NEURONAL UNDERPINNING OF REPRODUCTIVE STATE DEPENDENT OLFACTORY BEHAVIOR IN DROSOPHILA

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I grow old always learning many things.

Solon

Abstract

A general question in neuroscience is how the flow of sensory information is encoded towards a behavioral response. These behavioral responses can be interpreted as decisions the organism needs to make to get a most beneficial outcome. Factors which can influence these decisions can be external or internal. Considering sensory information, external stimuli can elicit "innate" responses to a sensory input, which lead to a certain behavior. Interestingly, these responses can be overwritten given a certain experience or context. The internal state of an organism can be such a context.

Internal states, such as age, stress, hunger, or reproductive state can have effects on chemosensory decision making behavior. Such behavior usually manifests itself by attraction or aversion towards a certain odor or taste. Occasionally, transient neuromodulation can affect these behaviors, by focusing an animal's attention to relevant sensory stimuli in its environment. This might facilitate remembering relevant vs. irrelevant stimuli. Here, we are investigating the role of such a sensory neuromodulation and the formation of memory in the female fruit fly, *Drosophila melanogaster*.

Previous work from our lab has shown that mating changes the sensitivity of olfactory and gustatory neurons with the help of specific neuromodulators that act directly on these chemosensory neurons. However, this very transient neuromodulation leads to a long-term behavioral change in females: for instance, while virgin flies usually prefer low concentrations of polyamines, mated flies will prefer higher concentrations after the mating experience and will continue this behavior for up to two weeks until falling back to a virgin-

like state. *Drosophila*'s genetic toolset allows us to test the hypothesis that this transient sensory enhancement facilitates the formation of a long-lasting memory.

Using a quantitative olfactory choice assay, my collaborators and I silenced and activated neuronal activity in different parts of the fly's associative memory center (i.e. the mushroom body). We revealed a possible neuronal pathway and its modulatory switch between virgin and mated state. These findings suggest that dopaminergic neurons, which are innervating the mushroom body, control virgin vs. mated female behavior by processing sensory input differentially before and after mating, respectively. Furthermore, our data suggests that courtship and pheromones are highly important signals to trigger the reproductive state dependent change in olfactory preference behavior.

In addition, my collaborators and I wanted to use state-of-the-art techniques to shed light on the detection of nutrients valuable for the gravid fly by using bioinformatic tools and to promote these methods to the biological fields.

As two-photon laser scanning microscopy is an important tool for neuroscientific research in the fly and beyond, I built such a microscope. Harnessing this experience, I have, in collaboration, written a guide for life scientists wishing to build or purchase such a microscope.

A joint effort between established behavioral assays and technological advances, such as bioinformatic tools, can support and extend our understanding of neuronal circuits underlying reproductive state dependent behaviors.

Glossary

2PLSM 2-Photon Laser Scanning Microscopy.

AC adenylyl cyclase; enzyme to synthesize cyclic adenosin-mono-phosphate, which is required for synaptic plasticity, and learning and memory functions.

AD activation domain of the GAL4 transcription factor.

AL antennal lobe; primary olfactory processing center; in vertebrates analogous to olfactory bulb.

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; a class of ionotropic glutamate receptors.

cAMP cyclic adenosin-mono-phosphate; synthesized by adenylyl cyclase, and an important second messenger for synaptic plasticity, and learning and memory functions.

CSD contralaterally projecting, serotonin-immunoreactive deutocerebral neuron.

cVA cis vaccenyl-acetate; male pheromone in *Drosophila melanogaster*.

DAL dorsal anterior lateral neurons; these neurons are often octopaminergic.

DAN dopaminergic neuron; includes dopaminergic neuronal subclusters such as PAM and PPL1.

DBD DNA binding domain of the GAL4 transcription factor.

DNA desoxyribonucleic acid.

dsx *doublesex*; a sex-determination gene in *Drosophila melanogaster*.

dTrpA1 *Drosophila*-specific temperature-sensitive transient receptor potential channel A1; a cation channel able to increase sodium and calcium conductance in the neuron.

EDNH egg development neurohormone.

fru *fruitless*; a sex-determination gene in *Drosophila melanogaster*.

GAL4 transcription activator in yeast; defines a target for the GAL4-UAS directed gene expression.

GCaMP GFP-Calmodulin protein; in the GAL4-UAS system: enables to image neuronal activity via green fluorescent protein at calcium channels.

GFP green fluorescent protein; in the GAL4-UAS system: enables to image target cell populations.

GPCR G-Protein coupled receptor; GPCRs can detect neuromodulators and are found in various sensory systems in higher animals; can subsequently activate second messengers such as cyclic adenosin-monophosphate.

GR gustatory receptor.

GSN Graduate School of Systemic Neuroscience, Ludwig Maximilian Universität, Germany.

iACT inner antennocerebral tract; old nomenclature for medial antennal lobe tract; one path for projection neurons to reach higher brain centers.

iGluR ionotropic glutamate receptor.

IR ionotropic receptor, ancient class of chemosensory receptors.

JH juvenile hormone.

KC Kenyon Cell; intrinsic neurons which form the mushroom body lobes and synapse onto mushroom body output neurons.

Kir potassium inward rectifier; in the GAL4-UAS system: enables block of synaptic output.

lALT lateral antennal lobe tract; one path for projection neurons to reach higher brain centers.

LH lateral horn; a higher brain center for olfactory processing; in vertebrates analogous to amygdala; mainly considered for innate behaviors.

LHON lateral horn output neuron.

LN local neuron; local interneurons of the antennal lobe.

mACT medial antennocerebral tract; old nomenclature for mediolateral antennal lobe tract; one path for projection neurons to reach higher brain centers.

mALT medial antennal lobe tract; one path for projection neurons to reach higher brain centers.

MB mushroom body; a higher brain center for olfactory processing; in vertebrates analogous to piriform cortex; mainly considered for behaviors regarding associative learning and memory.

MBL Marine Biological Laboratory, University of Chicago, MA, USA.

MBON mushroom body output neuron.

MIP myoinhibitory peptide; ancestral ligand for the sex peptide receptor.

mlALT mediolateral antennal lobe tract; one path for projection neurons to reach higher brain centers.

NMDA N-methyl-D-aspartate; a class of ionotropic glutamate receptors; also a receptor involved in polyamine detection in rats.

oACT outer antennocerebral tract; old nomenclature for lateral antennal lobe tract; one path for projection neurons to reach higher brain centers.

OR olfactory receptor.

ORCO olfactory receptor co-receptor; universal co-expressed receptor in olfacotory receptors (not in ionotropic receptors).

ORN olfactory receptor neuron; primary sensory neuron for the binding of odorants.

OSH oviduct stimulating hormone.

OSN olfactory sensory neuron; alternative name for olfactory receptor neuron; primary sensory neuron for the binding of odorants.

PAM protocerebral anterior medial cluster; cluster of dopaminergic neurons innervating the mushroom body.

PBAN pheromone biosynthesis activating neuropeptide.

PN projection neuron; secondary order neurons in olfactory processing; synapses onto Kenyon Cells in the mushroom body, or onto lateral horn neurons.

ppk *pickpocket*; a proprioceptive neuronal marker in the reproductive tract of *Drosophila*.

PPL1 protocerebral posterior lateral cluster 1; cluster of dopaminergic neurons innervating the mushroom body.

PTSD post traumatic stress disorder.

RNA ribonucleic acid.

SP sex peptide; a seminal fluid protein which is transferred to females during copulation; involved in numerous post-mating responses.

SPR sex peptide receptor; potential ligands: sex peptide and myoinhibitory peptide.

UAS upstream activation sequence; defines effector for the GAL4-UAS directed gene expression.

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Chapter 1

Introduction

The following sections will provide a short fundamental introduction into relevant fields and methods necessary for analyses during this work. A deeper insight will be given in the respective papers and manuscripts throughout this cumulative-style dissertation.

1.1 Sensory systems and decision making in neuroscience

Senses guide us through our daily life and through every environment. They tell us what is good or bad, or when to reconsider our actions based on the feedback we gain from them. This is not only true for humans, but for any animal. This multi-modality on how we perceive our world is guided by our brain. It takes the different information provided by the sensory organs, puts it into context, and gives value to it.

Based on the following combined interpretations of the sensory modality, we formulate decisions. For animals, many decisions are based on survival instinct. What, where and how to move, to eat, to rest: sensory systems help to navigate through vision, audition, or olfaction. By using vision and

gustation, food sources can be evaluated. Somatosensation not only interprets surface structures as haptic feedback, but provides stimuli like pain to indicate damaging factors. Loosing one or multiple senses makes it more difficult to interpret an environment and can be dangerous, if not life threatening.

When forming a decision the simplest outcome is a yes if interpreted attractive, or no if it appears more aversive. This decision can be initially dependent on the sensory perception. Initial responses are considered to be innate. Innate behaviors are corresponding not only to reflexes, but also naïve actions and reactions. Some of those innate responses follow fixed action patterns and appear rather hard-wired. However, even an innate attraction can become aversive if put in context with certain experiences or vice versa. Habituation, imprinting or conditioning have been under investigation for many years and repetitive contradicting stimulation can modulate behavior by rewarding or punishing actions. Environments can be perceived through sensory systems. Context can have influence on values and subsequently guide and adapt decision making behavior.

1.1.1 Chemosensation

Chemistry builds the fundamentals of our world, which is why chemosensation is one of the oldest senses. The detection of chemical compounds and reacting to them can prove useful for different parts of survival. This is not only true for single-cell, but also for any more complex organism. Some species are using chemosensation not only to navigate through environments, but also to find food sources, communicate with possible mating partners, or find good oviposition sites for their offspring [1].

Particularly the sense of smell gives information over longer distances as well as within close proximity. A degradation of a certain food source can be detected via odors prominent for decay. This could be of interest to species which found their ecological niche in environments of decay, such as rats or

certain types of insects. Furthermore, chemosensory perception can be based on physiological needs of an organism and the metabolism can communicate such physiological or nutritional needs.

In medical applications, olfaction is further considered the state of mind and psychology: olfaction is highly emotional state dependent, and often associated with memories of past stressful events, e.g. in post-traumatic stress disorder (PTSD) [2–5].

1.1.2 Effects of internal state on decision making

Not just stress, as in PTSD, can affect chemosensory perception. Other internal states of an organism, for instance sickness, or hunger have the ability to adapt behavior. While hungry, some food sources become more attractive and less aversive as long as they appear eatable. A decision to hunt for a food source which may initially imply to spend a great amount of energy, weighs up against the nutritional value. This means that an internal state is able to shift the animal's attention to factors that really matter, e.g. based on nutritional or metabolic needs.

1.1.2.1 Nutritional value and polyamines

Metabolic state drives actions. An animal lacking certain nutrients, necessary for its survival, will eventually start searching for these nutrients. The lack of such nutrients may be due to a dysfunctional endogenous production. Hence, searching for these substances exogenously will be beneficial for the animal. Particularly in early developmental stages or during reproduction, the metabolic state of an animal is constantly changing, for instance the production levels of polyamines [6].

Polyamines, such as putrescine, spermidine or spermine are poly-cations known in prokaryotes and eukaryotes, respectively. They are able to bind to negatively charged molecules. These may not only be receptor molecules,

but also DNA and RNA. Thus, polyamines can directly alter gene expression and subsequently protein synthesis: It is known that the gene expression in the fetus seems highly dependent on the maternal nutritional intake [7, 8]. Although they are endogenously produced from argenine (via arginase and ornithinedecarboxylase), it seems that due to the increased need for cellular proliferation during pregnancy, many species are actively seeking for polyamines [9–14]. Thus, polyamines provide nutritional benefits for parent and offspring, respectively. On the other hand, there are studies reporting that high levels of polyamine can be detrimental or cancerogenic [12, 15, 16]. Finding the right balance of nutrients seems to be essential.

1.1.2.2 Neuromodulation

Neuromodulation is the key to constantly evaluate, adapt and integrate the combinations of sensory cues, innate and learned behaviors, internal states, and metabolic needs to provide a proper decision making behavior. Neuromodulators range from simple neurotransmitters (like dopamine or nor-epinephrine), over neuropeptides (allostatin or corazonine) to other small signal molecules, such as hormones. A given neuronal network can become more robust, be degenerated or modulated for a certain time frame [17]. Moreover, it can be influenced by internal state as well. In insects, neuropeptides have been shown to be involved in many roles for changing neuronal signaling: not only with respect to reproductive state dependent changes, but also in social interaction, learning and memory functions, addiction, or sleep [18, 19]. Effects of such neuromodulation can be spontaneous, reversible, and temporarily or permanently change circuit compositions [20–24]. While intrinsic neuronal properties can be changed, they may even have an effect on retrograde cells as well. Such neuromodulations happen all over the body and already on the sensory level [25, 26].

Many neuromodulators are detected by G-protein coupled receptors (GPCRs), which are a superfamily among regulatory receptor types [27]. Such GPCRs are found in any sensory system (opsins, odorants, etc.), but also in immunol-

ogy (histamines), or in above mentioned behavioral regulation (via neuromodulators) [27, 28]. Signal transduction by GPCRs is prominent in many higher organisms [29] and is induced when an agonist binds to the membrane-bound GPCR [30]. Subsequently, the conformation of the GPCR changes, which ultimately activates effectors such as secondary messengers. A prominent secondary messenger is cyclic adenosin-mono-phosphate (cAMP), which is responsible for the regulation of ion channels and can therefore lead to a depolarization of a neuronal cell, ensuring the firing of action potentials. cAMP is synthesized by the enzyme adenylyl cyclase (AC), and studies revealed that genes identified in learning and memory correspond to the cAMP pathway [31].

1.1.2.3 Reproductive state

While animals are constantly adapting to their environment, there are two major drivers for decision making: survival and reproduction. Particularly aspects of reproduction, such as finding the right mating partners, reproductive success and offspring survival are key elements in evolution. Mating partners are chosen by their appearance, odor, or ability to perform certain actions [32]. After copulation, reproductive success can for instance be influenced by the metabolic state of the female and may require certain nutrients, such as the above mentioned polyamines. Thus, chemosensory perception can be altered during pregnancy and often corresponds to changes in their physiological needs [33–35].

Interestingly, animals can show diverse switches in their behavior after the mating experience due to changes on the neuronal level: it has been shown in rats that the reorganization of maternal brain structures during pregnancy leads to a long lasting altered olfactory interpretation of the environment [36, 37]. Pup odors can thus trigger a behavioral response in the mother when it comes to decisions regarding rearing the offspring; if and when to leave the nest, recognition and communication. Such changes in the brain are also known in humans, where the gray matter of the brain is altered in areas

of social cognition, potentially implying the necessity to detect needs and mental states of the child [38]. Such adaptations of neuronal morphology or olfactory preference are not only specific to copulation, but can be dependent on the menstrual cycle [34, 35, 39–41].

However there are still many open questions regarding reproduction on a neuronal level. How is the signal of mating conveyed to neuronal circuits? How are sensory systems altered depending on reproductive state? To address these questions more research is necessary.

1.1.3 The model organism *Drosophila melanogaster*

Drosophila melanogaster is an excellent model organism in various fields as shown by numerous Nobel prizes [42–47]. Especially from a neuroscientific perspective, *Drosophila* has a nervous system with adequate complexity in order to perform animal-wide comparisons. The advantages of model organisms, such as *Drosophila melanogaster*, are more than just a "simplification of a neuronal network" based on the number of neurons involved. Though *Drosophila*'s genome is approximately 4-5 % the size of humans', one of its major advantages are the possible genetic manipulations. Molecular genetic tools are widely available and allow to manipulate this species even on a neuronal level [48–55]. Thus, researchers can study behavioral outcomes and find evolutionary coherences. An additional benefit are *Drosophila*'s simple living conditions, their short life cycle and cheap maintenance.

Drosophila melanogaster is classified to the genus of *Drosophila* in the family of *Drosophilidae* in the order of *Diptera* and belongs to the class of *insects* within the animal kingdom [56]. It develops over three larval, one pre-pupal, one pupal and finally towards the adult stage, and this development can be influenced by external factors such as surrounding nutrients, temperature, or population size [57]. *Drosophila* has four chromosomes: the first is the sex chromosome, the second and third are large autosomes and the fourth is a small autosome which is often neglected for genetic manipulations [55].

Genetic tools, which will be discussed in the following section, allow for comparative studies with other species. For instance, *Drosophila melanogaster* shares nearly 75% of human disease-causing genes and has allowed for the investigation of numerous metabolic and internal state dependent mechanisms, including neurodegenerative or psychiatric disorders [58, 59].

1.2 Technical features and advances in behavioral neuroscience with respect to *Drosophila melanogaster*

In 1910, Thomas Morgan discovered that a mutation in a certain gene (i.e. the *white* gene) leads to a change in the eye color of the fly [60]. Ever since then, diverse systems have been targeting the genotypic and phenotypic differences, particularly with biological driven questions regarding diseases or behavioral features. For instance, gain-of-function or loss-of-function experiments have been performed through spatio-temporal targeting systems, such as GAL4-UAS, LexA-LexAop, Flp-Frt, and QF-QUAS [48, 61–65], which allow for single cell targeting, as well as targeting whole cell populations. Similarly, RNA interference (RNAi) [66] or the rather recent CRISPR-CAS technology [67] are used in the field. Using these systems, we can also induce fluorescent markers, calcium indicators [68], or recently discovered trans-synaptic labeling techniques [69]. Electron microscopy and connectomics on larval and adult organisms reveal neuronal connectivity and need to be analyzed further for their functional units [70–74]. Researchers are able to target, manipulate, modify, visualize and monitor neuronal activity down to single cell level and up to whole brain imaging, *ex* and *in vivo*, and even in real time. These technical features and advances may bring us one step closer in addressing biological questions from different perspectives and angles.

1.2.1 Spatio-temporal targeting of neurons

Particularly the GAL4-UAS system is a well-established technique for spatio-temporal targeting [48,52,62]. This binary system is derived from yeast. The transcription factor GAL4 is under control of an endogenous promotor, often providing the spatial aspects of cell types and tissues to be targeted. GAL4 will recognize and subsequently bind to an upstream activation sequence (UAS), which itself activates the expression of a downstream effector [63,75] (see Figure 1.1 A). The activation can further be regulated temporally either via the GAL system (e.g. $GAL80^{ts1}$), or the effector response [76,77].

A broad selection of GAL4 and UAS driver lines are available from many fly stock centers [78–80]. Those lines represent the parental flies and only the combined filial F1 generation will have the desired expression pattern [50,81]. GAL4 rarely has an effect by itself, which is also true for UAS. The resolution of spatial targeting was increased further by the arrival of the split-GAL4 system [48, 82–84]. Here, the GAL4 transcription factor is split into two domains: the activation domain (AD) and the DNA binding domain (DBD), which can both be expressed under different promotors. Hence, each of these proteins is expressed separately. In order for this method to work, both proteins need to be expressed in the same target cell population. They heterodimerize via a leucine zipper motif leading to the formation of the functional transcription activator GAL4, which can be used as described above [48, 62] (see Figure 1.1 B). Both proteins expressed on their own are transcriptionally inactive. Often empty-GAL4 constructs, which have no regulatory fragment, are used as a control (see Figure 1.1 C).

The effectors and responders located downstream of the UAS are able to allow for different manipulations of the targeted subpopulation of neurons. To visualize cells, UAS-GFP can induce fluorescence; UAS-GCaMP is a calcium indicator to visualize neuronal activity in vivo [85–87]. Even more, targeted neurons can be silenced or artificially activated, especially in a temporal fashion using thermogenetic effector proteins.

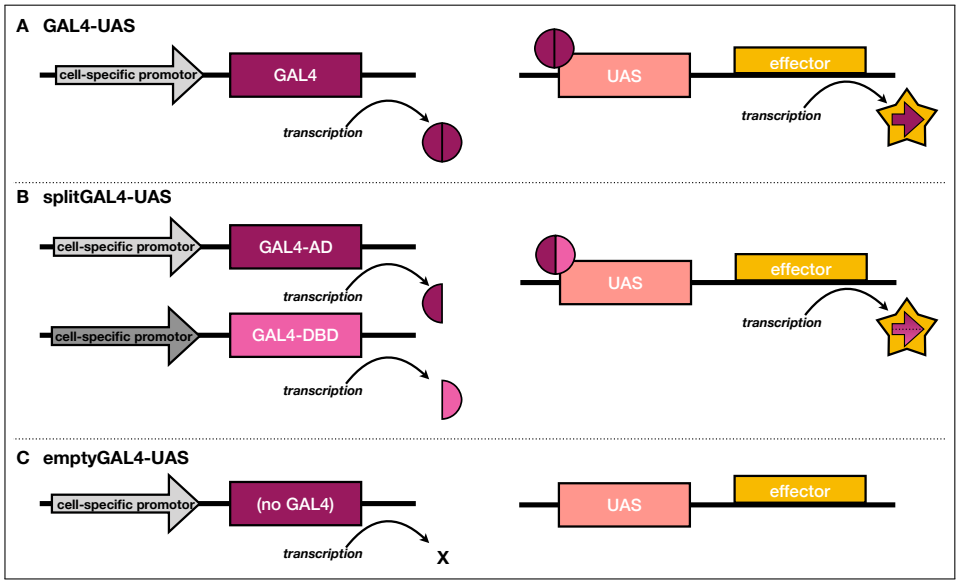

Figure 1.1: (**A**) After the promotor-specific transcription, GAL4 will recognize and bind to UAS which subsequently expresses a downstream effector. (**B**) The activation domain (AD) and DNA binding domain (DBD) of GAL4 can be transcribed under different promotors. If they are expressed in the same target cell population these proteins will be linked via leucine zippers and bind to UAS to express a downstream effector. (**C**) In an empty-GAL4 construct there is no regulatory fragment, thus the downstream effector cannot be expressed.

Such a temperature sensitive effector protein is UAS-Shibire^{ts1}. The gene *shibire* encodes the protein dynamin, which is involved in endocytosis and essential for synaptic vesicle recycling [88, 89]. The temperature sensitive allele version (abbreviated ^{ts1}) leads to a defective endocytosis at temperatures over 29 °C, by mis-folding of the vesicle scission protein dynamin, and therefore silences neuronal activity [62,88] (see Figure 1.2 left). *Shibire^{ts1}* belongs into the category of dominant-negative mutations. It is semi-dominant, meaning heterozygous flies are more sensitive to high temperatures than wild-type flies. This inhibitory effect of neuronal activity is reversible once the temperature is lowered to the permissive level of under 28 °C. Other silencing methods are tetanus toxin or the overexpression of inward-rectifier potassium channels (Kir) [89–91]. Channelrhodopsins allow for temporal silencing via light indicators as well [92].

To artificially activate a neuronal subset, a temperature-sensitive transient receptor potential channel A1 (dTrpA1) is inserted downstream of the UAS [50, 51]. A temperature over 25 °C leads to a depolarization of the cell (see Figure 1.2 right). Other prominent methods are using channelrhodopsins like CsChrimson or red-activateable channelrhodopsin (ReaChr) [93, 94].

The temporal and spatial control of neurons can therefore enhance behavioral studies to show the functional significance such as necessity, and sufficiency of neuronal sub-populations with respect to certain behaviors [51, 95]. The established system of spatio-temporal targeting in *Drosophila* is still growing and improving. These remarkable tools available will help future generations of researchers to get a good base understanding about how neuronal systems work and function.

1.2.2 2-Photon Microscopy

Among the first biological samples to be analyzed under a microscope were the polyamines spermine and spermidine by Antonie van Leeuwenhoek in the 17th century [96]. Ever since then microscopy developed rapidly to fulfill the

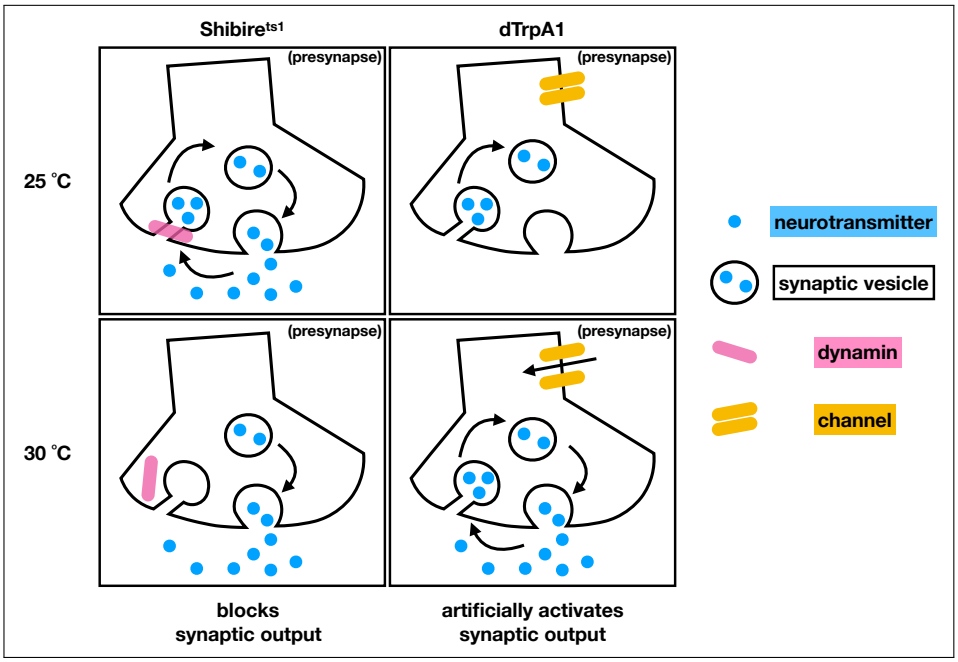

Figure 1.2: (**left**) The temperature sensitive effector Shibire^{ts1} expresses a functional dynamin protein for synaptic vesicle recycling. When the temperature is raised, dynamin cannot properly fold to its designated structure. Thus, there is no endocytosis of the synaptic vesicles; this ultimately blocks synaptic output. (**right**) Upon a temperature raise, the temperature dependent cation channel dTrpA1 increases sodium and calcium conductance in the neuron. This leads to a depolarization of the cell, i.e. activation of neuronal output.

needs for better *in vitro* and *in vivo* applications, especially regarding resolution and scale. Modern microscopy, particularly multi-photon imaging in *Drosophila*, has allowed scientists to formulate correlations between behavior and neuronal activity *in vivo* [97–101]. We are able to track neurons using photoactivatable GFP and subsequently gain knowledge, for instance, on the pheromone circuitry [102].

To compete in scientific environments, such modern tools have to be properly established for different purposes and easily adaptable to the need of a laboratory. Unfortunately this demands a broader scale of background knowledge not only in the biological systems, but also in the underlying physics. Advances should be made to transfer this knowledge in a simplified manner for the broader audience.

1.2.3 Bioinformatics

Similarly, over the past decade, bioinformatic tools, particularly those from the "omics" (i.e. transcriptomics, genomics, proteomics, etc.) fields have been rapidly evolving to fill the gaps between computational power and biological applications. From DNA sequence alone, these *in silico* bioinformatic tools are able to make accurate predictions and models for protein structures, functions, interactions, dynamics, and evolutionary connections. These tools also made it possible to create an interactive database for gene expressions [103], or to identify neuropeptide precursor genes and their use for characterizations in mass-spec analyses [104].

Computational approaches have been successfully used to analyze *Drosophila*'s chemosensation. After finding the genetic background for chemosensory receptors [105–107], bioinformatic tools were able to analyze these chemosensory receptors even further despite their lack of structural similarity to mammal olfactory systems [108–114]. Eventually, researchers were able to decipher structural scaffoldings and could make predictions on the sensitivity of chemosensory neurons [108–110,115–118]. Interestingly, the fly has taught

computer scientists how to improve the performance of computational similarity searches [119]. By comparing known computational algorithms to the olfactory processing of *Drosophila* the researchers were able to develop a novel strategy for similarity searches.

The advances of bioinformatic methods have not yet shown their full potential in the field. They could help future generations of researchers to find potential candidates for further investigations. Having knowledge of the genetic code of several generations of species can provide not only comprehension in an evolutionary perspective, but may also hint at functionally important residues in single proteins even on the level of an individual.

1.3 The olfactory pathway of *Drosophila melanogaster*

For *Drosophila melanogaster*, chemosensation is one possibility to scan its environment, e.g. to detect mating partners via pheromones, evaluate food sources, and find suitable oviposition sites. Using chemosensory organs, the fly is able to scan its environment. Olfaction can be used for long range cues using the antennae and the maxillary palp. Gustatory information is perceived using the labellum, legs and wing margins and is rather short ranged.

1.3.1 Early processing - from receptors to higher brain centers

Olfactory perception in *Drosophila* is initiated by the binding of an odorant to olfactory receptors on olfactory receptor neurons (ORNs) (also called olfactory sensory neurons (OSNs)), located at sensilla on the antenna or maxillary palp. There are three types of receptors in the olfactory system [105,107,108,120]: olfactory receptors (ORs), ionotropic receptors (IRs),

and a few gustatory receptors (GRs). ORNs express only one type of receptor [121–123], which can either be specific, or detect more than one odorant, while an odorant can activate multiple ORNs [120, 124–126]. This already shows the complexity of ORN systems.

When an OR is expressed, it is co-expressed with an another universal receptor, namely the OR co-receptor, ORCO (also known as OR83b) [121, 127]. A fly is almost anosmic if it has a mutation in ORCO [128]. However, ORs are not the only detectors for olfactory cues. Particularly amines have been found to be detected by a different class of ORNs [108,129,130]: IRs. IRs are also expressed with other co-receptors [131]. Interestingly, IRs can also act as gustatory receptors and are therefore not restricted to olfactory organs, implying a broader role for their functionality [108, 113, 132–134]. Furthermore, IRs represent a more ancient class of olfactory receptors, as they are evolutionary conserved among other insects, animals, but even bacteria and plants [108, 135]. Even though their name partially reveals that they are part of the ionotropic glutamate receptor (iGluR) family, IRs are still considered to be distinct from the standard three classes (i.e. AMPA, NMDA and kainate).

The interplay of OR, IR and the few GRs shows that chemosensation is an integrative multimodal task in *Drosophila* and may explain how different odors and their concentrations can be differentiated and processed [101,136, 137]. Odors, detected via the ORNs, are processed in approximately 51 glomeruli within the antennal lobe (AL) (see Figure 1.3), which leads to the ability to detect not only a variety of odors, but also their distinct features within a chemical space [121, 136, 138, 139]. The AL defines a topographical map, based on the OR-specific ORN tracing [138,140,141]. While ORNs send their dendrites towards the AL, they synapse either onto local interneurons (LNs) or projection neurons (PNs). LNs serve to connect the glomeruli of the AL to each other. The predominantly inhibitory LNs form dendritic connections to ORNs or PNs, while excitatory LNs only innervate PNs, both with modulatory functionality [142–146]. PNs connect the AL to higher brain processing areas: the mushroom body (MB) and the lateral horn (LH) [147–

152]. Inhibitory and excitatory PNs reach these higher brain centers on partially different pathways [101,153–155]: inhibitory PNs generally augment the innate discrimination of related odors and are mostly projected though the mediolateral antennal lobe tract (mlALT; previously mACT) towards the LH. Excitatory PNs are proposed to encode the characteristic of an odor and are projected through the medial antennal lobe tract (mALT; previously iACT) to both MB and LH. PNs may also reach the LH, MB and other regions via the lateral antennal lobe tract (lALT; previously oACT) (see Figure 1.3 right).

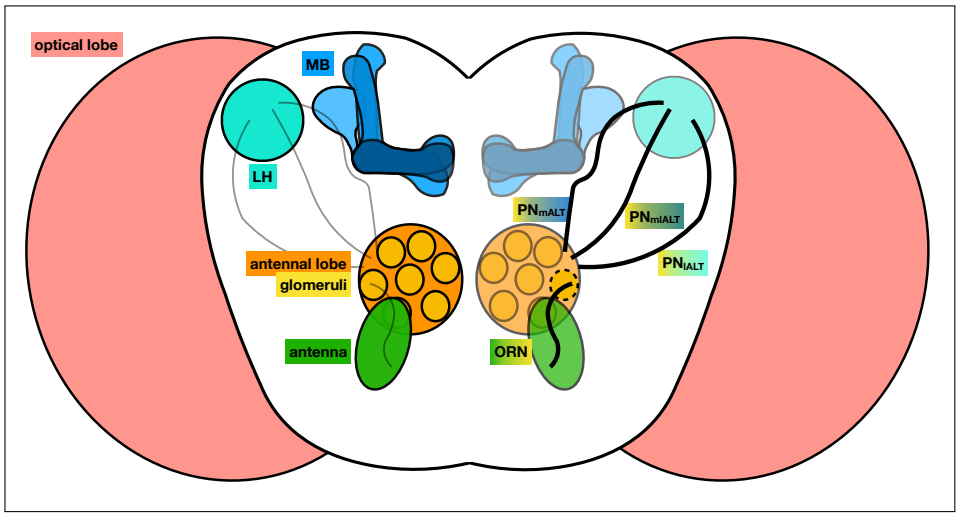

Figure 1.3: The olfactory pathway of *Drosophila melanogaster* progresses from odor detection at the antenna (green) via olfactory receptor neurons (ORNs) to glomeruli (yellow) in the antennal lobe (orange). Projection neurons (PNs) forward this olfactory information via the medial (m), mediolateral (ml), or lateral (l) antennal lobe tract (ALT) to the higher brain centers, the lateral horn (LH; cyan) and or the mushroom body (MB; blue).

1.3.2 Late processing - higher brain centers and their output

The LH and MB belong to the protocerebrum of the fly and are still not completely understood. Over the past years our initial understanding of

these brain areas has changed dramatically based on the findings of experimental studies and connectomics data: the invariant circuitry of the LH was long considered for innate behaviors like courtship, and sex-specific processes [153, 156, 157]. The LH is capable of odor responses in a segregated fashion and is involved in learning paradigms [153, 154, 158–161]. The MB was initially considered to be an associative learning and memory center [162–164]. More research has shown its involvement in context-dependent choice behavior [165–169]. The MB is further involved in the proboscis extension reflex or sleep regulation [170–172], innate food-seeking behaviors [173], and interestingly also in sex- and reproduction-specific behaviors like courtship, post-mating behaviors and oviposition [170, 174–176]. Connectomics revealed that both MB and LH are actually highly interconnected [84, 159, 160, 177, 178] allowing for interpretation of the interchange of information regarding context and value to certain odor cues.

The MB is intrinsically formed by over 2000 neurons, called Kenyon Cells (KC)s. From the MB calyx (see Figure 1.4), KCs progress in parallel through the peduncle towards a bifurcation point. Here, KCs form two vertical lobes α and α' and three horizontal lobes β, β', and γ [179]. KCs are proposed to be cholinergic (excitatory) neurons with three major projection patterns, $\alpha\beta$, $\alpha'\beta'$, and γ. These three classes of KCs can be divided into seven cell types that occupy specific layers within the MB lobes: the γ lobe is divided into main and dorsal layers by two cell types, the α'/β' lobes are divided by two cell types into middle and anterior-posterior layers, and the α/β lobes are divided into posterior, core, and surface layers by three different cell types. Even though KCs receive the input in the calyx from PNs in a randomized fashion, KCs can be sufficient for discriminating odors [179–183]. This likely occurs through either combinatorial and or distinct projection patterns via the PNs [163, 184–186].

KCs have *en passant* synapses with MB output neurons (MBONs). Interestingly, there are only 34 MBONs with 21 distinct subtypes [178, 179]. This means that the high-dimensional KC representation of odor identity is transformed into a low-dimensional MB output [179, 187, 188]. MBONs are defined

by their compartment within the MB structure. The cell types can either be cholinergic (excitatory), glutamatergic (inhibitory), or GABAergic (inhibitory). MBONs send their dendrites to areas outside the MB as well as other MBONs [182]. MBONs can show odor responses and often represent a first valence categorization into aversive and attractive odors [179, 187, 189, 190].

The synapses of and between KCs and MBONs have been shown to adapt during associative learning paradigms using modulatory dopaminergic neurons (DANs) [166,179,191–195]. KCs can also directly synapse onto DANs [72]. The axon terminals of the DANs project to specific compartments of the MB, where 17 out of the 20 DAN types project to only one single compartment. There are two main clusters of DANs innervating the MB and their names correspond to their location within the brain: the protocerebral anterior medial cluster (PAM) and the protocerebral posterior lateral cluster 1 (PPL1) [179] (see Figure 1.4). In associative appetitive learning paradigms PAMs seem to act as positive re-enforcers to negatively categorized odors [194, 195] and PPL1s vice versa as negative re-enforcers to positively categorized odors [179, 193, 196]. Thus, contextual cues, or internal state have an effect on the MB via these DANs [165, 166, 197], at both level of KCs or MBONs [70, 72, 189]. Hence, the DAN network information is processed in parallel and additive and there is crosstalk and feedback in dopaminergic circuits and within the MB [165, 198, 199].

Dopamine, and correspondingly DANs in the MB have been linked to olfactory memory formation, retrieval, re-evaluation and forgetting [200–203]. Such learning and memory paradigms require plasticity in the MB and DAN network [204]. It has been shown that the release of dopamine requires adenylyl cyclase (AC) activity [187, 201, 205]. AC is encoded by the gene *rutabaga* and has been shown to be a key player in associative conditioning and long-term memory [76, 164, 206–209].

