (RNAi) directed against *SPR* to identify the set of sensory neurons in the female reproductive tract sufficient to trigger two important post-mating behaviors: increased egg-laying and rejection of males [13,14]. We induced RNAi against *SPR* (*UAS-SPRi*) specifically in olfactory and gustatory neurons that sense polyamines, using the driver IR76b-Gal4 [33]. Importantly, this driver was not expressed in the internal sensory neurons that require SPR to induce the mating switch (S2A and S2C Fig). Mated females of the genotype *IR76b-Gal4;UAS-SPRi* showed a significantly reduced attraction to polyamine odor in the T-maze assay as compared to controls (Fig 2A). Remarkably, this reduction was similar to the reduction seen in *SPR* mutants (see Fig 1). Importantly, *SPR* RNAi did not reduce the attraction of virgin females further, showing that the regulation by SPR is indeed mating-state-dependent (Fig 2B). Similarly, expression of *SPR* RNAi in IR76b neurons fully abolished the taste-dependent egg-laying preference behavior of mated females (Figs 2C and S1C). We then refined the experiment with another, significantly more specific Gal4 driver, IR41a-Gal4, targeting only the small number of olfactory neurons sensing polyamine odor (*IR41a-Gal4;UAS-SPRi*). We observed a similar reduction in attraction to polyamine odor in the T-maze compared to knockdown with IR76b-Gal4 in mated females (Fig 2D). By contrast, egg-laying preference was comparable to control mated females (Figs 2E and S1D). This result was consistent with the absence of IR41a-Gal4 expression in IR76b gustatory neurons [33]. These data were consistent with the hypothesis that SPR in chemosensory neurons is necessary to modulate the attraction of females to the smell and taste of polyamines after mating.

Given the central role of SPR in the classical post-mating switch, we asked whether SPR in chemosensory neurons was not only necessary but also sufficient to modulate their sensitivity. To this end, we re-expressed SPR in *SPR* mutant females in all IR76b neurons (IR76b-Gal4, polyamine taste and olfaction), in bitter taste neurons (GR66a-Gal4), or just in the olfactory subset of IR76b-expressing neurons that express IR41a (IR41a-Gal4) and assayed olfactory behavior (T-maze) and taste-dependent oviposition behavior. We found that re-expression of SPR in IR76b neurons fully rescued the *SPR* mutant phenotype of mated females in olfaction as

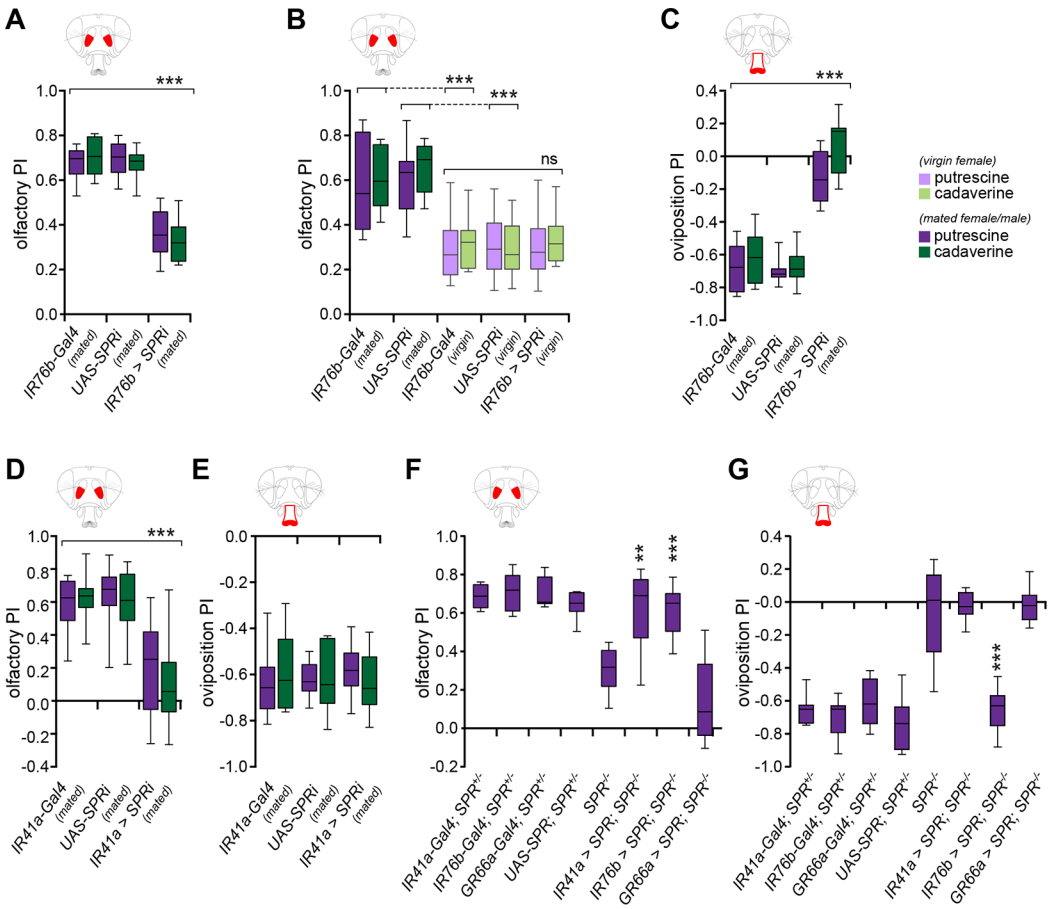

Fig 2. GPCR signaling in chemosensory neurons modulates female perception. (A) Knockdown of *SPR* in IR76b polyamine chemosensory neurons using RNAi (*IR76b-Gal4;UAS-SPRi*) significantly reduces olfactory preference to 10 ppm putrescine or cadaverine in mated females as compared to mated controls. ($n = 8$, 60 mated (♀) flies/trial). (B) The effect of SPRi in IR76b neurons is mating state-dependent, as knockdown of *SPR* (*IR76b-Gal4;UAS-SPRi*) does not further decrease the olfactory attraction of virgin females to polyamines compared to control virgins. ($n = 8$, 60 mated (♀) or virgin (☿) flies/trial). (C) Oviposition avoidance of a 1 mM polyamine/agarose substrate compared to a plain agarose substrate is strongly reduced upon knockdown of *SPR* in IR76b neurons (*IR76b-Gal4;UAS-SPRi*) ($n = 8$, 60 mated (♀) flies/trial). (D) Knockdown of *SPR* in IR41a neurons (*IR41a-Gal4;UAS-SPRi*) leads to a similar decrease in attraction to the odor of polyamine (10 ppm) in the T-maze as compared to knockdown of *SPR* with IR76b-Gal4, suggesting that SPR is required in olfactory neurons to enhance the attraction of mated females to the polyamine odors ($n = 8$, 60 mated (♀) flies/trial). (E) Knockdown of *SPR* in IR41a neurons (*IR41a-Gal4;UAS-SPRi*) did not affect oviposition behavior, and female behavior remained like their genetic controls. This result is consistent with the lack of expression of IR41a in taste neurons. (F) Re-expression of SPR using either IR41a-Gal4 or IR76b-Gal4 neurons fully rescued the *SPR* mutant phenotype of mated females in olfaction behavior to 10 ppm polyamines. ($n = 8$, 60 flies/trial). (G) Re-expression of SPR in IR76b taste neurons using IR76b-Gal4 fully rescued the *SPR* mutant phenotype of mated females in oviposition behavior. Conversely, re-expression of SPR in IR41a olfactory neurons or GR66a bitter taste neurons did not rescue oviposition preference behavior. ($n = 8$, 60 flies/trial). Box plots show median and upper/lower quartiles. All *p*-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

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well as in oviposition behavior (Fig 2F and 2G). Expression of SPR in GR66a bitter neurons, by contrast, had no effect on the *SPR* mutant phenotype in either of the two choice behaviors (Fig 2F and 2G). Re-expression of SPR selectively in IR41a OSNs did not rescue oviposition behavior of *SPR* mutant females, consistent with the fact that the egg-laying choice is mediated by taste neurons (Fig 2G). It did, however, rescue the olfactory attraction of *SPR* mutant females

to polyamine odor in the T-maze (Fig 2F). This suggests that SPR plays a cell-autonomous role in a specific set of peripheral chemosensory neurons independent of its function in the cells in the female reproductive system.

Altogether, based on these data, we propose that SPR regulates choice behavior in a mating-state-dependent manner directly in chemosensory neurons, providing a mechanistic link between mating state and the neurons that process odors and taste.

Mating and SPR Signaling Enhance Sensitivity of Gustatory Neurons

SPR signaling in chemosensory neurons appears to be required for the change in choice behavior after mating. This genetic mechanism could influence neuronal physiology at several levels of olfactory and taste processing starting at the peripheral level.

We have previously shown that IR76b taste neurons on the labellum are of particular importance for egg-laying choices on polyamine substrates [33]. Loss of IR76b completely abolishes the egg-laying preference of a mated female [33].

To test whether mating modulates the sensitivity of gustatory neurons, we examined the activity of IR76b chemosensory neurons by recording their Ca^{2+} responses to polyamines at the level of their axon terminals in the SEZ of the central brain (Fig 3A). Because mating induces short-term (<24 h) and long-term (~1 wk) effects [46,47], we performed these experiments at two different time points: at 1–6 h or at 1 wk post-mating (Fig 3B–3F). We measured Ca^{2+} increases by recording GCaMP6f signals in IR76b axon terminals in the SEZ (*IR76b-Gal4;UAS-GCaMP6f*), which we divided based on the innervation pattern of IR76b neuron subsets into two broader innervation zones, region of interest (ROI) 1 and ROI 2 (Fig 3A). At 1–6 h post-mating, labellar IR76b neurons projecting to ROI 1, the primary response area for polyamines [33], responded significantly more strongly to a putrescine taste solution in mated females than in virgin females (Fig 3C and 3D). Interestingly, this was not the case for ROI 2, which responded significantly only to higher concentrations of putrescine (10–100 mM). IR76b neurons projecting to this region of the SEZ of virgin and mated females showed a similar response (Fig 3E). Interestingly, at the later time point (1 wk post-mating), the difference observed for axons projecting to ROI 1 was no longer significant. Hence, we conclude that mating transiently increases the sensitivity of polyamine-detecting IR76b labellar taste neurons after mating.

Is this shift of sensitivity in the GRNs mediated by SPR signaling directly in chemosensory neurons as the behavioral data suggests? To answer this, we recorded GCaMP signals from polyamine-sensitive taste neurons of mated females, in which we triggered RNAi against *SPR*. Knock-down of *SPR* in IR76b GRNs (*IR76b-Gal4,UAS-GCaMP5;UAS-SPRi*) of mated females led to a significant decrease in the presynaptic calcium increase of these neurons in response to polyamine taste compared to the response of mated controls (Fig 3G–3I). Notably, *SPR* knock-down had no effect on the response of IR76b neurons projecting to the ROI 2 region of the SEZ. These neurons responded like control neurons (Fig 3H and 3I), suggesting that SPR modulation only occurred in neurons that were affected by the mating state.

These results provide a mechanistic explanation for behavioral change occurring in the oviposition choice behavior of females upon mating, and they are consistent with our model that SPR in GRNs directly modulates sensory neuron sensitivity and thereby regulates choice behavior.

Mating and SPR Signaling Decreases Responsiveness of Olfactory Neurons to Polyamines

Olfactory preference behavior appears to undergo a similar shift as gustatory preference behavior after mating. We therefore carried out a set of experiments in the olfactory system similar

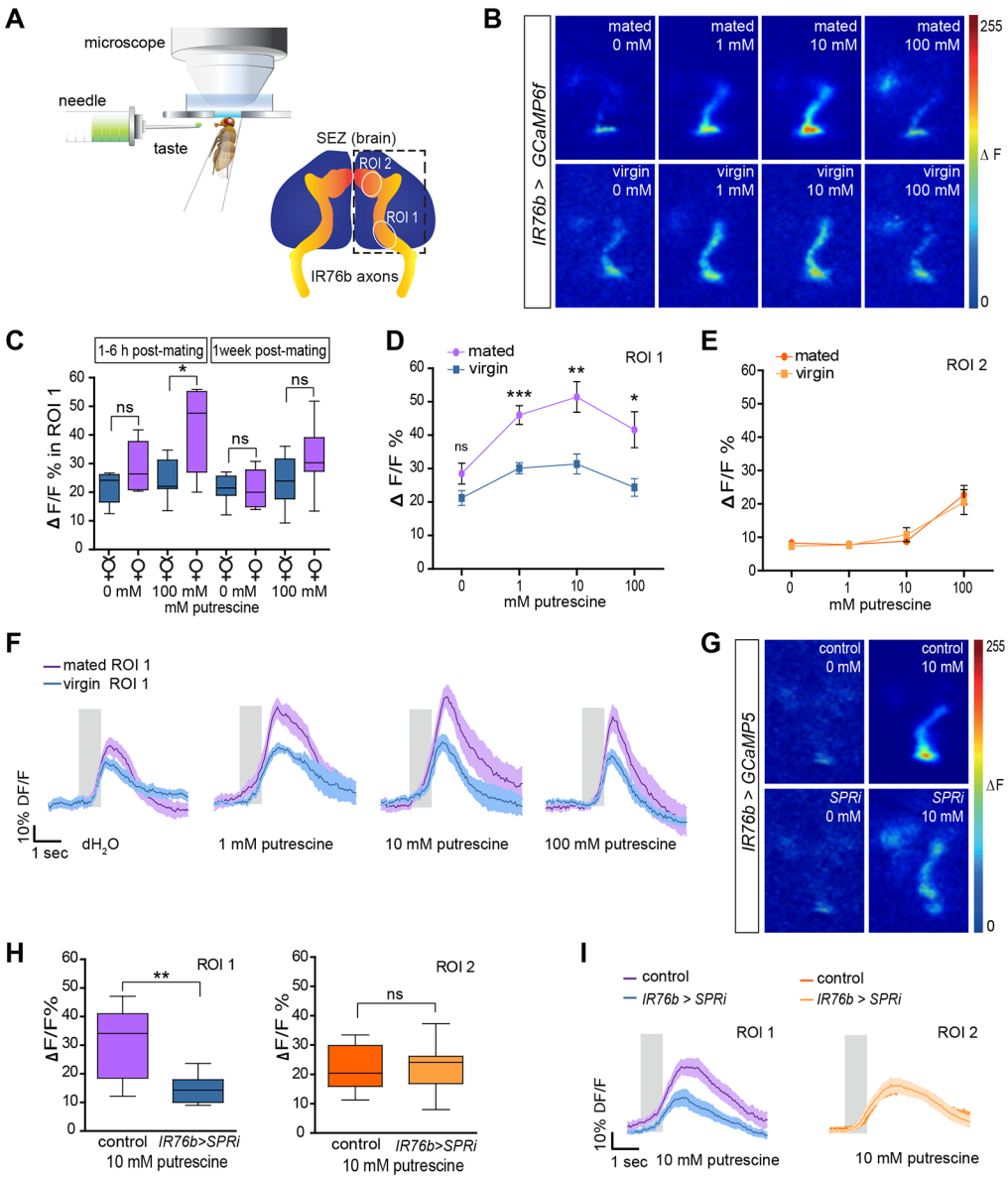

Fig 3. Mating increases sensitivity of taste neurons through SPR. (A) Scheme of the SEZ in vivo calcium imaging setup (top). Illustration of the SEZ area showing the innervation pattern of IR76b taste neuron axons (bottom). ROI 1 and ROI 2 delineate the regions of interest (ROI) used for quantification of the relative change in GCaMP-fluorescence (%ΔF/F). (B) Representative images of SEZ imaging of *IR76b-Gal4; UAS-GCaMP6f* mated and virgin female flies stimulated with distilled water (0 mM), 1 mM putrescine (1 mM), 10 mM putrescine (10 mM), and 100 mM putrescine (100 mM), respectively. (C) IR76b taste neuron terminals of mated females show a significantly increased response to putrescine after mating. While the response is highly significant at 1–6 h post-mating, it remains only a trend at 1 wk post-mating ($n = 7$). (D) Females at 1–6 h post-mating show higher IR76b taste neuron responses. GCaMP6f-fluorescence peak responses were quantified (in %ΔF/F) in the ROI 1 area. Flies were stimulated with increasing concentrations of putrescine ($n = 7$). (E) IR76b taste neurons of the same females as in (D) show no difference in the ROI 2 area. (F) Average response trace of the ROI 1 area ($n = 7$). The gray bar illustrates the stimulation period. The dark colored line in the middle presents the average value, and the light shade presents the SEM. (G) Representative images of IR76b GRN axons in the SEZ of test (*IR76b-Gal4*, *UAS-SPRi;UAS-GCaMP5*) and control (*IR76b-Gal4;UAS-GCaMP5*) females at 1–6 h post-mating. Flies were stimulated with distilled water (0 mM) and 10 mM putrescine (10 mM). (H) Quantification of peak responses (in % ΔF/F) of IR76b axon terminals of *IR76b-Gal4,UAS-SPRi;UAS-GCaMP5* and control (*IR76b-Gal4;UAS-GCaMP5*) females at 1–6 h post-mating ($n = 8$). Box plots show median and upper/lower quartiles, and whiskers show minimum/maximum values. (I) Average response trace of ROI 1 and ROI 2 area of IR76b axons in the SEZ of *IR76b>SPRi* and control females ($n = 8$). All *p*-values were calculated using an unpaired t-test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

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to those described above. Axons of OSNs project centrally to the AL, the functional equivalent of the vertebrate olfactory bulb. This first-order olfactory information is further processed by local interneurons and then transferred by projection neurons (PNs) to higher brain centers (Fig 4A) [48]. Recent studies have shown that hunger enhances olfactory sensitivity to food odor by increasing presynaptic responses of OSNs via the OSN-resident short neuropeptide F (sNPF) and its receptor, sNPFR [42,43]. Metabolic state thereby regulates the efficacy of the synapse between OSN and PN similarly to what we have observed for mating state and GRNs.

