Chapter

Neuro-Olfactory Regulation and Salivary Actions: A Coordinated Event for Successful Blood-Feeding Behavior of Mosquitoes

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Abstract

The synergistic actions of the nongenetic and genetic factors are crucial to shape mosquitoes' feeding behavior. Unlike males, adult female mosquitoes are evolved with unique ability to take blood meals from a vertebrate host for reproductive success which eventually makes them a potential vector. Processing and integration of chemical information in the neuro-olfactory system followed by salivary actions facilitate blood meal uptake process. Thus, deciphering the underlying molecular mechanism of odor sensing through the detection machinery (olfactory system), odor processing and decision-making by decision machinery (brain), and regulation of saliva secretion by the action machinery (salivary gland) is likely to reveal molecular pathways which can be targeted to disrupt mosquitoes' feeding behavior. Here we summarize how smart actions of highly specialized neurosensory systems guide and manage feeding behavior associated complex events of (i) successful navigation to find a suitable host, (ii) making food choice decisions, and (iii) regulation of the salivary gland actions in mosquitoes.

Keywords: mosquito, olfaction, brain, feeding decision, salivary gland, host-seeking, blood feeding

1. Introduction

Mosquitoes that belong to the order Diptera and Culicidae family account for large biomass of insects' community and are one of the most notorious animals on earth which transmit many blood-borne pathogens. It is only the adult female mosquitoes which bite on human and other vertebrate hosts to access the blood and thus have strong impact on epidemiological consequences. So far about 3540 species of mosquitoes have been recognized that are divided into 2 subfamilies and 112 genera [1] which are inhabiting throughout the temperate and tropical regions. *Anopheles* species biodiversity and species richness seem to be one of the dominantly evolved blood-feeding insect species race on earth, impacting millions of lives through transmitting deadliest disease malaria around the globe. In tropical areas, *Anopheles gambiae*, *Aedes aegypti*, and *Culex quinquefasciatus* are the most notorious mosquito vectors of infectious diseases such as malaria, dengue, and filariasis, respectively. The main causative agents of malaria are *Plasmodium falciparum* and *Plasmodium* *vivax* which infects millions of people each year, posing a major threat to society [2]. Arboviruses, viz. dengue, Zika, chikungunya, and yellow fever viruses, are also significant mosquito-borne pathogens that are mainly vectored by *Aedes* mosquitoes.

The mosquito-borne diseases not only are restricted to underdeveloped countries but also escalate in the developed world. Urbanization, continuous climate change, global warming, and other environmental factors are facilitating mosquitoes' adaptation and survival during adverse situations [3, 4]. Taken together, it is not hard to predict the situation of mosquito and other insect-borne diseases becoming exacerbate in the coming century [5, 6]. Even the diverse ecological and epidemiological settings within Southeast Asia favor the association of diverse Anopheline fauna which makes malaria prevalence and malaria eradication more challenging. In order to save humans from the mosquito's infectious bites, advanced chemical insecticide(s) still play a central role; however, fast emergence of insecticide resistance and increased toxicants to the environment demands the development of new molecular tools. Thus, it is challenging to understand the complex biology of mosquitoes which popularize themselves as the most dangerous animals on earth (https://www.statista.com/chart/2203/the-worlds-deadliest-animals/).

Disease transmission by mosquitoes is restricted to the blood-feeding behavior of adult female mosquitoes which takes blood meal from humans and other vertebrate hosts for the completion of their gonotropic cycle. In order to carry out the successful blood-feeding event, the integration of the "olfactory system," the receiver of the chemical/environmental stimuli; the "central nervous system," the hardware system with high processing efficacy; and the "salivary gland," the output/feedback device are obligatory. Here we provide an overview and update the current knowledge on how the sensory system of mosquito detects essential chemical information which are then processed by the central nervous system for successful navigation and stimulate the salivary gland for salivation to facilitate the feeding event.