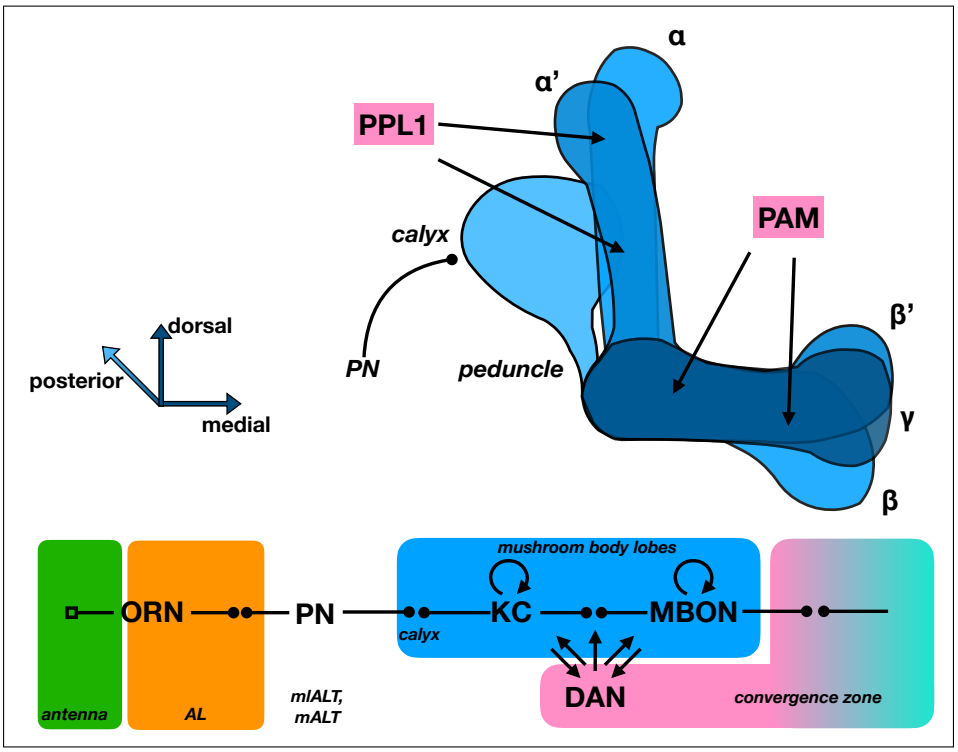

Figure 1.4: After olfactory signals are detected at the antenna (green) and sent via ORNs into the AL (orange), PNs forward the information to the calyx of the MB (blue). KCs, which form the lobular structure of the MB are progressing in parallel through the peduncle towards a bifurcation point at which the diverge into the distinct lobes (the vertical α and α' lobe, as well as the horizontal β, β', and γ lobe). Here, KCs synapse onto MBONs which eventually send their dendrites to the convergence zone (i.e. areas surrounding the MB; or the LH(cyan)). DANs of two clusters (i.e. PPL1 and PAM; pink) can innervate KCs and MBONs depending on their lobular location, respectively.

1.4 Neuromodulation in olfaction in *Drosophila melanogaster*

In general, it can be stated that dopamine is one of the driving factors in learning and memory functions [210]. However, some olfactory associative memory functions have been shown to be affected by octopamine [194, 211]. Octopamine seems to be present in dorsal anterior lateral (DAL) neurons, which also have effects on long-term memory. They are required in the interplay of memory consolidation (MB to DAN), storage (DAL) and retrieval (DAL to MB) [212], and express proteins known for long-term memory functionality (e.g. N-Methyl-D-Aspartate (NMDA) Receptors) [213].

Within the antennal lobe (AL), contralaterally projecting, serotonin-immunoreactive deutocerebral neurons (CSDs) are receiving glomerulus-specific modulatory information from the ORNs, as well as LNs and downstream PNs. CSDs themselves synapse onto LNs and PNs [214].

Even at the olfactory sensory level, responses of ORN are a target for modulation. Olfactory sensation of the environment can be modulated by neuropeptides or biogenic amines to unite the chemosensory circuit with the internal state of the organism [18, 215–217]. Innate habits like feeding, courtship, and post-mating behaviors can thus be adapted according to the animal's needs.

Furthermore, olfactory neurons are able to recognize microbiomes within the microbiota of the *Drosophila* gut, which has subsequent effects on the nutritional preference or foraging behavior [218]. Modulatory neurons can even target astrocytes which can tweak downstream dopaminergic neurons [219].

This broad array of neuromodulatory inputs to odor processing at any level of the olfactory pathway will be elaborated further in the review I wrote with my collaborators (**manuscript 1** [217]).

1.5 Reproductive state in female *Drosophila melanogaster*

Particularly in reproductive state, *Drosophila melanogaster* undergoes a series of modulations, as well as physiological and behavioral changes. Seminal fluid proteins have been shown to cause changes in the uteri and reproductive tracts of *Drosophila* [220, 221]. Sperm, as well as other seminal fluid proteins, e.g. sex peptide (SP), is transferred to the female during copulation and can be stored for several days [19, 216, 222–224]. SP has been shown to be a key player for post-mating responses. If SP is not present, female flies do not elicit post-mating responses [222, 225, 226]. Even more, injected SP can drive post-mating responses in virgin flies [226], such as the rejection of males, changes in oogenesis and oviposition as well as altered nutrient consumption [227–229].

SP activates its receptor, SPR, in sensory neurons that co-express the sex-determination genes *doublesex* (dsx) and *fruitless* (fru) as well as the proprioceptive neuronal marker *pickpocket* (ppk) in the reproductive tract, which is subsequently projected to higher brain areas [32, 156, 230, 231]. fru expressing neurons have also been found in ORNs and PNs responsible for the detection of the male pheromone cis vaccenyl acetate (cVA) [153, 232–235]. cVA is also transferred to the female during copulation. While cVA is initially responsible for social communication and courtship behaviors [236–238], it changes its functionality after mating. On the one hand, males are rejected after mating as detected via the now aversively interpreted pheromones [239], on the other hand, the transferred cVA can be used as olfactory cue for good oviposition sites [240]. Furthermore, cVA can be interpreted differently depending on the reproductive state of the animal and its surrounding nutrient sources [235].

Nutritional needs and behavioral preferences correlate with reproductive state [241]. Previous studies revealed that while virgin flies prefer low concentrations of polyamines, mated females prefer higher concentrations [13, 116],

possibly because polyamines are giving extra nutritional value for the gravid female. Polyamines are detected via co-expression of two IRs, namely IR76b and IR41a. These ORNs co-innervate the VC5-glomerulus (i.e. ventral central glomerulus 5) in the AL. Polyamine attraction is modulated by SPR directly on these ORNs, though not via SP, but its alternative ancestral ligand, the myoinhibitory peptide (MIP) [216, 242]. A fly which has mated has an approximately ten fold higher expression for SPR and subsequently modulates ORN output [13, 116] (see Figure 1.5 A). This modulation is only very transient and happens within the first 6-12 hours after the mating experience. The attraction behavior towards polyamines, however, is changed for an elongated time period of up to two weeks before returning to classical virgin-like attraction. This long-term behavioral change allows for experiments on the involvement of a reproductive state dependent neuromodulation, underlying changes in synaptic plasticity, and its relationship to learning and memory functions [95] (see Figure 1.5 B).

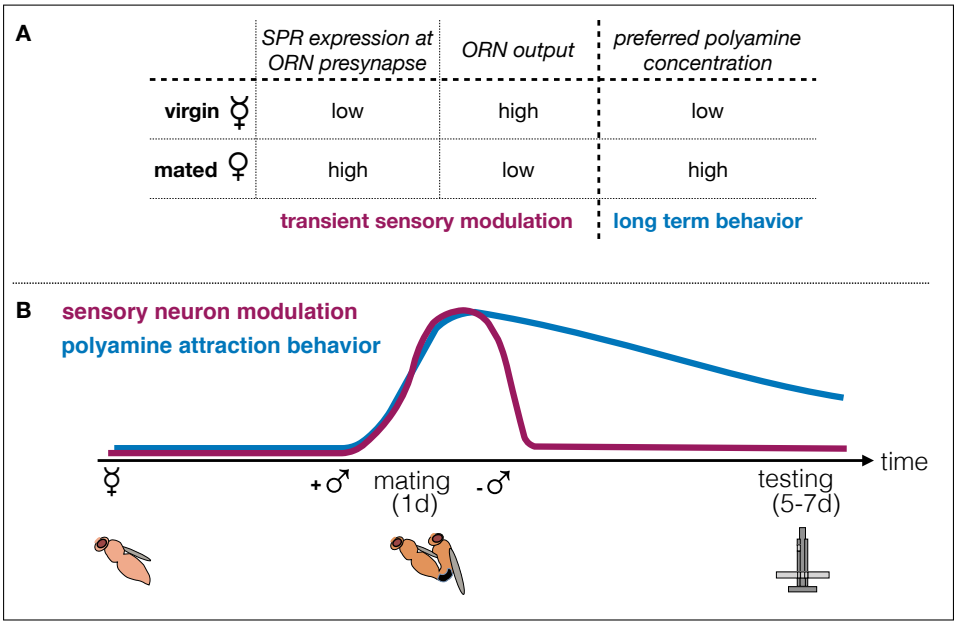

Figure 1.5: (**A**) Previous findings from Hussain et al. [13, 116] have shown that the expression of SPR at the presynapse of polyamine-sensing ORNs increases by 10-fold within the first 6-12 hours after mating, ultimately leading to a decrease in ORN output. This transient sensory modulation leads to a long term change in behavior: virgin flies prefer a low concentration of polyamines, while mated flies prefer a high concentration. (**B**) Long term polyamine attraction behavior is tested in a dual-choice assay, approximately 4-6 days post-mating (i.e. where the sensory neuron modulation took place). Spatio-temporal targeting (i.e temperature-sensitive blocking or activation of neuronal subsets at different times) allows for experiments in reproductive state dependent plasticity, modulation, and memory functions.

1.6 Aims

The observation that a transient neuropeptidergic modulation leads to a long term preference behavior towards polyamines in females, with respect to their reproductive state, may imply a long lasting plasticity of an involved neuronal network.

Using diverse techniques I have addressed the following questions in this dissertation: (1) how does sensory modulation lead to long term behavioral

changes? (2) which signals tell the sensory neurons that mating took place? (3) how do polyamines activate the IRs in the first place? (4) what are neuronal keys to modulate internal-state dependent behaviors?

1.6.1 Aim 1: Deciphering a network for reproductive state dependent olfactory behavior

In **manuscript 2** my collaborators and I have focused our attention on what happens downstream of the ORNs responsible for polyamine perception. The long-term modulation after mating indicated the involvement of synaptic plasticity. We have furthermore addressed the PNs leading to the higher brain centers (MB and LH) using imaging experiments. By applying genetic silencing and activation experiments in a spatio-temporal fashion we have analyzed the involvement of the MB from its intrinsic neurons towards its output and modulation via DANs.

1.6.2 Aim 2: Finding triggers for switch in reproductive behavior

Within **manuscript 2**, my collaborators and I have further tested different triggers at the time of mating to determine the role of known post-mating drivers, such as pheromone detection, courtship, seminal fluid proteins or oogenesis. A review on known neuromodulation systems within the internal state dependent pathways of olfactory processing, **manuscript 1**, has also contributed to the understanding of components within reproduction.

1.6.3 Aim 3: Unraveling a molecular scaffolding of polyamine detection

The detection of polyamines via olfactory receptors, particularly IRs, is still under investigation. These nutrients, which are endogenously produced, still require exogenous ingestion. Bioinformatic tools have presented a foundation for a molecular scaffolding of the ORNs. **Manuscript 3** has promoted the use of bioinformatic tools in the nutritional fields by providing an example of comparative studies, structure-function analysis and three-dimensional models of polyamine receptors.

1.6.4 Aim 4: Building a 2-Photon microscope

Lastly, the general promotion and sharing of technical advances require more and more understanding with the development of new tools and setups. Researchers need to keep up with state-of-the-art techniques, often without deeper understanding of the systems involved. I wanted to close this gap of knowledge by contributing a simple explanation on multi-photon microscopy for general life science audiences, **manuscript 4**, while I was building a two-photon microscope to integrate and advance a running setup myself.

1.6.5 Intention

With the experimental conclusions achieved by this work, I have gained insight into the neuronal underpinning of reproductive state dependent behaviors. I have shed some light onto polyamine detection, triggers for mating state dependent switches, as well as neuronal pathways and their modulation. To achieve this, I have used different approaches and perspectives including long established methods such as the GAL4-UAS system, as well as state-of-the-art techniques like multi-photon imaging.

Chapter 2

Results

2.1 Peer-reviewed and published paper

Manuscript 1:
"Internal State Dependent Odor Processing and Perception - The Role of Neuromodulation in the Fly Olfactory System." [217]

Authors: Sercan Sayin*, **Ariane C. Boehm***, Johanna M. Kobler*, Jean-François De Backer, and Ilona C. Grunwald Kadow
Journal: Frontiers of Cellular Neuroscience
* equal contribution

Author Contributions: SS, **AB**, JK, and IG drafted, wrote and revised the manuscript. J-FD implemented all figures and drafted and revised the manuscript. All authors approved to the final version and its publishing.

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REVIEW
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Internal State Dependent Odor Processing and Perception—The Role of Neuromodulation in the Fly Olfactory System

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Animals rely heavily on their sense of olfaction to perform various vital interactions with an ever-in-flux environment. The turbulent and combinatorial nature of air-borne odorant cues demands the employment of various coding strategies, which allow the animal to attune to its internal needs and past or present experiences. Furthermore, these internal needs can be dependent on internal states such as hunger, reproductive state and sickness. Neuromodulation is a key component providing flexibility under such conditions. Understanding the contributions of neuromodulation, such as sensory neuron sensitization and choice bias requires manipulation of neuronal activity on a local and global scale. With *Drosophila's* genetic toolset, these manipulations are feasible and even allow a detailed look on the functional role of classical neuromodulators such as dopamine, octopamine and neuropeptides. The past years unraveled various mechanisms adapting chemosensory processing and perception to internal states such as hunger and reproductive state. However, future research should also investigate the mechanisms underlying other internal states including the modulatory influence of endogenous microbiota on *Drosophila* behavior. Furthermore, sickness induced by pathogenic infection could lead to novel insights as to the neuromodulators of circuits that integrate such a negative postingestive signal within the circuits governing olfactory behavior and learning. The enriched emporium of tools *Drosophila* provides will help to build a concrete picture of the influence of neuromodulation on olfaction and metabolism, adaptive behavior and our overall understanding of how a brain works.

Keywords: *Drosophila melanogaster*, neuromodulation, internal state, hunger, reproductive state, sickness, microbiota, olfaction

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1. INTRODUCTION

Some odors elicit fast, almost reflexive behaviors such as fear and escape, others attract an animal already at the very first time it perceives them. Arguably, there might be behaviors that are appropriate at any life stage and in every situation and are therefore hard-wired into the nervous system. The large majority of behaviors, however, including innate odor reactions do make sense at one time, but should be suppressed at others. Or in other words, they strongly depend on an

animal's internal state, its current goals and sensory surroundings. These internal states comprise sleep. Sleep, a so-called global state, is essential in most animals (Lee and Dan, 2012). It affects all brain areas and conceivably most other organs in one way or another (Albrecht, 2012). Other internal states might be less exclusive, but probably similarly global. Here, we review recent works in *Drosophila* olfaction research on three important behavioral and internal states: hunger, reproductive state, and the state of sickness or better, the state of an activated immune response. All these states share that they start in one or few organs of the body, and slowly or rapidly, for a short or longer time, affect the rest of the body and in particular its nervous system.

Being able to smell and recognize odors as specific environmental signals is important to humans and absolutely essential for many other animals including *Drosophila melanogaster* (Ashburner et al., 1986). Odors signal food, danger or mating partners without direct contact to their source. Some odors are initially meaningless and remain so unless experienced with a salient cue or object, but some, often species-specific odors elicit a behavioral response such as appetite or repulsion. Nevertheless, how naïve and experienced animals perceive a given odorant depends on their internal state (Leinwand and Chalasani, 2011). For instance, food odors smell better when we are hungry (Rolls, 2006). Male pheromones are only of interest to the ovulating female mouse (Dey et al., 2015). *Drosophila* not only shares with humans and other mammals that odor valence depends on context, it also processes odors with an olfactory system that is highly conserved among different species (Bargmann, 2006). Different studies in the fly over the last decade have greatly improved our understanding of how odors are processed, perceived, categorized and learned (Masse et al., 2009; Wilson, 2013; Sachse and Beshel, 2016). Nevertheless, how flexibility and the ability to adapt to a particular behavioral or internal state is built into the olfactory system of any animal remains poorly understood at the molecular, neuronal and circuit levels (Bargmann, 2012; Taghert and Nitabach, 2012; Bargmann and Marder, 2013). While many neuromodulators have been long identified, a causal relationship between a particular neuromodulator or a group of modulators, their neuronal targets in a neural circuit, and the animal's behavior was established only for few reported cases (see below). Therefore, we focus in the coming paragraphs on the role and possibilities of *Drosophila* neuroscience in providing these causal links between the neuromodulator(s), a neural circuit, and behavior.

1.1. The *Drosophila* Olfactory System

As mentioned above, the *Drosophila* olfactory system resembles in many ways the mammalian olfactory system (Vosshall and Stocker, 2007) **Figure 1**. Peripheral olfactory receptor neurons (ORNs) located in hair-like structures, the so-called sensilla, on two of the fly's external appendages, the third segment of the antenna and the maxillary palp, detect the airborne cue via specific receptor molecules. Insects possess three classes of olfactory receptors, the olfactory receptors (ORs) (Vosshall et al., 2000), the gustatory receptors (GRs) (Jones et al., 2007; Kwon et al., 2007), and the ionotropic receptors (IRs) (Benton et al., 2009). While ORs and GRs are, like their mammalian counterpart, seven transmembrane receptors, IRs are related to glutamate receptors and share their structure of ion channels (Abuin et al., 2011). In contrast to the mammalian seven transmembrane receptors, ORs and GRs function as (primarily or exclusively) ion channels rather than as classical G-protein coupled receptors (Sato et al., 2008; Wicher et al., 2008). Nevertheless, similar to mammals, each ORN expresses usually only one ligand-specific receptor and therefore is tuned to few types of odors (Vosshall et al., 2000). ORs always require another OR, the so-called olfactory receptor co-receptor or ORCO, to function (Benton et al., 2006). Similarly, most IRs also appear to function as heteromers with another co-IR (Abuin et al., 2011).

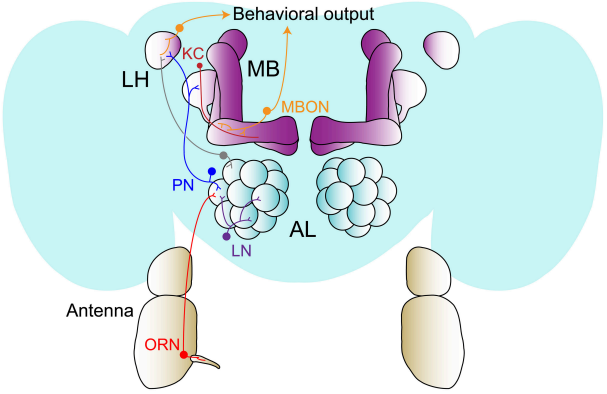

FIGURE 1 | Organization of the olfactory system in *Drosophila melanogaster*. Odors are detected by olfactory receptor neurons (ORN) located in the sensilla of the antennae (and the maxillary palp, not shown). They send axons to specific glomeruli in the antennal lobe (AL) where they form synaptic contacts with projections neurons (PN) and local interneurons (LN). PNs project to higher brain centers, the mushroom body (MB) and/or the lateral horn (LH). In the MB, PNs form en-passant synapses with the Kenyon cells (KC) that convey olfactory information to MB output neurons (MBONs).

ORNs expressing the same receptor or receptor pair send their axons from the peripheral sensilla through a common nerve bundle into the brain, where they innervate glomerular structures in the antennal lobe (AL), the equivalent to the olfactory bulb, in a receptor-type specific manner. Optogenetic activation of one distinct glomerulus is in some cases sufficient to replace an odor in eliciting an attractive or aversive behavioral response [e.g., CO_2 can be replaced by optogenetic activation of the V-glomerulus (Suh et al., 2007)]. More frequently, however, odors and natural odor blends bind and activate multiple receptors and glomeruli, and only the combined activation of all glomeruli represents the complete perception of a smell. These glomerular activation patterns are further shaped by local interneurons (LNs), which in the fly can be inhibitory and excitatory, to presumably strengthen or weaken similarities and concentration-dependent effects (Wilson, 2013).

In the antennal lobe, projection neurons (PNs) receive this processed information from the ORNs and pass it on to two higher brain centers, the mushroom body (MB) and the lateral

horn (LH) **Figure 1**. The mushroom body is essential for learning, storing, and re-calling odor associations (Aso et al., 2014b), but more recent work has also implicated it in the modulation of innate odor responses (Bräcker et al., 2013; Cohn et al., 2015; Lewis et al., 2015; Owald et al., 2015). While beautiful anatomical and physiological data suggests an important role for the LH in innate odor valuation (Jefferis et al., 2007), very few studies provide compelling behavioral evidence for this role up to now (Strutz et al., 2014). The mushroom body consists of cholinergic Kenyon cells (KCs) that receive sparse and primarily random odor input from PNs, and provide synaptic output to cholinergic, GABAergic and glutamatergic MB output neurons, the so-called MBONs (Aso et al., 2014a). The relative activity of these MBONs, which is highly plastic, is thought to control state- and experience-dependent behavioral output (Aso et al., 2014b; Hige et al., 2015b). Dopaminergic neurons (DANs) that are situated in two primary clusters in the fly brain (PAM, protocerebral anterior medial and PPL1, protocerebral posterior lateral) govern this synaptic plasticity between KCs and MBONs by responding to and integrating of internal and external sensory cues (Owald and Waddell, 2015). At this point, we know most about their role as teaching signals during associative appetitive and aversive memory formation (see for instance Yamagata et al., 2015). Nevertheless, they do modulate behavior instantaneously (Lewis et al., 2015), and potentially play a much greater role in internal state-dependent olfactory processing and behavior as previously thought (Krashes et al., 2009; Siju et al., 2014; Cohn et al., 2015). Finally, to date little is known about the neurons downstream of MBONs and upstream of DANs.

Thanks to great community efforts, we are beginning to appreciate the complexity of the neural connections within the MB circuit (Eichler et al., 2017; Takemura et al., 2017a), the AL (Berck et al., 2016), and other areas of the fly's nervous system (Takemura et al., 2017b). How this complex connectome interacts with a presumably equally complex network of the around 100 neuromodulators present in the fly is a fascinating question without a conclusive answer. The olfactory system of the fly, nevertheless, is a powerful model. It offers many genetic tools, a connectome and a selection of odor-dependent behaviors, which are easy to assess and score. This can tackle the complexity and provide important insights and pointers for research in higher animals. Several fundamental principles beyond the mere architecture of the olfactory system seem likewise conserved. For instance, hunger states and hormonal changes modulate early olfactory sensory processing in worms, flies, mice, and likely in humans (Root et al., 2011; Jang et al., 2012; Palouzier-Paulignan et al., 2012; Dey et al., 2015; Hussain et al., 2016a). Similarly, the mammalian olfactory bulb or its functional equivalent, the antennal lobe in insects, contain a large number of neurons expressing neuromodulators or their receptors (Carlsson et al., 2010; Giessel and Datta, 2014; Linster and Cleland, 2016). How the behavioral role and circuit mechanisms of higher brain centers such as the amygdala and piriform cortex relate to the insect mushroom body and lateral horn is one of the exciting questions that remain to be fully elucidated.

2. MODULATION HAPPENS AT MANY SITES

When observing an animal such as *D. melanogaster*, one can notice different facets of its behavior. The disruption of specific genes or a group of genes can change these behaviors and thereby indicates the importance of particular gene networks. Among such genes are genes encoding for neuromodulators, e.g., neuropeptides, enzymes for the generation of monoamines and other types of neurotransmitters.

Neuromodulators can act as control systems such as open and closed loops and feed-forward or feed-back motifs. Furthermore, neuromodulation can happen at many sites within a particular neural network. In the olfactory system, ORNs, secondary PNs, inhibitory neurons, and different types of neurons in the higher brain centers can be targets of modulation. Likewise, this modulation can concentrate on the pre-synaptic/axonal or post-synaptic/dendritic part of a neuron. The effects range from modification of synaptic strength, i.e., inhibition or facilitation, to changes in intrinsic properties, i.e., altering membrane potential or components of the synapse. By doing so, distinct modulators can have independent effects and can rearrange the network into functional units and subcircuits (Marder and Thirumalai, 2002).

In *D. melanogaster*, a large body of work has been published over the last years with respect to neuromodulators and their impact on behavior. One of the most prominent examples is the role of dopaminergic neurons in the MB. These neurons play a key role in olfactory learning and memory and exemplify the importance of neuromodulation in these processes (Berry et al., 2012; Aso et al., 2014a; Hige et al., 2015a; Owald et al., 2015; Aso and Rubin, 2016; Felsenberg et al., 2017; Hattori et al., 2017; Kaun and Rothenfluh, 2017). While DANs are influencing the pre-synaptic efficacy of synapses between KCs and their output neurons, KCs also synapse directly onto DANs, and DANs synapse directly on the output neurons (Takemura et al., 2017a). This means that within the DAN network, information is processed in parallel and in conjunction, with unaccounted opportunities for feed-back and feed-forward loops resulting in multiple layers of neuromodulation. On top of dopamine, octopamine has been shown to govern olfactory associative memory, as it can affect these dopaminergic circuits via dorsal anterior lateral neurons (Burke et al., 2012; Chen et al., 2012; Wu et al., 2013; Guven-Ozkan and Davis, 2014). A contextual cue or internal state allows a direct effect on the modulatory neurons, such as DANs (Cohn et al., 2015; Lewis et al., 2015; Musso et al., 2015). How different modulators like octopamine and dopamine act together to tune a nervous system to a specific state is not well understood.

An important effort in *Drosophila* neuroscience is to map these neural circuits using high-resolution electron microscopy (EM) and data reconstruction. In the *Drosophila* larva, the dense connectome of the MB was recently published. It unraveled expected and unexpected circuit loops and motifs, such as lateral inhibition, feed-back and feed-across circuits (Eichler et al., 2017). The dense interconnectivity of different modulatory neurons and their circuitry even includes a variety of other areas

or cell types in the fly brain, such as astrocytes, with even more neurotransmitters, e.g., serotonin (Huser et al., 2012; Ma et al., 2016; Coates et al., 2017, Zheng et al., in review).

EM circuit reconstruction and other modern tools allow the fly community to target and identify the importance of neuromodulators and their effects on behavior within the framework of a known synaptic network, where downstream and upstream neurons of neuromodulatory neurons can be readily identified. Nevertheless, it does not answer the important question of the biological and ethological role of different neuromodulators and how they convey and orchestrate experience, context, and different internal states such as hunger to guide adaptive behavior and ensure optimal chances of survival and success. We will focus on this question for the rest of the present review and provide examples for different internal states and a variety of neuronal targets of neuromodulation.

3. MODULATION BASED ON INTERNAL STATE

3.1. Modulation in Hunger

Metabolic state or hunger are arguably the best studied and understood examples of neuromodulation in *Drosophila* neurobiology. A hungry animal desires food. Hunger governs locomotion, perception, motivational state, and tightly links metabolic conditions and behavior. Starved animals show enhanced locomotor activity due to increased aminergic signaling in the fly central and peripheral nervous system (Yang et al., 2015; Yu et al., 2016). However, flies do not solely rely on this hyperactive locomotion to simply increase the likelihood of encountering food. Instead, flies like other animals use olfaction as a proxy, long-distance cue to identify palatable food patches. Therefore, it is no surprise that neuromodulatory effects allow metabolic states to tightly govern the sense of smell. These modulations help the hungry animal to alter its sensory and behavioral thresholds, to filter and sort the spectrum of sensory cues and to integrate innate odor responses with novel food indicators via associative, appetitive learning.

One important mechanism of hunger-dependent regulation of olfaction is the modulation of peripheral sensory neurons, which presumably changes odor valence representation at the level of the AL (Knaden et al., 2012) and in higher brain centers such as the LH (Strutz et al., 2014). Vinegar, as a food cue, activates ORNs and their respective glomeruli, which drive aversive as well as attractive behaviors (Semmelhack and Wang, 2009). The relative level of "push and pull" between low odor concentration driven attraction and high concentration dependent aversion has been shown to be controlled by two parallel modulatory systems at the level of ORNs (Root et al., 2011; Ko et al., 2015). In a paradigm with freely walking flies, starvation decreases the time required to find a food patch. The behavioral increase is accompanied by a rise in signal amplitude in ORNs projecting to three different glomeruli that respond to the lower appetitive concentrations of vinegar; DM1, DM2, and DM4. By contrast, the neuronal activation induced by aversive higher vinegar concentrations in the DM5 glomerulus was reduced in hungry flies (Semmelhack and Wang, 2009). These changes were due to the cohort activity of short neuropeptide F (sNPF) and tachykinin (DTK), respectively (Root et al., 2011; Ko et al., 2015). Removing sNPF via RNA interference (RNAi) or expression of a dominant negative mutant of the sNPF receptor rendered starved fly behavior indistinguishable from satiated flies (Root et al., 2011). Conversely, the induction of fed behavior in starved flies occurred when sNPF signaling was removed from DM1 glomerulus-innervating OR42b neurons. Moreover, removal of sNPF receptor (sNPFR) in secondary order PNs did not alter foraging behavior, suggesting that sNPF functions in an autocrine mechanism (Root et al., 2011). RNAi and overexpression experiments exclusively in OR42b neurons showed that sNPFR was necessary and sufficient for starvation-induced receptivity. What is the link between metabolic state and sNPFR expression in ORNs? mRNA levels of sNPFR were elevated in ORNs after 4 h of starvation, whereas sNPF levels did not change (Root et al., 2011). A parallel mechanism was observed for the modulation of the aversive vinegar channel (Ko et al., 2015). The DM5 glomerulus was found to be under control by Tachykinin (DTK), a player in metabolism, locomotion, aggression and pheromone detection (Winther et al., 2006; Birse et al., 2011; Song et al., 2014; Shankar et al., 2015). Down-regulation of aversive output from the OR85a/DM5 glomerulus was due to increased DTK receptor (DTKR) in the ORNs during food-deprivation (Ko et al., 2015). Starved flies phenocopied fed flies in the absence of DTKR from ORNs. Furthermore, requirement of DTKR was specific to DM5, while loss of DTKR in attraction-mediating OR42b neurons/DM1 had no effect. In contrast to sNPF, Tachykinin was previously reported to be expressed in LNs (Winther and Ignell, 2010). DTK knock-down in LNs mimics DM5 under fed conditions. Importantly, sNPFR and DTKR mRNA levels are under direct control by a common mechanism, insulin. Inducing insulin signaling constitutively abolished odor approach and downregulated sNPFR and DTKR expression from their respective neurons (Root et al., 2011; Ko et al., 2015). Its worthwhile noting that analogous mechanisms have been implicated in mammalian systems (for a review, McIntyre et al., 2017). The firing rate of mitral cells, secondary order neurons equivalent to PNs, were modulated by insulin-mediated inhibition of potassium Kv1.3 channels (Fadool et al., 2000, 2011).

While insulin seems to be the common regulator of some ORNs, do all ORNs respond to the same modulators? An analysis of antennae from starved and fed flies revealed 34 upregulated and 11 downregulated G-protein coupled receptors (Ko et al., 2015). Why there are so many putative modulators and how they contribute to olfactory processing remain open questions. Another study revealed more than 200 genes that are upregulated in the antenna upon starvation, including neuromodulators such as sNPF, allatostatin and CCHamide (Farhan et al., 2013). One of the underlying reasons potentially explaining such a plethora of neuromodulators is that starvation also alters non-food odor and OR independent food odor responses. For instance, cis-vaccenyl acetate (cVA), a pheromone best known for its role in mating, induces attraction in starved flies, even in the absence of potential mates in both sexes during the

experiments. This increased behavioral attraction is accompanied by base-line and odor-dependent amplified firing rates. This was equally observed for ethyl acetate encoding ORs, cVa responsive OR67d and ionotropic receptor IR84a that responds to food cue phenylacetaldehyde. Starved mutants of the peptide CCHamide also displayed a decreased attraction to all of these odors (Farhan et al., 2013). Interestingly, sNPF receptor knockdown in ORNs was not effective in reducing ethyl acetate attraction. In another study, a function for SIFamide (SIFa) at the level of projection neurons has been described, adding another neuromodulator involved in hunger-dependent modulation (Martelli et al., 2017). Here, the authors show that activation of SIFamide producing cells does not elicit any changes in activity at the level of peripheral olfactory receptor neurons, but at the level of olfactory projection neurons. This modulation acts through interneurons of the antennal lobe, LNs. Using ethyl acetate as a cue, artificial activation of SIFa neurons transforms a fed fly's odor response from indifference to attraction, mimicking the situation in starved flies. The removal of SIFa from SIFaminergic neurons abolished the differential activation in a specific glomerulus (DM3) between fed and starved animals. Anatomical and physiological data suggests that the neuropeptides hugin and myoinhibitory peptide (MIP, or AllatostatinB) might have opposite effects on SIFa-dependent modulation of appetitive behaviors. While thermogenetic activation of MIP expressing cells weakens SIFaminergic neuronal activity, hugin positive neurons enhance intracellular calcium levels (Martelli et al., 2017). In addition, while activity of sNPF alone did not induce a change in SIFa positive neurons, sNPF was found to be co-expressed with hugin in hugin-positive cells (Martelli et al., 2017). Hugin also plays a role in the Drosophila larvae, where it was recently shown to act as an inhibitory modulator of feeding behavior and as a promoter of locomotion (Melcher and Pankratz, 2005; Schoofs et al., 2014). The observed behavioral differences between larvae and adults may derive from the difference in the developmental/life stage of the animal or other aspects related to behavioral context. Furthermore, heterogeneity in neuromodulatory profiles could further enrich and explain modulatory flexibility of a circuit via concerted action, and might therefore lead to different and context-dependent behavioral outcomes. Neuromodulation of olfaction upon starvation is not restricted to attractive odors, food cues and pheromones. After starvation, behavioral attraction to benzaldehyde, a potent aversive odor for flies, was observed at low odor concentrations, while fed flies still showed odor aversion. In correlation with this behavioral switch, benzaldehyde responsive receptor neurons, which express the receptor OR7a, also showed increased firing rates during odor stimulation in starved animals (Farhan et al., 2013). However, in projection neurons innervating the OR7a-targeted DL5 glomerulus, starvation-dependent modulation was not observed in calcium imaging experiments. Nevertheless, higher benzaldehyde concentrations were used in this study, which might explain the different results (Martelli et al., 2017). Another aversive odor that is present in the context of the fly's preferred food source, overripe and fermenting fruits, is carbon dioxide. Why CO_2 is aversive is not fully understood, but it is produced by the flies themselves in response to stress or increased metabolic activity (Suh et al., 2004). The release of this odor in the context of food creates a conflict, where CO_2 aversion must either be overcome during food seeking or its valence must switch from aversive to attractive. Mimicking the context of food, the behavioral response to a mixture of CO_2 and vinegar was, nevertheless, indistinguishable from a response to CO_2 alone (Bräcker et al., 2013). Only the additional context of starvation reduced this aversion behavior with the help of the mushroom body (Bräcker et al., 2013; Lewis et al., 2015). In particular, CO_2 aversion was dependent on a distinct region of the MB lobes, the MB-β'2 lobe region. This region gave output to aversion-driving MBONs, MBON-β'2mp and MBON-β'2mp_bi, which reacted to CO_2. In the presence of vinegar, however, this CO_2 response was significantly dampened (Lewis et al., 2015). Although dopamine had been primarily studied in the context of olfactory memory, this study found that certain DANs in the PAM cluster responded to vinegar in a starvation state-dependent manner and inhibited the output of this lobe region (Lewis et al., 2015). Therefore, during starvation, it appears that innate odor responses, too, are under the control of higher brain centers, in particular the MB. Why this modulation does not take place earlier in the circuit, for instance in the ORNs, is not known. It is possible that modulation at the sensory level would be too slow to allow for fast execution of aversive and escape behavior without the context of other odors hinting at the presence of anything else but putative danger. These studies provide evidence that integrated responses of peripheral and higher brains centers are necessary to maximize flexibility and efficacy in behavioral execution.

Motivational thresholds provide an additional mechanism for hunger-dependent olfactory neuromodulation, via the unpaired1 (upd1) - neuropeptide F (NPF) axis (Beshel and Zhong, 2013; Beshel et al., 2017). Three members of the unpaired gene family, fly homologs of the satiety hormone leptin, are expressed in the brain and fat body of the fly and act through JAK-STAT receptor domeless (Tartaglia et al., 1995; Rajan and Perrimon, 2012; Beshel et al., 2017). While fat body-specific downregulation of upd2 leads to decreased body size, reduced upd1 activity selectively in the central nervous system triggers a significant increase in appetitive olfactory behavior in a 4-arm olfactory area assay (Rajan and Perrimon, 2012; Beshel et al., 2017). Lack of upd1 activity also augmented feeding behavior (Beshel et al., 2017). What are the downstream targets of upd1? In an immunohistochemistry experiment, dome was found to colocalize with NPF, the homolog of human NPY (Brown et al., 1999; Beshel et al., 2017). Of the 25 NPF positive neurons in the fly brain, only four have been shown to be essential for olfactory behavior (Beshel and Zhong, 2013). While NPF neurons responded to both food and non-food odors, neuronal activity was increased only for food odors under starvation when compared to the response in the fed state. NPF positive neuron activation levels correlated with the attraction that flies showed toward an odor in behavior. In an olfactory choice arena, given the choice between air and an odor, or two odors to compare, flies accumulated in the quadrant where the odor eliciting higher NPF activity was present. Furthermore, inducing NPF activity artificially was sufficient to facilitate attraction toward non-food odor in a graded fashion (Beshel and Zhong,

2013). When dome expression was targeted specifically to NPY expressing cells, satiated flies showed increased attraction to the food odors (Beshel et al., 2017). Likewise, internal state-dependent differential neuronal activity to the food odors in NPY neurons was abolished with the disruption of upd1/dome signaling. Reduction of upd1 in all neurons in the fly brain and dome knockdown in NPY neurons resulted in higher calcium signaling in fed state, thus mimicking starved condition (Beshel et al., 2017).