To test whether OSNs are modulated in a similar manner as GRNs, we imaged calcium increases of IR41a axon terminals at the level of the AL (*IR41a-Gal4;UAS-GCaMP6f*) (Fig 4B). Surprisingly, we observed that mating significantly decreased the response of these neurons to behaviorally relevant concentrations of polyamines (Fig 4C–4E). As in the gustatory system, this decrease was strongly significant at 1–6 h and remained only a trend at 1 wk post-mating (Fig 4C). In contrast to GRNs, however, mating transiently suppresses the sensitivity of OSNs. How does this result explain the behavioral shift toward higher polyamine levels after mating? Virgins show highest attraction to very low levels of polyamines and reduced attraction or enhanced aversion at levels preferred by mated females (Fig 1B). By contrast, mated females show the highest attraction to relatively high amounts of polyamine, which roughly corresponds to decaying fruit (10 ppm/1 mM; Fig 1B). It was previously shown that different odor concentrations can have differential behavioral effects and can even recruit different PNs downstream of the same OSNs [49,50]. Such a mechanism could also explain the change of behavior to polyamines, whereby a reduction of olfactory sensitivity may change higher olfactory processing and consequently shift the mated female's preference to increased levels of beneficial polyamines for egg-laying.

Again, we asked whether this change in sensitivity was mediated by SPR signaling in OSNs themselves, as the behavioral data would suggest. As in the gustatory system, this appeared to be the case for the olfactory system, as knockdown of *SPR* in IR41a OSNs resulted in a significant change in presynaptic calcium responses of these neurons (Fig 4F–4H). As predicted from the comparison of mated and virgin OSN responses to putrescine, we observed a greater increase of GCaMP fluorescence in OSN axon terminals of mated females with *SPR* knockdown (*IR41a-Gal4,UAS-GCaMP5;UAS-SPRi*) compared to mated genetic controls (Fig 4H).

Together, we interpret these data to mean that SPR in chemosensory neurons regulates the sensitivity of OSNs and GRNs to polyamines directly at the level of these chemosensory neurons. This change in sensitivity follows two different neural mechanisms, i.e., increased calcium responses of GRN and decreased responses of OSN axon terminals. This, in turn, appears to alter the mated female's perception and adjusts her choice behavior to polyamines.

Myoinhibitory Peptides Regulate Polyamine Sensitivity in the Context of Mating

We showed that polyamine perception changes upon mating and that this change is mediated by SPR signaling in chemosensory neurons. How SPR signaling is triggered in chemosensory neurons, however, remains unclear. The best-characterized SPR ligand is SP itself. A role for SP in feeding behavior was demonstrated previously. For instance, SP provided by the male stimulates feeding in mated females, and *SP* mutant male-mated females do not show this increase [10]. Furthermore, the mated female's feeding preference for yeast and salt depends on SP provided by the male during mating [9,41]. Here, SP activates the canonical SPR pathway through *ppk*-positive SPR neurons in the female's oviduct, which leads to a change in feeding preference. Whether and how mating and/or SP alter the sensitivity of taste neurons to yeast or salt or their higher-order chemosensory processing is not known. Furthermore, in the present context, if SP were to

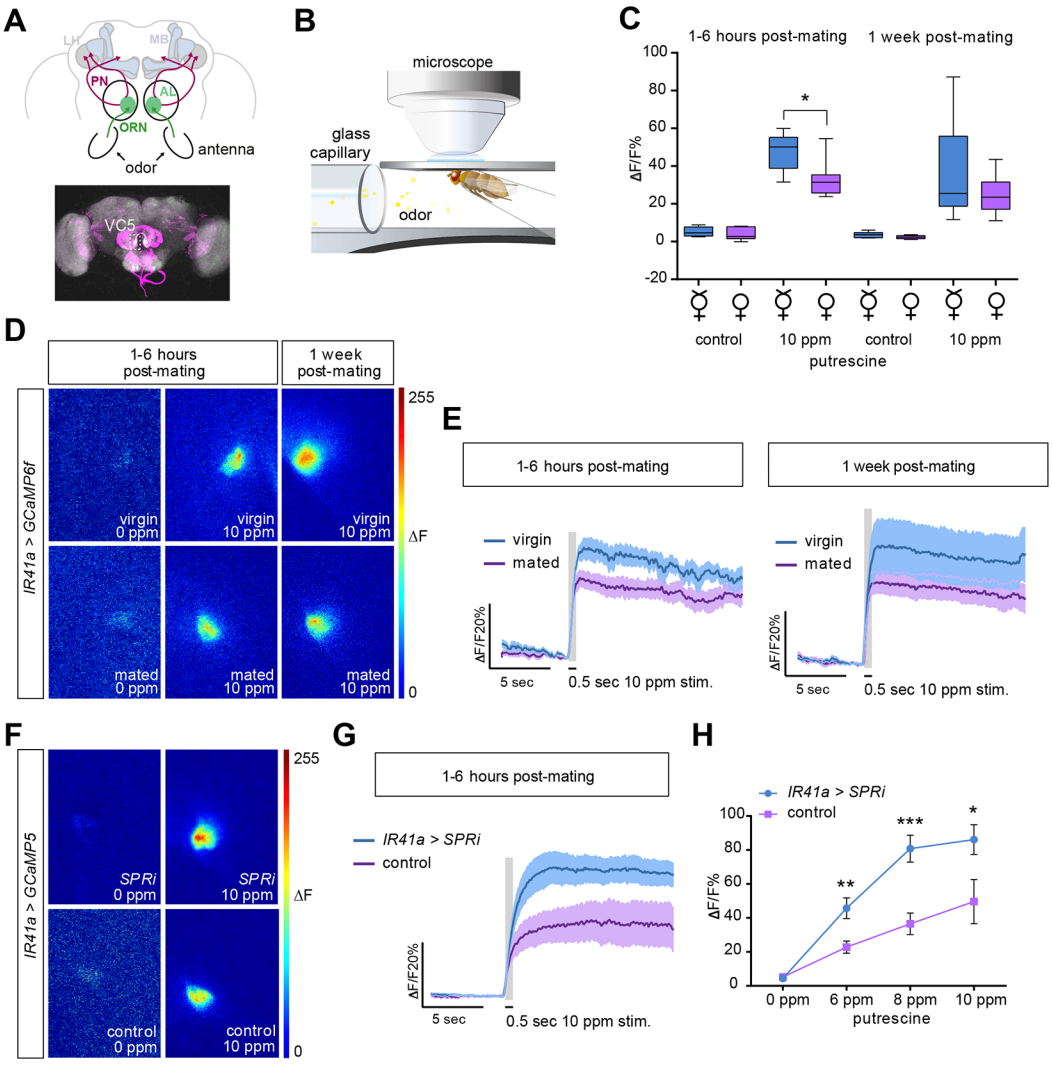

Fig 4. SPR decreases sensitivity of olfactory neurons to polyamines after mating. (A) Schematic diagram of a fly brain and its antennal appendages with olfactory sensory neurons (OSNs). OSNs project into the antennal lobe (AL), where they innervate a specific glomerulus (green). Projection neurons (PN) send the information mainly to two higher brain centers, the mushroom body (MB) and the lateral horn (LH) (top). Illustrative confocal image stack showing the IR41a and IR76b OSN innervation in the AL (bottom). VC5 is the glomerulus innervated by the polyamine-responding IR41a/IR76b sensory neurons. (B) Illustration of the in vivo calcium imaging setup. (C–E) In vivo calcium imaging of *IR41-Gal4;UAS-GCaMP6f* flies stimulated with water and 10 ppm putrescine, respectively. Mated females' OSN axon terminals show a significant reduction in their sensitivity to putrescine at 1–6 h post-mating. (C) Quantification of peak ΔF responses (in %ΔF/F) in virgin and mated females. Boxes show median and upper/lower quartiles, and whiskers show minimum/maximum values. *$p < 0.05$, unpaired *t* test ($n = 8$). (D) Representative pseudo-color images showing the response to water and 10 ppm putrescine in virgin and mated flies at 1–6 h and 1 wk post-mating. (E) Average response trace (in %ΔF/F) of the VC5 glomerulus peak response at 1–6 h and 1 wk post-mating compared to traces from virgin females. The dark colored line in the middle presents the average value and the light shade presents the SEM. (F–H) In vivo calcium imaging of test (*IR41a-Gal4*,*UAS-SPRi;UAS-GCaMP5*) and control (*IR41a-Gal4;UAS-GCaMP5)* mated female flies. OSN axon terminals of *IR41a>SPRi* females show significantly enhanced responses to putrescine compared to control females. (F) Representative pseudo-color images showing the response to water and 10 ppm putrescine in *IR41a>SPRi* and control females, respectively. (G) Average response trace of the VC5 glomerulus in *IR41a>SPRi* and control females at 1–6 h post-mating for 10 ppm putrescine. (E,G) The gray column represents the 0.5 s stimulation period. Dark colored line is the average response and the light shade is the SEM. (H) Quantification of peak ΔF responses (in %ΔF/F) in *IR41a>SPRi* and control females for 0 ppm, 6 ppm, 8 ppm, and 10 ppm putrescine, respectively ($n = 7 \pm$ SEM). All *p*-values were calculated using an unpaired *t* test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

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act directly on the chemosensory neurons, some SP would have to be transferred from its point of delivery, the female reproductive tract, to SPR in chemosensory neurons on the head. To test the requirement of SP in the sensitivity to polyamines, we crossed males that were mutant for the *SP* gene (SP^0), and thus lacking SP from their semen, to wild-type virgin females [51]. We compared the behavior of these females to that of females mated to wild-type males. Interestingly, the attraction of females mated to SP^0 males to polyamine odor in the T-maze was not significantly different from females mated to wild type males (Fig 5A). This suggested that SP was not the key to mating-state-dependent olfactory sensitivity modulation. Furthermore, it also indicated that changes in feeding behavior as reported by Carvalho et al. [10] are not necessary for the observed olfactory modulation. We also analyzed the contribution of SP to oviposition preference. SP^0 mated females appeared to show the same lack of preference as virgin flies and laid their very few eggs on either side of the assay (Fig 5B). Nevertheless, the olfactory preference data as well as the site of action of SP indicated that another additional ligand was involved in mating-state-dependent chemosensory changes in females. Moreover, this result was in agreement with our data showing that re-expression of SPR in gustatory or olfactory neurons was sufficient to modulate their responses to polyamines.

We therefore asked whether MIPs could be the functional ligands of SPR at the level of the chemosensory neuron central projections and could mediate the modulation of polyamine behavior. The expression of MIPs in the vicinity of IR41a axon terminals in the AL and in the vicinity of IR76b axons and axon terminals in the SEZ (Fig 5C) is consistent with their possible requirement in the chemosensory neurons themselves. We employed four different, independent RNAi-triggering transgenic lines to knockdown the expression of MIPs in IR76b-positive sensory neurons and tested fly behavior in the T-maze (olfaction) and oviposition (taste) assays. RNAi-mediated suppression of MIP expression in chemosensory neurons (*IR76b-Gal4; UAS-MIPi*) reduced the expression of MIP in chemosensory processing centers, but not in the rest of the brain as compared to controls or knockdown with a pan-neural driver (S5A Fig). Importantly, this manipulation (*IR76b-Gal4;UAS-MIPi*) also significantly lowered the attraction of mated females to polyamines in the T-maze as compared to genetic controls (Fig 5D and 5E). Notably, although MIP expression appears highly similar between males and females (S4 Fig) [52], this reduced olfactory attraction was only observed in females, but not in male flies (Fig 5D and 5E). These data mirror the lack of olfactory phenotype in the *SPR* mutant male (see Fig 1G) and further supported our model of a gender-specific role for SPR signaling. Furthermore, similar to what was observed upon *SPR* knockdown (see Fig 2C), virgin female attraction to polyamines was not further decreased when MIPs were down-regulated by RNAi, showing that the effect of MIP was mating-state-dependent (Fig 5F).

Finally, a similar analysis in the context of oviposition behavior showed that knockdown of MIPs in IR76b neurons (*IR76b-Gal4;UAS-MIPi*) had the same effect on female oviposition behavior as knockdown of *SPR* (Fig 5G). Female flies laid their eggs in equal numbers on polyamine-rich and control substrates (S5B Fig).

These data describe a role for MIPs in female reproductive behavior and indicate that they regulate polyamine-mediated chemosensory behavior presumably as ligands for SPR. Furthermore, similar to sNPF and its receptor [43], MIPs and SPR appear to be required directly in gustatory and olfactory neurons. In contrast to sNPF and sNPFR, SPR and MIPs are only required in the female.

Interaction of Mating State and SPR/MIP Signaling

Mating appears to induce a change in SPR signaling not only in the female reproductive tract as previously shown [15], but also in her chemosensory neurons. In the female reproductive

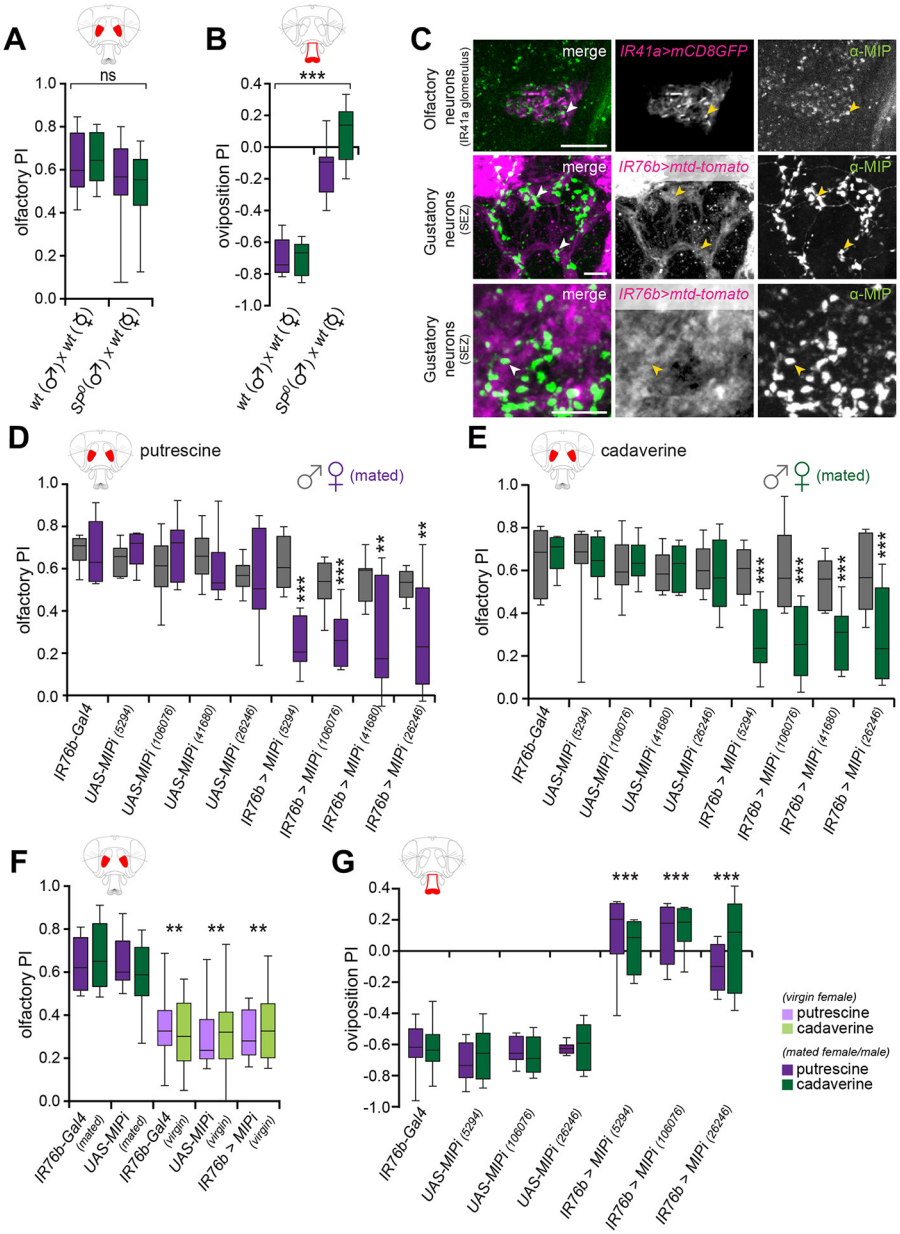

Fig 5. Myoinhibitory peptides regulate polyamine sensitivity in the context of mating. (A) Loss of sex peptide (SP) in the sperm of the male does not significantly affect chemosensory attraction of mated females to 10 ppm of polyamines. Wild-type (wt) Canton S females mated to wild-type or sex peptide mutant (SP^0) males do not show a significantly altered level of attraction to the odor of putrescine or cadaverine. ($n = 8$, 60 flies/trial). (B) SP^0 male-mated Canton S females lay their low numbers of eggs on either site of the oviposition assay and show no preference behavior. ($n = 8$, 60 flies/trial). (C) Myoinhibitory peptide (MIP) expression in the AL and SEZ regions in the female brain. In the AL, the glomerulus innervated by IR41a OSNs is displayed (*IR41a-Gal4;UAS-mCD8GFP*). Note that MIP staining is detected in close proximity to IR41a axon terminals. In the SEZ, anti-MIP staining (green) localizes close to IR76b neuron axons and axon terminals (magenta) consistent with MIPs being secreted by IR76b neurons (*IR76b-QF;QUAS-mtd-tomato*) (see arrowheads). (D,E) MIPs modulate olfactory attraction to polyamines selectively in mated females but not males. RNAi-mediated knockdown of MIPs with four different RNAi transgenic lines in IR76b neurons (*IR76b-Gal4;UAS-MIPi*) selectively reduces the olfactory preference of mated females but not of males to 10 ppm of putrescine (D) or 10 ppm of cadaverine (E) ($n = 8$, 60 flies/trial). (F) The effect of MIP knockdown (*IR76b-Gal4;UAS-MIPi*) depends on the mating state of the female, as the low attraction of virgin females to 10 ppm polyamine odor was not further reduced in virgin females with MIP knockdown compared to virgin controls without RNAi against MIPs. Box plots show median and upper/lower quartiles ($n = 8$, 60 flies/trial).