2. Mosquito feeding behavior

Feeding is a fundamental need of every animal to achieve their optimal growth, survival, and reproductive requirements. But the strategy of food intake and the feeding preference largely vary depending on the internal metabolic needs, i.e., whether they are starved or satiated; on the internal physiological state, i.e., whether they are virgin or mated and gravid or unfed; and also on the external sensory stimuli. In the case of mosquitoes, plant sugars such as nectar and honeydew are the primary energy source for survival, flight, and foraging activities of both males and females. Only adult female mosquitoes take blood meal as an optional dietary supplement, and this specialization is predicted to evolve for better fitness [7–9]. Genetic architecture and the allelic polymorphism of different mosquito species influence their traits towards selection and preference for feeding hosts. Apart from that other internal factors such as circadian rhythm and physiological status including nutritional and mating status, as well as environmental factors such as temperature and humidity, affect mosquito feeding behavior cumulatively [10, 11] (**Figure 1**).

Each feeding event of mosquitoes is initiated by random navigation from a long distance, which becomes specific when triggered by a certain group of chemicals such as CO_2, lactic acid, 1-Octen-3-Ol, acetone, ammonia, etc. The detection of other additional cues such as visual and thermal factors facilitates the downstream events of host localization, landing over the host and searching for a suitable site for probing by the proboscis to initiate blood-feeding. But, successful navigation does not always corroborate with a successful feeding event, because it involves another level of regulation of the central nervous system by discriminating the

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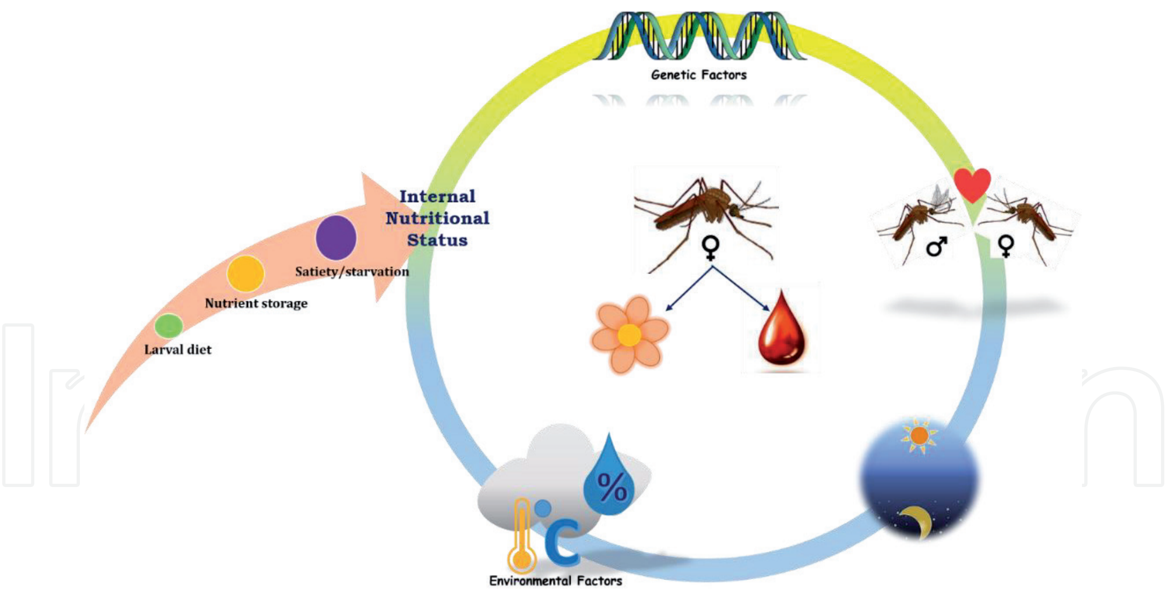

Figure 1.
Factors affecting mosquitoes' feeding behavior. The genetic structure, circadian cycle, mating status, internal nutritional status, and external environmental factors such as temperature and humidity cumulatively work to shape the mosquitoes' feeding preference toward either sugar meal or blood meal. The internal nutritional status is dependent on larval nutrition, the amount of nutrient storage, and the feeding condition of the mosquito, i.e., either starved or satiate.