Apart from innate odor responses, starvation also modulates appetitive associative learning and memory. Flies are capable of pairing a neutral or aversive odor cue with positive reinforcing stimuli, for example sugar, which provides sweet taste as well as calories (Huetteroth et al., 2015). However, the expression of this memory is suppressed if flies had access to food between odor training and the memory test, suggesting that hunger gates the degree of memory expression and prevents it when the fly does not require food (Krashes et al., 2009). This gating mechanism is provided by NPF. Artificial activation of NPF signaling overrides this suppression and leads to expression of appetitive memory in fed flies. RNAi mediated knock-down of NPF receptor revealed that a subset of dopaminergic PPL1 neurons was critical for this hunger-dependent learning (Krashes et al., 2009). In line with this, these dopaminergic neurons suppressed learning in starved flies when artificially activated. Therefore, hunger and NPF led to disinhibition of mushroom body output, which drives appetitive behavior. A follow up study showed that specific MBONs are modulated through a subset of PPL DANs. PPL1-γ1pedc targets MBON-γ1pedc>α/β (Krashes et al., 2009; Aso et al., 2014a). MBON-γ1pedc>α/β in turn acts as an inter-neuron, selectively inhibiting MBON M4/M6 cluster activity (Aso et al., 2014a; Perisse et al., 2016). This feed-forward inhibition was also found to be hunger regulated with MBON-γ1pedc>α/β showing higher calcium responses to odor in the starved animal. Interestingly, MBON-γ1pedc>α/β and M4/M6 are involved in innate odor aversion (Lewis et al., 2015; Perisse et al., 2016).

Hunger-induced metabolic changes in the nervous system influence ORN responses and modulate higher brain centers for effective foraging and appetitive learning. Strengthening of attractive channels and inhibition of aversive olfactory pathways therefore appears to occur at several (or every) stage of the olfactory circuitry. **Figure 2A** recapitulates the neuromodulation on the first level of olfactory processing, the sensory level. The involvement of the MB as the next-higher processing center is summarized in **Figure 2B**. Why such multi-layered modulation is used is unclear, but it suggests that foraging is under tight control to ensure behavioral expression only when it is in the animal's best interest.

3.2. Modulation in Reproductive State

For most animals, it is important to master the three components of reproduction: courtship, mating and reproductive success with respect to reproductive fitness. These behaviors are often influenced by neuromodulation and induced downstream of a chemosensory cue such as a pheromone. Courtship and sexuality regulation has previously also been linked to neuromodulators, such as dopamine and octopamine (Certel et al., 2007, 2010; Keleman et al., 2012; Zhou et al., 2012; Rezával et al., 2014; Kuo et al., 2015; Montague and Baker, 2016; Chen et al., 2017; Lim et al., 2017). Mating, however, appears to correlate more often with neurotransmitters engaging glutamate and GABA signaling (Pavlou and Goodwin, 2013; Pavlou et al., 2016; Lim et al., 2017). In the so-called post-mating switch, which includes suppression of re-mating and induction of egg-laying, dopamine, octopamine and certain hormones adapt the sensory perception of females to their reproductive state needs (Ribeiro and Dickson, 2010; Rezával et al., 2012, 2014; Walker et al., 2015; Corrales-Carvajal et al., 2016; Hussain et al., 2016a,b). To ensure reproductive fitness, sensory neurons are in addition modulated by neuropeptides to detect best nutrients and conditions for their offspring. To optimize the action sequence from courtship to mating and finally to offspring fitness, several layers of neuromodulation appear to be necessary to presumably provide sufficient flexibility and stability to the reproductive process of the species.

3.2.1. Modulation during Courtship

Courtship behavior in *Drosophila* is based on multiple sensory cues including vision, audition and chemosensation (Greenspan and Ferveur, 2000). Even though vision is a factor, flies can mate in the dark (Payne, 1911; Spieth and Hsu, 1950), indicating that there is more happening than meets the eye. Neuromodulation plays an important role during pheromone and olfactory processing in this behavioral context.

Courtship and subsequently mating requires the detection and recognition of a potential mating partner as a first step. Visual cues such as shape and size are indicators of attractiveness (Agrawal et al., 2014). Odor cues such as pheromone-scented fly dummies are able to modulate the duration of chasing behavior of males significantly, too (Agrawal et al., 2014). This is measured by looking at the important steps within courtship behavior: approach, chasing time and wing extensions.

One may argue that the efficacy of courtship behaviors such as chasing time and the movement of wings can also be age-dependent. Indeed, sexual function of flies has been shown to decrease with age. However, certain dopaminergic neurons of the protocerebral posteriolateral cluster, i.e., PPL2ab, compensate and enhance courtship behavior and therefore presumably also the sexual drive of aged male flies (Kuo et al., 2015). Interestingly, another study has shown that increasing dopamine levels in these PPL neurons can even drive inter-male courtship behavior (Chen et al., 2017) given that visual cues are present.

Not only higher brain areas undergo changes to ensure mating. Also chemosensory cues may guide the way. Cuticular hydrocarbons act as female pheromones. At the sensory neuron level, OR47b has been identified as a key player in the detection of these pheromones (van der Goes van Naters and Carlson, 2007; Dweck et al., 2015; Lin et al., 2016). In older males, the sensitivity of OR47b is augmented via juvenile hormone (Lin et al., 2016). More specifically, the binding partner for juvenile hormone is *Methoprene-tolerant* (Met). If the expression of Met is knocked down in OR47b sensory neurons, a significant reduction

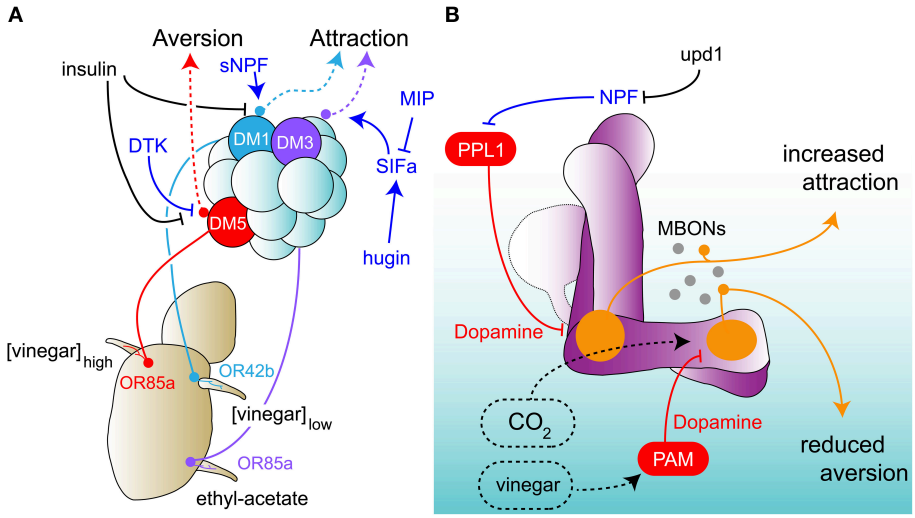

FIGURE 2 | Neuromodulation of olfactory perception in hungry flies. **(A)** Modulation of the peripheral olfactory sensory neurons. Low and high concentrations of vinegar promote attractive and aversive behaviors in *Drosophila*, respectively. In starved flies, the attraction toward low vinegar concentrations is upregulated whereas the repulsive effect of high vinegar concentrations in downregulated. This modulation occurs at the level of ORNs and is mediated by neuropeptides (blue). In fed flies, insulin counteracts these mechanisms by downregulating neuropeptide receptors mRNA. Isoamyl-acetate detection, an other attractive odorant, is upregulated in hungry flies by SIFa, under the control of the neuropeptides MIP and hugin. **(B)** Modulation of olfactory information processing in the mushroom body. In hungry flies, the repulsive effect of CO_2 is reduced through the activation of PAM dopaminergic neurons by vinegar. Conversely, NPF promotes attraction toward food odors by inhibiting dopaminergic PPL1 neurons. In fed flies, upd1 counteracts this effect. AL, antennal lobe; MB, mushroom body; MBONs, MB output neurons; DTK, tachikinin; NPF, neuropeptide F; sNPF, short NPF; MIP, myoinhibitory peptide; SIFa, SIF amide; PPL1, protocerebral posterior lateral; PAM, protocerebral anterior medial; upd1, unpaired1.

in courtship success has been observed. Therefore, older males have an advantage in detecting a female before a young male.

In addition to OR47b, there are further ORs which are able to detect pheromones. While OR47b and OR88a can detect male and female specific pheromones, OR65a and OR67d are specific to male pheromones, i.e., cis-vaccenyl acetate (cVA) (van der Goes van Naters and Carlson, 2007). A deeper analysis of OR67d neurons showed that cVA works in both sexes to inhibit courtship between males and to push courtship in females. This opposing effect is conceivably linked to the GABAergic and cholinergic PNs coming from the dimporphic glomerulus in the AL and leading toward the LH (Kurtovic et al., 2007; Ruta et al., 2010). Since the LH still is a largely uncharacterized area of the adult fly, its potential neuromodulation remains to be investigated.

Another step in successful courtship behavior is the reduction of competition from other males, solved by aggressive behavior. This behavioral switch in displaying either courtship or aggression has been associated with the neuromodulator octopamine (Certel et al., 2007, 2010). Even though a reduction of octopamine triggered enhanced levels of courtship toward other males, an activation of octopaminergic neurons led to the same behavioral outcome. A more detailed analysis needs anatomical precision: In *D. melanogaster*, one gene responsible for gender differentiation is *doublesex*. The development of sex-morphology is *doublesex*-dependent (Rideout et al., 2010). A gene which is often co-expressed with *doublesex* is *fruitless*. When *fruitless* is disrupted in octopaminergic neurons of the subesophageal ganglion (SEZ), male flies display a higher tendency to court other males (Certel et al., 2010). Since the SEZ is the primary gustatory processing center, there is a chance that pheromone detection through GR neurons is the key to this neuromodulation. However, even though GRs, such as GR32a and GR68a (Bray and Amrein, 2003; Miyamoto and Amrein, 2008) have been implicated in courtship success, a connection between GR32a-expressing neurons and the *fruitless*-expressing neurons has not been found (Miyamoto and Amrein, 2008). Therefore, the exact role of octopamine in courtship behavior needs refinement.

Octopaminergic neurons play yet another role within courtship behavior apart from aggression: After mating, females reject males. Hence, it is essential for a male to find a female that has not been mated. As mentioned earlier, females are presenting cVA due to previous mating with another male. These females should now be considered unattractive targets for mating by other males. Olfactory detection of cVA leads to a strong suppression of courtship and mating attempts in males that have previously experienced rejection by an already mated female (Ejima and Griffith, 2007; Ejima et al., 2007). Surprisingly, activation of octopaminergic neurons also induces this effect in virgin males (Zhou et al., 2012), suggesting that octopamine can substitute as a teaching signal during courtship conditioning. In line with this, silencing of octopaminergic neurons during a display of female rejection had a significant effect and males courted irrespectively of the previous rejection (Zhou et al., 2012). Knock-down of the octopamine receptor in the MB (OAMB) also led to a reduction in courtship learning. Furthermore, OAMB-expressing neurons responded to cVA stimulation, thus strengthening a direct role of OAMB.

Dopamine, similar to octopamine, regulates courtship and the males' experience of it. If olfactory detection of cVA is blocked, e.g., OR67d mutant flies, the courtship learning effect vanishes (Keleman et al., 2012). Activation of dopaminergic neurons in males reduces the courtship learning effect. More specifically, *fruitless*-expressing dopaminergic neurons of the class aSP13 are responsible (Keleman et al., 2012). These aSP13 neurons synapse onto the γ lobe of the MB and modulate its output. However, using a screening approach, another study has shown that the γ KCs themselves are not involved in courtship learning, but rather α/β KCs and PAM dopaminergic neurons (Montague and Baker, 2016). Even though *Drosophila* has four dopamine receptors, namely dDA1, DAMB, dD2R, and DopEcR, only one, i.e., dDA1, was identified to modulate courtship in naïve males (Lim et al., 2017). Whereas naïve males without the learning experience court normally, dDA1-mutant naïve males showed a prolonged courtship initiation. An analysis of α/β and γ KCs revealed that restoring dDA1-expressing neurons in these KCs rescues the effect. However, independently expressing dDA1 in either α/β KCs or γ KCs had no rescuing effect. Due to these ambiguous findings, the authors postulate that the neurotransmitters glutamate and GABA, which can be co-transmitters of dopamine in mammals, have a neuromodulatory effect (Lim et al., 2017).

In summary, dopamine and octopamine seem to be crucial modulators for courtship behavior. While juvenile hormone may modulate the sensitivity of sensory neurons, a significant amount of modulation happens at higher brain centers. The potential opposing effects of pheromones on males and females may even be linked to the classical neurotransmitters, e.g., GABAergic, glutamatergic, and cholinergic neurons. **Figure 3A** encapsulates the summary of neuromodulation in courtship behavior.

3.2.2. Mating-Induced Neuromodulation

As described above, *doublesex* and *fruitless*-expressing neurons play an important role in courtship behavior. From the ORNs which detect pheromones, through different glomeruli in the AL, projections reach higher brain centers: the LH and the MB. To link this with the summary statement of the previous part, it should be mentioned that *doublesex*-expressing neurons are of two types: glutamatergic and GABAergic (Pavlou and Goodwin, 2013; Pavlou et al., 2016). On the one hand, the glutamatergic motor neurons innervate the genitalia and enable attachment and intromission for the copulation itself. On the other hand, GABAergic inhibitory neurons mediate the uncoupling likely by inhibition of motor neurons. In combination with mechanosensory neurons, which innervate and activate both types, this leads to initiation and end of the copulation process (Pavlou et al., 2016).

Among the known behavioral changes that occur upon mating in *Drosophila* females is a change in their food substrate preferences, presumably due to changed nutritional requirements necessary for egg-production and/or identification of appropriate oviposition sites. Older and more recent work has shown that neuromodulatory mechanism are involved in changing the female's appetite post-mating. For instance, one of the seminal proteins transfered during copulation is sex peptide (SP). If SP is not transferred during copulation, for instance by mating with SP mutant males, female food intake post-mating is reduced compared to their wildtype male-mated peers (Carvalho et al., 2006). Furthermore, mating not only changes the amount, but also increases the female's appetite for yeast and salt again through the transfer of SP and the inhibition of SP receptor expressing neurons in the female's reproductive tract (Ribeiro and Dickson, 2010; Walker et al., 2015; Corrales-Carvajal et al., 2016). Although chemosensory neurons are responsible for the detection of yeast and salt (Walker et al., 2015; Corrales-Carvajal

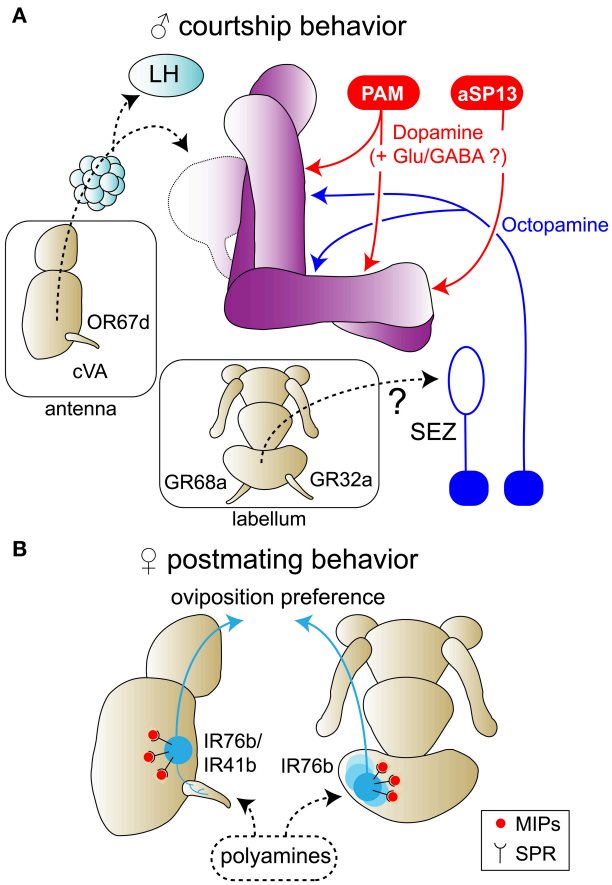

FIGURE 3 | Neuromodulation in reproductive state. **(A)** Modulation of male courtship behavior by pheromones. Pheromones can be detected by olfactory or gustatory receptor neurons in the fly antennas or labellum, respectively. Gustatory neurons are projecting to the subesophageal zone (SEZ) where they might form connections with octopaminergic neurons (blue) known to regulate courtship behavior. The cis-vaccenyl acetate (cVA) pheromone is presented by mated females and inhibits courtship behavior in males that already experienced rejection from an other mated female. This courtship learning is modulated by octopamine in the MB. DANs from PAM and aSP13 clusters (red) also regulate courtship behavior and learning. This might involve co-transmission of glutamate (Glu) or GABA. **(B)** Modulation of female oviposition preference. Mating increases female preference for polyamines. Polyamines detection in both olfactory and gustatory sensory neurons is enhanced through an increase in myoinhibitory peptide (MIP) signaling. This occurs via an upregulation of the expression of its receptor, the sex peptide receptor (SPR), at the membrane of sensory neurons.

et al., 2016), how and where those neurons are modulated remains unknown.

As mentioned before, *doublesex*-expressing neurons play an important role during mating. These neurons are downstream of SP signaling and have been shown to play an important role in the induction of post-mating behaviors in *Drosophila* females (Rezával et al., 2012). Expression of a membrane-bound form of SP in virgins elicits post-mating behaviors including the display of rejection behaviors toward males and an increase in egg-laying behavior. Furthermore, it is sufficient to inhibit SP receptor (SPR) in *doublesex*-expressing neurons to reduce the post-mating behaviors. In a later paper, the neuromodulator octopamine in *doublesex*-expressing neuron has been shown to modulate post-mating behaviors (Rezával et al., 2014). In virgins, feeding octopamine evoked post-mating responses. In comparison, silencing octopamine in *doublesex*-expressing neurons in mated females revealed a reduction in the post-mating behavior.

The final stage of successful reproduction is reproductive success. In female *Drosophila*, cVA detection may be beneficial for reproductive success, since females may use this olfactory cue to find and mark good oviposition sites (Wertheim et al., 2002). In addition, the consumption of polyamines such as putrescine, spermidine and spermine, can improve reproductive success (Lefèvre et al., 2011). Mated females appear to actively seek out these nutrients (Hussain et al., 2016a,b). Their detection is based on both olfaction and gustation; the ionotropic receptors 76b (IR76b) and IR41a are necessary to detect polyamine odor, while IR76b and the bitter receptor GR66a mediate taste perception (Hussain et al., 2016b). These sensory neurons are modulated at the ORN pre-synapse via endogenously produced MIPs, which are alternative and presumably older ligands of SPR (Hussain et al., 2016a). This peptidergic modulation of peripheral sensory neurons, which appears to be induced by mating, is necessary and sufficient to induce the mated female's increased interest in polyamines. Nevertheless, what triggers the increase of SPR expression in these peripheral neurons initially is not known. It could be a different component of the ejaculate, mating itself or another unknown factor. Finally, this sensory modulation only lasts for a few hours post-mating, while the modulation of female egg-laying behavior and her attraction to higher levels of polyamines are maintained for at least 1 week after mating. It is possible that neuromodulation at other chemosensory processing levels and/or learning play important roles.

This leads to the conclusion that a mixture of allocrine, endogenous and peptidergic modulators are responsible for reproductive success, especially at the different levels of olfactory processing. A recap of the the polyamine detection is depicted in **Figure 3B**.

3.3. State-Dependent Modulation by Sickness and the Immune System

Microbial organisms are abundant in the environment and can be found practically everywhere. While a large variety of pathogenic microbes can pose a threat to an animal's survival, many microorganisms also live in close association with animals. This so-called commensal microbiota comprises non-pathogenic microorganisms that reside both in and on an animal's body and play an important role for the host's physiology. Hence, animals must be able to detect microorganisms and distinguish beneficial from potentially harmful or even life-threatening ones when navigating their environment.

Drosophila melanogaster feeds on rotten and fermenting fruit, where it is exposed to a variety of different microorganisms including nutritious microbes, which possibly also benefit its microbiota and overall health, as well as pathogenic microbes. Just like hunger or reproductive state govern the animal's motivational state or how it perceives certain chemosensory stimuli, beneficial and harmful microbes and the induced immune response can modify *Drosophila* behavior, too. While much is already known about the composition of the microbiota and its effects on host physiology or the immune processes following pathogenic infection, more recent research is now starting to explore the modulatory influence of the microbiota or pathogens on behavior as well as the underlying neural circuits. Much of this research focuses on the chemosensory senses, and in particular on olfaction, since olfaction constitutes a central mechanism through which *Drosophila* perceives and evaluates its environment and adjusts its behavior in turn.

3.3.1. Modulation via the Microbiota

Drosophila possesses a relatively simple multispecies microbiota and intestinal structure, thus making it a useful model organism to study host-microbiota interactions and their influences on host behavior. So what does the *Drosophila* microbiota consist of? It mostly comprises yeasts and bacteria from the Enterobacteriaceae and Acetobacteraceae families as well as from the order Lactobacillales (Chandler et al., 2011, 2012; Wong et al., 2011; Broderick and Lemaitre, 2012). The gut bacterial microbiota of natural *Drosophila* populations is very restricted, with laboratory-raised flies exhibiting an even more limited microbiome (Chandler et al., 2011). The acquisition of the microbiota has been proposed to be determined by diet and host physiology (e.g., the pH of the intestine) as well as chance (Chandler et al., 2011). Yet ingestion of exogenous microbiome members, e.g., from decaying fruit, is not only suggested to be a means for the establishment of the *Drosophila* microbiota, but is also required for its maintenance (Broderick and Lemaitre, 2012; Blum et al., 2013).

How does the microbiota interact with its host? The *Drosophila* microbiota can have a variety of effects on its host's physiology, including development, immunity, nutrition, growth and metabolism, epithelium renewal and longevity (e.g., Buchon et al., 2009; Shin et al., 2011; Storelli et al., 2011; Ridley et al., 2012; Combe et al., 2014; Wong et al., 2014; Clark et al., 2015; Li et al., 2016). Interestingly, however, the microbiota can also affect *Drosophila's* behaviors such as nutritional, olfactory and mating preferences as well as oviposition. For example, it has been shown that isogenic *Drosophila* populations raised on either starch or molasses medium develop different microbiota and, when mixed, prefer mating partners reared on the same medium. This preference lasted for 37 generations (Sharon et al., 2010). Antibiotic treatment abolished this medium-induced mating preference, suggesting that fly-associated commensal bacteria

are responsible for this effect; a hypothesis that was further corroborated by showing that re-infection of antibiotic-treated flies with either the medium-specific microbiota, a mix of *Lactobacillus* sp. or with *Lactobacillus plantarum* alone, restored mating preferences. As they observed an altered cuticular hydrocarbon composition in the different fly groups, the authors propose that the microbiota influences mating preferences by changing sex pheromone levels (Sharon et al., 2010).

Furthermore, it is known that the microbiota impacts on the nutritional and metabolic phenotype of *Drosophila*. Removal of the resident microbiota for example disturbs energy homeostasis and carbohydrate allocation patterns (Ridley et al., 2012), and the microbiota also affects how nutrients are utilized, e.g., by promoting protein nutrition, modulating lipid/carbohydrate allocation and by provisioning B vitamins (Wong et al., 2014). Thus, in addition or due to the microbiota-host interactions on the physiological level, the microbiota also determines the nutritional preferences of *Drosophila*. In fact, commensal bacteria together with essential amino acids have been posited as central modulators of *Drosophila* food choice (Leitâo-Gonçalves et al., 2017). Flies increased their yeast and amino acid preference as well as their yeast appetite as a reaction to essential amino acid deprivation. Commensal bacteria, specifically the microbiome members *Acetobacter pomorum* and *Lactobacilli*, abolished this increased yeast preference, i.e., the appetite for proteinaceous food. In addition, the microbiota also influences egg-laying behavior: the same study showed that the presence of commensal bacteria similarly rescued the deficits in egg-laying brought about by depriving the flies of essential amino acids.

Another study that elucidates how the microbiota brings about behavioral changes focuses on the consequences of microbe-microbe metabolic exchange on *Drosophila* olfactory and egg-laying behaviors (Fischer et al., 2017). Here, flies preferred a co-culture of two representative microbiome members, i.e., yeast and an acetic acid bacterium, to the same mixture grown separately and combined before testing; a preference mostly mediated by the olfactory receptor OR42b. This divergent response is explained by metabolites that are produced exclusively in microbial communities due to microbial interactions, namely ethanol provided by yeast and converted to acetate by acetic acid bacteria. A second behavior affected by this mechanism is oviposition, as flies similarly preferred to lay their eggs in the co-culture. The emergent metabolites hence serve as an indicator for the presence of a beneficial multispecies microbial community, and by detecting those, *Drosophila* is able to adjust its behavior appropriately.

Furthermore, the gut microbiota can affect its host's chemosensory responses by modulating food preferences and foraging behavior (Wong et al., 2017). It has been demonstrated that *Drosophila* changes its olfactory guided microbial preferences depending on past host-microbe association and the gut microbiota composition. Specifically, microbial preferences in axenic flies were different from those of conventional flies in a foraging assay, and flies reared in monoassociation preferred food seeded with the corresponding bacteria. These diverging preferences for beneficial microbes were shown to be contingent on early-life microbial exposure, since inoculation of eggs was sufficient to alter microbial preferences in freshly emerged larvae. The attraction to these microbiota members was mediated mostly by olfaction. The gut microbes further affected flies' nutritional preferences, as the preference for a balanced diet was abolished in flies offered an imbalanced diet with microbial supplementation, suggesting that *Drosophila* is able to balance nutritional needs with the acquisition of beneficial microbes.

Taken together, apart from its impact on host physiology, it is obvious that the microbiota is also able to modulate a variety of *Drosophila* behaviors, such as oviposition, nutritional or olfactory preferences and foraging. However, so far, not much is known about the mechanisms underlying the formation of these behaviors. Future research will thus have to elucidate how this modulation of behaviors is implemented on the neural circuit level, including for example the necessary communication between gut and brain or the involved neuromodulators.

3.3.2. Modulation Due to Pathogenic Infection and Sickness

While it is crucial for *Drosophila* to find suitable food sources that contain beneficial microbes, it similarly has to be able to avoid harmful pathogens, which pose a potential threat to survival. These behavioral strategies that are employed in response to pathogenic microbes to minimize the adverse effects of an infection, such as negative chemotaxis, a reduction in feeding or oviposition in the case of *Drosophila*, can be subsumed under the term 'behavioral immunity' (de Roode and Lefèvre, 2012) and provide the animal with a powerful protection mechanism against sickness. Olfaction plays an essential role for these behaviors, as the olfactory system and the associated neural circuits are mainly responsible for the detection and avoidance of harmful stimuli.

Besides pathways which describe how *Drosophila* senses and responds to attractive microbes such as yeast (e.g., Christiaens et al., 2014), one specific olfactory circuit has also been found for the detection of detrimental, pathogenic microbes. Geosmin, a microbial odorant produced by some fungi, bacteria and cyanobacteria, has been shown to specifically activate a single class of sensory neurons that express OR56a and target the DA2 glomerulus in the antennal lobe, where they synapse on projection neurons that are similarly specific for geosmin (Stensmyr et al., 2012). The geosmin circuit hence forms a specific, functionally segregated pathway through the antennal lobe to higher brain centers. Interestingly, it can also modulate and even override innate attraction to potent attractive odors such as vinegar. Activation of the dedicated geosmin circuitry prompts feeding aversion and a reduction in egg-laying; suggesting that geosmin as a powerful indicator of toxic microbes helps *Drosophila* avoid potential sites of infection.

In addition, pathogenic bacteria have recently been shown to manipulate host behavior by increasing the pheromone production of infected flies, thereby attracting healthy flies that are in turn infected themselves and hence further spread the bacteria (Keesey et al., 2017). In particular, flies avoided feeding and egg-laying on a food source containing *Pseudomonas entomophila*, a bacterial strain highly pathogenic for *Drosophila*, but did not respond to the odor of *P. entomophila*. In contrast, *Drosophila* was highly attracted to the odor or the feces of infected flies compared to that of healthy flies; a behavior that was

shown to be due to an increase in fatty-acid-derived pheromone release via both immune and insulin signaling pathways upon infection with *P. entomophila*. These findings somewhat parallel the results from Sharon et al. regarding the effects of microbiota on *Drosophila* behavior, as that study similarly proposed a change in sex pheromone levels as the reason for the impact of beneficial microbiome members on *Drosophila* behavior (Sharon et al., 2010). Thus, the ingestion of both beneficial and harmful bacteria might, via immune and metabolic mechanisms, cause alterations in physiology that are in turn detected by conspecifics and provoke behavioral changes.

So far, little is known about the precise mechanisms underlying the behavioral changes prompted by pathogenic infection. More recently, however, it has been demonstrated that an altered egg-laying behavior upon infection in *Drosophila* was due to peptidoglycan sensing by octopaminergic neurons (Kurz et al., 2017). A systemic infection with *E. coli* lead to a reduction in female oviposition that was shown to be elicited by peptidoglycan, a component of the bacterial cell wall that also activates the IMD and Toll innate immunity pathways. Detection of peptidoglycan by the fly induced this decrease in egg-laying via NF-κB pathway activation in octopaminergic neurons, which led to a retention of mature oocytes in the ovaries of infected flies. Additionally, oviposition upon bacterial infection was further modulated by a specific isoform of a peptidoglycan-degrading enzyme that counteracts the reduction in egg-laying to prevent an extreme and thus harmful decrease. This study thus highlights a potential mechanism that allows flies to adapt their egg-laying behavior in response to detrimental environmental conditions, with the modulation of octopaminergic neuron activity playing a central role.

Regarding infection avoidance behavior, some pathogens are detected by their odor such as geosmin (Stensmyr et al., 2012). Nevertheless, not all pathogens smell or are in sufficiently high concentrations in a food to be detected. Hence, it is crucial for an animal to form a memory of the chemosensory perception of food that made it sick in order to be able to avoid it in the future and ensure survival. This acquired avoidance of a particular character of a food such as taste or odor (e.g., of the bacteria) after its pairing with an aversive postingestion effect (i.e., the malaise) is known in vertebrates, but also in invertebrates such as *Caenorhabditis elegans*, *Drosophila* melanogaster larvae or the honeybee.

C. elegans exhibits a range of behaviors in response to pathogenic bacteria; it can for example differentiate between beneficial and harmful bacteria and avoid the latter (Pradel et al., 2007; Schulenburg and Ewbank, 2007; Anyanful et al., 2009; Chang et al., 2011). Interestingly, *C. elegans* can actually learn to avoid pathogens: after exposure to and consumption of pathogenic bacteria, *C. elegans* has been shown to avoid odors from the harmful bacterial strain, while its attraction to odors from familiar non-pathogenic bacteria was increased; a process that required the upregulation of serotonin expression in chemosensory neurons (Zhang et al., 2005). This indicates that the rise in serotonin serves as the negative reinforcing stimulus upon infection with harmful microbes. Interestingly, exposure of *C. elegans* to harmful bacteria in the first larval stage led to long-term aversion of these bacterial odors that was maintained throughout adulthood; and this imprinted aversion was shown to depend on distinct circuits for both formation and retrieval of the imprinted memory (Jin et al., 2016).

Such behaviors have also been shown in insects like the honeybee, which can learn to associate the negative post-ingestive consequences of toxins with the taste of those toxins and the odor present at feeding (Wright et al., 2010). This paradigm mimics the avoidance behavior upon bacteria-induced malaise and similarly required serotonin, since blocking of serotonin receptors abolished the ability of honeybees to associate the odor with the onset of sickness. In line with the results from *C. elegans*, these findings also point to serotonin as a neuromodulator of the circuits that integrate the negative postingestive signal caused by pathogen infection within the circuits regulating olfactory learning **Figure 4**.

Research in *Drosophila* has so far not been able to show a similar involvement of serotonin or unveil other underlying mechanisms in more detail; however, there is evidence for learned pathogen avoidance behavior in *Drosophila*. Fruit flies, too, may be able to associate an odor with the intestinal malaise caused by pathogen infection (Babin et al., 2014). Following feeding on a food substrate that was supplemented with an odorant and the highly pathogenic bacterial strain *Pseudomonas entomophila*, flies decreased their attraction to this odorant in comparison to an odor not present during infection. No effect

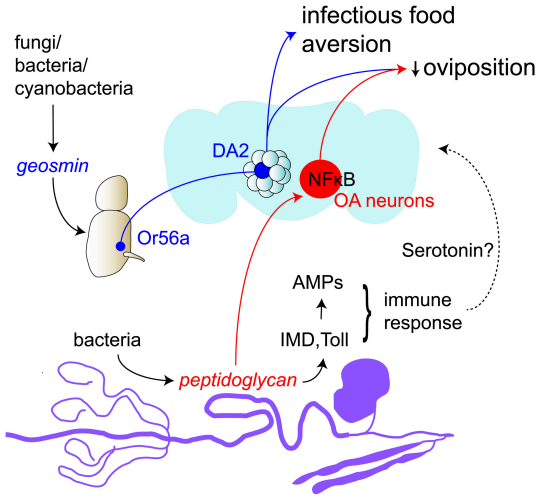

FIGURE 4 | Neuromodulation in sickness and behavioral immunity (blue) Drosophila can avoid feeding or laying eggs on infected food by detecting geosmin produced by some fungi, bacteria and cyanobacteria. Geosmin activates ORNs expressing OR56a that project to the DA2 glomerulus in the antennal lobe. They form synaptic contacts with projection neurons specific for this pathway. (red) In infected flies, octopaminergic neurons (OA neurons) can detect peptidoglycan, a component of the bacterial cell wall, and induce a decrease in egg-laying in infected flies. (black) Peptidoglycan also activates the innate immune response via the IMD and Toll pathways that lead to the production of antimicrobial peptides (AMPs). Studies from *C. elegans* and honeybees suggest that serotonin could form the link between the malaise caused by the ingestion of a pathogen or a toxin and the learned avoidance of the infected food.

was seen in flies conditioned with a harmless version of the same bacterial strain, suggesting that this behavior was in fact due to bacteria-induced malaise.

Drosophila larvae employ a comparable defense strategy in response to pathogenic bacteria: when exposed to a mixture of yeast and *P. entomophila*, *Drosophila* larvae moved away from the food source; a behavior that was not seen when a harmless mutant version of the strain or the less virulent bacterial strain *Erwinia carotovora carotovora* (Ecc15) were used (Surendran et al., 2017). This evasion behavior was diminished in starved larvae and was shown to be reliant on the release of hugin neuropeptide by hugin neurons, whose activity was decreased upon starvation. This puts hugin forward as a modulator of the larval response to harmful pathogens, with a decrease in hugin making starved larvae more prone to overcome their aversion of potentially detrimental food sources.

Therefore, while there is evidence that *Drosophila* can remember the malaise caused by infection, the detailed mechanisms underlying pathogen avoidance behavior in *Drosophila* and the putative memory formation caused by the corresponding negative postingestive effects remain to be elucidated. Nevertheless, studies from other organisms such as *C. elegans* or the honeybee point to a role of serotonin as a neuromodulator of the involved neural circuits. Future studies in *Drosophila* will hence have to address the interplay between sensory information, physiological change and the neural circuits involved in forming a memory of negative postingestive effects as well as the contribution of neuromodulators such as serotonin.

4. METHODOLOGY AND OUTLOOK

Neuromodulation is a testament to the fact that the nervous systems is not a static map (Bargmann, 2012). In order to understand comprehensively how the nervous system is rerouted under modulation, the scientific community needs better tools. These tools can be beneficial for expansive circuit mapping, transgenic access to critical nodes of circuits, monitoring activity over time in neuromodulatory cells and recording the impact of those cells on behavior. Recent developments in *Drosophila* expanded such tools drastically (Venken et al., 2011).

The road map of *Drosophila's* nervous system is about to be completed in larval and adult stages. Of the crucial centers of olfaction, the larval antennal lobe and mushroom body connectome has already revealed unsuspected features of wiring in these centers (Berck et al., 2016; Eichler et al., 2017). The adult mushroom body connectome has also been unraveled (Takemura et al., 2017a). However, without thorough understanding of the connectome's road signs, our vision will be only fractional.

Transcriptomics will be helpful in filling these gaps of knowledge, especially in revealing a particular neurons' arsenal of neuromodulators and receptors over time and different internal states. A recent study published the transcriptome of the 6000 cell *Drosophila* embryo (Karaiskos et al., 2017). Although collecting single-cell subtype neurons is arduous, automated cell collection methods have been utilized previously (Tirouvanziam et al., 2004; Salmand et al., 2011; Berger et al., 2012). For such procedures, the ability to repeatedly and reliably target any cell type is crucial.

The arrival of intersectional genetics, the split-Gal4 system, greatly enhanced the resolution one can achieve with transgenic manipulations (Luan et al., 2006; Pfeiffer et al., 2010; Dolan et al., 2017). The majority of mushroom body output neurons and the innervating dopaminergic neurons are now available as transgenic lines, thanks to the split-Gal4 system (Aso et al., 2014a). The split-Gal4 system also enables elegant physiological and behavioral analyses. Calcium integrators that label neurons with sustained responses over time may reveal differential neuromodulator activity between various internal states (Masuyama et al., 2012; Fosque et al., 2015; Gao et al., 2015).

Activity-dependent and specific immunolabeling of dopaminergic and serotonergic neurons is available (Inagaki et al., 2012; Watanabe et al., 2016). A new version of GRASP (i.e., GFP-Reconstitution Across Synaptic Partners), which is based on reconstitution of split-GFP between pre- and post-synaptic neurons, promises to label synapses dependent on synaptic activity (Macpherson et al., 2015).