(G) Knockdown of MIPs in IR76b neurons abolishes oviposition preference to 1 mM putrescine and cadaverine using three different MIPi transgenic lines (*IR76b-Gal4;UAS-MIPi*). Females laid their eggs on either side of the assay. All box plots show median and upper/lower quartiles ($n = 8$, 60 flies/trial). All p-values were calculated via two-way ANOVA with the Bonferroni multiple comparison post-hoc test (ns > 0.05, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

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tract, SP is only available upon mating. How is this change brought about in peripheral neurons or in regions of the central brain such as the AL or SEZ? The most straightforward mechanism would be an alteration in the expression of MIP or SPR upon mating such that there is more functional SPR or available MIP in the mated female compared to the virgin. Notably, MIP expression cycles with the circadian rhythm of the fly, in line with the role of SPR and MIP in maintaining a sleep-like state in flies [25]. As the authors did not observe any change in MIP mRNA levels, a post-transcriptional regulatory mechanism could be involved [25]. To test whether SPR and MIP expression was being modulated, we used quantitative PCR to compare mRNA levels of SPR and MIP before and after mating (Fig 6A). To this end, we dissected antennae and brains of virgins and mated females at 1–6 h after mating and compared the expression of MIP and SPR to a control mRNA not expected to change upon mating (see Materials and Methods). We found that SPR expression increases about 10-fold upon mating in the antenna but to a lesser extend in the brain (~3-fold, Fig 6A). By contrast, MIP expression after mating remained more similar to the expression before mating in both antenna and brain (Fig 6A). These data are consistent with our hypothesis that SPR expression is selectively increased in chemosensory neurons upon mating and modulates female preference behavior. Furthermore, it strengthens the conclusion reached by genetic experiments that SPR signaling is required in chemosensory neurons.

While we were not able to challenge or confirm this result by using antibody staining against SPR, both a previously published antibody [15] and another antibody that we produced ourselves showed similar stainings in wild-type and *SPR* mutant brains (S6A–S6C Fig), we sought to quantify MIP protein expression at the level of the OSN terminals in the AL. This was especially important because MIP expression was previously suggested to be regulated at the level of the protein and not at the level of the mRNA [25]. Antibody staining against MIPs reveals central neurons as well as axon tracts of peripheral neurons projecting into the brain (Figs 5C and S4). In the SEZ, passing neuronal tracts of central neurons dominate (S4 Fig) and unfortunately mask the MIP-stained axons projecting from peripheral taste organs, including the proboscis (Figs 5C and S4; see arrowheads). This situation prevented us from quantifying MIP expression selectively in GRNs. In the olfactory system, nevertheless, MIP expression was defined and appeared to stem only from OSNs and from local interneurons. Although MIP protein expression analysis did not show any gross differences between mated and virgin females (S4 Fig), using more detailed image quantification we observed a significant increase of MIP expression in the AL in mated compared to virgin females (Figs 6B and S7A). While this increase appears small, it is statistically significant.

These results suggest that mating leads to a marked increase of SPR in chemosensory organs. This increase in SPR expression, accompanied by a small increase in MIP expression, might be the trigger for the mating-state-dependent modulation of polyamine taste and smell neurons. Of note, hunger modulates levels of the receptor sNPFR but not the expression of the neuropeptide itself [43].

Based on these results, we tested the effect of overexpression of SPR or MIP in chemosensory neurons in virgin females. We overexpressed SPR and MIP under the control of the IR76b enhancer (*IR76b-Gal4*) in all IR76b neurons (taste and olfaction) as well as only in OSNs under the control of the IR41a enhancer (*IR41a-Gal4*) in virgin females and tested their preference

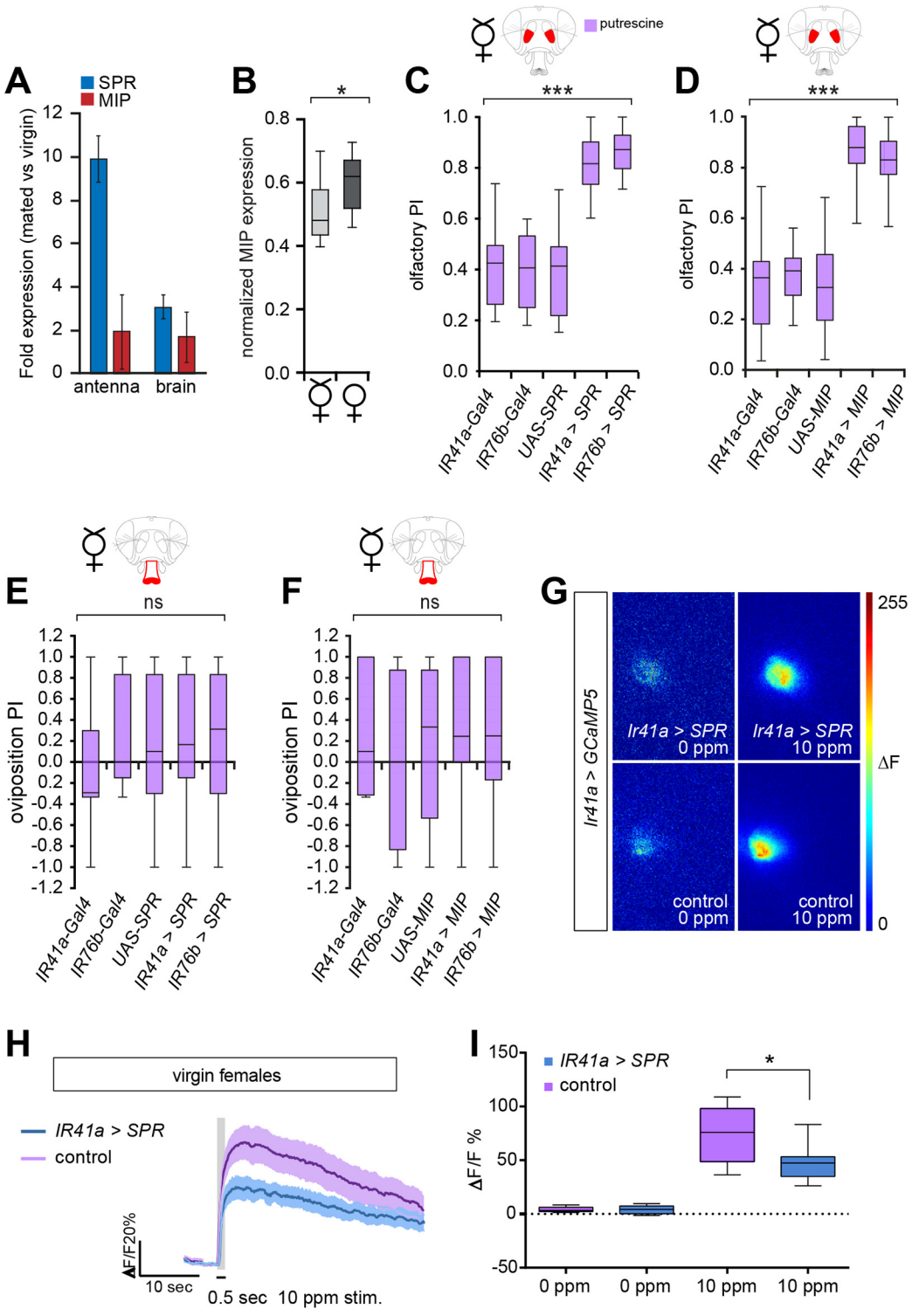

Fig 6. MIP expression is increased in the AL upon mating. (A) SPR and MIP expression analysis before and after mating of antenna and brain of virgin or mated females. Quantitative PCR ($n = 3$ genetic variants with 200 females per n and condition) of the antenna and brain of virgin and mated flies reveals that SPR expression upon mating increases upon mating ~10-fold in the antenna and ~3-fold in the brain. Graph displays 2^ΔΔCT ± SEM (see Materials and Methods for details). (B) Quantification of MIP protein expression in the AL. Mated flies show a small but significant increase of MIP expression in the AL. $n = 20$ flies per group. *$p = 0.0113$, unpaired t test. (C) Overexpression of SPR under the control of the IR41a enhancer (*IR41a-Gal4;UAS-SPR*) or IR76b enhancer

(*IR76b-Gal4;UAS-SPR*) in virgin females increases their attraction to polyamine odor in the T-maze assay ($n = 8$). (D) Overexpression of MIP under the control of the IR41a enhancer (*IR41a-Gal4;UAS-MIP*) or IR76b enhancer (*IR76b-Gal4;UAS-MIP*) in virgin females induces a strongly increased attraction to polyamine odor in olfactory T-maze assay ($n = 8$). (E,F) No egg-laying preference was observed in virgin females overexpressing SPR or MIP under the control of the IR41a enhancer (*IR41a-Gal4;UAS-SPR* or *IR41a-Gal4;UAS-MIP*) or IR76b enhancer (*IR76b-Gal4;UAS-SPR* or *IR76b-Gal4;UAS-MIP*) in oviposition assays compared to controls ($n = 8$). Virgin females overexpressing SPR or MIP laid very few eggs, similar to control virgins, which results in the high variability observed in the data. (G–I) In vivo calcium imaging of presynaptic terminals of OSNs in the AL expressing *IR41a-Gal4,UAS-SPR;UAS-GCaMP5* or *IR41a-Gal4;UAS-GCaMP5* (control). Virgin females overexpressing SPR in IR41a OSNs show significantly suppressed calcium signals to putrescine compared to virgin control females. (G) Representative pseudo-color images showing the response to 0 ppm and 10 ppm putrescine in SPR-overexpressing and control virgin females, respectively. (H) Average activity trace of the VC5 glomerulus in SPR-overexpressing and control virgin females for 10 ppm putrescine. (I) Quantification of peak ΔF responses in SPR-overexpressing ($n = 7$) and control ($n = 8$) females for 0 ppm and 10 ppm putrescine. Boxes 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, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$) except for Fig 6B and 6I, where *p*-values were calculated via an unpaired t-test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$).

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for polyamines. These manipulations had no effect on the number of eggs that virgin females laid, and egg numbers remained very low and similar to control virgins (S7B Fig). In contrast to the unchanged egg-laying activity, virgin females overexpressing SPR in chemosensory neurons showed a strongly increased attraction to polyamine odor (Fig 6C). This was true regardless of whether SPR was overexpressed under the control of IR76b-Gal4 or selectively in OSNs using IR41a-Gal4 (Fig 6C). We observed similar results in a reminiscent experiment, in which we overexpressed MIP instead of SPR. Also in this case, virgin females with increased levels of MIP in their chemosensory neurons showed a significantly increased preference for high polyamine levels compared to control virgins (Fig 6D). We also tested oviposition behavior. Given the low numbers of eggs, however, these data were less revealing and very variable, as small changes in egg-placing preference lead to large changes in preference index. In spite of these limitations, no clear preference was observable in virgins overexpressing SPR or MIP and control virgins (Fig 6E and 6F).

Our data would predict that the observed change in choice behavior upon overexpression of SPR is triggered by sensory neuron modulation. To analyze this, we used in vivo calcium imaging as described above. Indeed, we found that in virgins, overexpression of SPR selectively in IR41a OSNs significantly reduced the presynaptic response of IR41a neurons to polyamines compared to controls (Fig 6G–6I). This result was the exact opposite of the effect seen when SPR expression was knocked down using RNAi in IR41a OSNs (see Fig 3) and correlated well with the observed behavior of virgins overexpressing SPR.

In conclusion, expression analysis in conjunction with behavioral and imaging analysis leads us to propose that mating induces primarily an increase of SPR expression in chemosensory neurons. Boosted levels of SPR activated by mildly increased levels of MIPs modulate chemosensory neuron output in response to polyamines and thereby increase female preference for higher concentrations of polyamines. Thus, SPR/MIP signaling in chemosensory neurons seems not only necessary and sufficient but, as these data indicate, an instructive signal adjusting choice behavior to reproductive state.

Discussion

Here, we describe a novel mechanism that enhances the sensitivity of chemosensory neurons to match the choice behavior of a gravid female to her increased nutritional needs. Female *Drosophila* use polyamines to identify and evaluate beneficial food and egg-laying sites with specific olfactory and taste receptor neurons. We demonstrate that this multisensory detection of

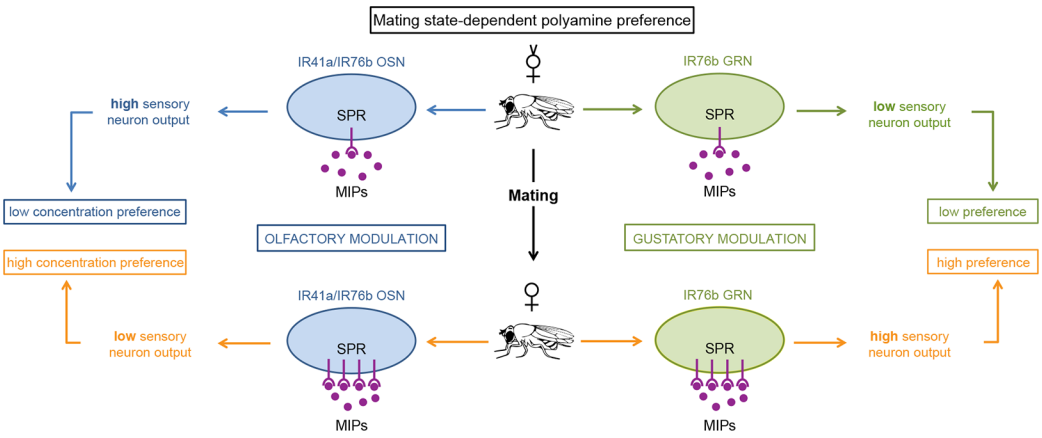

Fig 7. SPR/MIP signaling in chemosensory neurons adjusts female preference behavior upon mating. Model for mating-state-dependent modulation of olfactory and gustatory polyamine preference. (Left side) Upon mating, increased amounts of SPR in the sensory neuron suppress the output of IR41a/IR76b olfactory neurons, thereby increasing the female's preference for higher concentrations of polyamine. (Right side) Mating increases the SPR amount in gustatory sensory neurons, and in contrast to the situation in OSNs increases the presynaptic output of IR76b taste neurons. This change increases the mated female's preference for polyamine taste. In conclusion, mating increases SPR expression in chemosensory neurons and by two different cellular mechanisms enhances the mated female's preference for beneficial polyamines.

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polyamines undergoes reproductive state-dependent peptidergic modulation. Mechanistically, we show that virgin females, or mated females lacking the G-protein coupled receptor SPR, display reduced preference for polyamine-rich food and oviposition sites. Using targeted gene knockdown, mutant rescue, overexpression, and in vivo calcium imaging, we thus unravel a new role for SPR and its conserved ligands, MIPs, in directly regulating the sensitivity of chemosensory neurons and modulating taste and odor preferences according to reproductive state (Fig 7). Together with recent work in the mouse [6], our results emphasize that chemosensory neurons are potent targets for tuning choice behavior to reproductive state.

Neuropeptidergic Modulation of Chemosensory Neurons Regulates Mating State-Dependent Choice Behavior

Reproductive behaviors such as male courtship and female egg-laying strongly depend on the mating state [8,9,29,31,53]. While previous work has suggested that mating modulates odor- or taste-driven choice behavior of *Drosophila* females [9,29,31,41,54], how mating changes the processing of odors and tastes remained elusive. We show here that a female-specific neuropeptidergic mechanism acts in peripheral chemosensory neurons to enhance female preference for essential nutrients. Our data suggests that this modulation is autocrine and involves the GPCR SPR and its conserved MIP ligands. Notably, MIPs are expressed in chemosensory cells in the apical organs of a distant organism, the annelid (*Platynereis*) larvae, in which they trigger settlement behavior via an SPR-dependent signaling cascade [22]. Importantly, as SP and not MIP induces the SPR-dependent canonical post-mating switch [15,19], our findings report the first gender and mating-state-dependent role of these peptides [25]. Whether this regulation is also responsible for previously reported changes in preference behavior upon mating [9,29,31,41] remains to be seen, but we anticipate that this type of regulation is not only specific to polyamines. On the other hand, mating-dependent changes for salt preference—salt preference is also dependent on IR76b receptor but in another GRN type—might undergo a different type of regulation, as RNAi-mediated knockdown of SPR in salt receptor neurons had no effect

on salt feeding [41]. Instead, the change in salt preference is mediated by the canonical SP/SPR pathway and primarily reflects the fact that mating has taken place. The mechanism of how salt detection and/or processing are modulated is not known. In contrast to salt preference and polyamine preference, acetic acid preference is strongly modulated by egg-laying activity and not just mating [31]. The extent to which changes in salt or acetic acid preference are similar to the modulation of behavior to polyamine that we describe here can currently not be tested, because the olfactory neurons that mediate acetic acid preference have not been determined [31].