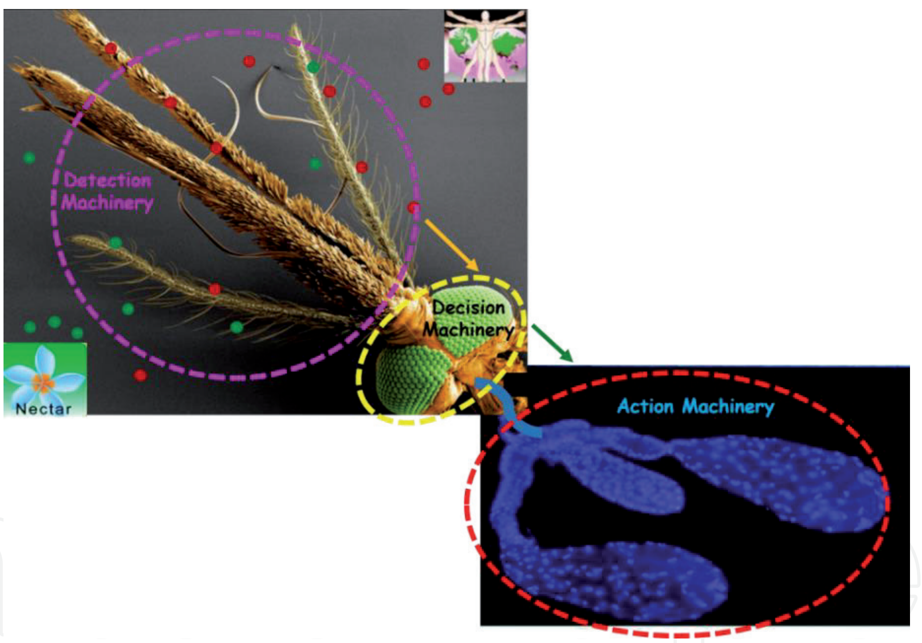

Figure 2.
The path of signal processing for achieving successful mosquito navigation and feeding. The tripartite inter-organ communication among three tissues, viz., olfactory tissue, central nervous system (brain), and the salivary gland, is crucial for the completion of feeding events. The olfactory tissue (highlighted by purple circle) senses and binds to odor molecules emanating from either plant or vertebrate host and sends the respective signal towards the brain system (highlighted as yellow circle). After processing the initial signal of odor in the central nervous system, the decision-making process occurs, and then the brain sends the signal towards the salivary gland (highlighted as red circle), and the process of salivation started to facilitate feeding. Photo credit goes to Zwiebel Lab, Vanderbilt University, for the olfactory system of Anopheles *mosquito. The salivary gland picture was taken from the research article by Ghosh et al. [12].*

odor molecules and making a decision for either to feed or not. Post landing and piercing on a particular site of the vertebrate host, successful uptake of blood meal largely depends on the proper functioning of the salivary gland which acts as the final action machinery by facilitating the feeding process through salivation. Thus, here we provide a detailed integrative description of (1) how mosquitoes detect and discriminate among odorant molecules through olfactory system, (2) how the initial signal of odor is processed in the central nervous system/brain and the molecular factors responsible for feeding decision-making process, and (3) how the brain influences and regulates distant tissue such as salivary gland for salivation and consequently helps in food acquisition (**Figure 2**).

3. Mosquito navigation

A sophisticated olfactory system of mosquitoes enables them to communicate and responds to the diverse array of biological and environmental chemical stimuli throughout their life cycle. They use olfactory cues for locating a food source (nectar sugar), finding a mate partner, locating oviposition site, and most importantly selecting a vertebrate host for blood-feeding. Among these olfactory-guided behaviors, searching and locating the desired plant for nectar-feeding involves both visual and chemical cues emanating from different plant species [13]. Volatiles such as mono- and bicyclic monoterpenes are major floral odors for mosquito attraction, and lighter-colored plant flowers have an additional benefit for successful sugar feeding [14]. But, detection of blood-feeding host requires the integration of olfactory, visual, thermal, and humidity cues [15, 16]. The pattern of host-seeking behavior and selection of a certain host are strictly species-specific. However, the navigation trajectory of all the blood-feeding mosquitoes may have some common events (**Figure 3**).