In acute monitoring of neuronal activity, advances in imaging techniques provide new opportunities, especially in freely behaving animals. Photo-activatable-GFP (PA-GFP) to track neurons or regions has already successfully been used to for example decipher the pheromone circuit (Ruta et al., 2010). In addition to two-photon imaging in head-fixed flies, transcutical multi-photon imaging and calcium-imaging through cutical windows in freely walking flies allows to directly correlate neuromodulation and behavior (Seelig et al., 2010; Grover et al., 2016; Tao et al., 2017; Zheng et al., 2017).

Large-scale behavioral analyses by themselves are highly valuable, too. For instance, a recent study altered and analyzed more than 2,000 lines that innervate the fly central nervous system in a machine vision based non-supervised fashion (Robie et al., 2017). Ultimately, computational modeling will converge anatomical, behavioral and physiological data to form the basis of our understanding of neuromodulation, from a single protein's three dimensional structure to universal models (Hussain et al., 2016b; Richter and Gjorgjieva, 2017).

Drosophila melanogaster with its sixth Nobel prize won recently in 2017 (Morgan, 1933; Muller, 1946; Lewis et al., 1995; Axel and Buck, 2004; Beutler et al., 2011; Hall et al., 2017) shows that this organism remains a highly relevant model organism for research. Maybe the next discovery can unravel fascinating insights into neuromodulation.

5. CONCLUDING REMARKS

In the quotidian environment of any animal, the influence of sensory stimuli is constantly present. Chemosensation and particularly olfaction can play an important role in how the animal perceives this environment. The driver of environmental perception is survival. To survive, an animal need to rely on its internal states. Issues including hunger, reproductive state and sickness are needed to be resolved. Neuromodulators are key to behavioral effects seen under these internal state conditions. Often changes are investigated using behavioral approaches.

Hunger has been shown to impact on the sensory level as well as higher brain centers in *D. melanogaster*. In courtship behavior, opposing effects of pheromones may be explained with meticulous modulation. Novel research also includes the effect of the microbiome on changes in behavior. If these changes are based on inner modulation remains to be elucidated. Compared to multiple papers that are out there on olfactory memory, it is moreover worth investigating the effects of negative post-ingestive memories like getting sick. The fly community celebrates its sixth Nobel prize. Let's continue on this and focus on unraveling the multi-layered modulation and its interplay with internal states.

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SS, AB, JK, and IG drafted, wrote and revised the manuscript. J-FD implemented all figures and drafted and revised the manuscript. All authors approved to the final version and its publishing.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.2 Manuscripts in preparation for submission

Manuscript 2:
"Learning underpins reproductive state-dependent decision making in *Drosophila* females"

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Author Contributions: **AB** and IGK wrote the manuscript with help of AF (immunohistochemistry), KPS (calcium imaging), and CD (food analysis). **AB** and IGK designed the experiments. **AB** conducted all single-fly T-maze experiments. JC conducted the MBON experiments at testing with Shibire^{ts1} in multiple fly approaches. MHL conducted the dTrpA1 at testing experiments in multiple fly approaches. **AB** trained students for validation of experiments. AF and **AB** did the immunohistochemistry of lines. AF contributed the brain images. SKP did the Calcium imaging experiments. CD and TH provided the analysis of polyamine concentration in our fly food. All authors approved to the final version and its publishing.

Learning underpins reproductive state-dependent decision making in *Drosophila* females

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Abstract

Reproduction is costly for the female body, inducing, in part lasting, physiological and behavioral adaptations. Here, we show that lasting changes in preference behavior rely on long-term synaptic plasticity in defined neurons in the female brain. Using *Drosophila* genetics, we show that a long-term increase in female preference for the nutrient polyamine, requires *rutabaga*, an adenylyl cyclase with a highly conserved role in associative learning and memory. We demonstrate through *in vivo* two-photon imaging that a change in reproductive state induces a lasting change in odor representation in the female's mushroom body (MB). We uncover two different MB pathways required for reproductive state-dependent choice behavior and implicate a role for the $\beta'1$ region. Dopaminergic neurons (DANs), re-enforcers in associative learning and memory, are not only necessary, but also sufficient to replace mating and induce the lasting behavioral switch in female preference. Our data in the fly provides mechanistic insights how a change in reproductive state can induce synaptic plasticity and with it, lasting changes in female choice behavior.

Keywords: *Drosophila melanogaster*; reproductive state; learning; mushroom body; modulation

1 Introduction

External stimuli and internal needs guide decisions and influence interpretations of the environment. Such decisions not only influence the individual itself, but may also have severe effects on their offspring. A female animal, therefore, adapts her choices to her reproductive state. This includes not only the decision to mate, but also a preference for certain, nutrient-rich food sources or, in case of egg-laying animals, oviposition sites (Chapman and Wolfner, 2017; Sayin et al., 2018a). Choices pertaining to food, nutrients or egg-laying sites frequently depend on chemosensory cues such as odors or tastes. Changes in olfactory and gustatory perception have even been reported for pregnant women (Ochsenbein-Kolble et al., 2007). The cellular and neural mechanisms responsible for reproductive state-dependent behavioral changes remain incompletely understood, however. A powerful mechanism for adapting preference for an odor or taste according to internal state acts within the very sensory neurons that detect the sensory cue (Leinwand and Chalasani, 2011). For instance, hunger increases the sensitivity of sweet taste and quenches the one of bitter taste neurons in different animal species (Rolls, 2007; Palouzier-Paulignan et al., 2012). The estrous cycle influences how a female mouse perceives a putative mate (Dey et al., 2015). Outside of estrus, the hormone progesterone strongly inhibits the female's male pheromone sensitive olfactory sensory neurons (OSNs), and thereby, completely blunts her interest in males.

In the genetically-tractable insect, *Drosophila melanogaster*, reproductive state also induces dramatic shifts in the female's chemosensory perception and choices (Hussain et al., 2016a; Ribeiro and Dickson, 2010; Walker et al., 2015; Gou et al., 2014). We have previously shown that a mating-induced transient neuropeptidergic modulation of OSNs strongly increases a female fly's preference for higher concentrations of the important nutrient polyamine (Hussain et al., 2016b,a). Importantly, this rather short-lasting modulation (max. 24 h) leads to a long-term change in the female's choice behavior (> 1 week), indicating that mating induces additional and longer lasting changes in the female brain. How does a short-term experience such as mating induce a long-lasting brain and behavioral change? Reproductive state-dependent behavior to polyamines is well suited to address this question. Polyamines, namely putrescine, spermine and spermidine, play essential and conserved roles in most eukaryotic cells and organisms, ranging from DNA replication, cell proliferation, embryonic development to healthy aging (Miller-Fleming et al., 2015). More importantly, olfactory detection of polyamines is relatively well characterized and depends on two co-expressed ionotropic receptors (IR), IR76b and IR41a on the fly's antenna (Hussain et al., 2016a,b). Within the first several hours after mating, the expression of the neuropeptide receptor, sex peptide receptor (SPR) increases by ten fold in the OSNs leading, when bound to its ligand myoinhibitory peptide (MIP), to a depression of polyamine OSN presynaptic output to the second order neurons of the olfactory system (Hussain et al., 2016a). Though this modulation disappears within 24 h, it triggers a long-term effect: While virgin flies show a rather exclusive attraction behavior towards low concen-

tration of polyamines, mated flies are highly attracted toward high concentrations, for at least one week after mating (Hussain et al., 2016a).

A prevalent way to change behavior in the long-term is learning. Therefore, we tested the compelling possibility that mating and a change in reproductive state not only induce changes at the level of sensory neurons, but also at the level of higher order circuits poised to undergo long-lasting synaptic changes. In insects, long-lasting synaptic plasticity is found in the so-called mushroom body (MB) (Fig. 1a left), a well-characterized brain region known for its role in associative memory formation (Aso et al., 2014a,b; Heisenberg, 2003; Owald and Waddell, 2015). Associative memory comprises the type of memory where a neutral stimulus is paired with a meaningful, positive or negative, signal and elicits a positive or negative memory, respectively. The pairing induces a long-lasting change in synaptic efficacy in specific mushroom body neurons. The availability of highly selective genetic tools provided an unprecedented insight into the underlying cellular and neural mechanisms of associative memory formation (Aso et al., 2014a,b). Moreover, more recent work revealed an essential function of the mushroom body in regulating the expression of innate behavior in an internal state-dependent manner (Sayin et al., 2018b; Grunwald Kadow, 2018; Lewis et al., 2015; Bracker et al., 2013; Tsao et al., 2018). Dopaminergic neurons (DAN) (Fig. 1a right top), as in other animals, provide the teaching or contextual signal that modulates the synapse between the mushroom body intrinsic Kenyon cells (KCs) (Fig. 1a right bottom) and MB output neurons (MBONs), which ultimately bias behavior (Owald and Waddell, 2015). Interestingly, the response of DANs to internal or external stimuli and their force to induce learning is internal state-dependent (Tsao et al., 2018; Lewis et al., 2015; Perisse et al., 2016; Cohn et al., 2015; Berry et al., 2015). Whether and how a change in reproductive state influences dopamine signaling in this plastic neural circuit, however, remains unknown. Previous work has implicated dopamine and DANs innervating the MB in the female's decision of where to place her eggs (Azanchi et al., 2013). The authors proposed that the two main subsets of DANs in the fly's central brain, namely PPL1 (protocerebral posterior lateral cluster 1) and PAM (protocerebral anterior medial), act in competition to guide the female's choice to place her eggs on an ethanol enriched egg-laying substrate. The function of the DANs and the role of the reproductive state of the female, however, have not been addressed. In contrast to female reproductive behavior, a crucial role of dopamine and the MB has been shown for male reproductive behavior (Keleman et al., 2012). Naïve males quickly learn to not court already experienced, mated females, presumably due to the negative experience of being rejected (Keleman et al., 2012). This so-called courtship memory can last for several days depending on the intensity of repetitions of the male's experience. Interestingly, activation of distinct DANs is sufficient to mimic courtship experience in naïve males in the absence of actual courtship or rejection (Keleman et al., 2012). Moreover, the recurrent architecture of a particular module of the MB network including the DAN and a specific MBON was required to maintain this - potentially associative - type of memory (Zhao

et al., 2018).

Apart from the MB, another higher olfactory brain center has been implicated in the control of reproductive behaviors, such as responses to sex pheromones, the lateral horn (LH) (Jefferis et al., 2007; Ruta et al., 2010). Neurons innervating or providing output of the LH show, by contrast to neurons in the MB, largely stereotypic responses to odor categories - among them a class of LH neurons that respond primarily to amines including polyamines (Jeanne et al., 2018; Frechter et al., 2018). While some work implicated the LH primarily in innate valence decisions (Strutz et al., 2014), newer data paint a more complex picture indicating a role in odor classification and corresponding selection of appropriate, rather stereotyped behavioral programs (Dolan et al., 2018). Interestingly, output of the MB can modulate output from the LH upon associative learning, and thereby modulate and override innate behavior (Dolan et al., 2018).

Here, we have tested the role of long-term synaptic plasticity in mating-induced long lasting changes in female decision-making and choice behavior. We show that mated females mutant for the type I Ca2+/CaM-dependent adenylyl cyclase (AC) *rutabaga* show a virgin female like preference behavior for polyamines, strongly suggesting a requirement of learning-induced synaptic changes in reproductive state-dependent odor preference behavior. Importantly, using temporary inactivation of KC output, we pinpoint that the MB is required at two time points to induce the behavioral switch from virgin to mated female choice behavior. First, inactivation of MB output at the time of olfactory choice, ~5-6 days after mating, led to mated females behaving like virgins in a polyamine olfactory choice assay. More importantly, however, blocking synaptic output of the MB exclusively during mating, but not before or 24 h after, also resulted in mated females behaving like virgins, in spite of a functional MB at the time of the olfactory choice assay. These data strongly suggest that MB output during mating is required to induce a lasting change in female decision-making. Using genetic screening of the recently generated tool box for intersectional genetic analysis of the mushroom body, we behaviorally characterized the contribution of different subsets of KCs, MBONs and DANs, respectively. Our results implicate two major MB network modules involved during mating or for olfactory choice behavior. In particular, we find that $\alpha\beta$ - type KCs and a corresponding MBON, which projects to the LH, are required for polyamine preference of the mated female. Furthermore, DANs of the PPL1 innervating the $\alpha'2\alpha2$ or $\gamma1_{ped} > \alpha'2\alpha2\alpha3$ area induce mated female-like polyamine preference behavior in virgins without mating experience. MBONs with dendrites in the $\beta'1$ region promote mated female preference, presumably upon modulation by $\beta'1$-DANs, whose activation can replace mating experience. Interestingly, $\beta'1$-MBONs innervate $\beta'2$-MBONs known to induce innate odor aversion and undergo hunger-dependent dopaminergic modulation. Together our data suggest that mating and a change in reproductive state induce a lasting change in interconnected circuits in the MB. This change lastingly changes how the brain perceives a nutrient potentially valuable for the gravid female. More generally, our findings show that an internal state, such as reproduc-

tive state, not only transiently changes sensory perception, but also induces or facilitates long-term behavioral changes through the plasticity of a brain center for associative learning.

2 Results

2.1 The mushroom body and plasticity are required for long-term behavioral changes

Long-term behavioral changes are often a result of changes in the strength of synapses between neurons in the underpinning neural circuits. The activity of AC *rutabaga* is an evolutionary conserved key mechanism triggering synaptic plasticity. In *Drosophila*, *rutabaga* mutant animals cannot learn to associate an odor with an electric shock or sugar reward. We, therefore, tested whether the switch in polyamine preference behavior would be triggered in *rutabaga* mutant virgin females. To this end, we allowed two days old *rutabaga* mutant virgin females and controls to mate with wildtype males for 24 h. Five days after mating, we tested the preference of single mated mutant and control females for the odor polyamine in a T-maze assay. After mating, 94 percent of control wildtype females chose polyamine odor over control, while only 63 percent of *rutabaga* females preferred polyamines (Fig. 1b left; 50 percent preference represents change level, see methods). For comparison, virgin females of the same age chose polyamine odor only 56 percent of the time (Fig. 1b right). Thus, plasticity of synapses is required for the preference change of females upon mating.

Given the prevalent role of the MB in associative olfactory learning and memory, we next investigated the role of the MB in mating state-dependent polyamine preference. To this end, we took advantage of *Drosophila*'s genetic toolkit allowing the spatial and temporal inactivation of neurons via overexpression of a temperature-sensitive dominant-negative mutant of dynamin, Shibire^{ts1}. At a temperature of 30 °C, Shibire^{ts1} blocks synaptic output, while at 25 °C the same synapses function normally. We shifted females expressing Shibire^{ts1} in all KCs (*MB010B-Gal4;UAS-Shibire^{ts1}*) from 25 to 30 °C for 24 h at four different time points: (1) 24 h before mating, (2) 24 h during mating, (3) 24 h after mating, and (4) only during the olfactory preference test for 1 h (Fig. 1c). Blocking synaptic output before and between mating at test for 24 h had no effect on the mated female's preference for polyamines (Fig. 1c'). By contrast, blocking MB output only during the test significantly impaired the animal's choice, and mated females became almost indifferent to polyamine (Fig. 1c'). More interestingly, however, blocking KC output exclusively for 24 h around mating, prevented the change from virgin to mated female behavior completely (Fig. 1c'). Importantly, all females were analyzed for successful mating afterwards and females that did not produce viable offspring were discarded from the analysis (see Methods). Therefore, KC output is required at two times: First, it is necessary to induce the switch in behavior upon mating, and second, the choice requires KC output. In line with the data suggesting a requirement for KCs in polyamine-induced preference behavior, using the trans-synaptic tracing method *trans*-Tango, we found that projection neurons (PNs), projecting from the antennal lobe (AL) glomerulus that receives

input by the OSNs detecting polyamine odors (i.e. IR41a/IR76b OSNs), innervate not only the LH, but also the MB calyx (see Figures 2 and 3).

Together, these data suggest that KC synaptic plasticity is required for the mating-induced switch in female choice behavior and decision-making.

2.2 Behavioral screening pinpoints roles for distinct MB neurons in reproductive state-dependent female decision-making

Having shown a requirement of KCs at two crucial time points (i.e. mating and choice), we sought to identify the mechanisms underpinning this requirement. The MB network, put simply, consists of three main components: KCs, MBONs and DANs; however, each category can be further subdivided into multiple cell types. We used the published collection of Split-Gal4 lines (i.e. MB-Gal4) to identify subsets of KCs, MBONs, and DANs involved by initially screening their requirement at the time of the choice assay in virgin and mated females. Based on the results, we subsequently determined necessity of MB network neurons also at the time of mating. We behaviorally analyzed 16 different MB-Gal4 lines expressing in the 7 different subsets of KCs by overexpressing Shibire^{ts1}. In mated females, blocking KC output at the time of testing with 4 different lines resulted in significantly reduced preference for polyamine odor in mated females (Fig. 4a). In particular, blocking synaptic release out of $\alpha\beta$-type (i.e. MB008B, MB185B) and $\alpha'\beta'$-type KCs (i.e. MB005B, MB463B) strongly reduced mated female preference for polyamines (Fig. 4a). By contrast, inactivation of KCs did not change the virgin female's indifference to polyamine odor (Fig. 4a). MBONs, as major output of KCs, appear to guide valence-based decisions. MBON dendrites tile the vertical and horizontal lobes of the MB with their dendrites and thereby provide compartment-specific output of 15 different MB regions (Aso et al., 2014b). In addition, some MBONs receive input by all KCs, while others are specific to KC subsets. We behaviorally screened, using Shibire^{ts1}, 22 MB-Gal4 lines for MBON requirement at olfactory choice in mated females. From these tested lines, in particular, 3 lines had a significant impact on female choice behavior. While inactivation of MBON-α2sc (i.e. MB080C-Gal4) during olfactory test significantly reduced attraction to polyamine, blocking synaptic output of MBONs-α'1 α'3m α2p3p (i.e. MB542C-Gal4) even increased the mated females polyamine preference (Fig. 4b). Similarly, blocking synaptic output of MBON-$\gamma 1_{pedc} > \alpha\beta$ (i.e. MB112C-Gal4) also increased the mated females attraction to polyamine odor (Fig. 4b). Furthermore, Shibire^{ts1} expression in MBON-β'1 also reduced the mated females attraction to polyamine - this phenotype was, however, only a trend that did not reach significance. Given the phenotypes observed upon inactivation of synaptic output of some KC-types and distinct MBONs, we argued that DANs might modulate synaptic output of these neurons, and thereby modulate behavior. We, hence, behaviorally screened 17 MB-lines expressing in different DANs of the PAM and PPL1 clusters. We observed phenotypes in either mated or virgin female in 6 lines (Fig. 4c). Blocking output of certain PAM neurons reduced the mated female's attraction

to polyamine, while, with one exception, blocking PPL1 output increased the virgin's attraction to it (Fig. 4c). This result suggested that PAM neurons contributed to the attractiveness of polyamines after mating, and PPL1 neuron activity reduced attractiveness of polyamines in virgins. The results of behavioral screening confirmed a role for the MB in mating state-dependent polyamine odor preference, and further implicated specific regions of the MB in this function.

2.3 A role for a lateral horn innervating MBON in mating state-dependent odor attraction

Having identified putative MB modules involved in mating state-dependent odor preference, we next aimed at dissecting their role during the two different time points when KC output is important. Given the proposed role of the LH in reproduction-related behaviors, we first focused on a possible module involving MBON-α2sc, which projects from the MB to the LH. Inactivation of MBON-α2sc during preference testing of the mated fly significantly reduced the female's post-mating attraction to polyamine (see Fig. 4b). We next tested, whether MBON-α2sc output was required during mating. We observed a mild, but non-significant increase in polyamine preference when MBON-α2sc output was inhibited during mating (Fig. 5a left). Similarly, inhibition of MBON-α2sc output did not further decrease polyamine preference of virgins (Fig. 5a right). Given the requirement of MBON-α2sc output for the high attraction of mated females to polyamine, we wondered whether mating induced a long-term change in the synaptic output of this neuron. To probe this hypothesis, we expressed the temperature sensitive channel dTrpA1 in MBON-α2sc (*MB080C-Gal4;UAS-dTrpA1*) and activated the neurons by shifting the animals to 30 °C (Fig. 5b). The neurons were activated in virgin females instead of mating for a period of 24 h on regular fly food. As with the regular mating protocol, we then tested these females for their preference for polyamine odor 4 days after MBON activation. Activation of MBON-α2sc sufficiently replaced mating in inducing a mated female-like attraction level of polyamine odor in virgin females (Fig. 5b) indicating a mating-induced lasting change in the activity or synaptic efficacy of this MBON. To test this idea, we postulated that an increase of KC output to this MBON in virgins at the time of the polyamine choice test should increase preference in lieu of mating or a mating induced increased activity of synaptic output of the MBON. Thus, we activated KC output using dTrpA1-mediated activation during the choice test (Fig. 5c). We activated all KCs using a general KC driver (*MB010B-Gal4;UAS-dTrpA1*) and the subset of KCs that provides input to MBON-α2sc, $\alpha\beta$-type KCs (*MB008B-Gal4;UAS-dTrpA1*). While activation of all KCs or only $\alpha\beta$ KCs during the olfactory test indeed increased virgin polyamine preference significantly, activation of γ-(*MB419B-Gal4;UAS-dTrpA1*) or $\alpha'\beta'$-(*MB005B-Gal4;UAS-dTrpA1*) KCs had no effect on the preference of virgin females (Fig. 5d). Furthermore, activation of all KCs or $\alpha\beta$-type KCs in virgin females instead of mating was not sufficient to induce mated female like polyamine preference behavior (Fig. 5e). In sum, these data strongly indicate that mating induces a lasting change in

MBON-α2sc, which project from MB to the LH, and thereby enables the switch from virgin to mating female polyamine preference.

2.4 $\beta'1$ - A mushroom body reproductive state-dependent pathway

Our screening data identified another region of the MB of putative importance for mating state-dependent sensory perception. Inactivation of MBON-$\beta'1$ (i.e. MB057B-Gal4) output at the time of olfactory test reduced the mated female's preference for polyamine. Remarkably, blocking of synaptic output of MBON-$\beta'1$ during a 24 h period during and around mating, resulted in a strong and significant reduction of polyamine odor preference in these mated females at the time of odor test (Fig. 6a) suggesting that this MBON's output critically contributed to the induction of long-term behavioral changes. In line with this hypothesis, dTrpA1-mediated activation of MBON-$\beta'1$ in virgins in place of mating increased, although not significant, their polyamine preference at the time of choice (Fig. 6b). By contrast, activation of MBON-$\beta'1$ in virgin females only at the time of choice led to a significant increase in polyamine attraction compared to controls (Fig. 6c). Together, these results argue that MBON-$\beta'1$ synaptic output is required to induce a long-lasting change in MB output during mating. This lasting increase in synaptic output can be mimicked by activation of MBON-$\beta'1$ at the time the animal is making the actual olfactory choice.

In contrast to MBON-α2sc, little is known regarding the function and connectivity of MBON-$\beta'1$ that would explain the observed behavioral phenotypes. MBON-$\beta'1$ releases GABA as neurotransmitter and will, therefore, inhibit downstream neuronal activity. We again used the *trans*-Tango system to identify the putative inhibited neuron (Fig. 7a and b). Expression of *trans*-Tango under the control of MB057B-Gal4 strongly labeled another MBON(s) with dendrites in the $\beta'2$ region of MB (Fig. 7b and c). Interestingly, MBONs providing output from $\beta'2$ were previously shown to be required for innate and hunger-dependent odor aversion (Lewis et al., 2015). In line with this, appetitive olfactory learning or pairing of an aversive odor with an innately attractive odor led to a decrease in $\beta'2$ MBON activity. Based on these prior data, we postulated that MBON-$\beta'1$, as a GABAergic neuron, might counteract MBON-$\beta'2$ activity. Consequently, activation of $\beta'2$ MBONs should reduce the mated female's attraction, while blocking $\beta'2$ synaptic output would increase the virgin female's polyamine attraction. To test this, we chose line MB011B-Gal4, which drives Gal4 expression in three different MBON-types, namely MBON-β'2mp, MBON-β'mp_bilateral and MBON-$\gamma5\beta'$2a. As predicted, temporary thermogenetic activation of these MBONs (*MB011B-Gal4;UAS-dTrpA1*) at the time of olfactory choice, significantly reduced the mated females attraction to polyamine (Fig. 8a). Conversely, temporary synaptic output inhibition at the time of choice in virgins significantly increased their attraction to polyamine odor (Fig. 8b). By contrast, neither activation nor synaptic output blockage at the time or in place of mating had a significant effect of the female's preference behavior (Fig. 8c and d). These data suggest that $\beta'2$ output is crucial for the choice of the female at the time it is making it, but it

is insufficient and also not important to induce the mating-induced long-term change in choice behavior.

Altogether, we propose the involvement of a second MB module. This module consists of MBONs providing output from $\beta'1$, which directly inhibit output of the $\beta'2$ MB region to change virgin indifference to a lasting polyamine preference upon mating.

2.5 Specific dopaminergic neurons can mimic mating

Long-lasting changes in MB synaptic efficacy during associative learning are brought about by dopaminergic neurons. Based on our behavioral results and imaging data, we sought to identify DANs involved in the lasting mating-induced change in preference. As described above, blocking output of PAM DANs reduced attraction to polyamine in the mated fly, while blocking PPL1 synaptic output increased attraction of the virgin (see Fig. 4c). Activation of MBON-α2sc in lieu of mating was sufficient to change virgin behavior to mated female preference (see Fig. 5b). Hence, we asked whether the DAN innervating the same lobe region could be involved in this effect. To this end, we activated PPL1-α2-type DANs by expressing dTrpA1 under the control of MB058B (only PPL1-$\alpha'2\alpha2$), MB060B (PPL1-$\alpha'2\alpha2$, PPL1-$\alpha'3$, PPL1-$\alpha3$, PPL1-$\gamma2\alpha'1$), and MB438B (PPL1-$\alpha'2\alpha2$, PPL1-$\alpha'3$, PPL1-$\gamma1$pedc). Activation of neurons in none of these lines was sufficient to replace mating experience and change virgin to mated female preference behavior (Fig. 9a). Therefore, it appears unlikely that a DAN innervating the α2-lobe region was a sufficient signal to replace mating. We found, nonetheless, that blocking the output of α2-innervating PPL1 DANs at the time of olfactory test, using the same transgenic lines to express Shibire^{ts1}, led to a significant increase in attraction to polyamine odor in virgin females (Fig. 9b). We therefore concluded that the activity of PPL1 DANs innervating the same MB region as MBON-α2sc were necessary to maintain a low attraction to polyamine in the virgin. This left us still with the question of whether and where DAN activity lastingly modulated synapses to induce a lasting change in preference behavior. We, thus, next analyzed the putative role of DANs innervating the $\beta'1$ region of the MB. We chose line MB188B, because it labeled several PAM DANs with axons in the $\beta'1$ lobe, namely PAM-$\beta'1$ap, PAM-$\beta'1$m, PAM-$\gamma3$, and PAM-$\gamma4$. We activated these PAMs in virgin females as a replacement for mating. Remarkably, this treatment was highly efficient in switching virgin female preference to mated female preference for polyamine (Fig. 9c right) strongly suggesting that $\beta'1$ DANs were mimicking an important aspect of the mating experience. Surprisingly, activation of these PAM neurons during mating prevented the mating-induced switch and mated females behaved like virgins at the time of testing (Fig. 9c left). Hence, a certain level or at least relative level of DAN activity is important to induce lasting preference changes in female choice behavior. We next asked whether the activity of these DANs was strictly necessary to induce the switch. This was not the case, since blocking synaptic activity of the same group of PAM DANs by expressing Shibire^{ts1} under the control of MB188B during mating or during the olfactory choice test had no effect on the vir-

gin or mated female choice (Fig. 9d). Therefore, additional neurons, possibly DANs appear to contribute to the observed mating-induced long-term switch. However, the present data still argued for a critical and sufficient role of DANs innervating the $\beta'1$ region of the MB in transmitting a mating-related signal to the MB network, thereby inducing a long-lasting change in the polyamine odor response of MBON-$\beta'1$.

2.6 Mating signal does not solely rely on pheromone detection, male ejaculate or sterility

Given the sufficiency of specific DANs and MBONs in inducing a lasting change in polyamine preference in place of actual mating, we asked what these neurons are actually detecting and signaling to the MB network. We tested three possible triggers as switch inducers: courtship and courtship-related signals, copulation and male ejaculate, and finally oogenesis. Male flies perform a complex courtship before attempting to mount the female in order to mate. During this time, females will, among other signals, also smell the male pheromone cVA. Interestingly, cVA is transferred to the female upon mating and remains on her body for an extended period of time (Ejima et al., 2007). To test the role of courtship and its signals vs. actual mating, we allowed fly couples to go through the courtship ritual, but separated them just as the male attempted to mount the female. We then analyzed the polyamine preference behavior of these females that were technically still virgins. Remarkably, the behavior of these females was neither significantly different from virgins nor from mated females indicating that courtship related signals played an important role as behavioral change triggers (Fig. 10a). Thus, we next tested one particular aspect of the courtship ritual: pheromone detection. Pheromone detection relies on olfactory receptor OR67B and the general OR co-receptor, ORCO. We analyzed mated females mutant for ORCO, and hence unable to smell cVA, to investigate the role of pheromone detection as inducer of increased polyamine attraction. These mated females still underwent the mating-induced switch in polyamine preference (Fig. 10b). However, there was a trend toward a lower polyamine preference. This result indicates that pheromones might be involved in the process and contribute to a switch in preference behavior, but they are not the sole factor. We next addressed the role of male sperm or seminal fluid. During copulation the male fly transfers sperm and seminal fluid into the female reproductive tract. Among the factors transferred with the sperm is one of the ligands of SPR, sex peptide (SP). SP binds to neurons expressing SPR in the female's reproductive tract and induces the so-called canonical post-mating switch, a suite of behaviors associated with reproduction such as increase in egglaying and rejection of males attempting to mate. Females mated to SP mutant males do not switch to these behaviors. Although SPR is required for the change in the mated female's polyamine preference behavior, this appears to rely mainly on the other ligands, MIPs. In fact, females mated to SP mutant females still undergo the change in polyamine preference behavior. Therefore, we tested mating with sterile males. There are two previously used experimental methods to achieve such an effect: males that do not produce either seminal fluid protein

or sperm. To generate males incapable of producing seminal fluid protein, accessory glands were disrupted through induction of ER stress (see methods; *prd-GAL4;UAS-BiP-RNAi*). After copulation with an seminal fluid protein deficient male, females showed a normal increase in preference for polyamine odor as compared to controls (Fig. 10c). Similarly, copulated with sperm-deficient males, which were generated by using mutants of the gene *tudor*, also led to mated females that behaved indistinguishable from females that mated with wildtype males (Fig. 10d). This result was in line with our previous finding (Hussain et al., 2016a,b), and suggested that not only was SP not essential, but also that other sperm- and seminal fluid-associated factors were dispensable. To further support this conclusion, we tested females unable to produce eggs by generating *ovoD* mutant females. *OvoD* females mated with wildtype males underwent the switch in behavior and were attracted to polyamines to the same extent as controls (Fig. 10e). Taken together, our data indicates that a combination of courtship signals including the pheromones and the act of copulation itself are the most important factors to induce a change in behavior from virgin to mated female.

2.7 $\beta'1$ dopaminergic neurons respond stronger to cVA after mating

In the so-called courtship learning, male flies show a higher sensitivity to cVA, and therefore are able to distinguish experienced females, which carry some of the former male's cVA on their body, from virgins. In addition, pheromones are not only cues for mating partner, they represent signals for marking good oviposition sites (Dumenil et al., 2016). From the data above (see Fig. 10), we postulated that females might be undergoing a similar change, where cVA could act as one part of the experience that modulates female preference in the long run. To test this idea, we imaged cVA odor responses in DANs, which are known to modulate MB synaptic output as described above. We expressed GCaMP6f in all DANs innervating the MB, and imaged cVA responses from several MB lobe regions. Remarkably, the response to cVA odor changed between mated and virgin fly (Fig. 11a). In particular, DANs innervating the $\beta'1$ and the $\alpha'1$ region of the MB lobes showed a significant increase in cVA response in mated females as compared to virgins (Fig. 11b). By contrast, we observed no change in the $\beta1$ or $\beta'2$ innervating DANs (Fig. 11c). These data are in line with our data obtained through unbiased behavioral screening and confirm that DANs innervating the $\beta'1$ region of the MB undergo mating-induced changes. Furthermore, they provide evidence for a modulatory role for cVA as potent activator of $\beta'1$ DANs, which are sufficient to replace the experience of mating as triggers of the change in female polyamine preference.

2.8 Polyamines are present in high concentrations in fly food

Our data reveals some interesting parallels with male courtship learning. In male courtship learning, it is thought that males associate cVA with the experience of rejection by experienced females. This could be similar for the females in our paradigm; females associate cVA with mating. Another interpretation could be

that cVA represents a main aspect of the mating experience, and modulates the perception of polyamine odor. We, therefore, wondered whether and what the females were actually associating, and in particular, if the presence of polyamines during mating mattered. We generally carried out all mating on standard fly food without the addition of extra polyamines (see methods). Does standard fly food contain polyamines? To answer this question, we analyzed standard fly food using chemical analysis for the presence and concentration of biogenic amines (concentration in μg/100 g of fly food). The results revealed substantial amounts of polyamines in the food (Fig. 12): histamine (5.49), ethanolamin (676.65), phenylethylamin (5.16), putrescine (172.50), β-alanine (930.84), tyramine (166.64), spermidine (18120.77) and spermine (492.81). Thus, the total amount of polyamines in standard fly food is $\sim$20 mg in 100 g food, which is comparable to levels in high polyamine foods such as oranges (Okamoto et al., 2014).

3 Discussion

The transition from a sexually immature to mature, reproducing parent is a major step in the life of most animals. With this step come new needs and demands reflected in a significant change in an animal's behavior and preferences. Failure to behave in a state-dependent manner could well mean the ebbing of new offspring, such as a gravid female mosquito no longer interested in obtaining a blood meal (Corrales-Carvajal et al., 2016; McMeniman et al., 2014) In addition, sexually mature animals spend a major proportion of their lives with generating or caring for their offspring. In line with this, some of the behavioral changes initiated upon mating will last for a significant fraction of an animal's lifespan. Here, we have identified a neuronal and circuit mechanism underlying a long-term behavioral change in gravid *Drosophila* females. We show that AC *rutabaga* and activity of the MB network, the primary brain center for associative learning and memory in insects, is required during mating to effectively switch virgin behavior to mated female behavior, which lasts for several days after mating. We identify two specific MB circuits critical during mating and at a later time when the female makes an olfactory choice, respectively. Moreover, mating can be substituted by the activation of a small group of DANs or connected MBONs. Finally, *in vivo* functional imaging suggests that mating induces long-lasting changes in the representation of odors in DANs and MBONs. We propose a model where mating and mating-related signals, such as cVA, trigger associative memory formation in specific MB neurons, which induces long-term synaptic changes leading to a lasting alteration in female olfactory choice behavior (Fig. 13).

3.1 A change in reproductive state lastingly changes the female brain

Soon after mating, a female fly starts to look for places to lay her eggs. Although this behavior is generally viewed as innate and not experience-dependent, it is induced by mating and the experiences surrounding it. Furthermore, earlier work has shown that oviposition decisions require the output of the MB (Zhao et al., 2018), a brain center classically viewed as redundant

for innate behavior. In line with a role during reproductive state-dependent choice behavior, MB output is necessary for the expression of metabolic state-dependent innate food search behavior. Moreover, similar to mating, hunger states also induce a neuropeptidergic modulation of OSNs, which is sufficient to increase the animals sensitivity and interest in food odor. However, at least in the context of modulation of behavior due to changes in reproductive state, the MB not only regulates immediate behavior, but, as we have shown here, determines future behavior of the gravid female. This lasting modulation, similar to classical associative learning, requires the action of AC *rutabaga* and dopamine at the time of mating. In other words, mating appears to facilitate the formation of an associative memory and induces long-term attraction to the important nutrient, polyamine.

While our data suggests that the increase in polyamine attraction from virgin to mated female requires the association of multiple cues and/or events, we have not been able to pinpoint a single signal by itself. Usually, SP by binding to SPR in the female reproductive tract induces a number of post-mating behaviors. We have previously shown, however, that SP appears to play a rather redundant role in the increase in polyamine attraction. Instead, SPR in OSNs together with its conserved ligand MIP depress OSN synaptic output upon mating and thereby trigger the switch to higher polyamine attraction. Similarly, egg production plays a similarly redundant role in this behavioral switch. Surprisingly, courtship in the absence of mating was sufficient to increase virgin female polyamine preference to a level non-significantly different from mated females. In line with this result, ORCO mutant females showed a similar intermediate attraction suggesting that the male pheromone, i.e. cVA, contributes to the change in behavior from virgin to mated female. Given the high concentration of polyamines in fly food, which serves as the environment during mating, it is conceivable that the coincedent detection of cVA with polyamines leads to the long-term more positive perception of this nutrient. Moreover, neuromodulation in OSNs upon mating tunes the female's preference toward high polyamine at the same time as she is exposed to cVA. It is attractive to speculate that short-term tuning of sensory neurons to a certain cue (e.g. polyamine concentration) facilitates the formation of a long-lasting associative memory. On top of the mutant behavioral data, our *in vivo* calcium imaging data argues for a critical role of cVA as one of the coinciding signals. cVA activates DANs, and in particular, $\beta'1$ innervating DANs. Activation of these DANs was sufficient to replace mating in inducing mated female behavior in virgins. Naturally, additional cues are available to females to recognize an interested male. For instance, visual cues (e.g. color or size of a mating partner) or auditory cues (e.g. wing flicks) during the courtship are presented to the female by the male (Greenspan and Ferveur, 2000; Agrawal et al., 2014). Mating itself stimulates mechanosensory neurons in the female's reproductive tract and thereby appears to contribute to a switch in her behavior (Haesemeyer et al., 2009). Finally, other hormonal or peptidergic signals, such as steroids or juvenile hormone (JH) could play a role.