G-protein Coupled Receptor Signaling Has the Opposite Effect on Olfactory versus Gustatory Neurons

While SPR regulates the neuronal output of both olfactory and gustatory neurons, our behavioral and our physiological data surprisingly revealed that it does so through two opposite neuronal mechanisms. SPR signaling increases the presynaptic response of GRNs and decreases it in OSNs. Behaviorally, these two types of modulation produce the same effect: they enhance the female's attraction to polyamine and tune it to levels typical for decaying or fermenting fruit. How these two effects are regulated by the same receptor and ligand pair remains open. GPCRs can recruit and activate different G-proteins. SPR was previously shown to recruit the inhibitory $G\alpha_{i/o}$-type, thereby down-regulating cAMP levels in the cell [19,55]. In the female reproductive tract, SP inhibits SPR-expressing internal sensory neurons and thereby promotes several post-mating behaviors [15]. This type of inhibitory G-protein signaling could also explain our data in the olfactory system. Here, mating decreases the presynaptic activity of polyamine-detecting OSNs, and conversely, RNAi knockdown of *SPR* increases their responses strongly. This decrease in neuronal output also shifts the behavioral preference from low to high polyamine levels. While the relationship between behavior and GRN activity is much more straightforward in the gustatory system (increased neuronal response, increased preference behavior), it implies that another G-protein might be activated downstream of SPR. G-protein $G\alpha_{i/s}$ increases cAMP levels and $G\alpha_q$ enhances phospholipase C (PLC) and calcium signaling [56]. In addition, Gβγ subunits regulate ion channels and other signaling effectors, including PLC [56]. Future work will address the exact mechanisms of this bi-directional modulation through SPR signaling. Nonetheless, it is interesting to speculate that different cells, including sensory neurons, could be modulated differentially by the same molecules depending on cell-specific states and the availability of signaling partners.

Modulation of Polyamine Perception and Its Relationship to Reproductive State

While our data provides a neuronal and molecular mechanism of how chemosensory processing itself is affected by mating, it remains unclear how mating regulates MIP/SPR signaling in chemosensory neurons. Our data indicates that SPR levels strongly increase in chemosensory organs upon mating. In addition, MIP levels appear to be mildly increased by mating. This suggests that mating regulates primarily the expression of the GPCR resembling the modulation of sNPFR expression during hunger states. On the other hand, MIP overexpression also induced mated-like preference behavior in virgin flies, suggesting a somewhat more complex situation. For instance, it is possible that overexpression of MIP induces the expression of SPR. Alternatively, active MIP levels might also be regulated at the level of secretion or posttranslational processing, and overexpression might override this form of regulation. In the case of hunger, sNPFR levels are increased through a reduction of insulin signaling [43]. SP could be viewed as the possible equivalent of insulin for mating state. Females mated to *SP* mutant males, however,

do not show a significant change in olfactory perception of polyamines. It is yet important to note that male sperm contains roughly 200 different proteins, some of which might be involved in mediating the change in MIPs/SPR signaling upon mating [7]. In the mosquito, which does not possess SP, the steroid hormone 20E serves as the post-mating switch [57]. Interestingly, mating or treatment with 20E induces in particular the expression of the enzymes required for the synthesis of polyamines in the female spermatheca, a tissue involved in sperm storage and egg-laying [57]. Whether such a mechanism also exists in *Drosophila* is not known.

In addition to mating and signals transferred by mating, it is possible that egg-laying activity contributes to the regulation of MIPs/SPR signaling in chemosensory neurons through a mechanism that involves previously identified mechanosensory neurons of the female's reproductive tract; such neurons may sense the presence of an egg to be laid [31]. Indeed, females that cease to lay eggs return to polyamine preferences as found before mating. On the other hand, *SP* mutant male-mated females and *ovoD1* sterile females still show enhanced attraction to polyamine odor, although they lay very few or no eggs. Conversely, knockdown of *SPR* in IR41a neurons reduced polyamine odor attraction but had a marginal effect on the number of eggs laid. We observed, nevertheless, somewhat reduced numbers of eggs laid upon inactivation of IR76b neurons. At this point, we can only speculate about possible reasons. Although IR76b receptor is not expressed in ppk-positive internal SPR neurons, we do find expression of IR76b-Gal4 in neurons innervating the rectum and possibly gut (data not shown). Hence, there might be an IR76b-mediated interplay between metabolism and nutrient uptake that influences egg-laying. However, females mated to SP-mutant males do not display an increase in feeding [10], indicating that preference for polyamines does not depend on the metabolic cost of egg-laying. This conclusion is strengthened by the data obtained with mated *ovoD1* sterile females, who show similar attraction to polyamines as compared to mated controls. Due to very few or no eggs laid by *SP* mutant male-mated females and *ovoD1* females, respectively, we cannot, however, fully exclude a contribution of egg-laying activity to taste-dependent oviposition choice behavior.

A further argument against an important role of egg-laying activity in our paradigm comes from the observation that the sensory modulation of OSNs and GRNs occurs rapidly after mating and is maintained only for a few hours. Similarly, SPR expression increases within the same time window shortly after mating. Egg-laying, however, continues for several days after this. In addition, overexpression of SPR was sufficient to switch virgin OSN calcium responses and female behavioral preferences to that of mated females without increasing the number of eggs laid. All in all, these data are more consistent with the hypothesis that mating and not egg-laying activity per se is the primary inducer of sensory modulation leading to the behavioral changes of females.

It remains that the exact signal triggered by mating that regulates odor and taste preference for polyamines through the here-identified mechanism needs to still be determined. Furthermore, the role of metabolic need and polyamine metabolism is not completely clear. This is similar to the situation found for increased salt preference after mating. While more salt is beneficial for egg-laying, sterile females still increase their preference for salt upon mating [41]. Regardless, in the case of polyamines, it is tempting to speculate that exogenous (by feeding) and endogenous (by enzymatic activity or expression) polyamine sources are regulated by reproductive state and together contribute to reach optimal levels for reproduction in the organism.

Modulated Sensory Perception Leads to Lasting Behavioral Changes

Our results bear some similarities to recent work on the modulation of OSN sensitivity in hunger states [43]. sNPF/sNPFR signaling modulates the activity of OSNs in the hungry fly. MIPs/

SPR might play a very similar role in the mated female. Overexpression of sNPFR in OSNs of fed flies was sufficient to trigger enhanced food search behavior [43]. Likewise, an increase in SPR signaling in taste or smell neurons converts virgin to mated female preference behavior. Therefore, different internal states appear to recruit similar mechanisms to tune fly behavior to internal state. Furthermore, such modulation of first order sensory neurons appears not only be conserved within a species, but also for regulation of reproductive state-dependent behavior across species. For instance, a recent study in female mice showed that progesterone-receptor signaling in OSNs modulates sensitivity and behavior to male pheromones according to the estrus cycle [6]. Also in this case, sensory modulation accounts in full for the switch in preference behavior. What is the biological significance of integrating internal state at the level of the sensory neuron? First, silencing of neurons in a state-dependent manner shields the brain from processing unnecessary information. As sensory information may not work as an on/off switch, it is possible that an early shift in neural pathway activation might reduce costly inhibitory activity to counteract activation once the sensory signal has been propagated. Second, another interesting possibility is that peripheral modulation might help to translate transient changes in internal state into longer-lasting behavioral changes that manifest in higher brain centers. This might be especially important in the case of female reproductive behaviors such as mate choice or caring for pups or babies. In contrast to hunger, which increases with time of starvation, the effect of mating decays slowly over time as the sperm stored in the female's spermatheca is used up [58]. We have shown that the effect of mating on chemosensory neurons mediated by MIPs/SPR signaling is strong within the first 6 h after mating and remains a trend at 1 wk post-mating. However, it triggers a long-lasting behavioral switch, which is observed for over a week. Therefore, this transient modulation and altered sensitivity to polyamines could be encoded more permanently in the brain when the animal encounters the stimulus, for instance, in the context of an excellent place to lay her eggs. Because polyamine preference continues to be high for as long as stored sperm can fertilize the eggs, we speculate that this change in preference might be maintained by a memory mechanism in higher centers of chemosensory processing. Thus, short-term sensory enhancement not only increases perceived stimulus intensity, it may also help to associate a key sensation to a given reward or punishment. These chemosensory associations are of critical importance in parent–infant bonding in mammals, including humans, which form instantly after birth and last for months, years, or a lifetime [59].

Materials and Methods

Fly Rearing and Lines

Drosophila melanogaster stocks were raised on conventional cornmeal-agar medium at 25°C temperature and 60% humidity and a 12 h light:12 h dark cycle. Following fly lines were used to obtain experimental groups of flies in the different experiments:

1. Canton S
2. w^{1118}
3. *w*;;UAS-Kir2.1::eGFP*
4. *w*;P[IR41a-GAL4.2474]attP40;TM2/TM6B*
5. *w*; P[IR76b-GAL4.916]226.8;TM2/TM6B*
6. *w*;GR66a-Gal4/Cyo;TM2/TM6B*
7. *Df(1)Exel6234* (*SPR* loss of function mutant); the mutation was verified using two different primer sets: Primer Pair-1: CCACCGTAATCTTGGCCCTTTTC, GTGGACCCCGAGT

GGAAAATAAAAG; Primer Pair-2: AAGGGAGTCGGTTACTTGCG, TTCGTTC GGGGGATGTCAAG (see S6 Fig)

8. *w**;*UAS-mCD8GFP*
9. *w**;*UAS-GCaMP6f*
10. *w**;;*UAS-GCaMP5*
11. For sex peptide mutant males: SP^{0}/*TM3 Sb* flies were crossed *to Δ130/TM3 Sb* (gift from Mariana Wolfner)
12. Lines for RNAi knockdown of MIP: #26246 (*y1 v1; P[TRiP.JF02145]attP2)*, #41680 (*y1 sc* v1; #P[TRiP.HMS02244]attP2*), #106076 (*P[KK106116]VIE-260B*), #5294 (w^{1118}*; P [GD2689]v5294*)
13. Line for RNAi knockdown of SPR: *#106804 VDRC P[KK103356]VIE-260B*
14. *w**;*P[IR76b-QF.1.5]2*
15. w^{1118};*UAS-mCD8GFP,QUAS-mtd-tomato-3xHA*
16. w^{1118};*UAS-SPR/CyO* (gift from Barry Dickson)
17. w^{1118};*UAS-MIP* (gift from Doug Allan)
18. *ovoD1* (#1309) sterile females were obtained by crossing *ovoD1* males to Canton S virgins

The majority of the lines were obtained from Bloomington (http://flystocks.bio.indiana.edu/) or the Vienna Drosophila Resource Center (VDRC) stock center (http://stockcenter.vdrc.at) except where indicated otherwise.

Behavioral Assays for *Drosophila melanogaster*

T-Maze Assay. The use of the T-maze assay is indicated in all figures with a fly head schematic with red-colored antennae to show that polyamine preference depends on olfactory sensory neurons on the antenna. 5–7 ds old flies raised at 25°C were used for all experiments, with the exception of experiments in which RNAi was used. In these experiments, experimental flies and genetic controls were raised at 30°C to enhance the effect of the RNAi. Flies were tested in groups of ~60 (30 females and 30 males or 60 females) in a T-maze and were allowed 1 min to respond to stimuli. Experimentation was carried out within climate-controlled boxes at 25°C and 60% rH in the dark. 50 μl of fresh odor solution at different concentrations diluted in distilled water applied on Whatman chromatography paper was provided in the odor tube, while 50 μl of distilled water (polyamine solvent) applied on Whatman chromatography paper was placed into the control tube. Unless otherwise indicated, 1 mM (~10 ppm according to measurements with a photo-ionization detector [PID]) of either putrescine or cadaverine were used. After experimentation, the number of flies in each tube was counted. An olfactory preference 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 by the total number of flies. Statistical analysis was performed using two-way ANOVA and the Bonferroni multiple comparisons post-hoc test using Prism GraphPad 6.

Oviposition Assay. The oviposition assay is indicated in all figures by an illustration of the fly head with a red-labeled proboscis showing that oviposition preference depends on labellar taste neurons. In addition, oviposition assays are shown in simple schemes in the relevant figures. Here, the gray circle shows the oviposition plate filled with 1% agarose, and the colored squares indicate the addition of putrescine or cadaverine on one-half of the plate. Unless

otherwise stated, 1 mM of polyamine was used in all assays. Mated female flies, reared on standard cornmeal medium at 25°C and 60% rH, were separated on ice from male flies at day 4 post-eclosion. Female flies were kept for 2 more days on fly food and used on day 6 for the oviposition assays. Flies raised at 25°C were used for all experiments, with the exception of experiments in which RNAi was used. In this experiments, experimental flies and genetic controls were raised at 30°C to enhance the effect of the RNAi. One percent low melting agarose was poured in a 60 x 15 mm petri-dish, and two halves were marked with a permanent marker on the bottom of the dish. Fifty μl of polyamine solution was applied on one side of the dish. In initial experiments, we also tested odor mixed into 1% low melting agarose compared to agarose only and obtained the same results as with applying the polyamine solution onto the hardened agarose. Sixty female flies were put in a gauzed top round cage, and the cage was closed with the test petri dish. Flies were kept for exactly 16 h in a light:dark cycle at controlled temperature and humidity conditions. An oviposition PI was calculated by subtracting the number of eggs on the test site from the number of eggs on the control site and normalized by the total number of eggs. Statistical analysis was performed using two-way ANOVA and the Bonferroni multiple comparisons post-hoc test using Prism GraphPad 6.

Anatomy

Adult fly brains were dissected, fixed, and stained as described previously [60]. Briefly, brains were dissected in cold PBS, fixed with paraformaldehyde (2%, overnight at 4°C or for 2 h at RT), washed in PBS, 0.1% Triton X-100, 10% donkey serum and stained overnight at 4° C or for 2 h at RT with the primary and after washes in PBS, 0.1% Triton X-100 with the secondary antibody using the same conditions. For SPR staining, a procedure previously published was followed [15]. All microscopic observations were made at an Olympus FV-1000 confocal microscope or at a Leica MZ205 epifluorescence stereomicroscope. Images were processed using ImageJ and Photoshop. The following antibodies were used: chicken anti-GFP (molecular probes, 1:100), rabbit anti-Dsred (Clontech, Living colors DsRed polyclonal AB, 1:200), rat anti-N-cadherin (anti-N-cad DN-Ex #8, Developmental Studies Hybridoma Bank, 1:100), mouse anti-Dlarge (4F3-anti-discs large-c Developmental Studies Hybridoma Bank, 1:50), mouse anti-MIP (gift of C. Wegener, 1:50), rabbit anti-SPR ([15], gift of Y.-J. Kim, 1:10), rabbit anti-SPR (generated by H. Ammer, Ludwig Maximilians University Munich, Germany against the same peptide as used in [15]). Secondary antibodies used were: anti-chicken Alexa 488 (molecular probes, 1:250) and anti-rabbit Alexa 549 (molecular probes, 1:250), respectively.

MIP expression was analyzed using antibody staining with the aforementioned MIP antibody. All brains were processed at the same time using the same conditions. Images were taken at an Olympus FV-1000 confocal microscope at the exact same settings. Seven single confocal sections were selected over the entire volume of the antennal lobe without knowledge of the mating state. ROIs were drawn around the AL in each section, and image quantification was carried out blindly using FIJI ImageJ software. All MIP quantifications were normalized to the intensity of anti-Ncad staining of the same ROI of the same section. Statistical analysis (*t* test) and data illustration were carried out using Excel and GraphPad Prism software.

In Vivo Calcium Imaging

For calcium imaging experiments, GCaMP6f (or for technical reasons GCaMP5 in experiments with SPR RNAi knockdown) were expressed under the control of IR41a-Gal4 or IR76b-Gal4. In vivo preparations of flies were prepared according to a method previously described [60]. In vivo preparations were imaged using a Leica DM6000FS fluorescent microscope equipped with a 40x water immersion objective and 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 all experiments with odor stimulation, the stimulus was applied 5 s after the start of each measurement. A continuous and humidified airstream (2000 ml/min) was delivered to the fly throughout the experiment via an 8 mm diameter glass tube positioned 10 mm away from the preparation. A custom-made odor delivery system (Smartec, Martinsried), consisting of mass flow controllers (MFC) and solenoid valves, was used for delivering a continuous airstream and stimuli in all experiments. In all experiments, stimuli were delivered for 500 ms, and during stimulations the continuous flow was maintained at 2,000 ml/min. For putrescine stimulations, 1 ml of a precise concentration was filled in the odor delivery cup and the collected airspace odor was injected into the main airstream to give 0 ppm, 6 ppm, 8 ppm, and 10 ppm final concentrations for 500 ms without changing airstream strength. To measure the fluorescent intensity change, 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 basal 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 pseudo-colored images were generated in MATLAB using a custom written program. All analysis and statistical tests were done using Excel and GraphPad6 Prism software, respectively.