a. Female mosquitoes are engaged in random, non-oriented navigation until they encounter a plume of host odorants including skin emanates consisting of hundreds of chemicals.

b. Random navigation became oriented when a female mosquito detects fluctuation in the carbon dioxide concentration above the atmospheric measurement, caused by the addition of ~4% CO_2 from human breath. The mosquito then follows the trail of odor plume and initiates to fly upwind in a zigzag pattern which drives mosquitoes to reach the odor source. The concentration gradient of different odorants, initiating from CO_2 from long distance (> 10 m), overlaps with other host odors such as lactic acid and 1-octen-3-ol available at closer vicinity, which acts in a synergistic way to make the navigation successful.

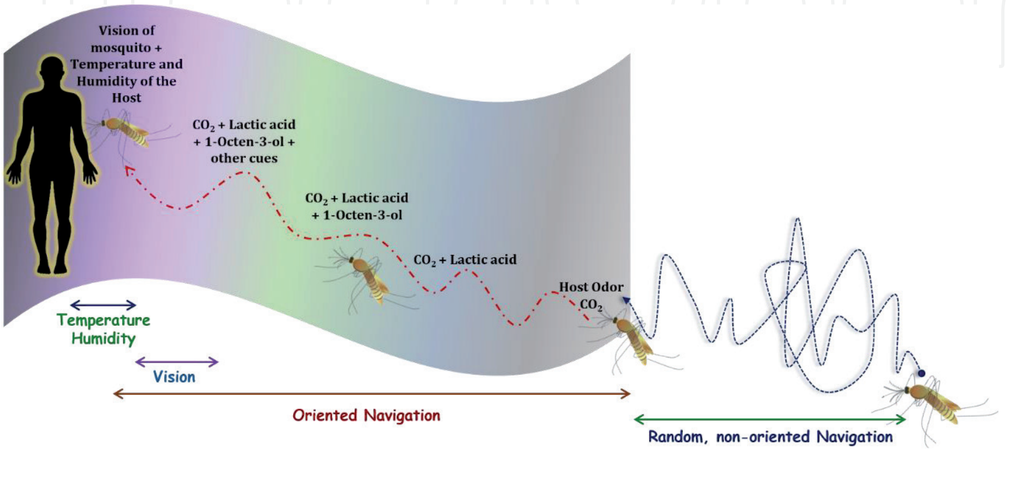

Figure 3.
Mosquito navigation trajectory according to odor plume. The random, non-oriented navigation becomes oriented when mosquitoes sense a gradient of different host odors such as CO_2, lactic acid, 1-octen-3-ol, etc. olfaction along with vision, thermosensation, and hygrosensation facilitates the navigation process and blood meal uptake.

c. When mosquitoes reach in the close vicinity of their potential host through olfactory-guided random to specific navigation, the cumulative role of visual thermosensors enhances. The compound eyes of mosquitoes enable them to visualize a particular host at a distance of 5–10 m by discriminating the light intensity and color of the respective host.

d. After host localization, a mosquito must first land over the host for biting. Temperature and humidity also play a crucial role in short-range host orientation and landing. Then, mosquitoes trace a suitable site for probing through mechanosensory system. The olfactory appendages labellum and stylets along with the peripheral appendages (legs) of mosquitoes finally determine the probing site for successful blood meal uptake.

4. Mosquito sensory system and olfactory signal transduction

It is not difficult to admire that the multimodal sensory system of mosquitoes is the critical regulator of different behavioral processes and thus has a potential impact on disease transmission. In addition, the wide diversity of host preference in mosquitoes is governed by the different genetic makeup of individual species which has strong epidemiological consequences. Therefore, decoding species-specific molecular factors of the mosquitoes' olfactory system may unravel the mechanism of their behavioral plasticity. Two primary components of the chemosensory system are the peripheral system where the chemical information is detected and the central processing unit where the initial signal of odor is processed. The appendages present on the head of the mosquitoes act as the principal detection system, which includes paired antennae, paired maxillary palp, and a labium [8, 17]. These peripheral appendages are equipped with fine hair-like structures called the sensilla, which are distributed nonrandomly across these antennae, maxillary palp, and labium. The type and number of sensilla present on the olfactory organ are highly species-specific [8, 17]. Odorants are thought to penetrate through the numerous pores present on the wall of the sensilla and then traverse through the aqueous sensillar lymph towards the array of molecular receptors present on the dendrites of olfactory receptor neurons (ORNs) [18]. Binding of the diverse odorants with their cognate receptors either activates or inhibits the receptors by changing the ORN action potential. More than two decades of research on insect olfaction uncover several molecular factors that are responsible for odor detection and downstream signal transduction processes. These include odorant-binding proteins (OBPs), odorant-degrading enzymes (ODEs), odorant receptors (ORs), sensory neuron membrane proteins (SNMPs), G proteins, arrestins, and other signaling molecules [8].