3.2 Specific pathways in the mushroom body underpin state-dependent behavioral change

KC synaptic output is required at the time of mating and later when the female makes the actual decision toward high polyamine content. Thanks to the large collection of specific transgenic driver lines for KCs, DANs, and MBONs, we begin to understand the mechanistic basis for this two time requirement.

The activation of DANs innervating the $\beta'1$ region of the MB is sufficient to induce mated female polyamine choice behavior in virgin females, while inhibition of MBON-$\beta'1$ output at the time of mating prevents the switch to mated female behavior. Similarly, activation of MBON-$\beta'1$ output instead of mating also increased the virgin female's polyamine attraction to mated female levels. We propose that a cVA and mating-induced dopaminergic modulation of MBON-$\beta'1$ lastingly changes the female's interest in the beneficial compound polyamine. Compared to other MB lobe regions, little is known about the role of $\beta'1$. A prior functional imaging study (Hige et al., 2015) suggested that $\beta'1$ MBONs are less broadly odor-tuned than other MBONs. Our data shows that DANs and MBONs of this region respond to cVA and polyamine possibly indicating a more specific function of the brain area in reproduction related behavior. Notably, $\beta'1$ MBONs have been implicated previously in courtship conditioning in *Drosophila* males (Montague and Baker, 2016). At this point, we can only speculate about what $\beta'1$ MB output does to these reproduction related behaviors. Our *trans*-Tango data implicated $\beta'2$ MBONs as target of MBON-$\beta'1$. We favor the model that GABAergic MBON-$\beta'1$ inhibits $\beta'2$ MBONs from driving aversive behavior as we and others have previously shown. In line with this, we find that blocking $\beta'2$ output indeed increases the virgin's attraction to polyamine.

The other MB area of functional importance is the $\alpha2$-lobe region. Based on our results we propose that mating modulates MBON-$\alpha2$sc, and thereby the output from MB to LH. MBON-$\alpha2$sc, also known as MB-V2α, received attention previously. For instance, it has been shown to be involved in the recall of aversive memories, i.e. taste memory (Masek et al., 2015) and olfactory memory (Séjourné et al., 2011). In other words, no output from MBON-$\alpha2$sc shifts a valence choice from more negative to more positive. This corresponds to our observation: inhibition of MBON-$\alpha2$sc synaptic output prevents mated females from being highly attracted to polyamine. In regard to our work, the most interesting results were obtained by Dolan et al. (Dolan et al., 2018), who showed that this MBON projects (next to SMP and CRE) directly to the LH, where it modulates stereotypic response behavior to polyamine. Hence, in the present scenario, MB output regulates the expression of stereotypic polyamine-induced behavior.

In the context of classical aversive odor conditioning, LH output neuron (LHON) cell types, PD2a1/b1, are postsynaptic to MBON-$\alpha2$sc. These LHONs are required for innate attraction to food odorant. While the same LHONs are redundant in the present paradigm (data not shown), MBON-$\alpha2$sc connects to a number of additional LHONs who might either compensate or be solely responsible. Of interest, nevertheless, appears the finding that PAM-$\beta'1$ and

PAM-$\beta'2$ DANs might receive LH input through a feedback connection from PD2a1/b1 axons. Whether this connection or other LH input to these DANs matter in the present context remains to be investigated.

Thus, upon mating, $\beta'1$-DANs increase their response to cVA and potentially other mating-related cues, which changes the output of the $\beta'1$-MBON. This in turn inhibits the connected MBON-$\beta'2$ and changes the net output of the MB network towards attraction. In parallel, MBONs providing output of $\alpha2sc$ promote attraction presumably through LHONs neurons. Taken together, we propose that a network of neurons primarily innervating the $\alpha2$ and $\beta'1$ regions of the MB lobes controls mating state-dependent preference of females to important nutrients for reproduction (Fig. 13).

3.3 Internal state and memory

Our data provides mechanistic insight into the relationship between internal state and long-term behavioral adaptation and memory. A state change could conceivably induce a period of increased synaptic plasticity, and hence, a window of opportunity for learning. Such a window of enhanced plasticity might arise through an increase in cAMP (through AC *rutabaga*) in the MB network (Louis et al., 2018) to facilitate the formation of memory at critical times in life. In such as context of high plasticity, sensory modulation, as seen in polyamine-detecting OSNs for a limited time after mating, could in parallel increase the salience of a specific sensory environment that should be remembered. Similar situations as we have characterized here might arise at times of high alertness such as hunger periods. While neuropeptides modulate sensory neurons to tune their sensitivity to a specific, likely food related cue, hunger state might facilitate plasticity in the MB and promote associations such as learning about a good food source. In other words, food that is being consumed when hungry not only tastes and smells better, it is also remembered better and more positively. Beyond insects, such a scenario could arise during hunger states, but also during other periods of important changes in the body. An example could be mother-child-bonding in mammals where mother and newborn are particularly sensitive to memorizing the sensory characteristics of each other for a certain period after birth. The orchestration of internal state-dependent sensory tuning and/or filtering with plasticity of neurons in higher brain centers could, thus, ensure that animals remember the most relevant information at key turning points in their lives.

4 Methods

4.1 Fly Husbandry

Flies were kept at 25 °C at 60 % humidity with a day:night cycle of 12 hours each. Flies were raised on standard cornmeal medium. Mushroom Body lines were received from the Janelia Fly-Light Split-GAL4 Driver Collection (Janelia, Research Campus, 2019) (i.e. for KCs: MB005B, MB008B, MB009B, MB010B, MB131B, MB152B, MB185B, MB364B, MB370B, MB371B, MB418B, MB419, MB463B, MB477B, MB594B, MB607B; for MBONs: MB002B, MB011B, MB018B, MB026B, MB027B, MB050B, MB051C, MB057B, MB077B, MB080C, MB083C, MB093C, MB110C, MB112C,

MB210B, MB242A, MB298B, MB310C, MB399B, MB433B, MB542B, MB549C; for PAM: MB040B, MB042B, MB043B, MB047B, MB087C, MB109B, MB188B, MB194B, MB213B, MB316B; for PPL1: MB058B, MB060B, MB296B, MB304B, MB438B, MB630B; other: MB013B, MB460B, MB465C, MB583B, empty-PBDP). *rutabaga* was ordered at Bloomington stock center (Bloomington, Indiana University, 2019). *UAS-BiP*, *prd-Gal4* were a generous gift from Anne von Philipsborn, the *trans*-Tango lines were a valuable gift from the Barnea Lab, the LH lines were thankfully provided by Greg Jefferis.

4.2 wild type experiments

After ecclosure, female virgins were kept in vial until testing one week later. As a second group, some female flies were kept with males for 24 hours. Then, the males were removed and the females were kept in vial until final testing 4-5 days later. Vials were checked for larvae to ensure mating took place.

4.3 Shibire and dTrpA1 Paradigms

For Shibire^{ts1} and dTrpA1, silencing or activation was due to a shift to 30-32 °C. For a control the flies were not shifted to higher temperatures at all.

For temperature shifts *at mating*, 1-2 day old virgin females were put on higher temperature for 30 minutes. Afterwards, experienced CS males were added for 24 h to ensure mating during this time period. The males were removed after the 24 hours. Females were tested 3-5 days later for putrescine attraction or avoidance behavior at 25 °C. The vials were checked for offspring to ensure the mating experience. In pure virgin experiments the CS males were not added and the virgins just put on higher temperature for 24 hours in the same time frame *instead of mating*.

For shift *at testing* 1 day old females were stored with males for 24 hours at 25 °C. Then, the males were removed and females were tested 3-5 days later for putrescine attraction or avoidance behavior. Virgins were not exposed to males. Flies were tested in a preheated T-maze chamber of 30-32 °C and 60 % humidity.

4.4 T-maze Experiments

T-maze experiment were performed at 60 % humidity and depending on the paradigm at either 25 °C or 30-32 °C. T-maze tubes were either prepared with 50 μl of 100 mM 1,4-Diaminobutan (Putrescine) or 50 μl ultrapure water on a piece of Whatman® filter paper and sealed with Parafilm® right before the experiment started.

In either case, females were transferred to the preheated T-maze chamber for 20 minutes before the experiment started. We performed single and multiple fly experiments in a reciprocal manner. For single fly experiments (data represented by single fly choice graphs) data was analyzed using a two-sided Fisher's exact test in R ((R Core Team, 2013)). For multiple fly experiments 15 ± 5 or 35 ± 10 flies were loaded in the T-maze. A preference index (PI) was calculated via the following equation 1, where ϵ refers to the number of flies, which are positioned in the elevator after the 1 minute choice time:

$$PI = \frac{(flies_{polyamine} + 0.5\epsilon) - (flies_{water} + 0.5\epsilon)}{flies_{total}} \quad (1)$$

The data is represented by box-plots of the PIs and analyzed using a Mann-Whitney-U test

with continuity correction. This has been done in Excel's RealStats Resource Pack (Charles, 2018).

For practicality, to ensure that mating took place in certain experiments, and to exclude group effect behaviors, experiments were performed in single and multiple fly experiments and have been validated by independent students. Furthermore, experiments were done blinded to ensure good scientific practice.

4.5 Courtship Experiments

Male flies were kept together in a vial for 2 days until exposure to 1-2 day old virgin females. Always one male was put into a vial with one female. The process of courtship steps as described in previous studies (Greenspan and Ferveur, 2000) was watched until the male began licking the female and making first attempts for copulation. Immediately, the male was removed from the vial. The female was tested 3-5 days later in a T-maze for putrescine attraction or avoidance behavior at 25 °C.

4.6 Immunohistochemistry

trans-Tango flies were kept for one week at 25 °C, then for another two weeks at 18 °C, as similarly indicated in the original paper (Talay et al., 2017). Flies were anaesthetized on ice, put in a glass staining cup with 80 % ethanol. After 30 sec flies were put in another glass staining cup filled with 1x phosphate buffered saline (PBS) by Roth. Flies were dissected in PBS under the microscope and brains were stored in a PCR cap with 1:4 solution of 4 % paraformaldehyde (PFA) by Roth and 0.1 % phosphate buffered triton (PBT = PBS +Triton X100, Roth) until final fixation. Brains were fixed in PFA and a drop of PBT for 60 min at room temperature. Brains were then washed with PBT three times for 20 min at room temperature. The PBT was removed and replaced by 3 % normal goat serum (NGS) in 0.1 % PBT for 30 min at room temperature.

Then, the first antibody mixture in 3 % NGS in 0.1 % PBT was applied for 24 h at 4 °C in darkness. Same for the secondary anitbodies. Brains were washed with 0.1 % PBT for 5 sec and then three times for 20 min at 4 °C in darkness. After washing with 0.1 % PBT for one last time for 1 h at 4 °C in darkness, brains were mounted on a glass slide with VectaShield (Vector Laboratories).

Antibody staining was done using as first antibodies, each in 1:200 dilution, a-GFP, clone N86/8 mouse (Neuromab), 3H9 a-GFP rat (Chromotek), a-RFP mouse (Dianova), Living colors DsRed polyclonal AB rabbit (Clontech), ChAT4B1mouse (DSHB) and DN-Ex#8 (a-nCad) rat (DSHB). As secondary antibodies goat anti-rat Alexa 488 (Invitrogen), Goat anti-mouse Alexa 488 (Invitrogen), Goat anti-mouse Alexa 633 (Invitrogen), Goat anti-rabbit Alexa 633 (Invitrogen) and Cy3-conjugated AffiniPure F(ab)2-Fragment goat anti-mouse IgG (H+L) (Dianova) and Cy3-conjugated AffiniPure F(ab)2-Fragment goat anti-rabbit IgG (H+L) (Dianova) in 1:200 dilution were used.

Imaging was done using a Leica SP8 confocal microscope. Image Processing and analysis have been performed using Fiji (Schindelin et al., 2012).

4.7 Calcium Imaging

All Calcium imaging experiments were conducted with a two-photon microscope. 5- to 7-day-old female virgin or mated flies of the genotype *TH-58E02-Gal4;UAS-GCaMP6f* were used. *In vivo* preparations were prepared according to a method described previously ((Bracker et al., 2013)). Preparations were imaged using an Olympus FV1000 two-photon system with a BX61WI microscope and a 40 x 0.8 water immersion objective. GCaMP fluorescence was excited at 910 nm by a mode-locked Ti:Sapphire Mai Tai DeepSee laser. Time series images were acquired at 210 x 210 pixel resolution with 3 frames/ sec speed using the Olympus FV10-ASW imaging software. A custom made odor delivery system with mass flow controllers were used for odor delivery. Throughout the experiments, a charcoal filtered continuous air stream of 1 ml/min was delivered through an 8mm Teflon tube positioned 10 mm away from the fly antenna. Odor was delivered into the main air stream by redirecting 30 % of main air flow for 1 s through a head-space glass vial containing 1 % 11-cis Vaccenyl Acetate (Pherobank AB, The Netherlands). In order to measure the fluorescent intensity change, region of interest was drawn manually, and the resulting time trace value was extracted for data analysis. The relative change in fluorescence intensity was defined by using the following formula 2, where F_n is the nth frame after stimulation, and F_0 is the averaged basal fluorescence of 5 frames (∼1 s) before stimulation.

$$\frac{\Delta F}{F} = \frac{100(F_n - F_0)}{F_0} \qquad (2)$$

The heatmaps were generated using a custom-written program in Python. All data processing and statistical tests were done using Excel and GraphPad Prism software, respectively.

4.8 Analysis of Fly Food

Chemicals

The following compounds were obtained commercially from the sources given in parentheses: formic acid, sodium hydroxide (Merck, Darmstadt, Germany), histamine, ethanolamine, 2-phenylethylamine, putrescine, β-alanine, tyramine, spermin, spermidine, trichloroacetic acid (Sigma-Aldrich, Taufkirchen, Germany), benzoyl chloride (VWR, Darmstadt, Germany), [2H4]-histamine, [2H4]-putrescine (CDN Isotopes, Quebec, Canada), [13C2]-ethanolamine, [13C3, 15N]-β-alanine (Sigma-Aldrich, St. Louis, MO, USA), [2H4]-phenylethylamine (Dr. Ehrenstorfer, Augsburg, Germany). The purity of all amines was checked by LC-MS and NMR experiments as described by Mayr and Schieberle (Mayr and Schieberle, 2012). Deuterated solvents were obtained from Eurisо-Top (Gif-Sur-Yvette, France). Solvents used for HPLC-MS/MS analysis were of LC-MS grade (Honeywell, Seelze, Germany); all other solvents were of HPLC grade (Merck Darmstadt, Germany). Water used for HPLC separation was purified by means of a Milli-Q water advantage A 10 water system (Millipore, Molsheim, France). [2H4]-Spermine, [2H2]-tyramine and [2H4]-spermidine were synthesized and purified as reported earlier (Mayr and Schieberle, 2012). Standard flyfood consisted of the following ingredients per 100 ml: 1.17 g agar, 10 g cornstarch, 1 g soy flour, 1.85 g yeast, 0.4 g diamalt, 0.4 g sugar beet molases, 0.25 g nipagin-salt, 10 % phosphoric acid and water.

Analysis of amines in fly food samples

Quantification of biogenic amines and polyamines in flyfood was performed by means of a stable isotope dilution LC-MS/MS method after derivatization as already reported by Mayr and Schieberle (2012). Stock solutions. Stock solutions of the internal standards [13C3]-ethanolamine (52 μg/mL), [13C3, 15N]-β-alanine (36.0 μg/mL), [2H4]-histamine (66.87 μg/mL), [2H4]-putrescine (28.86 μg/mL), [2H4]-spermine (91.56 μg/mL), [2H2]-tyramine (51 μg/mL) and [2H4]-spermidine (230 μg/mL) and [2H4]-phenylethylamine (109 μg/mL) were prepared in aqueous trichloroacetic acid (10 %) and stored at 7 °C until use. Sample workup. Flyfood was frozen in liquid nitrogen and grounded in a mill (Moulinette, Moulinex, Alenon, France). Aliquots (5 g) of each sample were spiked with an aliquot of the labled internal standards ([13C3, 15N]-β-alanine 50 μL, [2H4]-histamine 100 μL, [2H4]-putrescine 100 μL, [2H4]-spermine 50 μL, [2H2]-tyramine 100 μL, [2H4]-spermidine 50 μL, and [2H4]-phenylethylamine 109 μL), thereafter, aqueous trichloroacetic acid (10 %, 40 mL) was added, and vigorously stirred at room temperature. After an equilibration time of 30 min, the suspension was homogenated using an Ultraturrax (3 min, Jahnke and Kunkel, IKA-Labortechnik, Staufen im Breisgau, Germany), and ultrasonificated for another 10 minutes. The suspension obtained was centrifuged (10 min, 8000 rpm) and, finally, filtered (Schleicher & Schuell filter). The pH of the filtrate was adjusted to 10 with aqueous sodium hydroxide (1 M) and a solution of benzoyl chloride dissolved in acetonitrile (30 mL; 1 g/250 mL ACN) was added. After stirring for 2 h at room temperature pH was adjusted to pH 2-3 using HCl (conc.). The benzamides were then extracted with dichloromethane (3 x 20 mL), and the organic phases were combined, dried over Na2SO4, and evaporated to dryness at 30 °C. The residue was dissolved in a mixture of acetonitrile and 0.1 % aqueous formic acid (20/80, v/v) and filtered over a syringe filter (0.45 μm; Spartan 13/0.45 RC; Schleicher and Schuell, Dassel, Germany). The final filtrate was diluted with water (1/20, v/v). An aliquot (10 μL) of the prepared sample was injected into the HPLC-MS/MS system.

High Performance Liquid Chromatography-Triple Quadrupole Mass Spectrometry (HPLC-MS/MS). HPLC-MS/MS analysis was performed on a Surveyor high-performance chromatography system (Thermo Finnigan, Dreieich, Germany), equipped with a thermostated autosampler and a triple-quadrupol tandem mass spectrometer TQS Quantum Discovery (Thermo Electron, Dreieich, Germany). Temperature of the column compartment was set at 30 °C and autosampler temperature was 24 °C. After sample injection (10 μL), chromatography was carried out on a Synergy Fusion RP 80 Å column (150 x 2.0 mm id, 4 μm, Phenomenex, Aschaffenburg, Germany) using the following solvent gradient (0.2 mL/min) of acetonitrile (0.1 % formic acid) as solvent A and formic acid (0.1 % in water) as solvent B: 0 min, 0 % A; 1 min, 0 % A; 1.5 min, 35 % A; 20 min, 40 % A; 26 min, 50 % A; 27 min, 90 % A; 36 min, 90 % A; 37 min, 0 % A.; 52 min, 0 % A. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode, the spray needle voltage was set at 3.5 kV, the spray current at 5 μA, the temperature of the capillary was 300,

the capillary voltage at 35 V. Nitrogen served as sheath and auxiliary gas, which was adjusted to 40 and 10 arbitary units. The collision cell was operated at a collision gas (argon) pressure of 0.13 Pa. Mass transitions of the pseudo molecular ions ([M+H]+) into specific product ions are summarized in Mayr and Schieberle (Mayr and Schieberle, 2012). Calibration curves for the calculation of the response factors and linear ranges of the analytes were measured as described before by Mayr and Schieberle (Mayr and Schieberle, 2012).

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Author Contributions

AB and IGK wrote the manuscript with help of AF (immunohistochemistry), KPS (calcium imaging), and CD (food analysis). AB and IGK designed the experiments. AB conducted all single-fly t-maze experiments. JC conducted the MBON experiments at testing with Shibire^{ts1} in multiple fly approaches. MHL conducted the dTrpA1 at testing experiments in multiple fly approaches. AB trained students for validation of experiments. AF and AB did the immunohistochemistry of lines. AF contributed the brain images. SKP did the Calcium imaging experiments. CD and TH provided the analysis of polyamine concentration in our fly food. All authors approved to the final version and its publishing.

Declaration of Interests

The authors declare no competing interests.

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Figures, Tables and Schemes

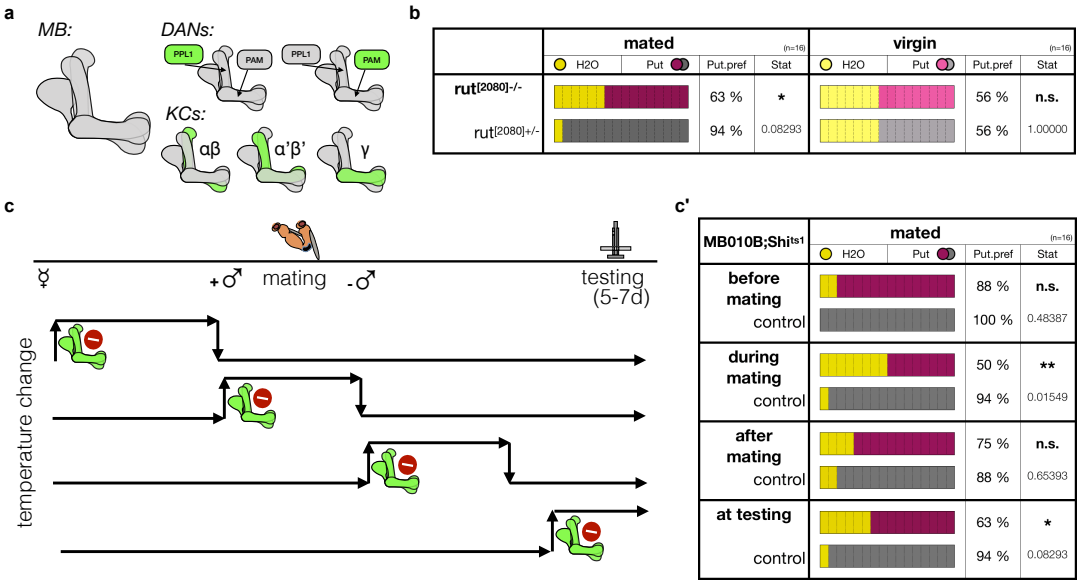

Figure 1: (**a**) left: scheme of a mushroom body (MB) from the left hemisphere; right top: dopaminergic neurons (DANs) synapse onto the MB from two clusters, PAM and PPL. While PAM innervates neurons in the horizontal lobe, PPL1 innervates the horizontal neurons; right bottom: Kenyon Cells (KCs) which form the intrinsic neurons and thus the lobular structure of the MB. (**b**) Mated females, mutant for adenylyl cyclase (*rutabaga*) show virgin-like behavior for polyamines. (**c**) Using the temperature-sensitive blocking system, Shibire^{ts1} (Shi^{ts1}), we are able to block all KCs at different times. (1) 24 h before mating, (2) 24 h during mating, (3) 24 h after mating, and (4) only during the test. The results (**c'**) show that KCs are required for the polyamine preference behavior at mating and during testing.

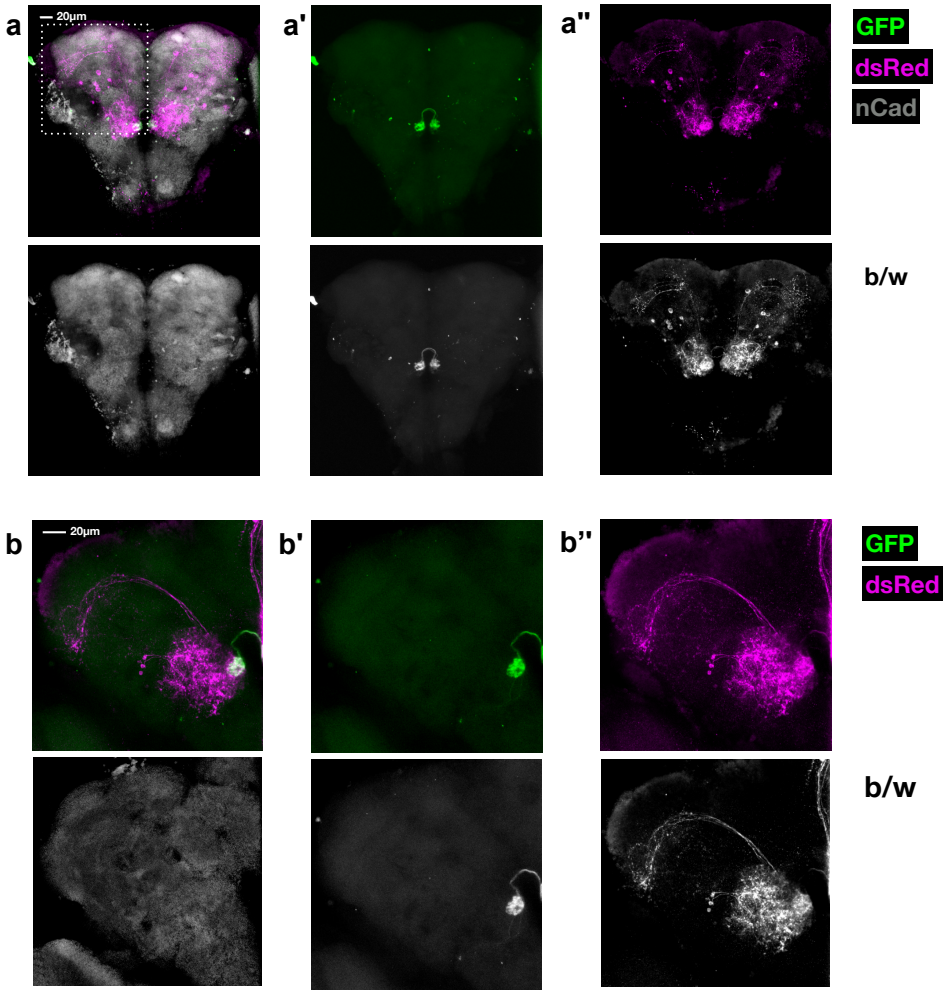

Figure 2: (**a; top**) IR41a (green, **a'**) and its postsynaptic projection neurons (pink, **a"**), labelled via the *trans*Tango system. (**a; bottom**) background staining. (**b**) sub-stack of the indicated dotted region in (**a; top**). The projection neurons innervate the MB and the LH.

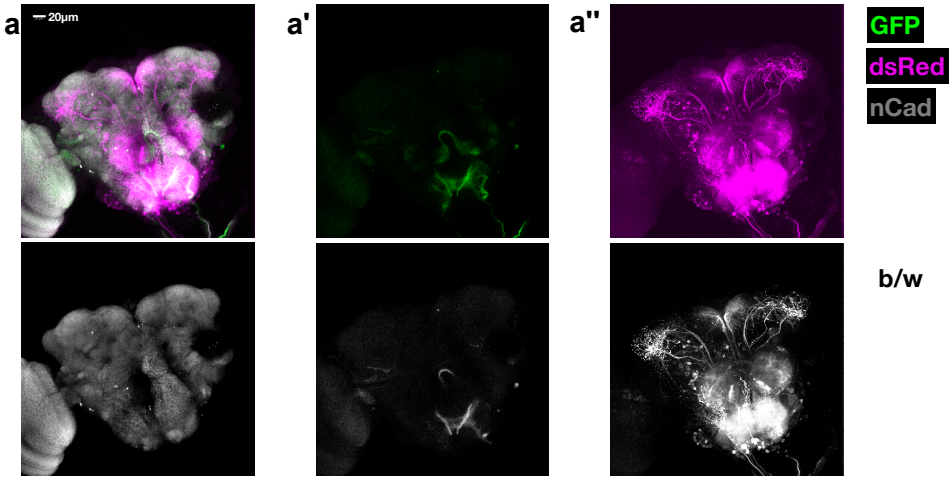

Figure 3: (**a; top**) IR76b (green, **a'**) and its postsynaptic projection neurons (pink, **a"**), labelled via the *trans*Tango system. (**a; bottom**) background staining. While a lot of innervations happen in the antennal lobe and the subesophageal zone, the projection neurons innervate the MB and the LH.

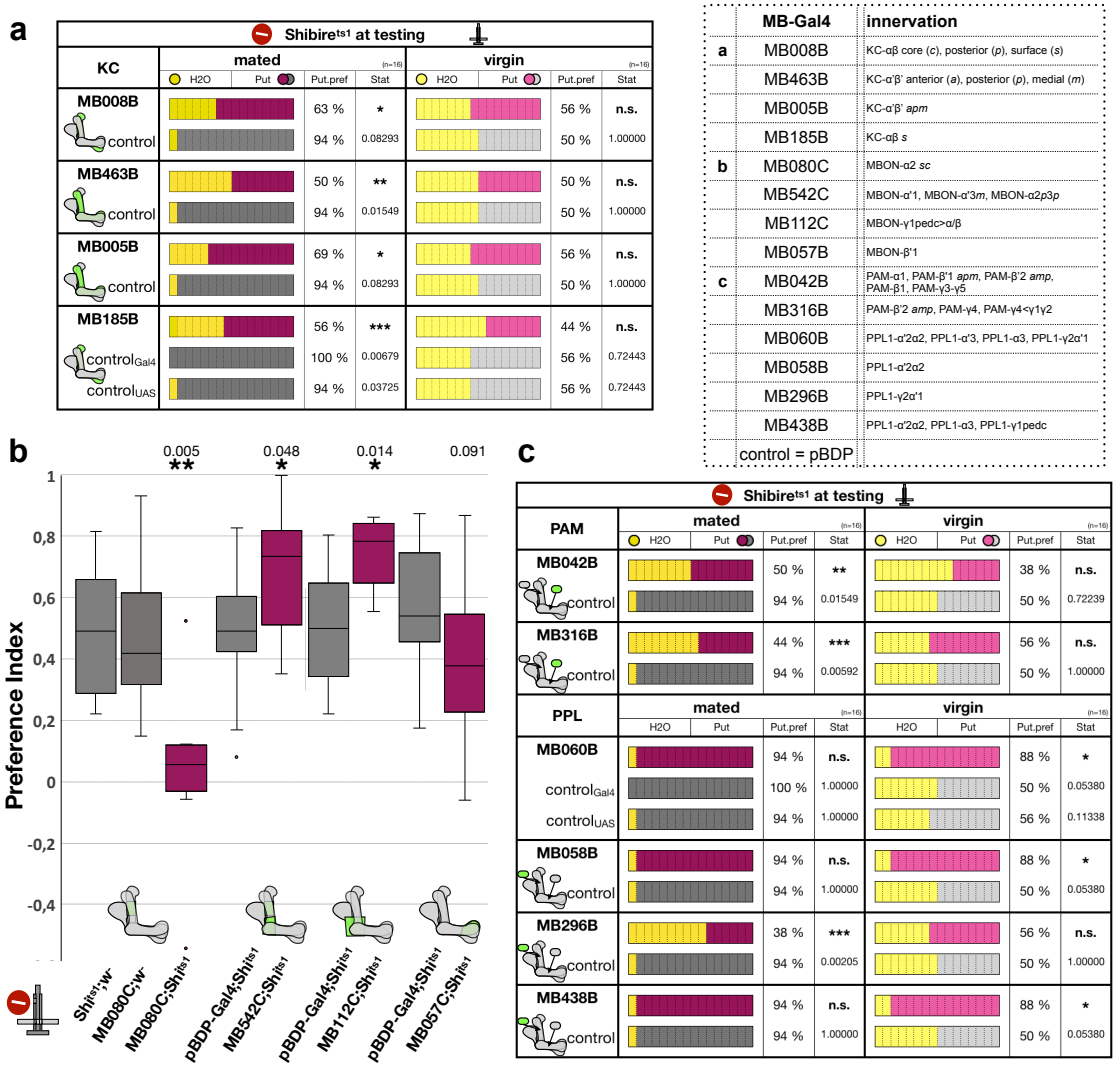

Figure 4: (**a**) Blocking KCs during testing in broad $\alpha'\beta'$, as well as $\alpha\beta$ and in particular αs led to a decrease in attraction behavior in mated flies. Blocking these KCs had no effect in virgins. (**b**) Blocking MBONs during testing only showed effects in cholinergic MBONs-α2sc (MB080C, decreased attraction), $\alpha'1\alpha'3\alpha2p\alpha3p$ (MB542C, increased attraction) and GABAergic MBONs-$\gamma1_p edc > \alpha\beta$ (MB112C, increased attraction). MBON-$\beta'1$ (MB057B) did not reach significance. (**c**) Blocking DANs during testing had effects on mated flies in PAM, but in virgin flies in PPL1. Only in one case in PPL1 DANs (PPL1-MB296B) a decrease in attraction behavior in mated flies was observed.

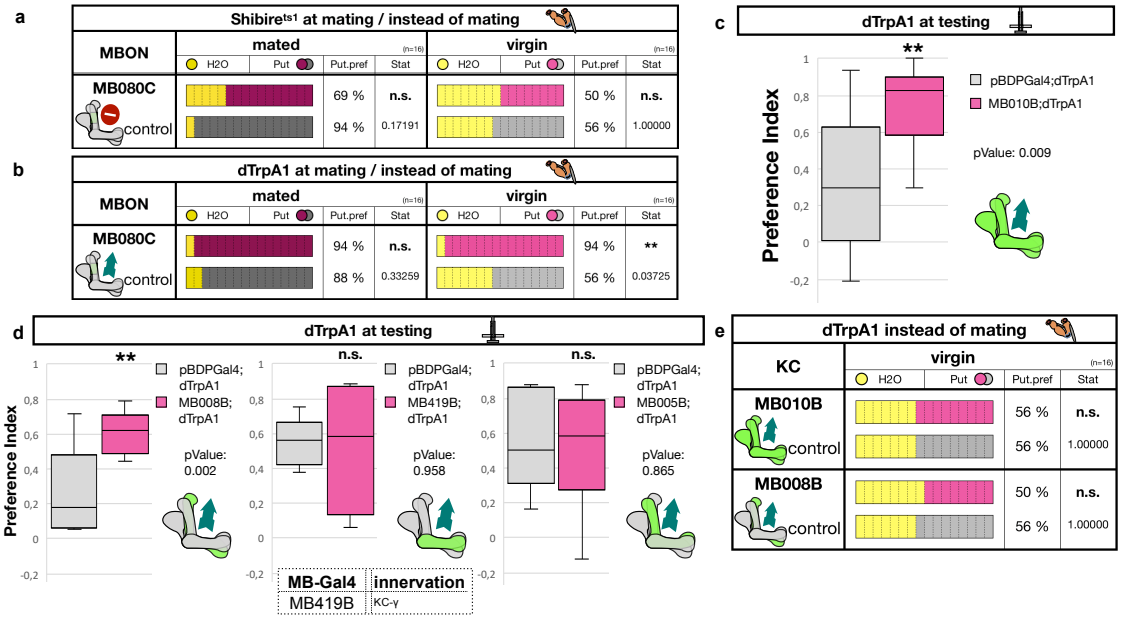

Figure 5: (**a**) Blocking MBON MB080C during mating was not sufficient to reduce attraction to polyamines. (**b**) Though activating MBON MB080C in virgins instead of mating increased polyamine attraction behavior in long term. (**c**) Activating KC output in all KCs (MB010B) at testing was sufficient to induce attraction behavior towards polyamines in virgins. (**d**) In particular this had an effect in $\alpha\beta$-KCs in virgins during testing. (**e**) The activation of these KCs instead of mating had no effect on behavior.

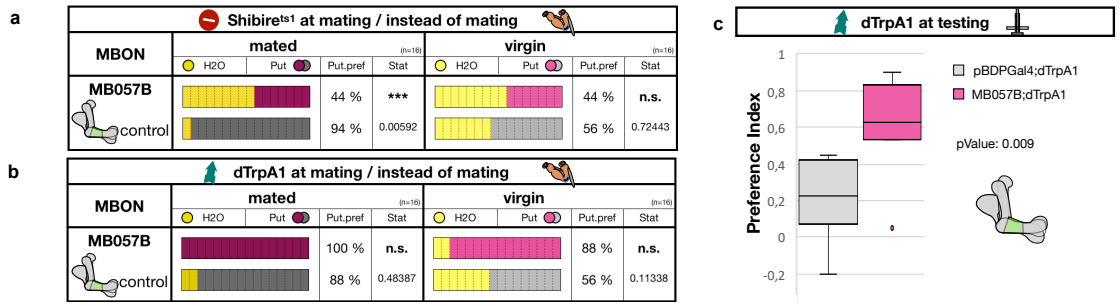

Figure 6: (**a**) Blocking MBON MB057B during mating decreased attraction to polyamines in mated flies. (**b**) Activating MBON MB057B instead of mating increased attraction to polyamines in virgin flies, though not significantly. (**c**) Activating MBON MB057B during testing is sufficient to increase attraction to polyamines in virgin flies.