Imaging with taste stimuli was performed in a similar setup as described above, with some modifications. The flies expressing GCaMP-fluorescence under IR76b-Gal4 were prepared according to a method previously described [61]. The proboscis of the fly was pulled out by suction and fixed by gluing to prevent it from going back into the head capsule. For taste stimulation, taste stimuli were diluted in distilled water and delivered by a custom-built 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 a stereomicroscope. A drop of taste was delivered to touch the labellum. The stimulus was applied for 1 s after the start of each measurement. All analysis and statistical tests were done using Excel and GraphPad6 Prism software as described above.

Quantitative PCR Analysis

Individual virgin female flies were mated with single males, observed, and separated after copulation. Two hundred of these mated females were kept for 4–6 h after mating following the same protocol as used for imaging. Antenna and brains of mated and virgin female flies of the same age were collected for RNA extraction. This procedure was repeated for three genetic replicates of 200 virgin and 200 mated flies. RNA was extracted using an RNA easy minikit (Qiagen) and used as a template for reverse transcription by superscript III reverse transcriptase (Invitrogen). Quantitative PCR was conducted using the following target gene primers: SPR (SPR-fwd: atgcacatcgtcagtagcct, SPR-rev: cagccgaccgaggaatatct) and MIP (MIP-fwd: ggacaatccgcacagcag, MIP-rev: ctgaacttgttccagccctg). H2A.Z, a histone variant (H2A.Z-fwd: tcgcatccatcgtcatctca, H2A.Z-rev: ctcggcggtcaggtattcc), was used as an internal control. All qPCR experiments were performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). All amplifications were done using SYBR Green PCR Master Mix (Applied Biosystems). The thermal cycling conditions included an initial denaturation step at 95°C for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s. Melting curve analysis of every qPCR was conducted after each cycle. C_T (cycle threshold) values were used for analysis. The $\Delta\Delta C_T$ was calculated as previously described [62] by subtracting the control ΔC_T

value of H2A.Z from the individual ΔC_T values of SPR and MIP for normalization (C_T mated–C_T virgin), respectively. The inverse logarithm was calculated to receive the expression fold change.

The numerical data used in all main and supplementary figures are included in S1 Data.

Supporting Information

S1 Data. The Excel spreadsheet contains, in separate sheets, the underlying numerical data and statistical analysis for the following figures with their relative panels: Fig 1, Fig 2, Fig 3, Fig 4, Fig 5, Fig 6, Fig 7, S1 Fig, S2 Fig, S3 Fig, S4 Fig, S5 Fig, S6 Fig and S7 Fig.
(XLSX)

S1 Fig. Polyamine behavior is modulated by mating state and SPR. (A) Graph shows number of eggs laid by Canton S mated and virgin females on agarose control (gray bars) or polyamine-rich substrates (putrescine: magenta, cadaverine: green) in 16 h oviposition assay. Number of eggs are averaged (n = 8± SEM, 60 ♀ flies/trial). (B) Average number of eggs laid by mated control and mated *Sex peptide receptor* mutant ($SPR^{-/-}$) females on agarose control (gray bars) or polyamine-rich substrates (magenta/green) in the oviposition assay. Number of eggs are averaged (n = 8± SEM, 60 ♀ flies/trial). (C) Average number of eggs laid by controls and flies with knockdown of *SPR* in IR76b neurons (*IR76b-Gal4;UAS-SPRi*). Number of eggs are averaged (n = 8 ± SEM, 60 ♀ flies/trial). (D) Average number of eggs laid by controls and flies with knockdown of SPR in IR41a neurons (*IR41a-Gal4;UAS-SPRi*). Number of eggs are averaged (n = 8 ± SEM, 60 ♀ flies/trial). (E) Bar graph shows average number of eggs laid by controls and flies with re-expression of SPR in IR41a, IR76b, and GR66a neurons. Number of eggs are averaged (n = 8 ± SEM, 60 ♀ flies/trial).
(TIF)

S2 Fig. IR76b is not expressed in ppk-positive neurons innervating the uterus. Expression analysis of IR76b compared to the ppk-Gal4 reporter in the female reproductive tract using *ppk-Gal4;UASmCD8GFP* (green in B and C), *IR76b-QF;QUASmdTomato-3xHa* (magenta in B and C), and *IR76b-Gal4;UASmCD8GFP* (green in D). Scale bars equal 200 μm. (A) Schematic drawing of the female reproductive tract showing the two ovaries, the snail-shaped seminal receptacle, the bilateral spermatheca and the uterus. (B–B″) Epifluorescent images of reproductive organs. White arrow points to ppk-positive neurons innervating the uterus just underneath the seminal receptacle. Note that the uterus contains an egg in this case. Magenta staining has been overexposed and the color seen is primarily autofluorescence. (C–C″) Magnified pictures of the boxed area in B of the same sample using confocal imaging. Tomato signal does not show positive cells but autofluorescence. (D) IR76b expression analysis with epifluorescence and confocal microscopy using the Gal4/UAS reporter system confirms the results obtained with the QF/QUAS system. The region of ppk-positive neurons beneath the seminal receptacle is devoid of GFP signal. The GFP signal does not show positive cells but autofluorescence.
(TIF)

S3 Fig. The role of SP in the modulation of chemosensation. Bar graph shows average number of eggs laid by wild-type (wt) Canton S females mated to wild-type (wt) Canton S males and of wild-type (wt) Canton S females mated to Sex peptide mutant (SP^{0}) males. Number of eggs is averaged (n = 8 ± SEM, 60 ♀ flies/trial).
(TIF)

S4 Fig. MIP expression in the brain. (A) Representative pictures of virgin and mated female and mated male brains. MIP is expressed in neurons in the central brain as well as on axon

tracts of peripheral neurons projecting into the brain (yellow arrowheads). Brains were stained with anti-MIP (yellow) and anti-NCad (blue). Images were taken at the confocal microscope.
(TIF)

S5 Fig. MIPs are the putative central ligands for SPR. (A) Representative pictures of anti-MIP/anti-Dlarge antibody stained female brains showing knockdown of MIPs using two different RNAi lines and corresponding controls. The pan neuronal driver *elav-Gal4* removes MIP from all neurons. In brains from crosses with the specific driver *IR76b-Gal4* MIP staining is still present in most brain regions, but reduced in the antennal lobes (AL) and in the subesophageal zone (SEZ), where IR76b positive neurons project their axons. Upper panels show brain overview, while lower panels show substacks of the AL and SEZ, respectively. Images were taken at the confocal microscope. Scale bars equal 50 μm. (B) Average number of eggs laid by controls and flies with knockdown of three different MIPi transgenic lines in IR76b neurons (*IR76b-Gal4;UAS-MIPi*) on agarose control (gray bars) or polyamine-rich substrates (putrescine: magenta, cadaverine: green). Number of eggs are averaged (n = 8 ± SEM, 60 ♀ flies/trial).
(TIF)

S6 Fig. Expression analysis of SPR using antibody staining. (A) Representative confocal images of brain and proboscis of $SPR^{+/-}$ and $SPR^{-/-}$ flies stained using two different SPR antibodies. No specific signal could be detected with either of the two antibodies. Arrows point to some staining in the SEZ and the labellum, which was also observed in the *SPR* mutants. Left panels show staining with a newly generated antibody against an SPR peptide (see Materials and Methods). Right panels show staining with a previously generated antibody [15]. Scale bars equal 50 μm. (B) Organization of the *SPR[Df(1)Exel6234]* ($SPR^{-/-}$) deletion region on X chromosome. Df(1)Exel6234 covers the entire *SPR* gene and neighboring genomic regions. (C) Agarose gel electrophoresis of PCR product of a ~1.5 kb SPR gene fragment. *SPR[Df(1)Exel6234]* homozygous samples ($SPR^{-/-}$) are negative for the *SPR* gene fragment amplification, showing that the *SPR* gene is deleted in those flies. By contrast, $SPR^{+/-}$ and $SPR^{+/+}$ flies show the expected band. (D) Agarose gel electrophoresis of PCR product of a ~500 bp region spanning the Df(1)Exel6234 deletion region. The band is visible in $SPR^{-/-}$ homozygous and heterozygous samples, but not in $SPR^{+/+}$ wildtype controls. (-)controls contain no genomic template DNA.
(TIF)

S7 Fig. SPR and MIP expression modulates chemosensory behavior. (A) Confocal images showing MIP antibody staining (green) in seven representative single sections of the antennal lobe of a virgin and a mated female fly at 1–6 h post-mating. Sections were used for image quantification, and MIP staining intensity was normalized to staining intensity of anti-Ncad antibody staining (magenta) of the same section (see Materials and Methods). (B) Average number of eggs laid by control virgins and virgin females with overexpression of SPR or MIP under the control of the IR41a enhancer (*IR41a-Gal4*) or IR76b enhancer (*IR76b-Gal4*) on agarose control (gray bars) or polyamine-rich substrates (putrescine: magenta) in oviposition assays (n = 8). Number of eggs are averaged (n = 8 ± SEM, 60 ♀ flies/trial).
(TIF)

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Author Contributions

Conceived and designed the experiments: ICGK AH HKÜ. Performed the experiments: ICGK AH LFL HKÜ MZ. Analyzed the data: ICGK AH LFL HKÜ MZ. Contributed reagents/materials/analysis tools: ICGK AH LFL HKÜ MZ. Wrote the paper: ICGK.

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Neuropeptides Modulate Female Chemosensory Processing upon Mating in Drosophila.

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Supplementary Figures

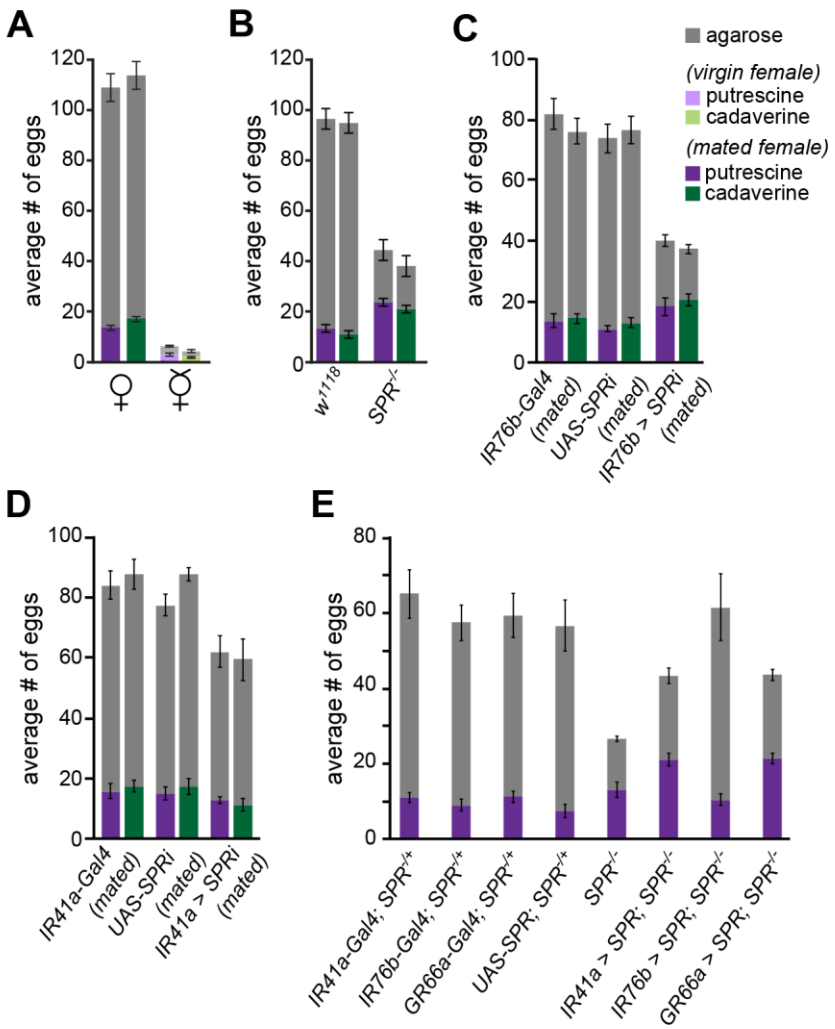


Fig. S1 Polyamine behavior is modulated by mating state and SPR.

(A) Graph shows number of eggs laid by Canton S mated and virgin females on agarose control (gray bars) or polyamine-rich substrates (putrescine: magenta, cadaverine: green) in 16 h oviposition assay. Number of eggs are averaged (*n* = 8± SEM, 60 ♀ flies/trial). (B) Average number of eggs laid by mated control and mated *Sex peptide receptor* mutant ($SPR^{-/-}$) females on agarose control (gray bars) or polyamine-rich substrates (magenta/green) in the oviposition assay. Number of eggs are averaged (*n* = 8± SEM, 60 ♀ flies/trial). (C) Average number of eggs laid by controls and flies with knockdown of *SPR* in IR76b neurons (*IR76b-Gal4;UAS-SPRi*). Number of eggs are averaged (*n* = 8 ± SEM, 60 ♀ flies/trial). (D) Average number of eggs laid by controls and flies with knockdown of SPR in IR41a neurons (*IR41a-Gal4;UAS-SPRi*). Number of eggs are averaged (*n* = 8 ± SEM, 60 ♀ flies/trial). (E) Bar graph shows average number of eggs laid by controls and flies with re-expression of SPR in IR41a, IR76b, and GR66a neurons. Number of eggs are averaged (*n* = 8 ± SEM, 60 ♀ flies/trial).

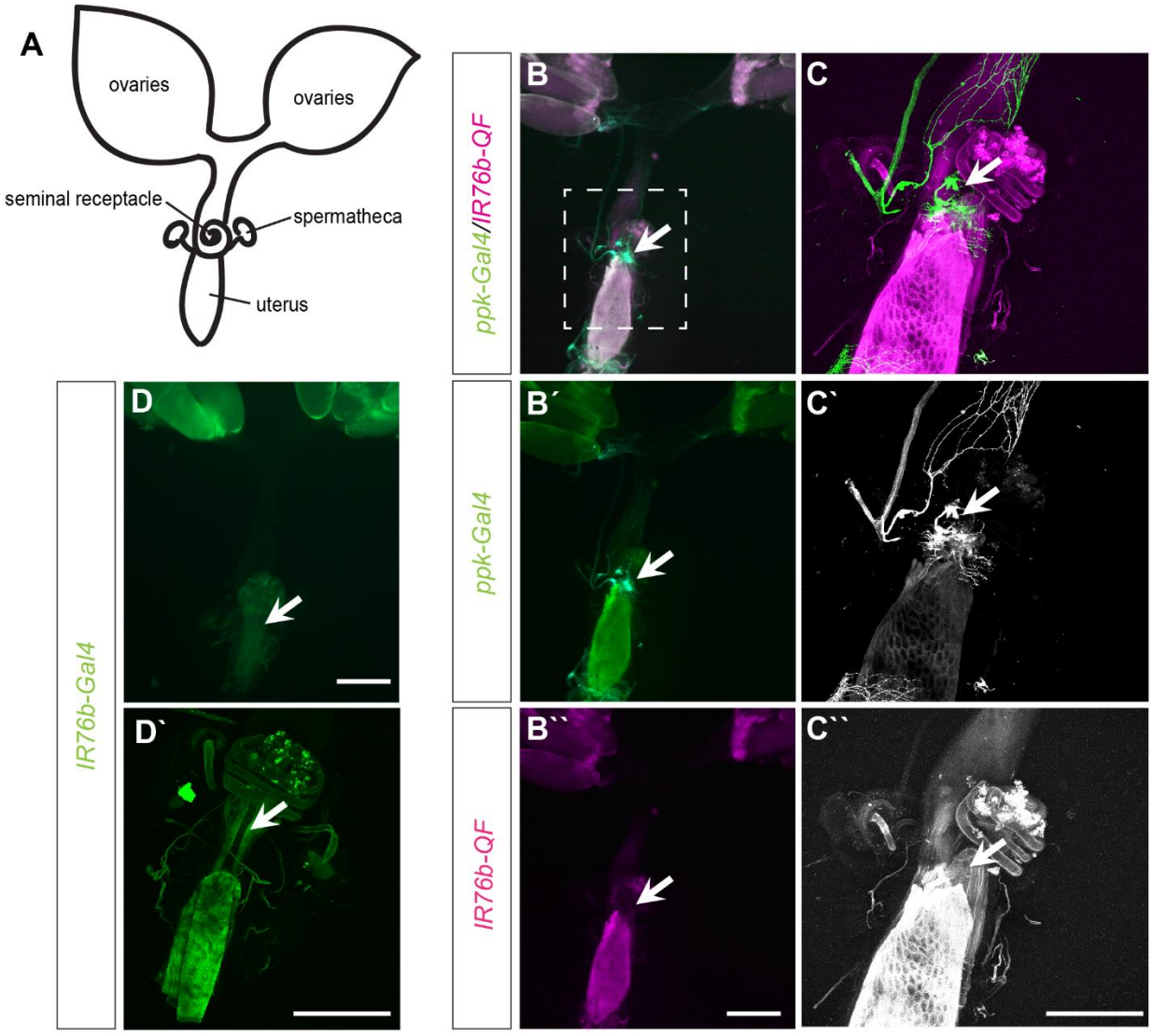


Fig. S2 IR76b is not expressed in ppk-positive neurons innervating the uterus.