4.1 Odorant-binding proteins and odorant-degrading enzymes

Odorant molecules are hydrophobic in nature which require cargo to traverse through the sensillar lymph to reach the receptor molecules, which are present on the dendritic membrane [19–21]. This role is carried out by the odorant-binding proteins (OBPs), which act as a passive carrier of the chemical odorant molecules. OBPs are water-soluble globular proteins containing six α-helical domains with conserved cysteine residues [22]. The number of genes encoding different OBPs varied across different mosquito species and is also dependent on the number of odorant receptors [22]. The availability of this wide and diverse spectrum of OBPs in the insect's tissues facilitates their rapid adaptation in distinct environment. The OBP family broadly includes the pheromone-binding proteins (PBPs) which transport pheromones and different chemosensory proteins (CSPs) which are smaller in size but can bind with a broad spectrum of semiochemicals. In mosquitoes, the OBP genes are classified in three subfamilies: (i) classic OBPs that carry a conserved motif consisting of six cysteine residues; (ii) Plus-C OBPs which contain six additional cysteine residues with novel disulfide connectivity along with three classic OBP motifs; and (iii) atypical OBPs, the longest OBPs that contain a single classic OBP domain in its N-terminal which is extended by a C-terminal extension. Among these three subfamilies, Plus-C OBP class is more divergent in nature and has only been identified from Diptera *Anopheles*, *Culex*, and *Drosophila*; however, Hymenoptera and Lepidoptera did not possess these OBPs. The first OBP of mosquito origin was isolated from the antennae of female *Culex quinquefasciatus* (CquiOBP1) in the early twenty-first century [20, 23]. The availability of genome sequence of several mosquito species in the public domain facilitates the identification and characterization of this large family of OBP genes from different mosquito species, for example, the total number of 69 OBPs from *A. gambiae*, 111 OBPs from *A. aegypti*, 109 OBPs from *C. quinquefasciatus*, *and* 63 OBPs in *A. culicifacies* [20, 24, 25]. Activation of the chemosensory receptors by odorants also requires timely termination and desensitization of peripheral signaling to maintain sensitivity of ORN-based signaling [26, 27]. In this process, odorant-degrading enzymes (ODEs), particularly several esterases and cytochrome p450s, play a crucial role by terminating the odor-induced signal transduction processes [28–30].

4.2 Odorant receptors

After the OBPs, the principal molecules in odor detection are odorant receptors that convert the chemical signal into electrical outputs and therefore ensure the continuous flow of information from the environment to the insect brain [31–33]. Within the insect phylum, odorant receptors (Ors) were first identified and characterized from the model insect *Drosophila melanogaster*, using an intensive bioinformatics approach [34, 35]. Insects' OR proteins consist of seven transmembrane domains with inverted topology, where N-terminus is intracellular, as compared to mammalian odorant receptors, which are the conventional G protein-coupled receptors (GPCR) [36, 37]. Further experimental evidence suggested that mosquito ORs act as ligand-gated ion channels comprising of heteromeric complexes of two subunits [38, 39]. One subunit is highly conserved and known as olfactory receptor co-receptors (Orco), and the other subunit is largely divergent in terms of number as well as amino acid sequences (Orx) [31, 40–42]. Pilot studies of the OR gene repertoire primarily in *Drosophila melanogaster* [35] and later in mosquitoes [42, 43] suggest that despite having a limited number of ORs, mosquitoes can respond to an array of varied chemicals depending on the specific demand at different life cycle stages [44]. This is possible due to the combinatorial coding mechanism of the insect's olfactory system which increases the perceived odor space of each species. Combinatorial coding increases odor sensitivity to several-fold, where each OR can respond to multiple ligands and a single ligand can activate more than one OR [45]. Moreover, a single odorant can either elicit attractive responses or activate repellent pathway depending on their quality and concentration which subsequently determine insects' behavior [46, 47].