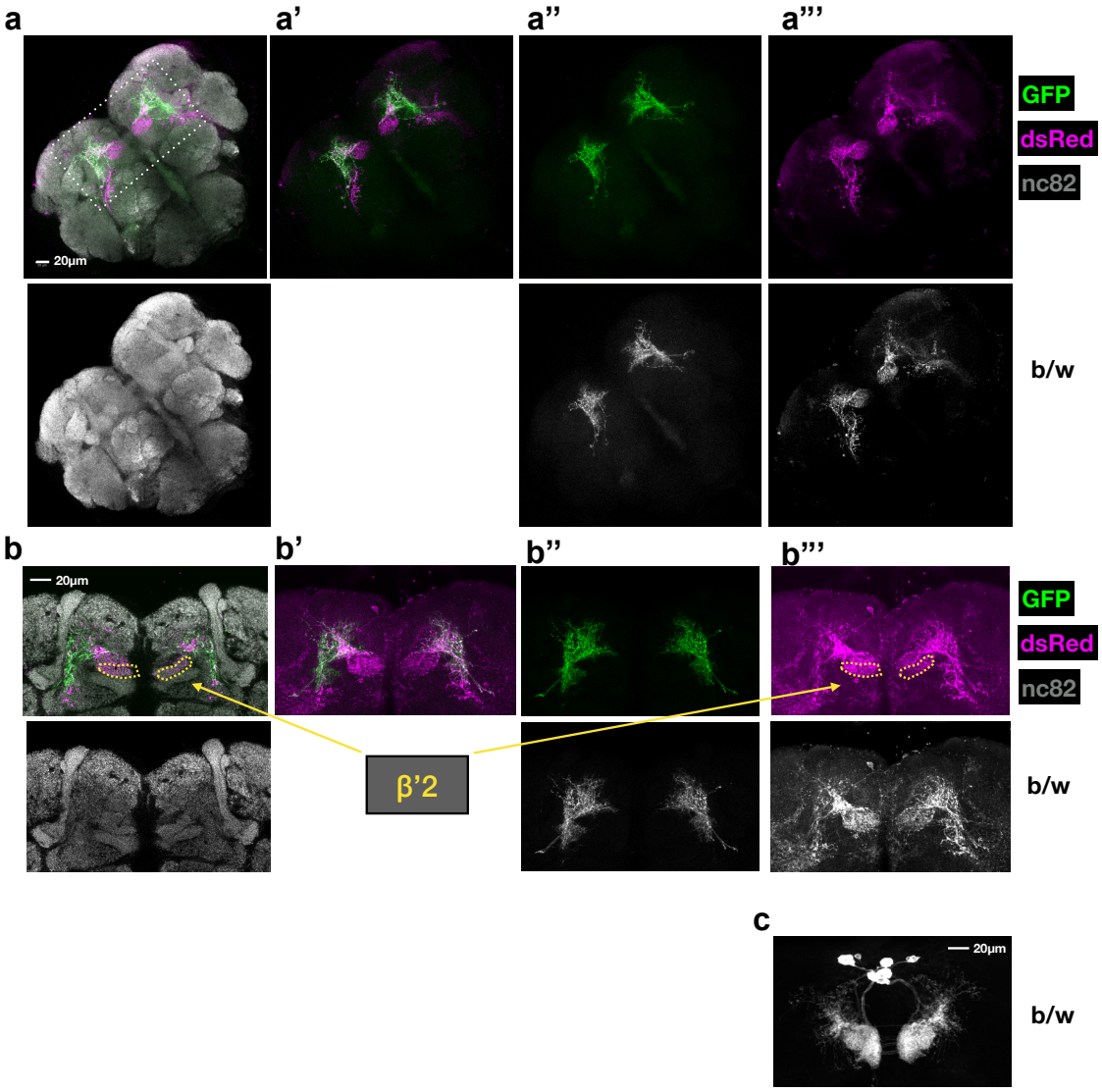

Figure 7: (**a**) MBON-$\beta'1$ (green, **a"**) and its postsynaptic innervation (pink, **a"'**), labelled via the *trans*Tango system. (**a; bottom**) background staining with nc82. (**b**) A sub-stack of the indicated dotted region in (**a; top**) indicates a possible dendritic connection to $\beta'2$-MBONs (yellow). (**c**) Image of MBON-$\beta'2$, similar to (Lewis et al., 2015).

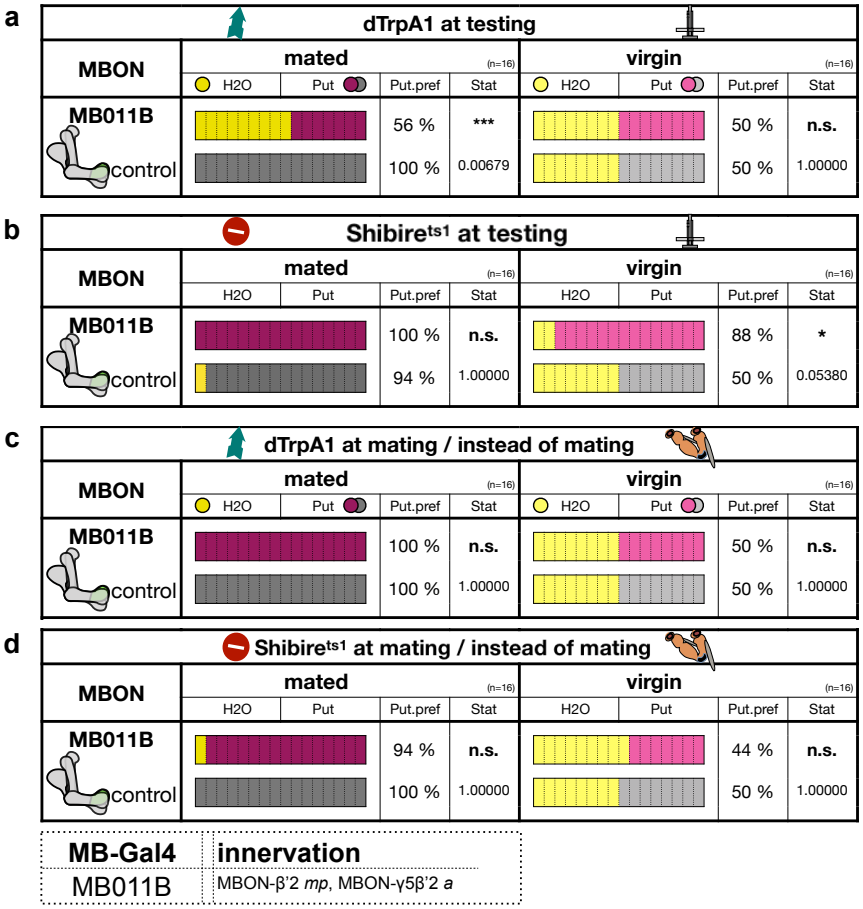

Figure 8: (**a**) Activation of β'2-MBONs (MB011B) at testing had a significant effect on polyamine preference in mated female flies. (**b**) Blocking of β'2-MBONs (MB011B) at testing had a significant effect on polyamine preference in virgin flies. (**c**) Activation of β'2-MBONs (MB011B) during and instead of mating had no effect on polyamine preference. (**d**) Blocking of β'2-MBONs (MB011B) during and instead of mating had no effect on polyamine preference.

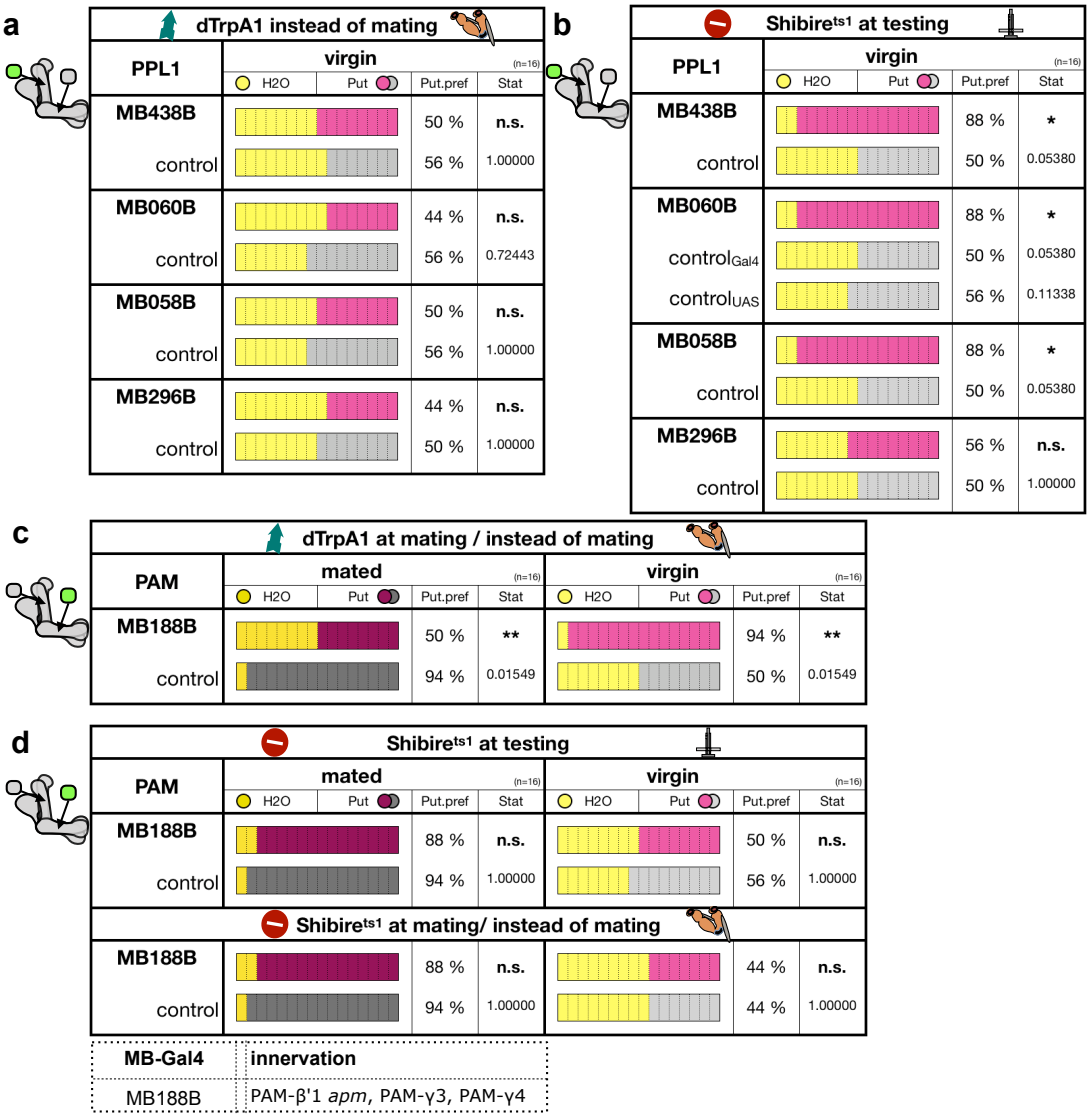

Figure 9: (**a**) Activation of PPL1-DANs instead of mating had no effect on polyamine preference in virgins. (**b**) Blocking of PPL1-DANs, particularly in regions innervating $\alpha 2$, during the test had a significant effect on polyamine preference in virgins. (**c**) Activation of PAM-DAN MB188B during and instead of mating had an effect on polyamine preference in both mated and virgin flies. In mated flies attraction was reduced and in virgin flies attraction was increased. (**d**) Blocking PAM-DAN MB188B at test or during and instead of mating had no effect on behavior.

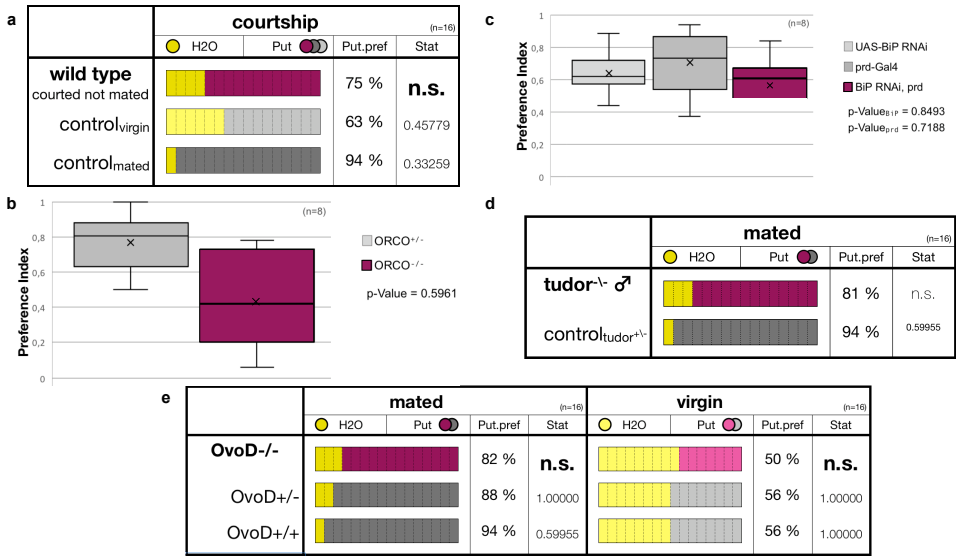

Figure 10: The analysis of different triggers for transition from virgin to mated preference behavior: (**a**) courtship; wild type flies were courted but not mated (so practically still virgins); there is no significant difference to mated flies, indicating that courtship already has an effect on polyamine preference. (**b**) As pheromone detection needs ORCO, we tested a mutant version and found a trend, but no significant difference to mated flies. (**c**) Females were mated with males incapable of producing seminal fluid proteins through ER stress in the accesory gland (*prd-GAL4;UAS-BiP-RNAi*); there is no significant difference in polyamine perception. (**d**) Females were mated with sperm-deficient males (*tudor* mutant); there is no significant difference in polyamine perception. (**e**) Females, unable to produce eggs (*ovoD* mutant) were mated with wild type males; there is no significant difference in polyamine perception.

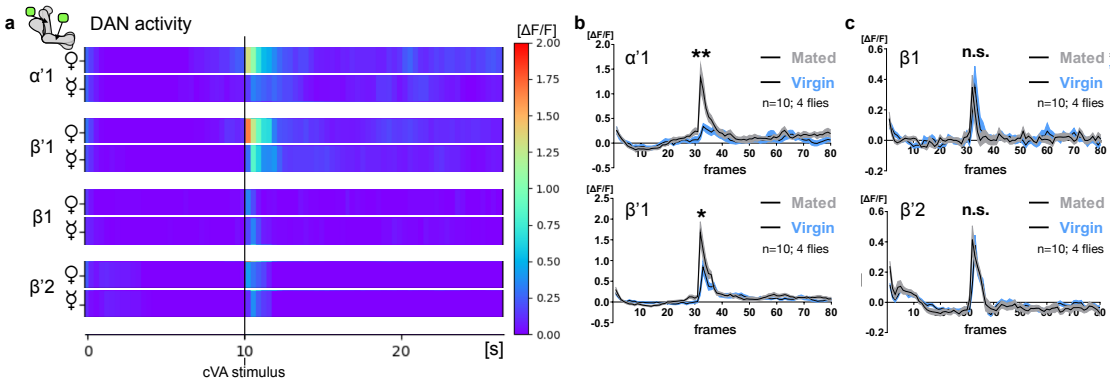

Figure 11: (**a**) DAN activity in certain regions of the MB ($\alpha'1$, $\beta'1$, $\beta1$, and $\beta'2$) upon stimulation of cVA after 10 seconds. (**b**) Statistical validation of significant differences in DAN activity upon cVA stimulation in virgin (blue) or mated (gray) female flies in MB-$\alpha'1$ and -$\beta'1$, respectively. (**c**) No significant difference was observed between virgins and mated in $\beta1$ and $\beta'2$.

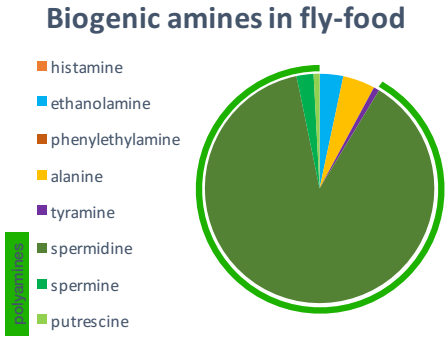

Figure 12: concentration of biogenic amines in μg/100 g fly food: histamine (5.49; orange), ethanolamin (676.65, blue), phenylethylamin (5.16, brown), β-alanine (930.84, yellow), tyramine (166.64, violet), spermidine (18120.77, dark green), spermine (492.81, green), and putrescine (172.50, light green). The total amount of polyamines (green) in standard fly food is $\sim$20 mg in 100 g food.

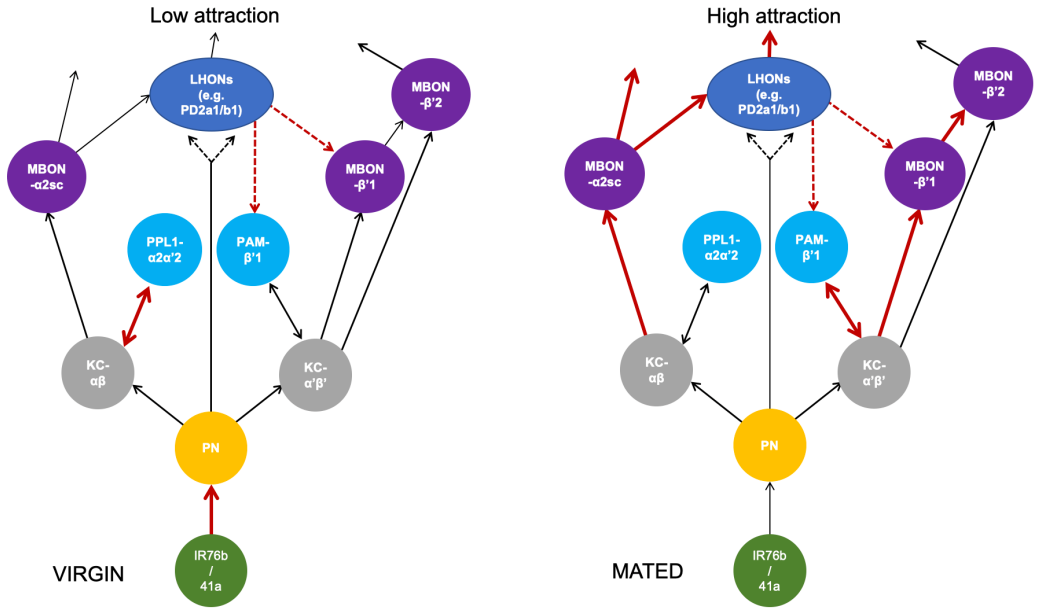

Figure 13: Neuronal network underpinning reproductive state dependent polyamine preference behavior. From OSNs (IR41 and IR76b) transsynaptic labelling revealed connections to the LH and the MB. In virgins (left) dopaminergic neurons innervating the $\alpha 2$ area (PPL1) are necessary to maintain a low attraction to polyamines. Likely through triggers during the mating experience such as cVA, $\beta'1$-DANs (PAM) have an effect on subsequent $\beta'1$-MBONs (right). At the same time, output from MBON-α2sc may advance this neuronal network even further, possibly through LHONs such as PD2a1/b1. This leads ultimately to an increased attraction to polyamines and conceivably other nutrients important for the gravid female.

Manuscript 3:
"Nutrition of the future - decoding polyamine receptors with nutri-informatics"

Authors: **Ariane C. Boehm**, Despoina Zapolgou, Dietrich Mostert, and Ilona C. Grunwald Kadow

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Author Contributions: conceptualization, **AB** and DZ; methodology, **AB** and DM; software, **AB** and DM; writing-original draft preparation, **AB** and DZ; writing-review and editing, **AB**, DZ, DM and IGK; visualization, **AB** and DM; supervision, **AB** and IGK; project administration, IGK; funding acquisition, IGK; All authors approved to the final version and its publishing.

Nutrition of the future - decoding polyamine receptors with nutri-informatics

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Abstract

Nutrition and diet are key modifiable lifestyle factors that have significant impact on growth, development, function, repair, and thus overall health maintenance and disease prevention. Over the past decades, the positive effects of polyamines have been intensively studied in a broad range of species. Even though these metabolites are endogenously produced, it is often necessary to include them in the diet. For instance, the beneficial effects of a polyamine-rich diet range from cognitive function over longevity to reproductive success. The chemosensory detection of polyamines may therefore be essential. Over the past two decades bioinformatics tools, such as the "-omics" fields (i.e. genomics, transcriptomics, proteomics, metabolomics) as well as modeling, have improved and contributed to more than just proof-of-concept theories. Could this be expanded and computational models be used to unravel nutrition-based questions, as in nutri-informatics? For instance, in model organisms, such as *Drosophila melanogaster*, polyamine detection starts at the receptor level which is still not completely understood. Could we indeed predict molecular scaffoldings for polyamine receptors? How much information can we use and does it show us how we can progress from there? In this work, we want to show that nutri-informatics can not only work, but it may also solve issues regarding how we perceive nutrition driven questions in the future.

Keywords: nutri-informatics; metabolism; iGluR; polyamines; olfactory receptor; *Drosophila melanogaster*

1 Introduction

1.1 Importance of Polyamines in Metabolism

The family of polyamines comprises naturally occurring polycations ubiquitous in all living cells with major representatives in eukaryotes the tri-amine spermidine, the tetra-amine spermine, and the di-amine putrescine that serves as their precursor (Igarashi and Kashiwagi, 2010). Due to their cationic nature at physiological pH polyamines interact directly with negatively charged molecules including DNA, RNA, proteins (such as kinases, phosphatases, and enzymes participating in histone methylation and acetylation), and ion channels (such as Kir and TRPC channels, and iGluRs) exerting their modulatory effects (Igarashi and Kashiwagi, 2010; Guerra et al., 2016; Pegg, 2016; Igarashi and Kashiwagi, 2019). Polyamines thus participate in a wide array of critical functions including gene expression, cellular growth and proliferation, apoptosis, autophagy, and stress response (Igarashi and Kashiwagi, 2010; Miller-Fleming et al., 2015; Wallace et al., 2003). They are present in high concentrations in rapidly growing and regenerating tissues and are essential for reproductive success, early embryonic and fetal development, as well as intestinal mucosal growth and regeneration (Lefèvre et al., 2011; Kalač, 2014; Pegg, 2016; Hussain et al., 2017; Igarashi and Kashiwagi, 2019). Polyamines are also involved in pathogen-host interactions, innate and acquired immunity, stress resistance, and the modulation of synaptic activity and neuronal excitation, among others (Kalač, 2014; Limon et al., 2016). The main sources of polyamines are dietary intake, *de novo* synthesis, and intestinal absorption of the small amount present in the metabolic products of the gut microbiota (Kalač, 2014). As their composition and concentrations vary among different organs and tissues and their role is multifaceted, tight homeostatic control of their intracellular levels is crucial for the maintenance of their physiological function and it is achieved through coordinated biosynthesis, catabolism, and transport (Igarashi and Kashiwagi, 2010).

Disruption of this homeostasis has been associated with disease in model organisms and humans. Extensive research has identified ornithine decarboxylase (ODC), the key enzyme in the biosynthesis of putrescine, as a target of the oncogene MYC and established the correlation between elevated levels of polyamines and various types of cancer. The concentration of spermine oxidase (SMO), that mediates the direct conversion of spermine back to spermidine, has also been found to be high in cancer (Miller-Fleming et al., 2015). Polyamines and their metabolic pathways have thus been the target of chemotherapeutic agents such as enzyme inhibitors and polyamine analogues(Nowotarski et al., 2013). Additionally, loss-of-function mutations in the gene encoding spermine synthase (SPMS), that transfers aminopropyl groups from decarboxylated S-adenosylmethionine (dcAdoMet) to spermidine for the synthesis of spermine, result in dysregulated spermidine/spermine ratios and have been linked to the Snyder-Robinson syndrome, a rare X-linked disorder that leads to intellectual disability and a variety of other abnormalities (Pegg, 2016; Murray-Stewart et al., 2018). Interconversion and degradation of higher polyamines, particularly spermine, generates reactive oxidative products such as hydrogen peroxide and acrolein that can cause toxicity and cell death; indeed, increased polyamine

catabolism has been implicated in the development of numerous pathologies including neurological diseases and stroke (Pegg, 2013; Wallace et al., 2003; Igarashi and Kashiwagi, 2019). Dysregulated concentrations of polyamines have also been associated with neurodegenerative diseases, mood disorders, major depressive disorder, suicidal behavior (Minois et al., 2012; Kalač, 2014; Miller-Fleming et al., 2015; Limon et al., 2016), and they have been found to increase with inflammation and decline with age (Minois et al., 2012). Recent work in patients with systemic lupus erythematosus has shown that spermidine, its acetylated derivative N1-acetylspermidine, and spermine are significantly lower than those of healthy controls (Kim et al., 2018). Studies have highlighted the significance of arginine and methionine, both precursors of polyamines, in embryogenesis, placental growth, and angiogenesis (Böhles et al., 1998; Rees et al., 2006; Mandal et al., 2013; Ramani et al., 2014; Bjørke-Jenssen et al., 2017). Apart from enhancing their *de novo* synthesis by increasing protein intake, polyamine levels can be directly elevated through the consumption of foods rich in these metabolites e.g. aged cheese, fruits, vegetables, cereal, and nuts. An increasing body of knowledge underlines the need for quantification of the polyamine content of foods for the development of databases to ultimately enable reliable dietary recommendations (Atiya Ali et al., 2011; Kalač, 2014; Handa et al., 2018). Pregnancy is a special period of elevated nutrient and energy demands for the female body as it adjusts to meet the increasing needs of the developing embryo and pregnant women characteristically report an altered preference towards certain foods. Maternal nutritional and metabolic status are the primary environmental factors influencing the expression of the fetal genome (fetal programming) (Wu et al., 2004; Kwon and Kim, 2017). Nutrient reference values, such as the Dietary Reference Values (DRVs) set for Europe by the European Food Safety Authority (EFSA), enable health professionals to make the necessary dietary recommendations to expecting mothers (European Food Safety Authority, 2019) and among those, sufficient protein intake is of primary importance. Indeed, polyamines participate in a multitude of critical functions including reproductive success and embryogenesis and are thus essential during pregnancy (Rees et al., 2006; Lefèvre et al., 2011; Bjørke-Jenssen et al., 2017).

1.2 Polyamine-sensing Receptors in Drosophila melanogaster

The fruit fly, *Drosophila melanogaster*, is a well established model organism to address scientific questions, as proven by the numerous Nobel prizes awarded to *Drosophila* researchers (Morgan, 1933; Muller, 1946; Lewis et al., 1995; Axel and Buck, 2004; Beutler et al., 2011; Hall et al., 2017). Approximately 75 % of human disease-causing genes have been identified in *Drosophila* allowing researchers to investigate even in the fields of psychiatric or neurodegenerative disorders, as well as metabolic processes (Reiter et al., 2001; Ueoka et al., 2018). It also constitutes a model well suited to the study of polyamines in varying contexts. The concentrations of polyamines are dynamic throughout the flys development, increasing during organogenesis and displaying tissue-specific accumulation (Burnette and Zartman, 2015). They have also been found to interact with the MAPK signaling pathway to modulate cellular response (Stark et al., 2011). Dietary supplementation of spermidine significantly increases its lifespan (Eisenberg et al., 2009), promotes stress resistance (Minois et al., 2012),

increases reproductive success (Lefèvre et al., 2011), and prevents age-related olfactory memory decline as a result of enhanced autophagy (Gupta et al., 2013). Recent work has further shown that female *Drosophila* alter their preference behavior towards polyamines depending on their reproductive state (Hussain et al., 2016a,b): gravid females display an enhanced detection of and attraction towards these molecules compared to virgin flies. Polyamines exert positive effects on the gravid female and its offspring in *Drosophila* (Ramani et al., 2014; Hussain et al., 2016a,b), which is why it is understandable that a female would search for this molecule. But how do polyamines activate sensory neurons in this reproductive state dependent manner?

In this work, we would like to investigate this question whose answer remains elusive. The initial olfactory and gustatory perception of a nutrient or molecule depends on receptors on chemosensory neurons. There are three major classes: gustatory receptors (GRs), olfactory receptors (ORs) and the more recently discovered ancient class of ionotropic receptors (IRs) (Croset et al., 2010; Benton et al., 2009). Contrary to their mammalian counterparts, olfactory receptors do not act as classical G-protein coupled receptors, but rather as ion channels (Benton et al., 2009). Hussain et al. (Hussain et al., 2016a,b) have shown, that polyamine perception on the olfactory level is not dependent on ORs, but particularly two IRs: IR41a and IR76b. Interestingly, the perception on the gustatory level is also dependent on IR76b, while a possible co-factor is still unknown. They particularly unraveled that while IR41a binds polyamines specifically, the function of IR76b appears more co-receptor-like, possibly through its expression in many different olfactory and gustatory receptor neurons (Benton et al., 2009; Hussain et al., 2016a,b), which may hint for more diverse function of this neuron.

However, while we now know behaviorally which neurons are involved in the process, we still lack the understanding of the molecular scaffolding of these receptors. In the past ten years computational methods have improved our understanding and provided paths towards solving these questions. Modern methods employ the "-omics" fields (e.g. transcriptomics, proteomics, metabolomics) and other bioinformatic tool sets (e.g. big data analysis, modeling); we would like to give a perspective towards how to address such a question in a "nutri-informatics" way.

2 Computational analyses of olfactory receptors in *Drosophila melanogaster*

Olfactory receptors in *Drosophila* have long been a target for computational approaches. Among the first papers that applied such methods, three focused on the putative receptor genes and their corresponding transmembrane region (Vosshall et al., 1999; Gao and Chess, 1999; Clyne et al., 1999). Through extensive bio-computational methods via amino acid evolutionary co-variation models and the analysis of locations for functionally important residues, it was possible to create three-dimensional models of ORs (Hopf et al., 2015). They have shown the hepta-helical structure of these channels. Evolutionary analyses via codon-based substitution models (Jeffares et al., 2015) could even reveal the functional flexibility of the OR co-receptor (Soffan et al., 2018). These predictions were quite accurate

as they can now be compared to the latest cryo-electron microscopy study of OR co-receptors (Butterwick et al., 2018).

Bioinformatics methods started to analyze IRs in 2009 (Benton et al., 2009) and showed their phylogenetic connection to the ionotropic glutamate receptors (iGluRs). Such iGluRs include AMPA, NMDA and kainate. This has been expanded to a more detailed phylogenetic survey of iGluRs and IR genes in different insect and drosophilic species (Croset et al., 2010; Liu et al., 2018). Nowadays, homology modeling and amino acid substitution analyses cannot only provide putative three-dimensional models of IR sub-structures (Hussain et al., 2016b), but also reveal specificity determinants for the sensitivity of neurons, such as olfactory sensory neurons (Prieto-Godino et al., 2017).

Combining these sources of knowledge, we should be able to make more detailed predictions regarding the molecular scaffolding for polyamine perception. The polyamine receptors are IR41 and IR76b (Hussain et al., 2016b). These IRs are closely connected in the phylogenetic tree and their next non-IR relative is NMDAR1 (Benton et al., 2009). Polyamines have effects on NMDA Receptors (Masuko et al., 1999; Igarashi and Kashiwagi, 2010; McGurk et al., 1990; Kashiwagi et al., 1996). Hussain et al. have shown that a structural similarity of IRs and NMDA receptors (NMDARs) indeed exists (Hussain et al., 2016b) and claimed that this can "provide hints for how polyamines could activate IRs". We used the protocol Hussain et al. (Hussain et al., 2016b) used in their first article on the putative structures of the IRs to take a closer look at their hypothesis.

3 Comparative Results based on previous work

Protein Sequence Acquisition

Using the protein data bank (PDB) (Berman et al., 2000) and previously published papers including the protein sequence (Masuko et al., 1999; Moriyoshi et al., 1991), the sequence for the NMDA receptor in rats sub-unit R1 has been used for further analysis, as done by Hussain et al. (Hussain et al., 2016b). PDB also provided the protein sequence for the olfactory sensory neuron IR76b and IR41a in *Drosophila*.

3.1 Protein Structure Prediction and Comparison

The protein structure was predicted via RaptorX (Källberg et al., 2012). We visualized the proteins in PyMol (Schrödinger, LLC, 2015) to get a first look at the structures and their overlap (Figure 1 a-c). The major domain regions are the R- (regulatory), S- (agonist binding site) and M- (membrane) domains (Figure 1 d), as already indicated in (Masuko et al., 1999; Moriyoshi et al., 1991). These regions are corresponding to the following nomenclature in iGluRs: The R-region would most likely be comparable to the amino-terminal domain. The S-region corresponds to the ligand binding domain. The M-region is comparable to the trans-membrane domain (Croset et al., 2010; Bowie, 2018).

While there is good structural overlap with NMDAR1 in the M- and S-domain (Figure 1 e), the secondary structures of the R-domain are not fully formed. This structural ambiguity also makes it

difficult to predict an ultimate function for the R-domain. There are indications that the R-domain is responsible for the formation of the channel molecule, similar to the hepta-helical appearance of such receptors in ORs (Hopf et al., 2015). Furthermore, we suggest that IR76b functions as an ORCO for a variety of olfactory receptors. This is probably a reason for the unspecific structure of the R-domain. However, the structural similarities observed between IR41a and the NMDAR1 sub-unit , as well as the overlap in S- and M-domains, could give indications about their activation by polyamines or similar mechanisms. Similar claims regarding the organization of the S-domain towards iGluRs (Croset et al., 2010) have already been made. Different approaches could help our understanding of the mechanistic background of how and where polyamines activate these IRs. Such approaches include the analysis of conformational changes based on protein sequence, as well as finding functionally important residues via for instance a sequence based mutation analysis.

3.2 Normal Mode Analysis

A common tool for predicting conformational changes or protein dynamics is a normal-mode analysis (Hollup et al., 2005; Lindahl et al., 2006; Tiwari et al., 2014). Using the web tool NOMAD (Lindahl et al., 2006) we analyzed the protein dynamics of NMDAR1, IR41a, and IR76b. They suggest a conformational change in which the R- and S-domains bend or rotate towards each other (supplementary video). The M-domain stays rigid, which is reasonable with its role as membrane anchor. The movement between S- and R-domain may be an indication of polyamine binding: Masuko et al. (Masuko et al., 1999) have shown that substituting the acidic amino acids E181 and E185 in NMDAR of rats eliminates polyamine binding. They hypothesized a mechanism in which these acidic amino acids bind at least one amino-group of the polyamine, leading to a conformational change. Could the binding of polyamines in IRs indeed follow the same mode of action?

3.3 Binding Pockets and Electrostatic Analysis

To address this question, it would be interesting to know about the electrostatic distribution of the molecule. A surface plot indicating contact potentials provides such a simplistic analysis e.g. via PyMol (Schrödinger, LLC, 2015). As polyamines are positively charged, they are likely to mask negatively charged binding pockets. In NMDAR1, two glutamate residues (E181 and E185) form a negatively charged binding pocket between the R and S domain (Figure 2 a). This is comparable to the identified positions of amino acid residues influencing polyamine sensitivity (Masuko et al., 1999) in NMDAR1. Such could lead to a conformational change similar to that seen in the normal mode analysis, even though this statement is quite speculative. A protein-protein interaction analysis for receptor-ligand docking (Zhang et al., 2017), as used in drug-target mapping, could confirm such binding sites. For IR41a potential binding pockets by the electrostatic distribution can be indicated (Figure 2 b). While the potential binding pocket towards the R-subunit is located to a position similar to that of NMDAR1, another potential binding pocket can be possible; according to another paper (Kashiwagi et al., 1996) the area of NMDAR1 in the residue regions 669-700 can display

sensitivity changes for polyamines. This is located between the S-subunit compartments, an area which forms a negatively charged spot for potential polyamine binding in IR41a (Figure 2 b, lower arrow). Regardless, to compare polyamine-binding of the NMDA receptor to that of IR41a and IR76b, the acidic amino acids of these receptors and their functional effects on these sites need to be elucidated.

3.4 Sequence Based Mutation Analysis

It is known that even single amino acid changes in IRs are sufficient to change the sensitivity of certain odors (Prieto-Godino et al., 2017). But apart from checking for evolutionary similarities or differences, we can use neural networks which have learned to predict if there are functional effects in general, independent of whether the functional effect represents a molecular difference or may provide a disease-causing interpretation.

SNAP2 (Hecht et al., 2015) is a trained classifier which can predict functional effects of single amino acid substitutions using a scoring system. This functional effect is likely to be of molecular basis, rather than displaying rare variants and can reach an accuracy of 88 % given a certain score of at least 75 (Mahlich et al., 2017). If we use SNAP2 (score larger 75) on the IRs, we can identify several amino acids as functionally important. It is possible to investigate these residues depending on their location within the different subdomains:

A lot of the predicted functionally important amino acids are located in the M-domain (Figure 3 a). Trans-membrane regions are highly conserved as their chemical environment in the membrane is quite complex (Clyne et al., 1999; Kaupp, 2010). Hence, amino acid substitutions can have strong effects on the integrity of the trans-membrane domain.

More interestingly, cystein residues at the outer fringes of the R-subunit have been classified as functionally important (Figure 3 a-c). Given that these proteins (IR76b, IR41a, and NMDAR1) are just subunits of even more complex n-helical structures, which form the ion channel, it is plausible that these cysteine residues are responsible for disulfide-linkage between the protein-subunits. The constructed functionality may thus even represent the conformational change from one protein-subunit to the other or between one-and-another.

The S-domain exhibits many amino acids, classified as functionally important. Many olfactory receptors in Drosophila bind their ligands in the S-domain (Prieto-Godino et al., 2017). In IR41a, most of the acidic amino acids that have been identified as functionally important by SNAP2 are clustered in the S-domain (Figure 3 b).

Similarly to a paper published by the Benton lab (Benton et al., 2009), which also checked for potential candidate regions for ligand binding domains, we could also identify the S-region around the hypothesized amino acid residues from the electrostatic surface plot (Figure 2 b) and the analysis of functionally important amino acids (Figure 3 b). The area for potential sensitivity regarding polyamines (Kashiwagi et al., 1996) (Figure 2 b, lower arrow) also includes some classified functional amino acid residues.

Taken together, this data gives a strong indication that the activation of receptors through polyamines is indeed comparable to that of NMDA receptors, and may even allow interpretation for an early adaptation of iGluRs to this task, as previously postulated by Hussain et al (Hussain et al., 2016b). Furthermore, we were able to identify potential binding sites for polyamines and unravel some detailed information on these receptors using nothing but its genetic code.

4 Discussion

Let food be thy medicine, and medicine be thy food, the famous aphorism attributed to the father of modern medicine Hippocrates of Kos reflects that the impact of diet on disease development, progression, and treatment has been recognized since Antiquity (Kleisiaris et al., 2014). When addressing nutrition-related questions, we need to combine advancements in technology and improvements in methodological approaches.