Expression analysis of IR76b compared to the ppk-Gal4 reporter in the female reproductive tract using *ppk-Gal4;UASmCD8GFP* (green in B and C), *IR76b-QF;QUASmdTomato-3xHa* (magenta in B and C), and *IR76b-Gal4;UASmCD8GFP* (green in D). Scale bars equal 200 µm. (A) Schematic drawing of the female reproductive tract showing the two ovaries, the snail-shaped seminal receptacle, the bilateral spermatheca and the uterus. (B–B'') Epifluorescent images of reproductive organs. White arrow points to ppk-positive neurons innervating the uterus just underneath the seminal receptacle. Note that the uterus contains an egg in this case. Magenta staining has been overexposed and the color seen is primarily autofluorescence. (C–C'') Magnified pictures of the boxed area in B of the same sample using confocal imaging. Tomato signal does not show positive cells but autofluorescence. (D) IR76b expression analysis with epifluorescence and confocal microscopy using the Gal4/UAS reporter system confirms the results obtained with the QF/QUAS system. The region of ppk-positive neurons beneath the seminal receptacle is devoid of GFP signal. The GFP signal does not show positive cells but autofluorescence.

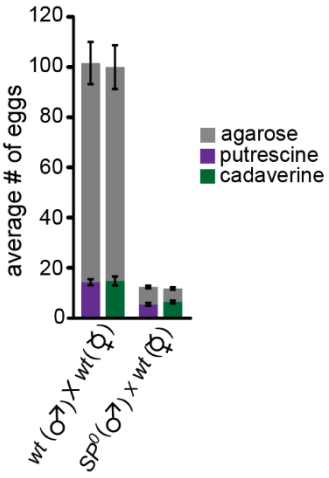


Fig. S3 The role of SP in the modulation of chemosensation.

Bar graph shows average number of eggs laid by wild-type (wt) Canton S females mated to wild-type (wt) Canton S males and of wild-type (wt) Canton S females mated to Sex peptide mutant (SP^0) males. Number of eggs is averaged (n = 8 ± SEM, 60 ♀ flies/trial).

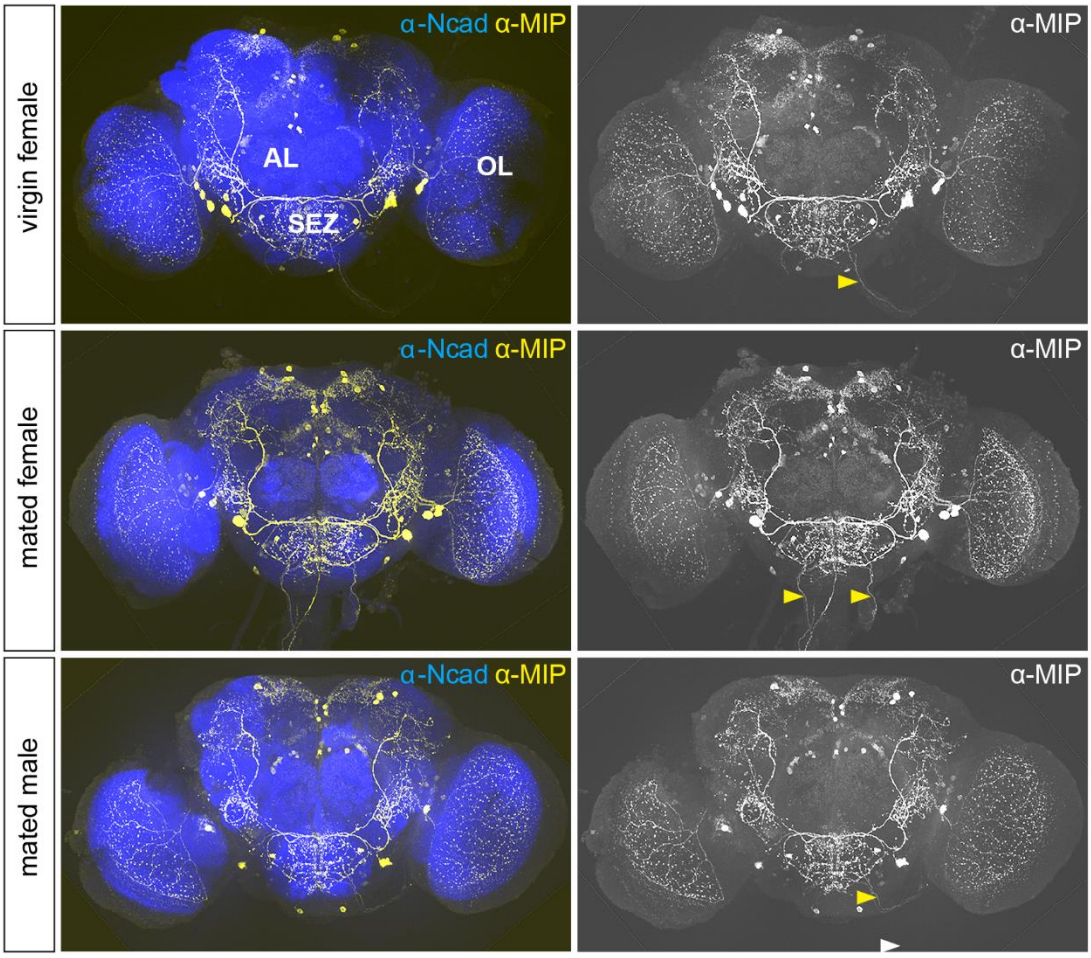


Fig. S4 MIP expression in the brain.

Representative pictures of virgin and mated female and mated male brains. MIP is expressed in neurons in the central brain as well as on axon tracts of peripheral neurons projecting into the brain (yellow arrowheads). Brains were stained with anti-MIP (yellow) and anti-NCad (blue). Images were taken at the confocal microscope.

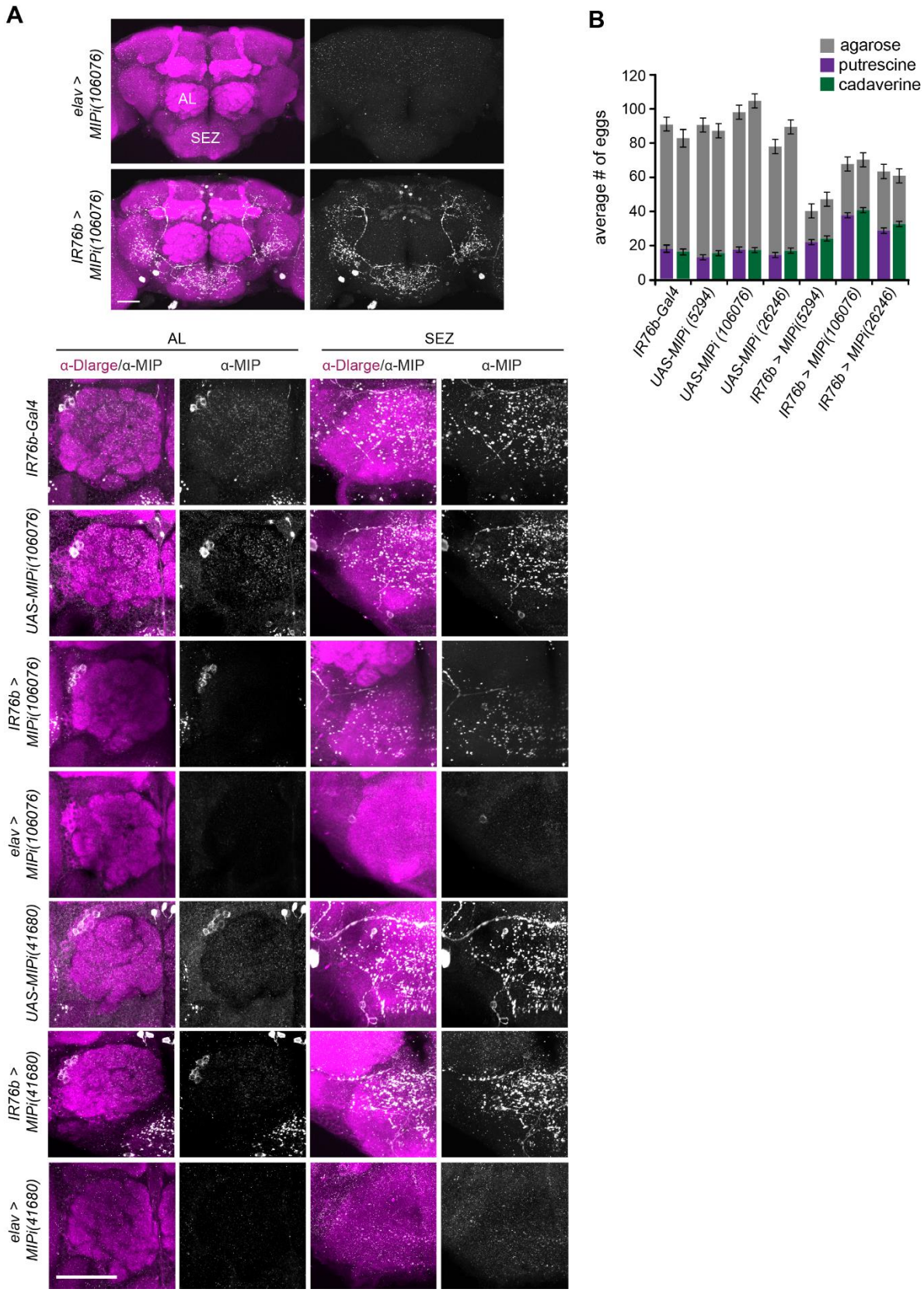
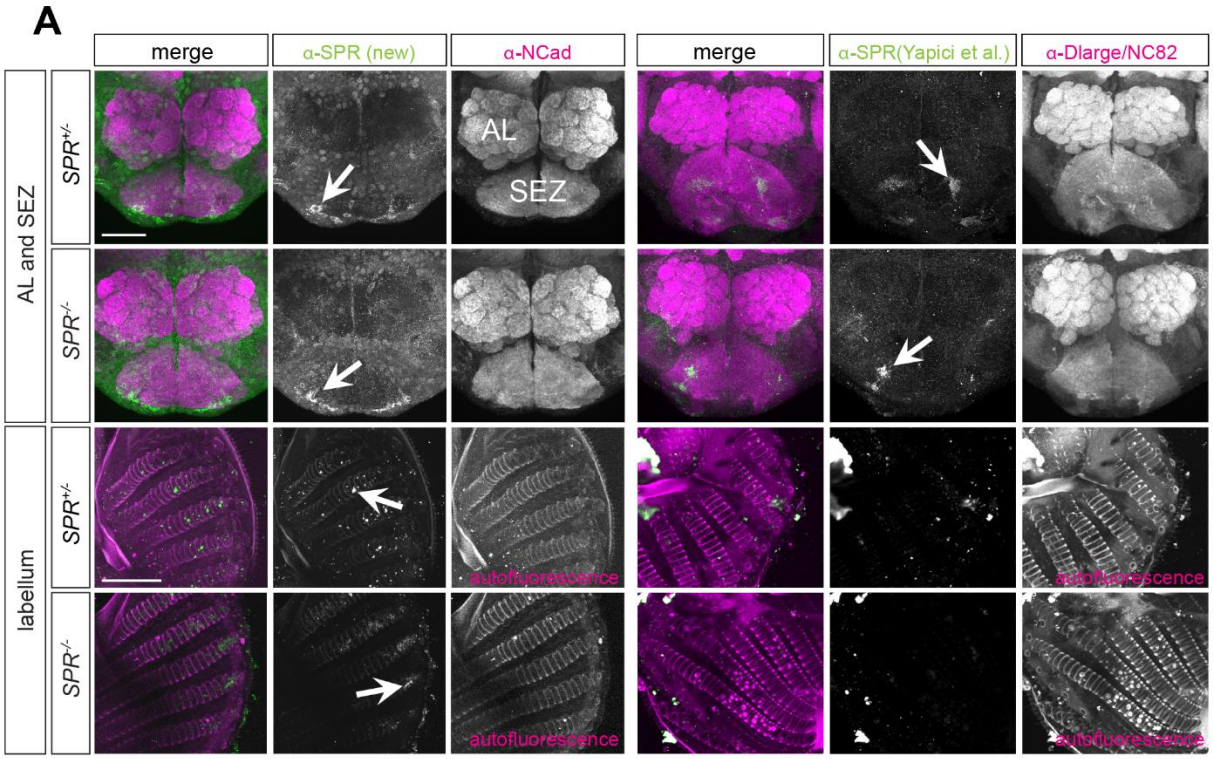

A
elav > MIPi(106076)
AL
SEZ
IR76b > MIPi(106076)
AL
SEZ
α-Dlarge/α-MIP
α-MIP
α-Dlarge/α-MIP
α-MIP
IR76b-Gal4
UAS-MIPi(106076)
IR76b > MIPi(106076)
elav > MIPi(106076)
UAS-MIPi(41680)
IR76b > MIPi(41680)
elav > MIPi(41680)
B
agarose
putrescine
cadaverine
average # of eggs
0
20
40
60
80
100
120
IR76b-Gal4
UAS-MIPi (5294)
UAS-MIPi (106076)
UAS-MIPi (26246)
IR76b > MIPi(5294)
IR76b > MIPi(106076)
IR76b > MIPi(26246)

Fig. S5 MIPs are the putative central ligands for SPR.

(A) Representative pictures of anti-MIP/anti-Dlarge antibody stained female brains showing knockdown of MIPs using two different RNAi lines and corresponding controls. The pan neuronal driver *elav-Gal4* removes MIP from all neurons. In brains from crosses with the specific driver *IR76b-Gal4* MIP staining is still present in most brain regions, but reduced in the antennal lobes (AL) and in the subesophageal zone (SEZ), where IR76b positive neurons project their axons. Upper panels show brain overview, while lower panels show substacks of the AL and SEZ, respectively. Images were taken at the confocal microscope. Scale bars equal 50 µm. (B) Average number of eggs laid by controls and flies with knockdown of three different MIPi transgenic lines in IR76b neurons (*IR76b-Gal4;UAS-MIPi*) on agarose control (gray bars) or polyamine-rich substrates (putrescine: magenta, cadaverine: green). Number of eggs are averaged (*n* = 8 ± SEM, 60 ♀ flies/trial).


A
merge
α-SPR (new)
α-NCad
merge
α-SPR(Yapici et al.)
α-Dlarge/NC82
AL and SEZ
labellum
SPR+/-
SPR-/-
AL
SEZ
autofluorescence

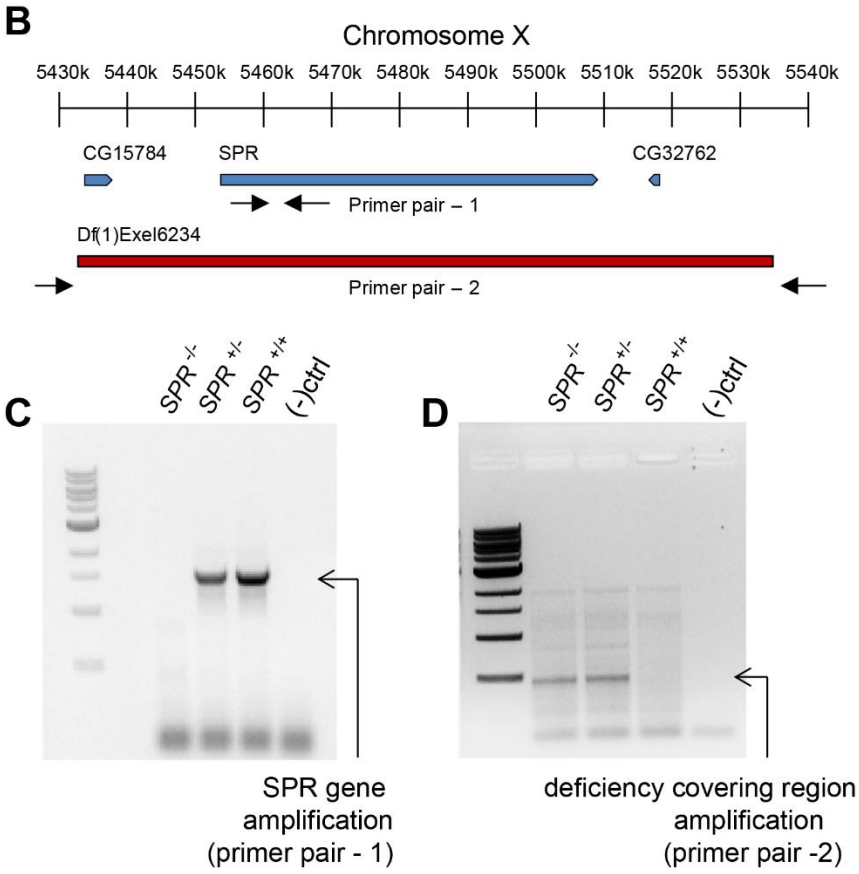

B
Chromosome X
5430k 5440k 5450k 5460k 5470k 5480k 5490k 5500k 5510k 5520k 5530k 5540k
CG15784
SPR
CG32762
Primer pair – 1
Df(1)Exel6234
Primer pair – 2
C
SPR -/-
SPR +/-
SPR +/+
(-)ctrl
SPR gene amplification (primer pair - 1)
D
SPR -/-
SPR +/-
SPR +/+
(-)ctrl
deficiency covering region amplification (primer pair -2)

Fig. S6 Expression analysis of SPR using antibody staining.