The number of receptors present in each mosquito species is highly variable, e.g., *A. gambiae* genome contains 79 OR genes, *C. quinquefasciatus* has 117, and *A. aegypti* possesses 110 OR genes [47]. Among them the receptor protein Or8 is predominantly studied in mosquitoes because of its conserved nature and specificity towards 1-octen-3-ol, which is a crucial component of human sweat [48, 49]. Deorphanization of other olfactory receptors of mosquitoes was performed using an in vivo heterologous expression system, the "empty neuron" system, originally established in the fruit fly *Drosophila*. The "empty neuron" system is a combination of a GAL4 driver line and a mutant ORN line (UAS—"OR gene") where endogenous odorant receptor is missing and thus gives the opportunity to express and functionally characterize mosquito olfactory receptor gene repertoire. The complexes of both *Drosophila* and mosquitoes using "empty neuron" model indicated that the "odor space" of mosquito and flies is significantly distinct [50, 51]. Furthermore, over the evolutionary time scale, the sensitivity of a particular mosquito OR either increases towards certain predominant hosts or decreases if the host odor profile changes [47]. Thus, it is not difficult to predict that ORs have evolved with highly sensitive and selective property for the detection of diverse odorants which consequently facilitate mosquito adaptation in diverse ecology.

4.3 Other sensory receptors

After detection of a particular odor through synergistic actions of OBPs and ORs, mosquitoes use other sensory modalities such as vision, thermosensation, and hygrosensation to make the differentiation between biting hosts [15, 47, 50]. For visualization, the photoreceptor cells expressing multiple UV-sensitive and long-wavelength sensitive opsin proteins are responsible for detecting and transmitting visual information towards optic lobe (the region of mosquito brain where optic information is processed) [52]. But how mosquitoes integrate visual information with other cues to differentiate hosts remains unclear. Following visual selection, the temperature and humidity are intricately linked to make biting decision [47, 50]. The thermosensory transient receptor potential (TRP) channel protein present on the tips of the antennae of mosquitoes can sense the variation of temperature associated with vertebrate skin [53]. For hygrosensory information processing, the ionotropic receptors (IRs) are reported to play a crucial role in *Drosophila* [47]. Although the role of IRs in humidity sensing in mosquitoes remains elusive, few recent studies highlight their sensitivities against narrow range of odorants such as amines and carboxylic acids and thus have potential function in host-seeking [54].

Once the host is located by the harmonious actions of all the sensory modalities, the mosquito first lands over the host and engages in a mission of locating a proper site for probing by repeated contacting of the skin with the labellum [50]. The gustatory receptors (taste receptors) (GRs), expressing on the labellum and the tarsae (the last segment of their legs through which mosquitoes make contact with the host), may play a pivotal role in biting behavior of mosquitoes [47, 55]. While the functional characterization of mosquito OR genes are of prime focus, a significant number of studies reported that the putative gustatory receptors (Gr1, Gr3 in *A. aegypti* and *C. quinquefasciatus*; Gr22, Gr24 in *A. gambiae*) of mosquitoes are sensitive to CO_2 and thus influence host-seeking behavioral activities [47, 50].