All senses participate in food evaluation and selection: vision (e.g. color, shape, size, quantity, overall appearance, packaging), hearing (e.g. sound during mastication), taste (i.e. bitter, sweet, salty, sour, umami), touch (e.g. texture, temperature, mouthfeel), smell (including flavor perception and aroma). The overlapping sensory cues enhance nutrient absorption and metabolism following food ingestion by priming the gastrointestinal tract through cephalic phase responses (Smeets et al., 2010). Influencing eating behavior, food consumption, satiation, and ultimately nutrient intake, the sensory properties of foods constitute targets for the development of products with improved nutritional characteristics (McCrickerd and Forde, 2016; Stone, 2018). The chemical senses, smell and taste, are those evolved to evaluate a food source as safe and nutritious or potentially harmful. Gustation often acts as a sensor of macronutrients while olfaction has been shown to induce appetite for the specific food that elicits the sensory cue (Zoon et al., 2016; Boesveldt and de Graaf, 2017).

Chemosensation, however, is not only dependent on sensory cues; it is highly modifiable by metabolic processes, the internal state of an organism. Hunger, disease, age, or reproductive state can all affect the detection of these cues. In the animal kingdom, the chemical senses are also modified by the internal state of the organism. Nutritious fermenting fruit or other high quality food sources can thus be located via polyamine detection. In the fruit fly, internal state exerts modulatory effects in the processing and perception of odors (Sayin et al., 2018). Diet provides macronutrients (carbohydrates, lipids, proteins), micronutrients (vitamins and minerals), and water necessary for tissue synthesis, growth, maintenance, and repair. Bioactive food components, such as dietary fiber and polyphenols (such as quercetin, and resveratrol) although not essential for growth and development, can have beneficial effects when consumed in sufficient amounts as part of a balanced diet. In particular, the levels of polyamines have been shown to be of high importance. We have mentioned the importance of polyamines on the metabolism and the negative effects if this homeostasis is disrupted.

Drosophila females seem able to find the right balance for the exogenous search for polyamines regarding their reproductive state. Gravid females display higher preference towards polyamines

compared to virgin flies (Hussain et al., 2016a,b), most probably due to their positive effects on parent and offspring. Although there is still no complete answer to the question of how polyamines activate olfactory sensory neurons, the use of computational "*in silico*" methods can give us more insights for upcoming behavioral and computational experiments. Taking into account all data gathered on the topic, species-specific information, information from evolutionary comparative studies or data sets from behavioral studies, it could reduce the number of model organisms used.

So, when we asked if bioinformatic tools could be expanded and used to unravel nutrition-based questions, as in nutri-informatics, we would say yes, it is possible. We just showed a great example for the molecular scaffolding for polyamine receptors. To reinforce this hypothesis, functional analyses of other olfactory receptors using SNAP2 should be undertaken. If they vary considerably from IR41a and do not show similarities in which polyamine-binding would be conceivable, the functional analysis of IR41a by SNAP2 would be strengthened. Long-term, simulated annealing experiments of polyamine with IR41a to predict binding pockets, combined with mutational analyses of the receptor can lead to the exact identification of the binding site of polyamines.

Nutri-informatics could be of significant value to nutritional research by predicting 3D structures, isoforms, subunit organization, and putative binding sites and mechanisms of receptors or transporters and enable comparisons across species using computational data from different fields. This would allow for functional analyses such as examination of putative interactions with a dietary compound of interest, whether a metabolite, a neutraceutical, or a non-nutrient food component. Furthermore, it would permit the prediction of compounds with structure similar to that examined which could act as its inhibitors, agonists, antagonists, or regulators; a more comprehensive approach and perhaps the development of synthetic analogues with the desired synergistic or competitive function would then be more feasible. Personalized medicine approaches and the interpretation of genetic backgrounds may not only be used to explain differences between disease-causing effects and molecular variation, but may also help advance research in clinical nutrition. Patients (whether hospitalized patients or outpatients) whose metabolic needs and perception of smell and taste are affected by their disease, its stage, and the medicines taken, the elderly, or people suffering from anosmia, parosmia and dysgeusia are at risk of malnutrition and identification of new targets for dietary interventions could further improve the therapeutic outcome. Dietary patterns along with specific nutrients have been the focus of intensive and extensive research to further elucidate their beneficial effects on the prevention and treatment of cancer, neuropsychiatric, and neurodegenerative diseases and ultimately lead to dietary recommendations and interventions (Bourre, 2006a,b; O'Neil et al., 2014; Huang et al., 2016; Vauzour et al., 2017; Mischley, 2017; Zwilling et al., 2018; Sánchez-Villegas et al., 2019). Here, we focused on polyamines as the metabolite of interest to exemplify the application of nutri-informatics; polyamines participate in a multitude of critical functions including reproductive success and embryogenesis and have been found to be required in pregnancy (Rees et al., 2006; Lefèvre et al., 2011; Bjørke-Jenssen et al., 2017). Dysregulated levels of these metabolites have been associated with several diseases including type 2 diabetes (Fernandez-Garcia et al., 2019) and cancer (Asai et al., 2018), and thus

polyamines have been under investigation for dietary interventions and nutritional approaches for disease management and prevention (Böhles et al., 1998; Estebe et al., 2017; Rondón et al., 2018), while their biosynthetic pathway has been a target for cancer therapy (Nowotarski et al., 2013; Casero, 2018; Casero et al., 2018).

A long history of scientific progress and technological advancements has enabled significant discoveries in the field of nutrition and studies are now highlighting the need for ever more rigorous, diverse, and interdisciplinary approaches (Mozaffarian et al., 2018), which may enable us to decipher a few more facets within the field and its impact on our daily dietary habits. Nutrition is not only essential to sustain life, but to improve the quality of life of the worlds population.

5 Figures, Tables and Schemes

Supplementary video: Normal Mode Analysis of polyamine receptors.

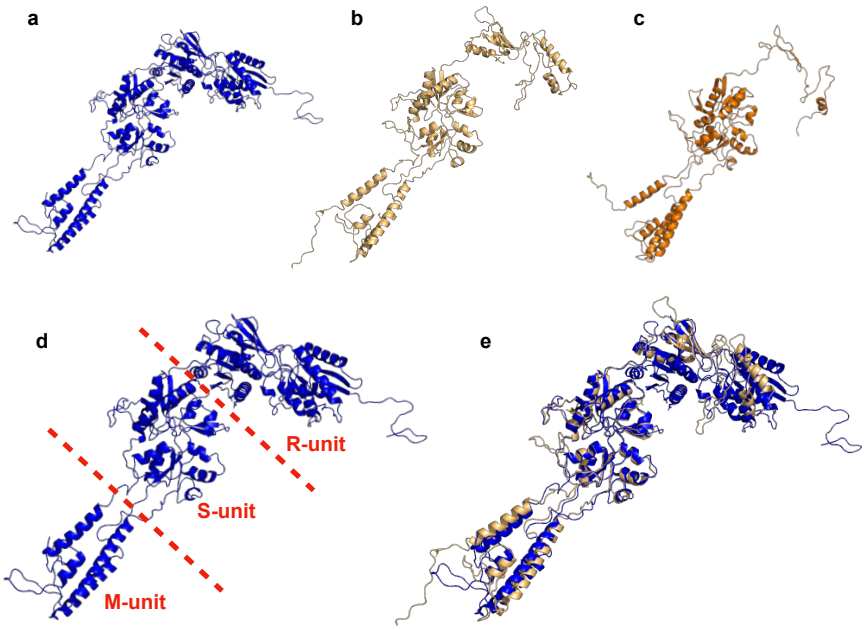

Figure 1: (**a**) putative structure of NMDAR1 (blue) (**b**) putative structure of IR41a (yellow) (**c**) putative structure of IR76b (orange) (**d**) nomenclature used for further description of the polyamine receptor protein (**e**) structural overlap of NMDAR1 (blue) and IR41a (yellow). Structures have been predicted by RaptorX (Källberg et al., 2012) and visualized by PyMol (Schrödinger, LLC, 2015).

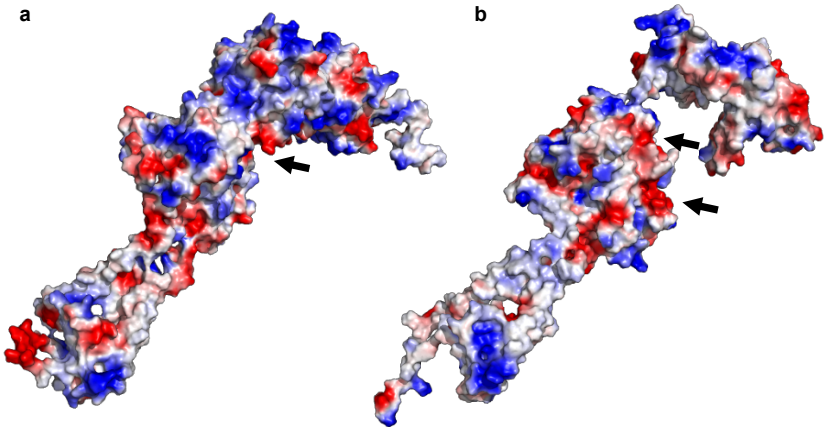

Figure 2: Electrostatical surface plots by PyMol (Schrödinger, LLC, 2015) of (**a**) NMDAR1 and (**b**) IR41a; potential binding pockets are indicated with an arrow. Negatively charged areas are colored in red, positively charged areas in blue.

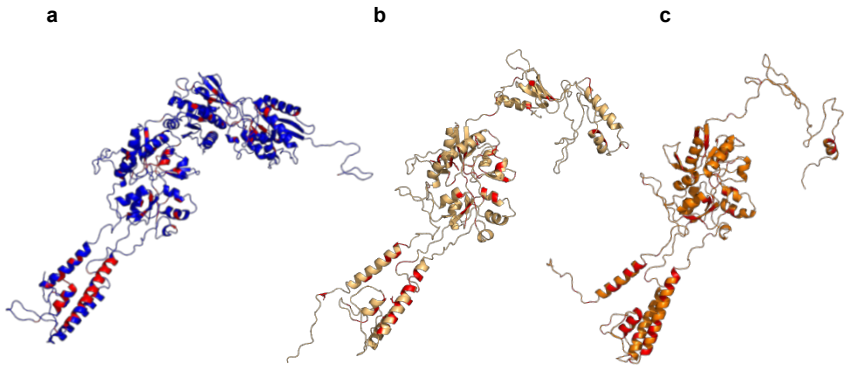

Figure 3: Functional important amino acid residues (red) as predicted by SNAP2 (Hecht et al., 2015) for (**a**) NMDAR1 (blue), (**b**) IR41a (yellow), and (**c**) IR76b (orange); with SNAP score larger or equal to 75.

6 Appendices

Author contributions

conceptualization, AB and DZ; methodology, AB and DM; software, AB and DM; writingoriginal draft preparation, AB and DZ; writingreview and editing, AB, DZ, DM and IGK; visualization, AB and DM; supervision, AB and IGK; project administration, IGK; funding acquisition, IGK; All authors approved to the final version and its publishing.

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Conflicts of interest

The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
dcAdoMet	decarboxylated S-adenosylmethionine
DNA	desoxyribonucleic acid
DRV	Dietary Reference Values
EFSA	European Food Safety Authority
GR	gustatory receptor
iGluR	ionotropic glutamate receptor
IR	ionotropic receptor
Kir	potassium inward rectifier
MAPK	mitogen-activated protein kinase
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
ODC	ornithine decarboxylase
OR	olfactory receptor
ORCO	olfactory receptor co-receptor
PDB	protein data bank
RNA	ribonucleic acid
SMO	spermine oxidase
SPMS	spermine synthase
TRPC	transient receptor potential cation

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Manuscript 4:
"How to Multi-Photon for Life Scientists"

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How to Multi-Photon Microscopy for Life Scientists

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Abstract

The development of the two-photon microscope has been an unprecedented story of success and its impact on today's biological questions and in particular neuroscience is still growing year by year. Since it is not only limited to two excitation photons we will call it Multi-Photon Laser Scanning Microscopy (MPLSM) throughout this paper.

MPLSM gives researchers the opportunity to build their own setups and adapt them to their specific experimental needs to particularly understand brain functions and disentangle neuronal networks throughout a wide range of species. Even commercial systems are adaptable to a certain degree. For someone to fully understand the proper terminology and functions of the main components requires effort and screening various sources across different scientific fields like biology, physics and even computer science.

This half-review, half-methodology paper gives a comprehensive overview and introduction to MPLSM and its main components, such as Laser, Filters, Pockel's Cell, Scanners or Detectors in a simplified way.

Keywords: multi-photon laser scanning microscopy; components; laser; conceptualization of MPLSM; Pockel's Cell; scanners; detectors; hardware; maintenance

1 Introduction

Even though one may argue about the exact start of microscopy, the terminology was first mentioned by Giovanni Faber to describe Galileo Galilei's compound microscope in the 17th century and it displays an unprecedented story of success. Even after more than 200 years it undergoes constant technical development and modification (Helmchen and Denk, 2005; Euler et al., 2009; Seelig et al., 2010; Helmchen et al., 2013; Zheng et al., 2017). One of the first microscopes by Antonie van Leeuwenhoek consisted of a mirror capturing sunlight, followed by a lens system to guide it through a specimen, followed by an rudimentary objective to focus the image through an eyepiece into the eye (Lane, 2015). It took roughly 100 years until Ernst Abbe published the underlying theoretical foundation of light and resolution (Zeiss-International, 2016). The diffraction barrier as physical dogma still holds true but today we can circumvent this limit and image with unprecedented resolution in light microscopy. In 1942 Frits Zernike (Zernike, 1942) contributed his findings on phase contrast and developed the phase-contrast microscope. For his work he received the Nobel prize in physics in 1953. Another important step was the invention of the confocal microscope by Minsky (Minsky, 1961) in 1957 (Figure 1).

Even though the technical tools changed a lot, the general concept of a microscope remains the same: light is focused into a sample and the created image, whether from transmitted, reflected light or generated fluorescence, is sent to a detector. The development of modern microscopy started: optical systems, such as lenses, mirrors or apertures were adapted to the system; image and particularly video processing improved the system; clearer pictures and videos could be analyzed; for light sources, the development of LED or laser systems had a great impact. Theodore H. Maiman, the inventor of the first laser (Maiman, 1967), claimed in a press conference in 1960 that "the laser could be useful in biology, medicine and industry" (Maiman, 1960). The first confocal laser scanning microscope was developed by Thomas and Christoph Cremer in 1978 (Cremer and Cremer, 1978), followed by the development of scanning tunnelling microscopes by Gerd Binnig and Heinrich Rohrer, who received the Nobel prize in physics together with Ernst Ruska, for the development of the electron microscope in 1986 (2019, 1986). Over the past three decades high-, and super-resolution microscopy evolved and were recently (2014) awarded with a Nobel prize in chemistry to Eric Betzig, Stefan Hell and William Moerner (2019, 2014).

Unfortunately, exposing specimens to light has its disadvantages. Particularly in conventional microscopy the effect of light could be severe: the samples and specimens suffer from photo bleaching and other photo-induced damages (Pawley, 2006). One main player inducing photo-damage is oxygen. Reactive oxygen species were reported in several cases to cause effects on other cellular mechanisms. Controlling the light, its features and exposure time can therefore effectively have a positive impact on the viability of the specimen or sample (Pawley, 2006). But even though with today's equipment photobleaching can be reduced dramatically we cannot prevent scattering of photons in the sample. And furthermore both, excitation and emission are effected by scattering. So

sample thickness is limited to 100- 200 um without modern clearing methods.

In 1930 already Maria Göppert Mayer reported in her dissertation that light quanta can be split mathematically (Göppert-Mayer, 1930). So, in principle, one should be able to split the energy necessary for electronic excitation into two individual photons. This builds the foundation for multi-photon microscopy. From its original first major publication as a microscope in Science in 1990 (Denk et al., 1990, 1991) through its developments (Helmchen and Denk, 2005; Euler et al., 2009; Seelig et al., 2010; Helmchen et al., 2013) two-photon imaging has started a new area of imaging techniques widely used in science laboratories all over the world. Now, we are generating multi-photon laser scanning microscopes (Zheng et al., 2017) and complete light sheets (Huisken et al., 2004; Krzic et al., 2012; Wolf et al., 2015) to image brains *in vivo* without the negative consequences of photo-induced damages or scattering on the living organism.

When considering building, using or buying such a system, it is useful to know the names and functionality of the different components within such a system. Unfortunately, when trying to find this information, several research areas from physics to biology have to be surveyed. Here, we want to give a brief introduction to and explanation of all major components including some background information within currently used multi-photon microscopes.

2 Fundamentals: Light, Laser, Fluorescence, and the Multi-Photon Effect

2.1 Light

The starting point of every microscope is the excitation light source. Light propagates in the form of waves and its color is determined by its wavelength, generally categorized in three major regions: within, above, and below our visible spectrum. The visible spectrum has wavelengths of 390 to 760 nm. In the range of 10 to 390 nm is ultra-violet light, which some species are able to see, but is not considered within range of visible light. On the other side is infrared light, which ranges from 0.75 to 300 um (Saleh and Teich, 2007) (Fig 2). One light particle is called a photon, and a photon can be described by its energy and thus subsequently by wavelength.

2.2 Laser (Light Amplification by Stimulated Emissions of Radiation)

To generate light of a very narrow wavelength range one needs a laser. Lasers emit coherent light, streams of photons of the same energy. Furthermore, lasers create a very narrow beam with low divergence. Imagine the difference between the effects of a light bulb, where photons can reach every part of the room, in comparison to a laser pointer, where the light is spatially narrow and clearly defined. Relative to other or simple light sources, **L**ight **A**mplification by **S**timulated **E**missions of **R**adiation (**laser**) emits light coherently. This coherence is spatially defined, i.e. collimated, and temporal,

which means it emits light with a narrow spectrum (e.g. focus on just one wavelength).

Nowadays, in ultra-fast lasers the system is modelocked: the laser light is rapidly turned off and on. The emission of such short laser pulses go down below ten femtoseconds, which is fast enough to be regarded as a single beam, but the photons are more likely to have the same phase.

2.3 Fluorescence

The excitation light source is needed to generate fluorescence. A given molecule has various internal states namely vibrational, rotational and electronic states. With excitation energy the molecule can be brought into the first excited state, a energetically non favorable state so that the molecule will return into the ground state via fluorescence by emitting a photon (Fig. 3 A). The energy is equivalent to the energy gap between the ground state and the first excited state. There are other conversion possibilities, see Jablonski Diagram (Fig. 3 B), that are not relevant for this paper. Todays fluorescent molecules, either organic dye molecules or fluorescent proteins are designed to maximize the conversion via fluorescent and cover the entire range from UV to infrared excitation.

2.4 Multi-Photon Effect

A big advantage is the scattering of the tissue which is significantly reduced for higher wavelength. So the excitation beam is able to travel deeper into the tissue. To still achieve the excitation effect two photons of appropriate energy have to hit the molecule almost simultaneously. This demands a laser system with high enough peak power at the appropriate wavelength.

3 Conceptualization of a Multi-Photon Microscope

Whether commercial or self-built MPLSM, the concept within the system is often very similar. To get a general idea of what an MPLSM looks like, please take a look at Figure 4. The setup is composed of three main compartments: laser beam arrangements (A), scanning process (B), and detection (C+D). The detection is split into ocular(C) and main detection(D). The single parts will be explained in detail throughout these sections.

Lasers used in MPLSM setups often consist of a power supply, a chiller and recirculating units. The chiller provides a constant temperature to prevent overheating of the laser. The recirculation unit filters the air (i.e. cleaning, dehumidifying, conditioning) to maintain stability inside the laser head.

Finally, the MPLSM is likely connected to a computer with an imaging software to analyze the data. While in this section we are focusing solely on the components within the beam path, the computational aspects will be discussed in the following section.

3.1 Laser Beam Arrangements

The first step to get the MPLSM working is to align the laser beam optimally for the task. This includes getting the beam where it should be, modulating the beam and widening it using a telescope. For this, several devices are necessary which will be briefly discussed.

3.1.1 Optical Components: Lenses, Mirrors, Apertures and Filters

Optical components help guide the light where we want it to be. The arrangement of these parts is called the beam path, because they guide the laser beam along its path all the way into the microscope. Possible ways of guiding light from one point to another are depicted in Figure 5: controlling the light in a specific way can therefore be achieved using lenses (a), mirrors (b) or internal reflection similar to optical fibres (c) (Saleh and Teich, 2007). Depending on individual experimental designs, the advantages and disadvantages of the different options to guide light need to be considered.

Lenses We usually see the world through lenses in our eyes, which ideally refract the light onto the retina. Lenses can achieve different functions, e.g. parallelizing a beam, narrowing a beam, or widening a beam (Hecht, 2002), as depicted in Figure 6.

An achromat is a compound lens and is corrected for achromatic aberrations, in short different wavelengths get focused differently and this is corrected so that all colors end up in the same spot (see Figure 7). Then, light can easily be bundled and and therefore manipulated.

Mirrors and Apertures Similarly to lenses, mirrors can have different shapes (e.g. convex, concave) to reflect the incoming light. Most commonly, planar mirrors are used to direct the laser light from one point to another. Here, the entry angle equals the exiting angle (Pawley, 2006).

To ensure that a beam is parallel or hits certain points the right way, apertures are used as alignment tools: arranging mirrors in two steps helps arranging the laser light into parallel beams. A first and second mirror should reflect the beam in the central area of the mirror. Then two apertures are placed in the beam bath: one close to the two mirrors, one far away. The first mirror corresponds to the first aperture and accordingly the second mirror to the second aperture, as depicted in Figure 9 A. To align the beam the aperture should first be wide opened and then slowly closed until a beam is visible (Fig. 9 B). If the beam is visible in e.g. the upper part of the aperture, it means that the mirror is still not correctly adjusted. The first mirror must be adjusted so that the beam passes through the middle of the iris. This procedure has to be repeated until the first and the second aperture can be almost closed and the beam hits the center spot in both apertures (Fig. 9 C and D).

In self-built microscopes, the two-step mirror arrangement gives enough degrees of freedom to include components for the laser setup. Also, when reflecting the beam or using lenses, a beam profiler can provide information about possible beam divergence and will indicate if it may be necessary to set up more lenses or telescopes to reduce it.

Filters Filters (see Fig. 8) come in different varieties and usually have a specific coating to achieve the filtering, e.g. density filters that influence the intensity of the beam. Controlling the intensity can help create a safer work environment and get rid of unnecessary intensity.

Excitation filters are selected for a certain wavelength area and filter out other unnecessary wavelengths. Usually this will either be a

short pass, long pass or band pass filter. Short pass filters let only shorter wavelengths through, long pass filters only longer wavelengths, while band pass filters select for a certain range and get rid of both shorter and longer wavelengths.

Emission filters can eliminate the excitation light and only focus on a very specific range, e.g. fluorescent green. Deep-blocking filters or notch filters are commonly used, which block a narrow range of the spectrum, while allowing the remaining wavelengths to pass. Dichroic filters let a certain range pass but at the same time reflect another, which is also why they are often called beam splitters.

3.1.2 Shutter and Beam Dumps

A shutter ensures a security and provides a fast way to block the laser beam for a certain time period. It should be included in the software handling the laser. While the shutter usually interrupts the laser beam briefly, a beam dump completely terminates it. The different kinds of beam dumps can be combined with density filters and even cooling units to terminate beams.

3.1.3 Pockel's Cell

In many MPLSM systems the power of the laser beam needs to be adjusted externally. An electro-optic modulator (EOM) is a device to modulate a beam of light based on phase, frequency, amplitude or polarization of the laser light (see Figure 10). The output power of the laser itself is usually fixed and they run in a modelocked state, so very short pulse width in the low femtosecond range that are phase locked. As a result the polarization of the laser is maintained as well and typically linear polarized. The laser output is linear polarized light of a certain wavelength. This is send into the Pockel's Cell. Polarization describes the wavefunction of the electric field along the laser beam. The field vector pints in 90 degrees to the direction of propagation. When this vector is only modulated in one plane the beam is linear polarized. This polarization can be used to modify the power of the laser beam. In a Pockel's cell a crystal is used to rotate the polarization based on the electro optical effect. Based on the electro optical effect one can rotate the polarization of the beam by applying voltage to a crystal inside the cell (Goldstein, 1968; Pockels, 1894). Combined with the polarization filter at the output of the cell one can now adjust the output power. The Pockel's Cell can achieve that very fast and accurate, which will be beneficial for the upcoming scanner unit.

The important step when aligning a Pockel's Cell is to make sure that the estinction ratio (ER) correctly corresponds to the one tested. The ER needs to be calculated and defines the "null power". It is a way to "turn off" the laser beam. Since it is not a complete turn off, rather than a very low power, this must be done very precisely. The ER is then the ratio between the maximum and minimum intensity, as also depicted in Figure 11.

3.1.4 Photodiode

Due to the voltage-dependent maxima and minima of the EOM as discussed in Figure 11, the laser beam intensity may fluctuate. However, one still needs to know the intensity of the laser beam, which can be achieved using a photodiode. Controlling beam intensity can provide valuable information about the functionality of the Pockel's Cell. Furthermore, knowing the

intensity ensures preservation of the specimens under the MPLSM.

3.1.5 Telescope

Another problem when dealing with light is divergence, a diverging beam will widen its diameter over traveled space. So usually, one of the first optical elements along the beam path is a telescope. It is a pair of lenses turns the beam into a parallel laser beam and depending of the focal length ratio either in or decrease the diameter of the laser beam. Using a pinhole at the focus point, any further divergence can be eliminated. The general concept is displayed in Figure 12. When using a pinhole in the focal point of the first lens after the beam passes through further increases the quality of the beam. By passing the pinhole the intensity of the beam follows a Gaussian distribution perpendicular to its optical axis.

3.2 Scanning

The main body of an MPLSM consists of a scanner unit with two mirrors, each of which corresponds to and scans only in either the X or Y axis. With the scanner module a field of view can be generated according to the needs of the specimen. Followed by a lens array consisting of a scan and tube lens, the objective lens this field of view is then transformed into the specimen. This second part of the MPLSM will ensure that a specimen is properly scanned, via the scanner unit, and that the focus is correct, via the lenses.

3.2.1 Galvanometer and Resonance Scanner

In the 1820s Danish physicist and chemist Hans Christian Ørsted realized that magnetic compass needles will change their direction, when they are close to an electric conductor (Oersted, 1820). This is also the main concept behind galvanometer scanners. They use magnetism to quickly switch the positioning of each of the two mirrors. The galvanometer scanners will scan each spot on the focal plane in a very fast manner. Another way of scanning would be the use of resonance scanners. While galvanometer scanners are slower and allow for point control, resonance scanners are faster and enable high scan rates. When scanning an area of interest, the galvanometer scanner essentially needs to stop before turning via magnetism, whereas the resonance scanner adapts to the directional change in a sinusoidal manner (see Figure 13). The best application for galvanometer scanners is probably morphological imaging, while resonance scanners thrive at real time functional imaging. This may also explain the higher cost of resonance scanners. In our configuration of the MPLSMs the distance of these two scanning mirrors is so narrow that they can be considered as one focus point, so only one scan lens is used.

3.2.2 Lensarray: Scan and Tube Lens

When the scanners change the direction of the beam it is necessary to realign the beam towards the objective lens. To achieve this, the combination of a scan lens (itself a lens array) and a tube lens, will change the beam direction accordingly and focus it the beam precisely onto the objective lens, as indicated in Figure 14.

3.2.3 Objective and Specimen

Depending on the specimen, a different size of the field of view may be needed based on the structures that need to be imaged. Appropriate objectives can be chosen out of a vast range of different magnifications and immersion media. One has to be aware of the special needs regarding transmission of infared light using MPLSM. Most commonly used are low magnification water immersion lenses. They usually combine a good magnification (16-40x), high numerical aperture, wide field of view and high focal length. Especially focal length becomes important when doing live imaging in living specimen.

3.3 Detection

After the laser light reaches the specimen, fluorescence is created, collected by the same objective and via a dichroic beamsplitter directed towards a detector, in our case a photon multiplier tube (PMT).

A collecting lens focuses the fluorescent light through an emission filter. The filter will let the corresponding emission pass through (in many cases GFP, RFP or YFP) and reflect the remaining fluorescence in the direction of a second emission filter. Photons from this fluorescence which pass through the emission filter will be bundled using another lens and are focused onto the detector unit (PMT). A second or third filter may or may not be used, depending on what range of fluorescence is desired. As previously described, only the corresponding fluorescence will pass through (bundled and focused) or will be reflected and eventually loose itself in the construction. Hence, multiple PMTs can work in parallel in the system to allow multicolor visualization of possible biological interactions via the different fluorescence channels, as defined by the emission filter.

The PMT transfers the incoming fluorescent photons into electrons. These electrons then will be amplified and the current directly depends on the fluorescent signal and can easily be measured. The PMT is very light-sensitive, as it multiplies the electronic signal given by the fluorescence photons to a certain degree that can be adjusted by the user.

3.4 Ocular

One can either use a regular ocular or replace it by a camera, in which case the integration of a wide-field one-photon microscope setup is necessary. Either option serves the task of aligning the specimen under the microscope before starting scanning with a two-photon laser. When this light reaches the specimen, the thus reflected fluorescence will be detected via a high-resolution camera or an ocular using an emission filter (e.g. GFP). This detection via the ocular is designed independently of the original multiphoton laser pathway and will therefore not pass to the PMT. The camera image can be seen on the computer screen and the specimen can be aligned accordingly. Since the light source uses relatively low energy and only for a brief time, the negative effects of standard one photon microscopes, such as photo bleaching, are greatly reduced.

4 Computational Hardware and Software

Buying or building a microscope also requires some computational background. Purchased systems often have the advantage that they

come with a predefined software to use the microscope. This software then handles the individual compartments, as well as produces the resulting images or videos.

Self-built systems, however, need to be set up first. With the manipulation of several systems and integrating components to the system, it is necessary to buy hardware components for the computer as well. In our setting, for instance, we use two Peripheral Component Interconnect (PCI) boards produced by National Instruments (NI), one of which is an express (PCIe) type. These boards can provide high speed exchange between a computer and the connected hardware. If the two PCI boards are integrated into the computer, it should be noted that they need to be connected to each other using a Real Time System Integration (RTSI) cable - also called a RTSI-bus. External devices can be plugged to PCI data acquisition (DAQ) devices using coaxial cables, i.e. BNC. Lastly, the hardware must be connected to a software to easily handle components or read out the data. A prominent open-source software package is for example ScanImage (Pologruto et al., 2003), which uses MatLab as a main programming language. Other requirements the computer should have, e.g. graphics, are often indicated by the software packages used. Though depending on the application commercial software or other offers should be considered.

Whether bought or built the data acquisition is independent of the image processing. In this paper we do not want to give further detail on image processing.

5 Maintenance and Security

The final step may be the most important: maintenance and laser safety. Multi-photon devices often require a laser safety officer, who should ideally be contacted before setting up such a system. They can give valuable input on creating a safe environment, or even how to position an MPLSM to ensure standards. In some countries, a laser must be registered at governmental institutions. A laser safety officer can help here as well.

When built, someone needs to ensure beam stops, security curtains or shielding at the right places. For instance, when using a Pockel's Cell the remaining laser light after modulation should be absorbed. Therefore, to get a terminal piece of the Pockel's Cell or any other optical system a beam trap should be introduced. To extinct the light, the beam trap is usually a black metal piece that absorbs the energy of the laser beam

To maintain a stable functionality of the MPLSM there are some things to take care of. This can either be done by the laser safety officer, or any person responsible for the functionality of the laser. At least yearly, the following things should be checked: all external devices for functionality, (i.e. PMT, micromanipulators, Pockel's Cell, shutter, laser table, connection of NI and PCI boards), internal beam path for divergences and laser safety, and laser safety goggles, hazard assessment, laser safety instructions for new laboratory members.

Next to laser hazards, secondary hazards should be addressed: when working with light, certain components transmit, reflect, or absorb light. Not only jewelry, but especially in a biological or chemical laboratory, it should be men-

tioned that certain chemical components react to light or heat with extreme reactions, e.g. explosions.

Modern laser safety can be ensured using augmented reality (Quercioli, 2018). Laser safety goggles need to be calculated specifically for a given laser system, however the quality depends on the supplier (Stromberg et al., 2017).

6 Concluding Remarks

Even though light microscopy itself has experienced dramatic advances over the years, the invention of the two-photon microscope has been a breakthrough. Biological studies are published weekly since MPLSM enables researchers to investigate the living brain in action. No other technique is able to dive as deep, as fast into the neuronal network of a living animal. Its easy adaptation to be combined with setups allows for long-term, whole-brain imaging even regarding calcium or voltage dynamics (Chamberland et al., 2017; Mann et al., 2017; Aimon et al., 2019; Huang et al., 2018). Fascinating examples can be seen in very different fields of neuroscience namely plasticity of the brain and the conceptual understanding of memory formation in the mouse brain, or deeper understanding of vision, or learning in mouse, fly and zebra fish (Seung et al., 2000; Helmchen and Denk, 2005; Kerr and Denk, 2008; Yuste and Bonhoeffer, 2004; Grienberger and Konnerth, 2012). Furthermore there are already successful examples of three-photon imaging to reach unprecedented depth or even imaging through the intact scull in mice (Wang et al., 2018; Horton et al., 2013).

This paper should help life scientists understanding the general concept behind building and constructing a two-photon microscope on their own. Of course more advanced technical changes can be and could be made to adapt the microscope to the special needs for a given sample. For instance, the Pockel's Cell can be exchanged with an motorized polarizer for power adjustment if speed is not an issue. The galvo/galvo scanner combination can be replaced by galvo/resonant combination or even two scanner pairs to achieve high speed and a large field of view. Even photomanipulation while scanning different brain regions is possible (Anselmi et al., 2011; Dal Maschio et al., 2017). However, by giving scientists insight into designing their own microscope in the first place, it becomes much easier to adapt and build their own microscope, best suited for their needs.

Upcoming challenges need to be addressed (Lichtman and Denk, 2011) and must be resolved if we want to continue working in high-resolution. While building and or buying a multi-photon microscope can be an enormous task, it is worth the effort. The future of microscopy will allow us to get deeper into tissues and progress on *in vivo* imaging and its application for modern advances in "biology, medicine and industry" (Maiman, 1960) to an extend far beyond the imagination of Galileo Galilei.

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Author Contributions

AB drafted the the manuscript. AB and RK wrote the manuscript. AB, RK and IGK revised the manuscript. IGK provided funding. All authors approved to the final version and its publishing.

Declaration of Interests

The authors declare no competing interests.

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Figures, Tables and Schemes

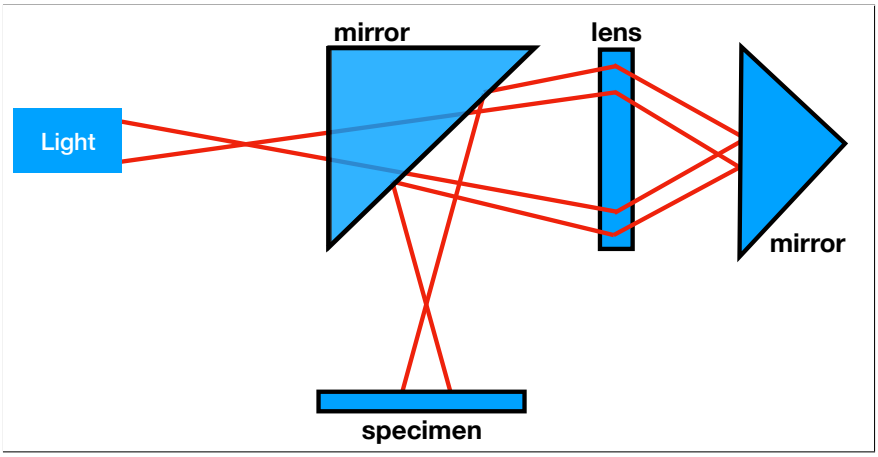

Figure 1: Minsky's Patent for the microscopy aparatus for confocal imaging as defined in (Minsky, 1961) (adapted from (Minsky, 1961)).

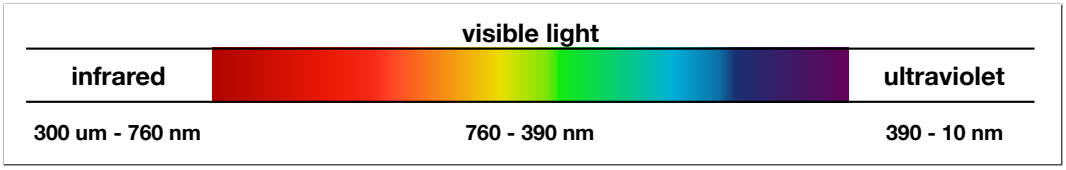

Figure 2: This depicts the light spectrum from infrared to ultaviolet.