(A) Representative confocal images of brain and proboscis of *SPR*$^{+/-}$ and *SPR*$^{-/-}$ flies stained using two different SPR antibodies. No specific signal could be detected with either of the two antibodies. Arrows point to some staining in the SEZ and the labellum, which was also observed in the *SPR* mutants. Left panels show staining with a newly generated antibody against an SPR peptide (see Materials and Methods). Right panels show staining with a previously generated antibody [15]. Scale bars equal 50 µm. (B) Organization of the *SPR[Df(1)Exel6234] (SPR*$^{-/-}$*)* deletion region on X chromosome. Df(1)Exel6234 covers the entire *SPR* gene and neighboring genomic regions. (C) Agarose gel electrophoresis of PCR product of a ~1.5 kb SPR gene fragment. *SPR[Df(1)Exel6234]* homozygous samples *(SPR*$^{-/-}$*)* are negative for the *SPR* gene fragment amplification, showing that the *SPR* gene is deleted in those flies. By contrast, *SPR*$^{+/-}$ and *SPR*$^{+/+}$ flies show the expected band. (D) Agarose gel electrophoresis of PCR product of a ~500 bp region spanning the Df(1)Exel6234 deletion region. The band is visible in *SPR*$^{-/-}$ homozygous and heterozygous samples, but not in *SPR*$^{+/+}$ wildtype controls. (-)controls contain no genomic template DNA.

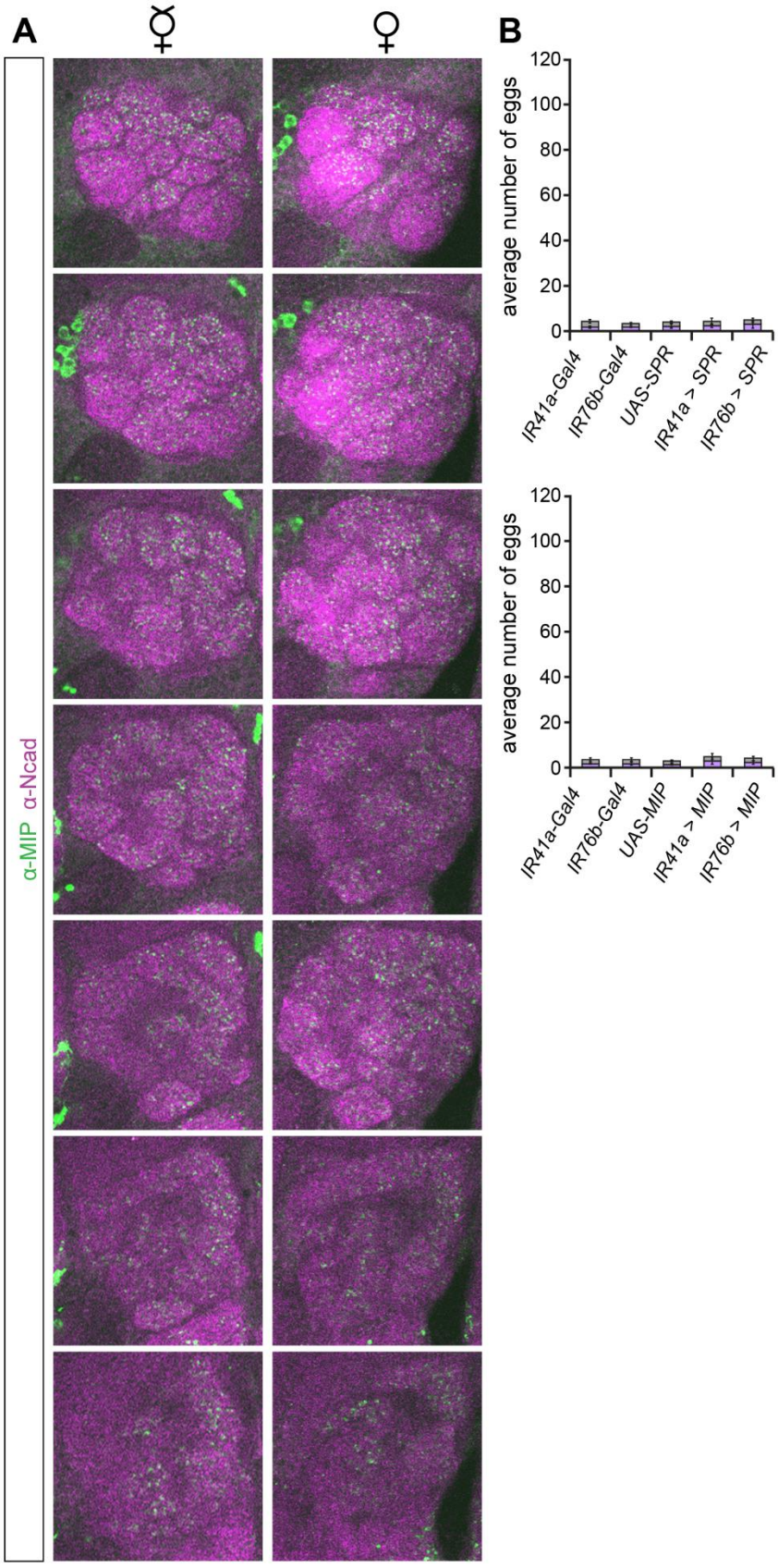

A
♂
♀
α-MIP α-Ncad
B
average number of eggs
120
100
80
60
40
20
0
IR41a-Gal4
IR76b-Gal4
UAS-SPR
IR41a > SPR
IR76b > SPR
average number of eggs
120
100
80
60
40
20
0
IR41a-Gal4
IR76b-Gal4
UAS-MIP
IR41a > MIP
IR76b > MIP

Fig. S7 SPR and MIP expression modulates chemosensory behavior.

(A) Confocal images showing MIP antibody staining (green) in seven representative single sections of the antennal lobe of a virgin and a mated female fly at 1–6 h post-mating. Sections were used for image quantification, and MIP staining intensity was normalized to staining intensity of anti-Ncad antibody staining (magenta) of the same section (see Materials and Methods). (B) Average number of eggs laid by control virgins and virgin females with overexpression of SPR or MIP under the control of the IR41a enhancer (*IR41a-Gal4*) or IR76b enhancer (*IR76b-Gal4*) on agarose control (gray bars) or polyamine-rich substrates (putrescine: magenta) in oviposition assays (n = 8). Number of eggs are averaged (n = 8 ± SEM, 60 ♀ flies/trial).

6. Discussion

6.1. Summary of Results

Flies use chemosensory cues for finding food sources and mating partners, and also staying away from toxic substances and predators. In this cumulative thesis, I, in collaboration with colleagues, studied the multimodal detection of an essential class of nutrients, polyamines, and the modulation of their detection in a mating state-dependent manner.

In the first study, we investigated the impact of polyamines on reproductive success and whether flies can detect them as chemosensory cues. In order to do that, we used a combination of behavioral genetics and *in vivo* calcium imaging, which showed that polyamine-rich food increases the egg number as well as survival of the progeny. Moreover, we also found that flies can detect polyamines via both their taste and olfactory modalities. In the gustatory system, opposing valences are encoded via two receptors, GR66a and IR76b. While GR66a expressing neurons mediates an aversive taste response, IR76b expressing neurons generates an attraction and leads flies to lay their eggs on polyamine rich substances. In the olfactory system, IR41a and IR76b are co-expressed to mediate attraction to the odor source of polyamines.

In the second study, we tested whether the reproductive state has an effect on choice behavior, given the positive role of polyamines on reproductive success that we showed in the first study. In line with the beneficial impact of polyamines on reproductive success, female flies are more attracted to polyamines once they are mated. A neuropeptidergic signaling mechanism modulates sensory processing of polyamine detection in both gustatory and olfactory systems. A GPCR, sex peptide receptor, and its ligand myoinhibitory peptides modulate polyamine attraction directly at the level of the sensory neurons. Following mating, SPR expression increases in sensory neurons and modulates their physiology to enhance the attraction to polyamines. This study constitutes an example of the important role of peripheral modulation of sensory perception according to changing internal states.

6.2.Polyamine Consumption has a Positive Effect on Reproductive Success

Polyamines are important components of basic cellular processes like cell proliferation, gene expression and stress responses (Miller-Fleming et al., 2015). Therefore, an imbalance in the amount of polyamine in cells might have severe effects in an organism. Low amounts of polyamine in the body cause reproductive problems (Lefevre et al., 2011). In our study we showed that polyamine supplemented food has a direct effect on the reproductive success of *D. melanogaster* supporting the essential role of polyamines in reproduction. Females fed with polyamine rich food laid significantly higher number of eggs and produced more progeny. This beneficial effect might explain why flies prefer to feed and lay their eggs on decaying and fermenting food sources which are rich in polyamine content. Mosquitoes also lay their eggs into polyamine rich areas like standing waters. In addition, polyamine synthesizing enzymes are upregulated in the spermatheca of female mosquitoes after mating, indicating an increased need of polyamines with reproduction in those insect species, too (Gabrieli et al., 2014). Interestingly, it has been shown that spermidine, a member of polyamine family, has a fundamental role in mating and egg fertilization in *C. elegans* and yeasts, suggesting a conserved role for polyamines in fertility and reproduction (Bauer et al., 2013).

Polyamines are either produced *in vivo*, or taken up by food. With advanced age, *in vivo* production declines and a polyamine rich diet becomes more important for a healthy reproductive system also in humans. If not eaten enough, polyamine deficiency leads to age dependent sterility problems (Lefevre et al., 2011). Interestingly, this problem can be reversed by just eating more polyamine containing food on a regular basis. Polyamine supplementation not only solves the age related reproductive problems, but also improves memory performance and extends lifespan (Eisenberg et al., 2009; Gupta and Sigrist, 2013; Kalac, 2014; Minois, 2014). However, an excess of polyamine in the body might be a sign of a problem, too. Elevated amounts of polyamines are found in cancer tissues, suggesting that polyamine amount in the diet should be carefully regulated (Miller-Fleming et al., 2015).

6.3.Independent Polyamine Detection by Olfactory and Gustatory Systems

Animals use the olfactory system to evaluate long range cues, and the gustatory system for the short range evaluation. By using a behavioral genetics approach, we discovered that flies can smell and taste polyamines, and choice behavior of polyamines mediated via both modalities. In the gustatory system, there are two independent sensory neuron groups detecting two different qualities of polyamines, bitterness and the polyamine taste, respectively. In the case of pure forms, the gustatory system is dominated by the bitter taste of polyamines and flies generate an aversive egg laying behavior. However, when the bitter taste is mixed with a sugar supplement – as it is found in the decaying fruits – flies prefer to lay their eggs more into polyamine enriched sweet side, indicating that the choice behavior for polyamines is regulated with the consideration of proper food context. Such a convergence in the attractive and repulsive taste qualities may protect animals from exploiting non-beneficial sources and concentrations of polyamines. A similar modulatory interaction between sugar and bitter sensing neurons has been observed. (Chu et al., 2014; French et al., 2015; Inagaki et al., 2014). Sufficient amounts of sugar contained in food or egg-laying substracte override the bitter taste and make polyamines very attractive to flies.

In addition to the gustatory system, flies also detect polyamines with their olfactory system as diffused in the air flow. While flies use their taste modality to evaluate the food source for egg laying and eating, they use olfaction to find the polyamine rich food sources from far away. With a real-time tracking experiment, we were able to show that flies use their olfactory system first to find the polyamine rich side, and their gustatory system to evaluate the relevant side to lay their eggs. Such a multimodal detection mechanism devoted for a single class of molecules stresses the importance of polyamines as an essential part of animals' metabolism.

6.4.Ir76b is Required in Both Olfactory and Gustatory Detection of Polyamines

Polyamines are found in increasing amounts in overripe and decaying fruits which are among the favorite foods of flies. We found that IR76b receptor is necessary for the polyamine detection in olfactory as well as in the gustatory systems. Behavior experiments with the IR76b mutant flies showed that polyamine attraction is significantly reduced in those flies. In the olfactory system, in addition to IR76b, another ionotropic receptor, IR41a, is necessary for the detection. IR41a is co-expressed with IR76b in a small subset of antennal OSNs which project to a single glomerulus, VC5, in the AL. Unlike the IR41a, IR76b has a broader expression pattern, which projects to several glomeruli. Although IR76b is expressed in multiple receptor combinations, It is not clear whether IR76b plays a co-receptor role in polyamine sensitive neurons similar to other IR co-receptors, IR25a and IR8a (Abuin et al., 2011). IR25a is also co-expressed in the polyamine sensitive neurons and it could be the co-receptor for polyamine detection. However, our behavioral data with IR25a mutant flies showed that an IR25a mutation does not affect polyamine detection, suggesting that IR25a has no function in the polyamine detection. Together, these results suggest that olfactory detection of polyamines mediated through IR76b and IR41a receptors, potentially forming heteromers to form a functional receptor complex.

Egg laying and functional imaging experiments showed that IR76b is necessary and sufficient for polyamine detection in the gustatory system. While behavioral experiments with the IR76b mutant flies showed a complete loss of the egg laying choice behavior, calcium imaging with mutant flies also showed a complete loss of calcium signal in polyamine sensitive neurons suggesting that IR76b is fundamental for polyamine gustation. IR76b is expressed in the labellar GSNs and our tip recording data excludes the L-type and S-type sensilla on the labellum for polyamine detection, leaving the IR76b expressing peg taste neurons as the potential polyamine detectors. Although IR76b receptor is also expressed on the legs, leg amputation experiments showed that legs are not necessary for polyamine taste- dependent egg laying decision. Together, our data shows that IR76b receptor neurons on the labellum detect the taste of

polyamines, although more experiments needed to be done to conclude whether the peg neurons are the polyamine sensitive ones.

Interestingly, IR76b has been suggested as the salt detecting receptor in a previous study (Zhang et al., 2013a). According to the study, IR76b receptors expressed in L-type neurons on the labellum mediate low-salt attraction and function as Na^+ channels. One possible explanation of how IR76b mediates two different tastes could be that IR76b functions as a co-receptor and there are additional receptors pairing up with IR76b for salt and polyamine detection, although so far our candidate approach has failed to find additional co-receptor for polyamine detection. Another explanation could be that the same receptor may detect more than one taste molecule similar to the olfactory system. However, in the olfactory system, receptors usually detect structurally similar ligands and polyamines and salt are not comparable with their structures. Given that IR76b is broadly expressed both in the olfactory and gustatory systems, it is more likely to be that IR76b acts together with another receptor and possibly as a co-receptor.

6.5.Polyamine Sensitive OSNs Show Atypical Temporal Dynamics

In their natural habitat, animals experience dynamically changing odor environments, in which each olfactory stimulus is presented in varying lengths of time. Previous physiology experiments showed that each odor has a unique activation/inhibition pattern at the level of olfactory receptor neurons. While some are activated during the whole course of the odor stimulation period, some only get activated at the initial phase of the stimulation. However, most of them show their effect only during the stimulation time (Hallem and Carlson, 2006). In our imaging experiments, we saw that polyamines depolarize the OSNs for an unusually long period of time. Animals were stimulated for 0.5 sec. and in response polyamine detecting OSNs got activated for longer than 15 seconds. We measured the polyamine release in the odor delivery system to see whether there is a remaining trace of polyamine in the delivery. Photoionization detector (PID) measurements suggested that there might be an odor trace up to 4 seconds, which in part can explain the longer response profile but still cannot explain fully a response profile of longer than 15 seconds. Potentially, such a long response can be a feature of IR expressing neurons,

which have also been observed in another study (S. Min et al., 2013). Interestingly, it has been shown that another neuropeptide, neuropeptide Y, also has a prolonging effect on mammalian hippocampal neurons. Together with the simultaneous release of glutamate, it causes a long-term depression in both electrical activity and intracellular calcium levels (van den Pol et al., 1996). Here, the authors argue that the prolonged neuronal response is a form of cellular learning and memory that prolongs the time window where the hippocampus can adjust to changing internal states. Whether a similar temporal activity structure for polyamine sensitive neurons has a memory-like function remains unclear (see 6.9).

6.6. Multimodal Polyamine Perception is Modulated in a Reproductive State-Dependent Manner

Flies use taste and olfactory organs to find food sources to feed on and lay their eggs. We showed that female flies use the taste and smell of polyamines as a signal for beneficial feeding and egg laying sites and lay their eggs on polyamine-rich foods. Once females are mated, the multimodal polyamine detection is modulated through a neuropeptidergic mechanism (Figure 5). An SPR-dependent mechanism enhances the attraction of females to a higher concentration of polyamines after mating. Notably, mated females missing SPR in their sensory neurons show a virgin-like, reduced preference suggesting that SPR acts directly on sensory neurons. Overexpression of SPR exclusively in taste and olfactory sensory neurons was sufficient to switch virgin to mated female behavior in the absence of mating. Thus, we found a novel mechanism that requires SPR, in addition to the classical post-mating switch, which had been described previously (Yapici et al., 2008). Interestingly, SPR does not interact with its post-mating switch mediating ligand, SP, for modulation of polyamine perception as females mated to SP mutant males still undergo mating-induced changes in polyamine perception. It interacts with its alternative, though highly conserved ligands, MIPs (Y.-J. Kim et al., 2010), in an autocrine way. MIPs are expressed endogenously in the central and peripheral nervous system and not transferred from males during copulation like SP. A previous study suggests that MIPs regulate homeostatic sleep in flies (Oh et al., 2014). Our study showed a reproduction related role for MIPs for the first time.