4.4 Olfactory signal transduction

The information, i.e., hidden within the odor molecules, are amplified by activating the sensory neurons. The activation of a different subset of sensory neurons to a different degree is the basis for neuronal coding. When compared with the vertebrate OR, the insect's ORs show a high degree of variation with different topologies, which strongly suggest a different signal transduction mechanism [8]. Some previous studies highlight that olfactory signal transduction in insects involving a ligand-gated ion channel that is formed by the hetero-dimerization of diverse odorant receptor and its co-receptors [27]. This fast ionotropic response does not postulate the involvement of any G proteins and any intracellular second messengers. In contrast, another study indicated the entanglement of G protein and the synthesis of cAMP, IP3, and other secondary messengers that consequently induce the downstream effector enzymes and also affect the membrane potential through activating the co-receptor protein [27, 56]. The resultant change in the membrane potential/permeability by either process causes the generation and propagation of action potentials along the ORN axon membrane towards the antennal lobes. In contrast to the rapid ionotropic pathway, the G protein-mediated metabotropic pathway is slower. However, it plays an important role when the odor cues are present in lower concentration, whereas high concentration directly involves the ionotropic pathway [8, 27, 57].

5. The decision-making unit: the brain

The discrimination and integration among the odor molecules and the exchange of electrochemical information consequently influence the neuronal decision-making abilities of the brain system [58]. When an animal is given preference for food, several decisions can be made such as whether to eat or not, what to eat, and when to eat, which not only depends on the internal physiological condition but also relies on the biological clock of the respective animal. In the case of mosquito species, making a choice among the different available foods requires a fine-tuning of the nasal system and strong integration of the decision-making machinery. The availability of diverse nature of blood-feeding hosts not only makes the decision-making process more complex but also has an impact on mosquito survival, fitness, and fecundity [59].

5.1 Structural basis of signal processing

The knowledge about insect olfactory coding is strongly rooted in the fruit fly *Drosophila melanogaster*. Over the last two decades, the cellular and molecular bases of *Drosophila* olfaction have been studied well with the assistance of varied genetic tools. The three milestones of olfaction have been documented comprehensively in the fruit fly on how odor information is received, concatenated, and processed by the peripheral and central nervous systems, respectively [60, 61]. Apart from that, "the parallel olfactory processing" and "feature detection" mechanism has also been unlocked in honey bee brain and sphinx moth, respectively [62–64]. Several studies on *Drosophila* and other insects (*Manduca sexta and Bombyx mori*) suggested that the primary brain structures responsible for receiving initial information of odor are the antennal lobes (ALs) [62, 63, 65, 66]. These antennal lobes consist of a specific number of spherical condensed neuropil structures, which are known as glomeruli. Depending on the nature and sex of the insect species, the number of glomeruli varied between 50 and 200, whereas each respective species possess the same number of glomeruli having identical features (shape, size, location) [67, 68]. Olfactory receptor neurons that express a particular type of receptor on their dendrites project their axons into the same glomerulus [8, 45, 67, 69]. Furthermore, each glomerulus is housed with the arms of the local interneurons (LNs) and the dendrites of the projection neurons (PNs) [69]. Thus, within the antennal lobe, a synaptic connection is formed between olfactory receptor neurons and antennal lobe interneurons. From the antennal lobe, the olfactory information is transmitted to a higher brain center by the projection neurons [8, 69, 70] (**Figure 4**). Horizontal innervation of the local interneurons within the glomerulus facilitates interglomerular communication. The primary neurotransmitter found to communicate between local interneurons is the gamma-aminobutyric acid (GABA) which facilitates the generation of Na^+-mediated action potential in response to olfactory stimulation [8].