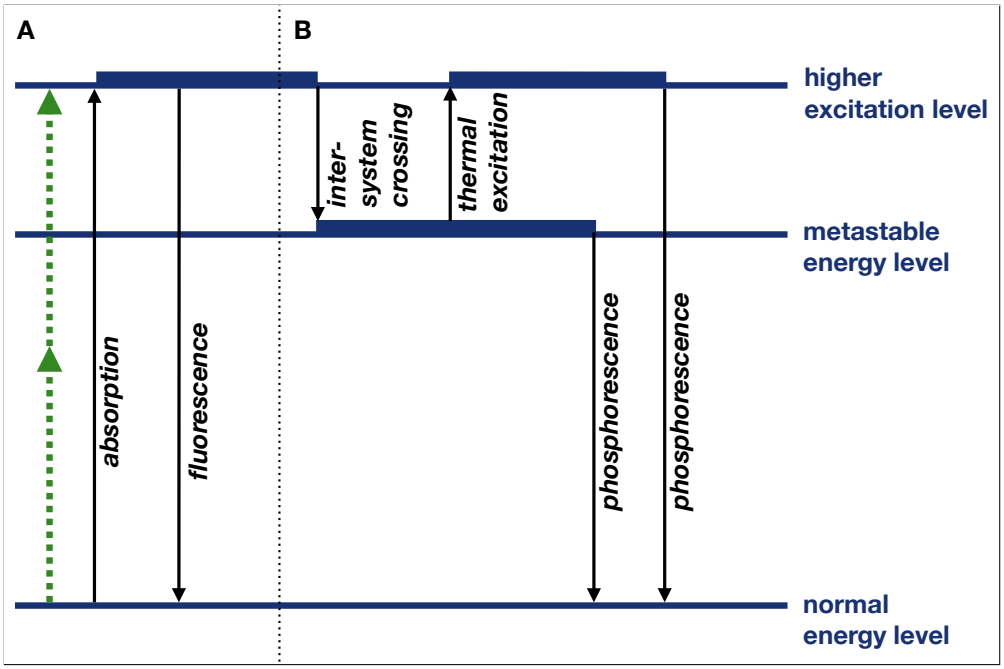

Figure 3: Jablonski Diagram. Coming from a normal energy level, light is absorbed and the molecule is therefore brought to a higher excitation level. Following that, the molecule can return to its normal state either using fluorescence (**A**) or internal conversion (**B**). Internal conversion follows the idea of vibrational relaxation, which means to release the spare energy to the surrounding environment. It is also possible for the molecule to get into a metastable energy level, such as a triplet state using intersystem crossing. From there phosphorescence is the process toward the normal energy level. The two-photon effect is indicated through the green dotted line (adapted from (Jablonski, 1933)).

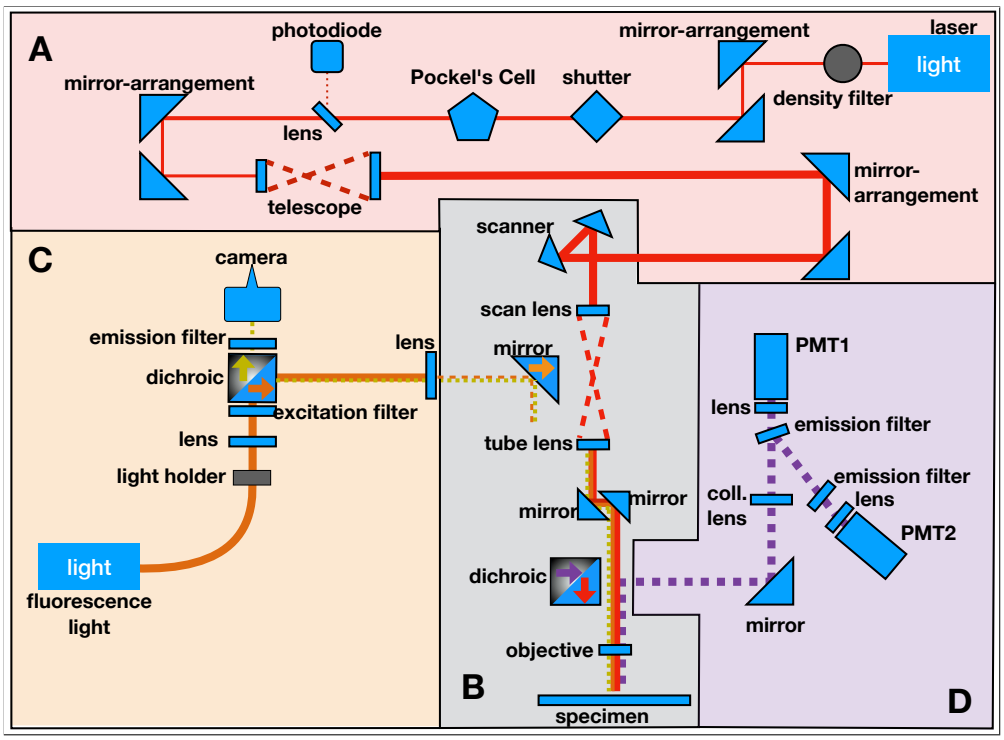

Figure 4: This is the beam path of our MPLSM. It can be divided into 4 general compartments. (**A**) shows the general laser beam arrangements, (**B**) the scanning part, (**C**) the so-called ocular, and (**D**) the detection part.

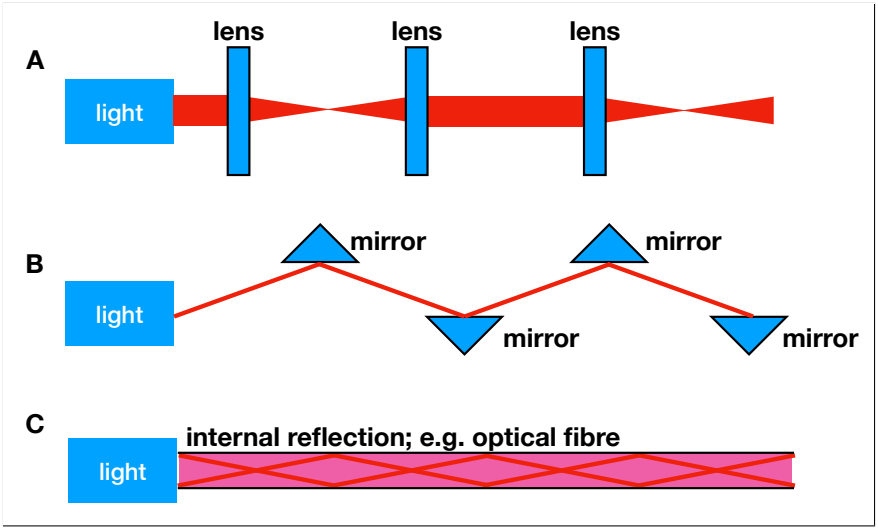

Figure 5: This depicts possible way to guide light from a source to a different point . (**A**) using lenses, (**B**) using mirrors, and (**C**) using total internal reflection (adapted from (Saleh and Teich, 2007)).

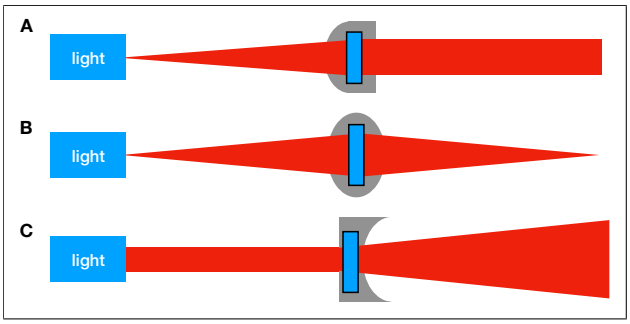

Figure 6: Shown are different kinds of lenses and their respective effects on either a point source of light (**A** and **B**) or a parallel beam (**C**), as well the the corresponding output (adapted from (Hecht, 2002)).

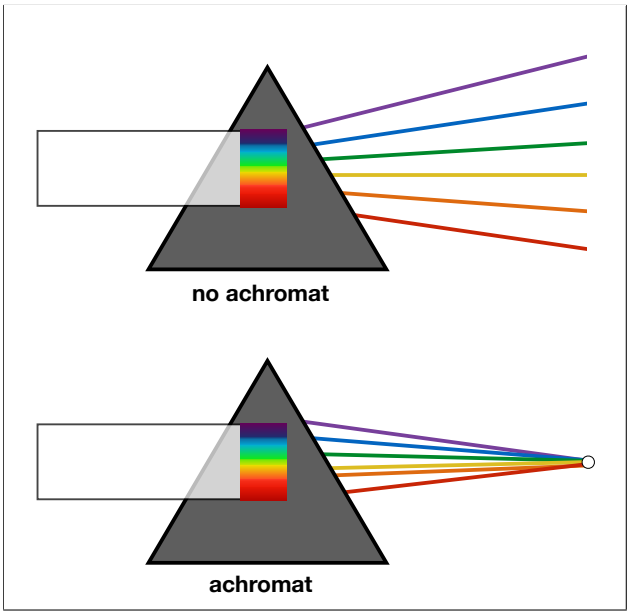

Figure 7: Focal points with and without an achromat type lens.

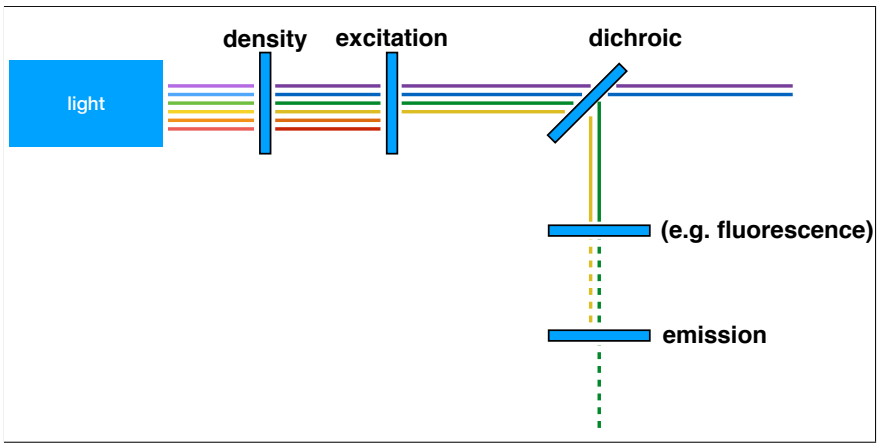

Figure 8: Different kinds of filters. Density filter reduce intensity. Excitation filters select for a certain range. Dichroic Filter split the beam and let either pass through or reflect, which is why thei are also calles beam splitters. Emission filters can eliminate excitation light and focus on a very specific range, e.g. fluorescent green

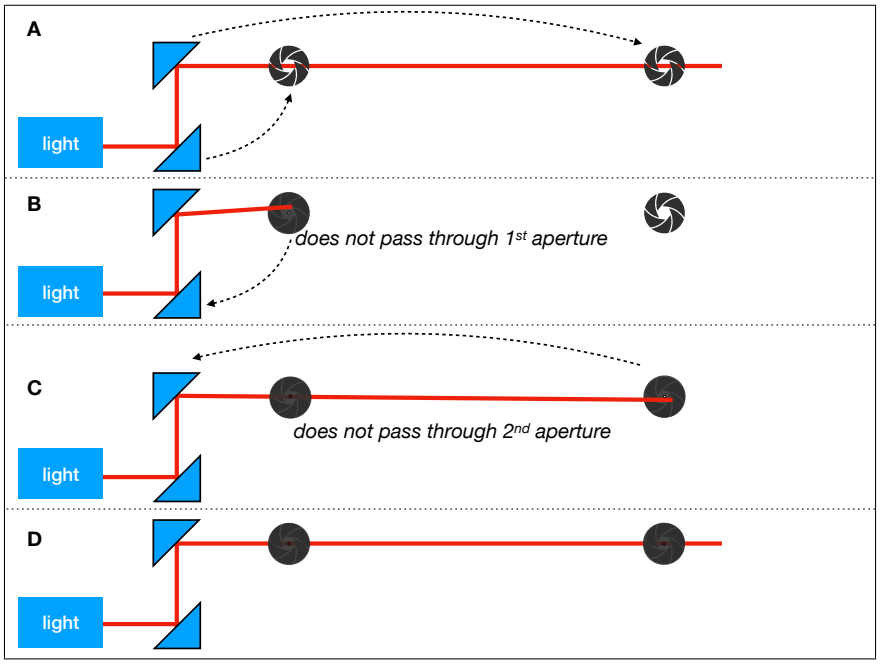

Figure 9: (**A**) A mirror (triangles) refers to (arrow) a corresponding aperture (circle). The apertures are open at first. (**B**) For alignments the first aperture is almost shut. If the beam does not pass through the first aperture, the first mirror needs to be re-aligned. (**C**) The same is done for the second aperture (**D**) This procedure is repeated until the beam passes through both almost shut apertures.

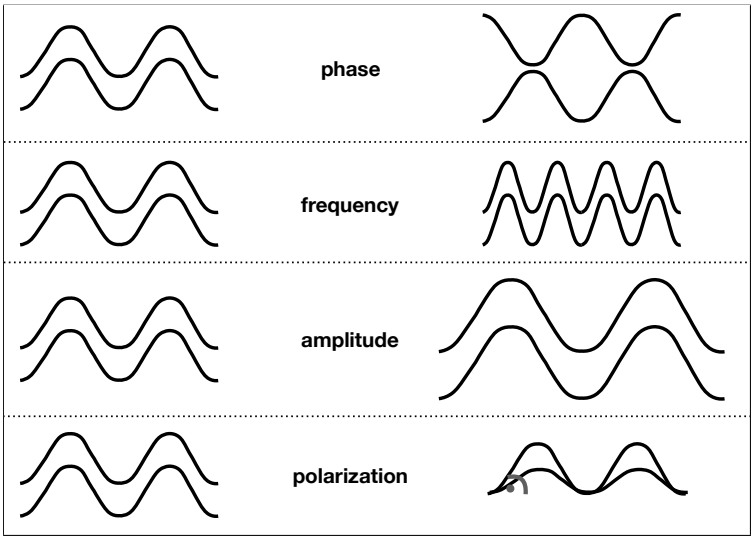

Figure 10: Given a certain photonic wavelength (left side), an electro-optic modulation based on phase, frequency, amplitude or polarization (right side) can also influence the output power.

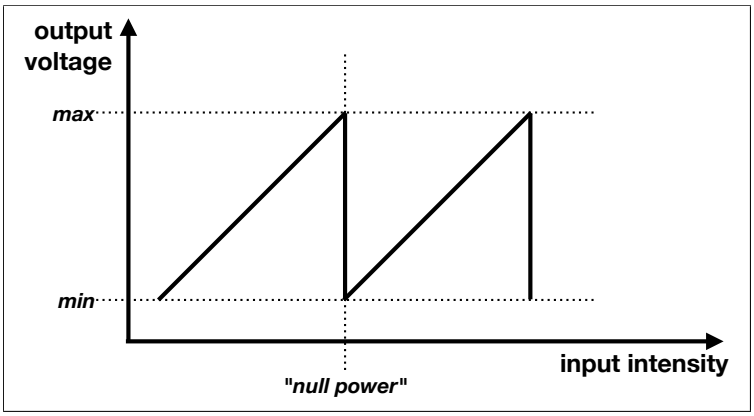

Figure 11: A Pockel's Cell has two intensity maxima. Somewhere along the input intensity is a region called null power where the output voltage of the Pockel's Cell is minimal.

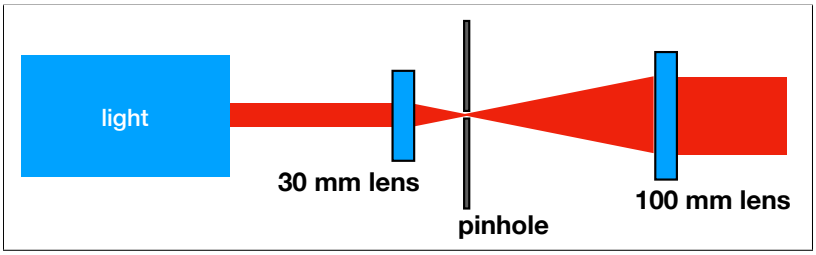

Figure 12: A simple telescope with a pinhole provides optimal arrangement of the beam in terms of parallelizing, widening and divergence reduction.

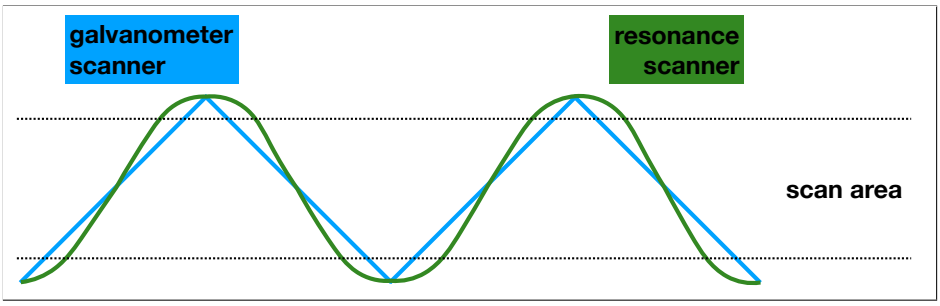

Figure 13: Galvanometer scanners (blue) have to stop to turn, while resonance scanners (green) can flow through the scanning process. During the scanning process only the linear movement is measured (scan area).

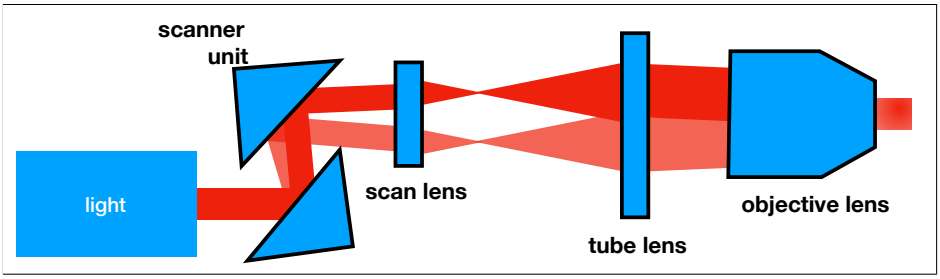

Figure 14: A laser beam reflected by the scanning unit. The two mirrors of the scanner will change the beam in X and Y direction (indicated by bright and dark red). To provide an optimal focus, scan and tube lens provide the background to reach the specimen behind the objective lens.

Chapter 3

Discussion

3.1 Summary of Aims and Results

Upon mating, female *Drosophila melanogaster* have been shown to change their attraction behavior towards polyamines [13,116]. This attraction is linked to a neuropeptidergic modulation at the polyamine sensing ORN presynapse. Even though this modulation is transient, it leads to a long term change in the female's preference behavior towards these polyamines. The aim of this dissertation was to find the neuronal underpinning of this long term behavioral change with respect to neuronal pathways and their modulation, as well as to find potential triggers for the switch in mating state using established and state-of-the-art techniques.

3.1.1 Sensory modulation leads to long term behavioral changes

My collaborators and I were able to find a reproductive state dependent neuronal pathway involved in the switch of long term preference behavior towards polyamines (**manuscript 2**). We focused our effort onto neurons

downstream of polyamine sensing ORNs. We were able to image the projection neuron and found its innervation in the MB and the LH via mALT and mlALT pathways. The long term behavioral change depends on synaptic plasticity via the AC *rutabaga*. Using spatio-temporal targeting at the level of the MB, we found necessary components for polyamine perception in α2sc and β'1 (see Figure 3.1 A and B).

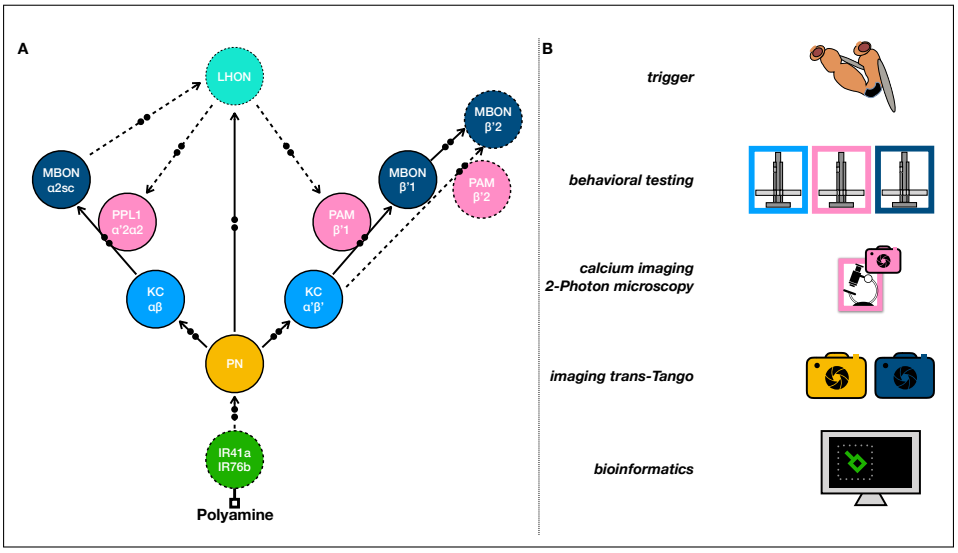

Figure 3.1: (**A**) In collaboration, I have unraveled a neuronal pathway for reproductive state dependent polyamine preference behavior. After the detection of polyamines at the ORNs IR41a and IR76b ([13,116]; green dashed), we transsynaptically identified PNs (yellow) and their innervation towards the LH (cyan) and the MB (blue). We particularly found two pathways along MBON α2sc and β'1 (dark blue). DANs innervating these areas (pink) are sufficient to modulate reproductive state dependent behaviors. The identified MBON α2sc has been shown to innervate the LH and corresponding output neurons (LHONs) which send their axons to DANs [159] (dashed line), potentially implying a feedback loop to the identified network. MBON β'1 synapses onto MBON β'2 which (together with its corresponding PAM, dashed) has been identified in previous studies with respect to hunger paradigms [166]. (**B**) In this study, my collaborators and I used various methods targeting different areas of the identified pathway (indicated by colors in A) to determine the neuronal underpinning of reproductive state dependent behavior.

3.1.2 Neuronal keys to modulate reproductive state dependent behaviors

Internal states highly depend on the input of neuromodulatory signals (**manuscript 1**, i.e. [217]). These modulations happen at many sites and are not restricted to first, second, or third order processing areas alone, but can rather have global effects. Classical neuromodulators, such as the transmitters dopamine, serotonin or octopamine are as important to consider in the process as neuropeptides or hormones [18,194,210,211,214–216,243]. My collaborators and I were able to find that modulatory DANs are sufficient to induce reproductive state dependent attraction behavior towards polyamines (**manuscript 2**).

3.1.3 Signals that tell sensory neurons that mating took place

Particularly in reproduction, which is a highly complex state consisting of several layers from finding the right partner, to successful copulation, and subsequent post-mating behaviors, neuromodulation has an impact (**manuscript 1**) [32–35, 170, 174–176, 217, 220–222, 225–229]. While seminal fluid proteins influence many post-mating behaviors, they have no effect on polyamine perception (**manuscript 2**). Alas to say, we were not able to decipher any sole component for the trigger towards the change in behavior, although we assume that rather a combination and interplay of multiple factors contribute to this switch (**manuscript 2**). The detection of the male pheromone cVA seems one of the most important players in this process.

3.1.4 Polyamine specific activation of IRs

Ultimately, reproductive success can not only be influenced by modulation and endogenous signals, but also by the perception of the external stimuli.

The detection of polyamines as an exogenous nutritional value is beneficial not only for the metabolic state of the animal itself but also for its offspring [6, 9–14]. My collaborators and I used bioinformatics tools to understand the molecular scaffolding for polyamine receptors using comparative studies, sequence based mutation analysis, electrostatic validation and the predictions for protein dynamics (**manuscript 3**).

3.1.5 Promoting and sharing technical advances

Generally, computational and technical advances over the past decades have had a great impact in scientific fields [97–103, 108–119]. Microscopy has developed to cover a wide range of specifically designed setups with the possibility for flexibility in experimental arrangements. Monitoring neuronal activity *ex vivo* and *in vivo* allows for the possibility to correlate behavior with it. However, constant changes and adaptations on experimental setups and microscopes need experienced professionals. My collaborators and I were able to methodologically describe the components and facets of multi-photon laser scanning microscopy for scientific audiences in a simplified manner (**manuscript 4**). Furthermore, such setups can be used in the context of reproductive state. We have shown reproductive state dependent changes in the perception of pheromones through *in vivo* calcium imaging on dopaminergic neurons (**manuscript 2**).

3.2 Sensory filtering and internal state dependent hierarchies

Sensory perception, and subsequently decision making depends on the interplay of external and internal stimuli. Sensory organs can detect environmental factors as well as metabolic states, and use them to form an internal state dependent behavior [6, 33–35]. This requires a strong adaptability and reciprocity between peripheral sensory neurons as well as higher brain circuits

and their potential neuromodulations. Interestingly, such modulations often already happen at the periphery and thus allow for sensory filtering before information reaches higher brain areas [244].

Fields et al. [2] have discussed how stress can have a direct effect on opioid receptors in noci-ceptive neurons responsible for pain perception. When pain is present in a stressful situation, the sensory filtering allows for either forwarding this signal, or to refrain from doing so by inducing analgesia, such that an adequate reaction to the situation can take place unhindered. This has not only been shown for stress, but also under the paradigm of hunger and inflammatory pain perception [245]. It clearly indicates that internal states are able to change sensory perception and use sensory filtering to prioritize the animal's needs.

When a fly is in a nutrient rich environment, the detection of the pheromone cVA can be modulated at the ORN level to make a virgin more responsive to male mating attempts [235]. Sensory filtering may also be of interest for polyamine perception given the reproductive state of an animal. After the mating experience, ORNs shift their input on the filtering process towards the detection of polyamines. Virgin flies prefer low concentrations of polyamines, while mated flies prefer high concentrations [13,116]. Polyamines can be beneficial within the framework of reproductive state, though high levels of polyamines have been shown to be be cancerogenic [9–12,12–16]. Thus, finding a right balance between internal polyamine levels and nutrient supplement is highly important. It remains to be elucidated, whether the sensory filtering for polyamine attraction depends purely on the transient neuropeptidergic modulation of ORNs via MIP, or if other neuromodulatory signals are part of it as well. My collaborators and I were able to show that DANs in the MB are able to sufficiently induce long lasting reproductive state dependent polyamine attraction behaviors (**manuscript 2**). It is possible that neuronal feedback loops top-down towards the AL [246] to preserve the attraction behavior until there is no further requirement for external polyamine consumption.

Another possibility is that internal states are validated against each other at the higher brain centers, hinting at the interpretation of an internal state dependent hierarchy. In the postulated network we see that the modulatory mechanisms for reproductive state dependent behavior (here $\beta'1$) are wired towards $\beta'2$. While this region plays multiple roles in various behaviors, it is also mentioned in hunger paradigms [166, 173] (**manuscript 2**). That a potential "hunger pathway" is retrograde of our "reproductive pathway" allows for different interpretations. Either the internal drive for reproductive success proves more useful than the survival of the individual itself, or is it *vice versa* that because the "hunger pathway" is retrograde, its impact will have the ultimate effect on the arising behavior. Both options are possible and may be driven by modulatory neurons. It has been shown before that the multi-modality of sensory perception is able to prioritize an emotional state with the interpretation of a stimulus [2, 4, 245]. Possible experimental designs may aim for the investigation of choice behavior when an animal is starved or fed, mated or virgin, or a combination of these, while presenting either standard food odors (e.g. vinegar) or polyamines. Whether modulatory neurons are capable of tweaking the importance of a particular internal state at a given time remains to be resolved.

3.3 Reciprocity of higher brain centers

The initial understanding of the higher brain centers, the MB as center for associative learning and memory functions, and the LH as center for innate driven behaviors and sex differentiation, has been revised over the past years [153, 153, 154, 156–172, 174–176]. Recent findings have shown that there are indeed direct connections between the two [84, 159, 160, 177, 178]. The reciprocity of these higher brain areas have the capability to integrate context and value to neuronal processing.

The LH has been shown to be involved in post-mating behaviors and is highly linked to courtship behavior or pheromone detection [102, 153, 156, 157]. Find-

ings from my collaborators and me revealed two interesting facets to this: (1) courtship was sufficient to induce attraction to polyamines; (2) virgin and mated flies shows different responses to the pheromone cue, cVA, in DANs innervating the MB; (3) one MBON necessary for reproductive state dependent polyamine attraction behavior (α2sc) is also connected to the LH (**manuscript 2**). The dopamine pathway has been shown to be involved in male and female courtship behaviors [247, 248]. Thus, as LH output neurons (LHONs) are innervating the MB and dopaminergic pathway [159], it is reasonable that other post-mating factors or cVA-specific information are exchanged between LH and MB, leading to the corresponding polyamine attraction behavior. An investigation of LHON driver lines needs to tackle whether or not the LH plays a role in the reproductive state dependent switch in olfactory behavior.

3.4 From external signals to neuromodulation

A major open question remains regarding the change in reproductive state dependent olfactory behavior: how does the fly in general, or the deciphered dopaminergic neurons in particular know that mating took place? My collaborators and I were able to exclude some factors: neither the exchange of seminal fluid proteins and sperm, nor oogenesis appears to be a sole trigger for the behavioral switch. Courtship, however, was sufficient to make the virgin behave not significantly different from the mated female (**manuscript 2**). This selection of triggers is surely incomplete. Follow-up experiments can be designed to check for the relevance of other triggers (e.g. auditory or visual inputs) or trigger combinations within this paradigm.

While my collaborators and I have seen that dopaminergic neurons are required for the behavior (**manuscript 2**), we are still lacking the information where these DANs get their input from. As mentioned, the LH may provide some insights [159], but current studies also focus their efforts on connec-

tomics [70–74]. However, even if connectomics provide a neuronal network, we still need to decipher the underlying neurotransmitters or co-released modulators.

Apart from dopamine, it is possible that other neuromodulators are involved in the reproductive state dependent switch in behavior. My collborators and I have tested the involvement of CSD (serotonine), DAL (octopamine), and amidergic neurons with no conclusive findings (**manuscript 2**). Potential future work can focus its efforts on the involvement of neuropeptidergic modulators responsible for the SPR and MIP signaling, and the change of the long term behavior to detect polyamines. A target for such an experiment can be for instance tachykinin (homolog of substance P), which has been shown to be involved in pheromone detection and is able to reduce ORN activity [18, 173, 216, 249–251].

3.5 Considering reproductive state in science

In comparison to other internal states, reproductive state is regarded as a more delicate one, as it is very characteristic for females and sometimes only during certain time frames (e.g. virgin vs. mated in flies, estrus in mice, etc.) [34, 35, 39–41, 252].

My collaborators and I have seen reproductive state dependent behavior in olfaction (**manuscript 2**). The reproductive state dependency shows the value and importance of this internal state in a broader context. A differentiation of menstrual cycles in females is a common practice in mammal studies and has for instance shown how pheromone sensitivity changes at the periphery dependent on estrus [252]. Particularly in fruit flies, the main differentiation is usually sex-based, but not reproductive state based. In future studies, the reproductive state of *Drosophila* should be considered as classifier for good scientific practice, as it is already done in mammals.

Prominent in estrus and menstrual cycles in mammals are hormones [40].

Therefore, the endocrine system of insects may provide a different approach to solving reproductive-state dependent changes. It has been shown that the transfer of SP influences the release of juvenile hormone (JH) by the *corpus allatum* [241]. Subsequently, JH affects shape and function of sexual organs [253], can reduce resistances to infections [254], and also has effects on courtship success in males [255, 256]. My collaborators and I have investigated that JH has no effect on polyamine perception in a reproductive state dependent manner (data not shown). In insects, other hormonal systems of interest relate to steroid or peptidergic pathways [257]. Potential targets for experiments are ecdyson as a steroid hormone, as well as for instance peptidergic hormones similar to oviduct stimulating hormones (OSH), egg development neurohormone (EDNH), pheromone biosynthesis activating neuropeptide (PBAN), or allostatin and allotropin, which also influence JH. This may also give more indication to what modulates ORNs or provides association to the DANs with respect to polyamine preference.

3.6 Neuronal systems and metabolism

The endocrine systems, particularly with respect to their function in metabolism, seem connected to chemosensory systems [258] and are able to regulate food intake: even though polyamines are endogenously produced, they seem to provide benefit for the gravid female in mosquitos [259], for fertility and reproduction in the worm *C. elegance* [260] and for placental and fetal growth in mammals [7] (**manuscript 3**). Interestingly, maternal nutrition can influence the metabolism of the offspring, which is defined by the term "fetal programming" [7]. This gives two interesting interpretations of the behavior of *Drosophila* towards polyamines: Is the gravid fly attracted to polyamines because it is beneficial (1) for itself; or (2) for the offspring? It would be interesting to analyze the dietary effects of polyamines in the long run not only towards behavior, but on the metabolic level and its influence on the offspring.

Particularly after our findings with respect to the olfactory pathway (**manuscript 2 and 3**), it would further be good to know how polyamines are perceived in the gustatory system. We have at least one candidate for the sensory perception on the gustatory level, namely IR76b [13], but are still lacking a potential co-receptor. It is difficult to pinpoint questions in the gustatory field, since we are still lacking tools for understanding the gustatory pathways in more detail. Maybe future methods will help to shed more light on the gustatory pathways.

Apart from the beneficial effects of endogenously produced polyamines and exogenous polyamine consumption there are high chances that our findings about reproductive state dependent changes in polyamine preference also hold true for other nutrients. Similar analyses of reproductive state dependent behavior towards ammonia have not shown a significant difference (data not shown). One may argue that the beneficial effects of ammonia are not as clear as the ones of polyamines. However, reproductive-state dependent behaviors can occur with different nutrients. Upcoming findings may be of great interest.

3.7 Upcoming methods for scientific thrive

Scientific technology and computational applications are more prominent than ever: from transcriptome studies, over predicting protein structures, to more than classical models to explain, test, and link neural networks, and activities. EM circuit reconstruction allows scientists to have full connectomes of brains in model organisms. Whole brain imaging over short and long duration grants scientists insight into neuronal activity patterns *in vivo* [261–264]. Computational and technological advances are developing faster than ever. Will we be able to keep up with the speed of constantly changing technologies around us? There are so many techniques out there that it is easy to loose track on the options. Even more, are these technological advances even useful for the biological questions we try to solve?

For instance, clear synaptic networks are helpful to understand the underlying network connections. Classic circuit features, such as loops, feed-across and feed-back circuits are under close investigation. Nonetheless, experimental biology focusing on how past experiences or internal states are represented in such connectomics data is necessary to fill these gaps [217]. Particularly modulatory neurons are of interest, as they are able to integrate different values of neuronal activity. With *Drosophila*'s genetic toolset, we are able to tackle these underlying biological features using the collection of driver lines, which are currently available [48, 50–52, 61–69, 78–80, 82–87, 89, 90, 92–94]. Nonetheless, we are still lacking some driver lines necessary for this research. My collaborators and I were able to image the PNs transsynaptically [69] to polyamine sensing ORNs (**manuscript 2**). There is still no driver line available for these particular PNs. Thus, we still have no information which neurotransmitter these PNs release. It will be of interest to know how reproductive state dependent modulation at the ORN presynapse affects these PNs and if these PNs are themselves modulated by a different source.

Such modulations can be revealed by *in vivo* calcium imaging of the neuronal activity using driver lines (**manuscript 2**). To get the most information out of these images, we need high resolution and microscopy power. However, it is often difficult to adapt high power systems to biological experimental designs. This interplay of experimental biology, and computational methods and technologies will help researchers unravel more about the neuronal systems to understand the brain. Researchers need to be able to understand the technological systems they are using (**manuscript 4**) and the computational methods available (**manuscript 3**) to design new ideas and perspectives on neuroscientific questions. Vice versa, computational and technical scientists can use biology and neuroscience to increase the performance of their underlying methodologies [119]. Rather "philosophical" questions, if biologists are able to fix radios or understand microprocessors [265, 266] (by asking if biological tools are able to solve these technological and clearly defined "model organisms", i.e. radios or microprocessors) should be extended by asking how greatly we could thrive, if we would just work much more closely

together.

3.8 Concluding Remarks

My collaborators and I were able to find a neuronal underpinning of reproductive state dependent behavior, particularly its neuronal modulation (see Figure 3.1 A). We further investigated potential triggers for the switch in mating state. In this research, we have used a broad range of tools, using established and state-of-the-art techniques, to allow for different points of view at various stages of the chemosensory pathway. While some findings have been validated through an array of various methods, others are still open for future investigations (see Figure 3.1 B). We are looking forward to how the topic of reproductive state and its modulatory aspects in the higher brain centers will shape our understanding of the neuronal networks in the brain of *Drosophila* and maybe even other species using the currently and soon available technological advances. The collaboration and interplay of technological features, computational analyses, and biological questions has the ability to raise a new renaissance in the scientific community.

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Appendices

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publications

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Internal state dependent odor processing and perception - the role of neuromodulation in the fly olfactory system.
Sercan Sayin*, Ariane Boehm*, Johanna Kobler* (* equal contribution), (...) Ilona Grunwald Kadow
Frontiers in Cellular Neuroscience *(Jan. 2018). 2018*

Homology-based inference sets the bar high for protein function prediction.
Tobias Hamp, (...) Ariane Boehm, (...) Burkhard Rost
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Cloud prediction of protein structure and function with PredictProtein for Debian.
László Kaján, (...) Ariane Boehm, (...) Burkhard Rost
BioMed research international *2013.3 (2013) pp. 398968–6. 2013*

A large-scale evaluation of computational protein function prediction.
Predrag Radivojac, (...) Ariane Boehm, (...) Iddo Friedberg
Nature Methods *10.3 (Mar. 2013) pp. 221–227. 2013*

artices in preparation for submission

Learning underpins reproductive state-dependent decision making in *Drosophila* females
Ariane Boehm, Anja Friedrich, (...) Ilona Grunwald Kadow

How to Multi Photon Microscopy for Life Scientists
Ariane Boehm, Ilona Grunwald Kadow, Robert Kasper

Nutrition of the future - decoding polyamine receptors with nutri-informatics
Ariane Boehm, Despoina Zapoglou, Dietrich Mostert, Ilona Grunwald Kadow

Eidesstattliche Versicherung/Affidavit

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation 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 this dissertation is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den 5.April 2019

Munich, April 5th 2019

Unterschrift / *signature*

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Declaration of author contributions

Manuscript 1:

"Internal State Dependent Odor Processing and Perception - The Role of Neuromodulation in the Fly Olfactory System." [217]

Authors: Sercan Sayin*, **Ariane C. Boehm***, Johanna M. Kobler*, Jean-François De Backer, and Ilona C. Grunwald Kadow

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Author Contributions: SS, **AB**, JK, and IG drafted, wrote and revised the manuscript. J-FD implemented all figures and drafted and revised the manuscript. All authors approved to the final version and its publishing.

Manuscript 2:

"Learning underpins reproductive state-dependent decision making in *Drosophila* females"

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Manuscript 3:

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