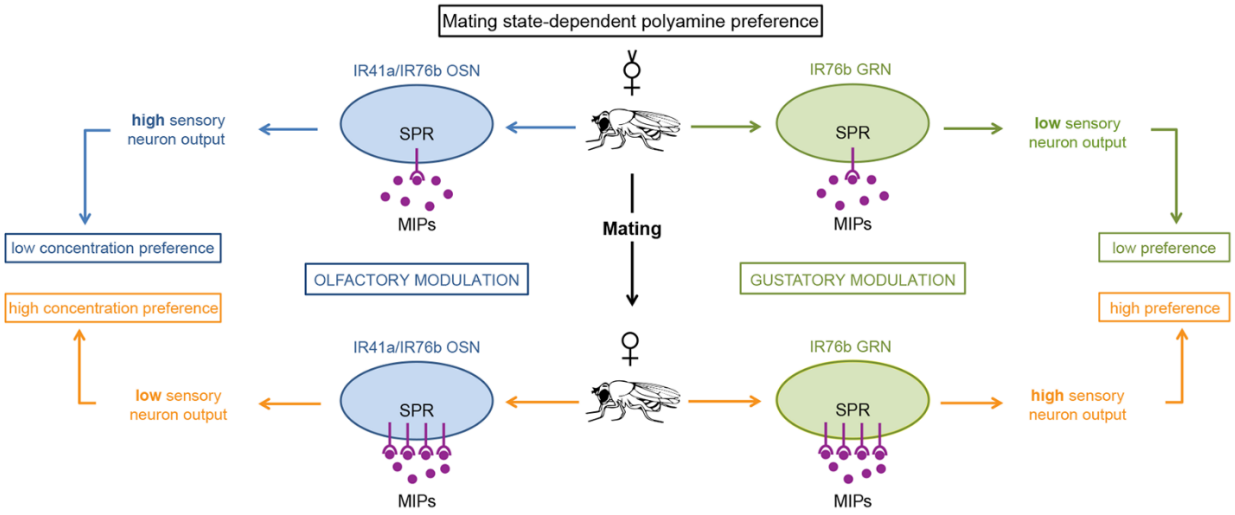


Figure 5: Model for mating state-dependent modulation of chemosensory polyamine preference.

Mating increases SPR expression in both olfactory and gustatory sensory neurons. Increased amounts of SPR in OSNs suppresses their output and shifts the females' preference to higher doses of polyamines (left side). By contrast, in GSNs, increased amounts of SPR enhance their presynaptic output thereby increasing the females' preference for polyamines as an egg laying site cue (right side). Altogether, SPR mediates two different physiological outputs in olfactory and gustatory sensory neurons to modulate the preference for polyamines towards higher concentrations.

Polyamines are not the only nutrients whose preference is modulated in a reproduction behavior-dependent manner. IR76b dependent salt preference is enhanced with mating through the classical postmating switch SPR/SP signaling pathway (Walker et al., 2015). In that study, RNAi knockdown of SPR in the salt sensitive neurons showed no effect suggesting that modulations of polyamines and salt follow different pathways although the same receptors are involved. However, the exact mechanism of how salt detection is modulated is still not known, but it is likely a consequence of mating-induced changes to the female's metabolism. In another study it has been shown that eggs to be laid in the reproductive tract of females induces acetic acid appetite by triggering mechanosensitive *ppk1+* neurons, which appear to be involved in egg-laying (Gou et al., 2014). Unfortunately, since the sensory neurons that mediate acetic acid sensitivity could not be clearly identified yet, it is not possible to go further and unravel the full pathway of this modulation. Together, these examples show that mating state-dependent neuromodulation of nutritional perception is utilized for multiple nutrients because a successful reproduction requires various adjustments in the diet of an animal.

6.7.SPR Regulates the Physiology of Olfactory and Gustatory Neurons in Opposite Directions

One of the most interesting findings of our study is that SPR mediated modulation in olfactory and gustatory sensory neurons results in opposing physiological outputs. Calcium imaging experiments by using either SPR overexpression or RNAi inhibition showed that SPR in the olfactory sensory neurons inhibits their presynaptic response and on the contrary, enhances it in gustatory sensory neurons. How can two opposing physiological effects be obtained through the same receptor/ligand pair? One possibility could be that SPR as a G-protein coupled receptor, may recruit different G-proteins in different types of sensory neurons. For instance, it has been shown that SPR recruits inhibitory $G\alpha_{i/o}$ in the canonical post-mating switch modulation and inhibits SPR expressing neurons (Yapici et al., 2008), which fits well with the polyamine sensitive OSN modulation as their presynaptic response also decreases with increased SPR expression/activation. How can an inhibition in the sensory neurons result in an induced attraction? In the olfactory system, the dose of a ligand is critical for its perceived valence. Flies detect different concentration ranges of different olfactory cues. For instance, an overdose of an attractive ligand may easily generate an aversive response. In the case of the polyamine sensitive OSNs, by inhibiting the presynaptic response, flies might be able to approach higher doses of polyamines without an overdose effect. Indeed, our behavioral experiments with different polyamine concentrations showed that mating shifts female flies' preference to higher doses. On the other hand, since in the gustatory system flies prefer polyamines in the natural food content, increased neuronal response may result a direct increase in preference behavior as the bitter taste of a high concentration of polyamines could be quenched by the sugar in the food. However, more experiments need to be performed to unravel the downstream pathways of the two ways of modulation in those two modalities.

6.8.Peripheral Modulation Mediates State-Specific Behaviors

Neuronal tuning and inter-neuronal computations are as important as the original sensory input for decision making processes. We discovered that an internal state, i.e. mating, has a

modulatory effect on the tuning of sensory neuron involved in polyamine chemosensation. Mating tunes flies to higher polyamine odor concentrations, and it also enhances polyamine preference in gustation. It is an interesting phenomenon that an internal state has control over sensory neuron physiology. Yet, it is a smart solution for a system that has to compute a multitude of inputs simultaneously. Internal state-dependent sensory neuron modulation can protect the nervous system from unnecessary noise and generates a cost-effective computing mechanism. Thus, prioritizing the inputs at the very beginning can help the organism to filter relevant sensory information dependent on its sensory context and internal state. Sensory neuron modulation is not limited to polyamine sensitive neurons and also not limited, as one would expect, to *Drosophila*. There is a similar sensory neuron modulating mechanism found for hunger in flies (Root et al., 2011). According to that study, sNPF and its receptor (sNPFR) are expressed locally in the food odor detecting sensory neurons and enhance the presynaptic activity in those neurons. The expression of sNPFR is suppressed by elevated insulin in the circulatory system when the animal is fed thereby inhibiting presynaptic activity. Decreased levels of insulin remove the suppression on the expression of sNPFR, which leads to increased presynaptic activity-mediated food search behavior. Likewise, MIPs and SPR are local signals modulating presynaptic activity of sensory neurons. We investigated SP as the potential equivalent of the insulin since it plays a major role in post-mating switch behavior. However, we did not find any significant change in the olfactory perception for polyamines in females mated to *SP* mutant males. Therefore, the global signal of mating and how mating information is integrated and transferred to polyamine sensitive sensory neurons still needs to be determined.

Targeting sensory neuron physiology for behavioral modulation is not limited to flies. A recent study in female mice showed that male pheromone sensitive sensory neurons are modulated in an estrus cycle-dependent manner via the female sex-steroid progesterone (Dey et al., 2015). Furthermore, stress-induced analgesia is another well-known example of changing the gain of peripheral sensory neurons according to changing internal states (Fields, 2004; Kane and Eyk, 2010). Stress modulates and temporarily suppresses perception of pain via presynaptic release of endogenous opoids and enkephalin, showing that principle of sensory input gating via neuromodulators extends to mammals.

6.9. Transient Modulation of Sensory Neurons Generates Long Lasting Behavioral Changes

Flies have a stereotypical mating behavior, which causes short and long term changes in female behavior and physiology through a well-defined mechanism called post-mating switch (Carvalho et al., 2006; Yapici et al., 2008). Through calcium imaging experiments performed in different time windows after mating, we found that polyamine sensory neurons are strongly modulated only for a short period of time (around 6-12 hours after mating), whereas the behavioral modulation for polyamine preference lasted for days after mating. Behavior experiments showed that polyamine attraction stays enhanced over a week after mating, which is comparable to the usage time of stored sperm in the female permathecae. This finding brings up the possibility that enhanced attraction to polyamines might be induced by egg laying behavior and not mating itself. To clarify the main reason, we tested *ovoD1* sterile mated females for polyamine attraction. Interestingly, although those flies cannot lay fertilized eggs, they still showed enhanced attraction to polyamine odor, suggesting that the mating itself and not the egg laying behavior mediates the behavioral shift. However, there is still an open question about the duration of the modulation in the physiology versus behavior. How can a sensory neuron modulation that lasts for a short period of time generate a long-lasting behavioral effect?

As discussed in a previous section, polyamines generate atypically long sensory responses following short stimuli. Such a long response of sensory neurons might generate a long-term potentiation-like effect on the postsynaptic partner of the polyamine sensitive neurons, which might lead to a memory-like effect for the enhanced odor response in the post-synaptic neurons. Thus, a transient modulation of the sensory neurons might have a longer effect in the higher brain centers. One possibility of testing this hypothesis is to image from the projection neurons that connect polyamine sensitive OSNs to higher brain centers. A Gal4-line, GH146, covers around 70% of the projection neurons that carry information from AL to higher brain centers. Unfortunately, projection neurons of polyamine sensitive OSNs are not included in this line, neither in other candidate Gal4 lines that we screened. Therefore, it was not possible to image

from projection neurons that at the post-synaptic side of the polyamine sensitive neurons to test the hypothesis. Nevertheless, it is still exciting to think that a short-term sensory enhancement may be a way of facilitating associative learning for a reward or punishment.

Altogether, previous studies and our work show that sensory neurons are not mere ligand detectors, but an important element of the homeostasis and the decision making process.

6.10. Conclusion and Outlook

How are polyamines, an essential group of nutrients, detected? And how do changing internal states affect the perception of those nutrients? In this cumulative thesis, I have presented two published studies investigating those intriguing questions. I, in collaboration with colleagues, found that IR76b, previously reported as salt receptor, detects polyamines in the olfactory system together with IR41. Moreover, polyamines are perceived differently by virgin and mated females, such that females increase their preference for polyamines upon mating. This modulation is mediated by the SPR and its ligand MIP right at the sensory neuron level. Although we suggested a complete mechanism at the sensory neuron level, there are several questions remained to be answered.

First, how does IR76b detect both salt and polyamines in the gustatory system? In the olfactory system of *Drosophila* often the same ORCO/OR-X pair detects more than one ligand. However, these ligands are usually similar to each other in terms of their chemical characteristics and/or structure. Polyamines and salt do not carry any obvious similarity in that respect leaving that possibility out. Therefore, it is possible that there is a co-receptor for the gustation of polyamines and/or salt, which still needs to be found.

Second, it is still unclear that how the reproductive system communicates with the sensory neurons to signal that mating took place. Sex peptide, the main ligand of the post-mating switch, is transferred from males locally to the reproductive organ of females and is not available in the brain. In addition, it is not required for the observed mating-induced modulation of behavior.

Instead, SPR levels change in chemosensory neurons shortly after mating and according to the behavioral experiments we conducted, the post-mating switch mediator SP is not the factor inducing it. On the other hand, we found a pair of IR76b expressing neurons in the gut area that might be an entry point to answer this question. These neurons are not ppk-positive, therefore are not expected to involve in the post-mating switch pathway. Instead, Ir76b expression in the gut may mediate internal polyamine level monitoring thereby providing a feedback to the metabolism for nutrient uptake to prevent developmental interruptions in growing embryos due to polyamine deficiency.

Third, how can a brief modulation of chemosensory neurons' physiology generate a long lasting choice behavior in females? We hypothesize that there might be a mechanism to encode the shift in the higher brain centers similar to learning mechanisms. However, more experiments need to be done to prove this claim. Preliminary experiments of a new PhD student in the lab suggest that the mushroom body, the fly's higher brain center for learning and memory is required in the mating induced decision to lay eggs on polyamine-rich media suggesting that learning might be indeed involved.

Altogether, this study contributes to our understanding on how internal states, in particular reproduction, which is much less studied as compared to other physiological states such as hunger, act on and modulate sensory systems at the peripheral level. Follow up studies hopefully will unravel the missing parts described above and provide a broader picture. Obtaining this will bring a new aspect to our understanding of the cross-talk between metabolism and physiology and the nervous system providing flexibility and adaptive properties to the nervous system, which are key features for the survival of an organism in changing conditions.

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9. Curriculum Vitae

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Education

2011 – 2016	**LMU, Munich, Germany** PhD at the Max Planck Institute of Neurobiology in the lab of Dr. Ilona Grunwald Kadow on neuromodulation of chemosensory systems in *Drosophila*.
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2007 – 2010	**International University of Sarajevo, Sarajevo, Bosnia and Herzegovina** BSc (high hons.) Biological Sciences and Bioengineering

Research Experience

2011 (March – May)	Department of Nuclear Medicine, **Klinikum Grosshadern Hospital**, Munich, Germany
2010 (Oct. – May)	Department of Pathology, **Clinical Center of the University of Sarajevo**, Sarajevo, Bosnia and Herzegovina
2009 (Aug. – Sept.)	Sensory Neurogenetics Research Group, **MPI of Neurobiology**, Munich, Germany
2008 (June – Aug.)	**INGEB Genetic Engineering and Biotechnology Institute**, Sarajevo, Bosnia and Herzegovina

Scientific Awards and Fellowships

2010 – 2016	International Max Planck Research School (IMPRS) Fast-track PhD Fellow
2010 – 2016	Ludwig Maximilians University (LMU) Graduate School of Systemic Neuroscience (GSN) Fast-track PhD Fellow
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Publications

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Hussain A*, Zhang M*, **Üçpunar HK**, Svensson T, Quillery E, Gompel N, Ignell R, Grunwald Kadow IC. (2016) Ionotropic chemosensory receptors mediate the taste and smell of polyamines. PLoS Biol 14(5): e1002454

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Conference Talks and Posters

2015	XXV ECRO – European Chemoreception Research Organization, Bogazici University, Istanbul, Turkey. (Poster presentation)
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2013	XIII ESITO- European Symposium for Insect Taste and Olfaction, Villasimius, Italy (Oral presentation)
2013	Harvard – LMU Young Scientists' Forum (YSF): From Molecules to Organisms V, Munich, Germany. (Oral presentation)
2011	INTERACT – PhD Symposium, Munich, Germany. (Poster presentation)

Teaching and Supervision

2011 – 2015	Theoretical and practical supervision of yearly masters practical course in molecular neurobiology
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10. Author Contributions

The author contributions to the papers presented in this cumulative thesis were as follows:

- Paper I: “Ionotropic chemosensory receptors mediate the taste and smell of polyamines.” Ashiq Hussain*, Mo Zhang*, **Habibe K. Üçpunar**, Thomas Svensson, Elsa Quillery,Nicolas Gompel, Rickard Ignell, Ilona C. Grunwald Kadow. - ICGK, AH, MZ, **HKÜ** and RI conceived and designed the experiments. AH performed the fly behavioral experiments (Fig. 1, 3 and 5), MZ performed the calcium imaging experiments at the SEZ and performed electrophysiological tip recordings (Fig. 4), **HKÜ** performed the calcium imaging experiments at the AL (Fig. 2), TS and EQ performed the mosquito behavior experiments (Fig. 6). AH, MZ, **HKÜ**, TS, EQ, RI and ICGK analyzded the data. AH, MZ, **HKÜ**, TS, EQ, RI, ICGK and NG contributed reagents/materials/analysis tools. ICGK, NG and AH wrote the paper with the help of the other authors.

  * Equal contributions.

- Paper II: “Neuropeptides modulate female chemosensory processing upon mating in *Drosophila*.” Ashiq Hussain*, **Habibe K. Üçpunar***, Mo Zhang, Laura F. Loschek, Ilona C. Grunwald Kadow. - ICGK, AH, **HKÜ** conceived and designed the experiments. AH performed behavior and Q-PCR experiments (Fig. 1,2, 5(except 5c), and 6a-f, **HKÜ** performed calcium imaging at the AL (Fig. 4, 6g-h and 7), MZ performed calcium imaging experiments at the SEZ (Fig. 3), LFL performed immunostainings (Fig 5c). ICGK wrote the paper with the help of the other authors.

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I hereby certify that the information above is true and accurate.

Habibe Üçpunar — Ilona C. Grunwald Kadow

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11. Declaration (Eidesstattliche Versicherung/Affidavit)

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation ‘A neuromodulatory mechanism for state-dependent nutrient detection in *Drosophila’* selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation ‘A neuromodulatory mechanism for state-dependent nutrient detection in *Drosophila’* is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den / Munich, date Unterschrift / signature