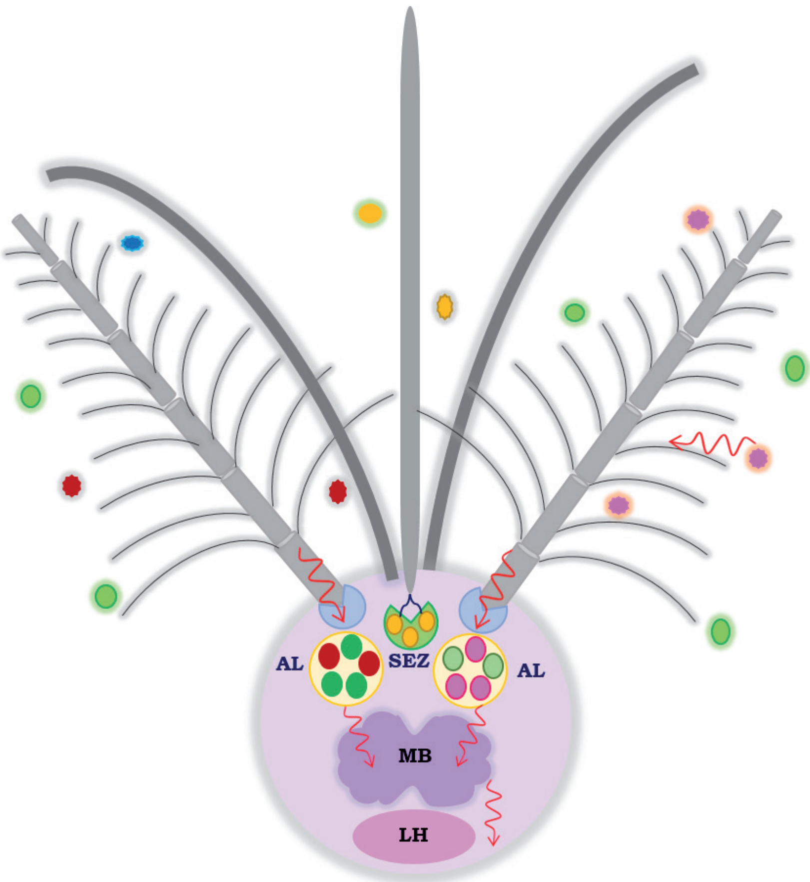

Figure 4.
Schematic presentation of flow of odor signals from the environment to the central nervous system. Odor molecules of diverse nature (highlighted as multicolored small circle) bind to their respective receptors present on the olfactory receptor neurons of antennae of mosquitoes. Then the initial signal of odor is transmitted to the antennal lobe (AL), and from the AL the signal is transmitted to higher brain centers, i.e., mushroom body (MB) and lateral horn (LH) for signal processing and decision-making. The red arrow indicates the path of signal flow.

Vertically arranged distinct fiber tract of the projection neurons connects the ALs to the higher brain centers such as calyces of the mushroom body and the lateral horn of the protocerebrum [8], where olfactory information is integrated with other sensory cues. The cell bodies of the PNs are located at the periphery of the antennal lobe glomeruli and their axons spread in the higher brain center. The branching pattern of PNs is either uniglomerular or multiglomerular [69]. The functional characterization of odor coding properties of individual ORN targeting each glomerulus revealed the existence of strong sexual dimorphism between male and female AL glomeruli which lead to a highly specialized odorant response towards general odorants and sex pheromones. PN odor response is not identical to the ORN odor response. The majority of the PNs are broadly tuned with respect to the general odors and send their dendritic arbor into the ordinary glomeruli (OG) (both uniglomerular and multiglomerular) which respond vigorously during the odor onset [69, 70]. This results from the high convergence of ORNs expressing the same odorant receptor into a single glomerulus. Generally, ORNs project its axon into a particular glomerulus, and PNs receive input from all of the ORN axons entering into that cognate glomerulus. As a result, the signal gets amplified many folds which makes the PNs very sensitive to small changes in the presynaptic ORN input. But the PNs associated with sex pheromone target different regions of the lateral horn in a sexually dimorphic manner, and thus the same pheromone elicits distinct behavior in males and females [32, 68, 71]. Most of the ORNs to PN synapses are cholinergic, and PNs respond more strongly to the fluctuating amount of odors in the odor plume [8, 32, 68]. Next, the PNs form synaptic connections with Kenyon cells, neurons of the protocerebrum, and mushroom bodies. Odor information from multiple glomeruli finally merges into narrowly tuned Kenyon cells which affect memory formation [65].

Despite the knowledge about the neuronal firing path during odor transmission, a pilot question arises in mosquito neurobiology on how discrete sensory inputs integrate and translate into varied behavior. An in-depth understanding of the neuronal circuitry involved in olfactory signaling and decision-making in mosquitoes is limited due to the absence of established neurogenetic methods. A recent study by Olena Riabinina et al. suggested that an integration of the olfactory and gustatory signals commenced within the antennal lobe and subesophageal zone of the brain, respectively [72]. Furthermore, Clement Vinauger et al. reported that despite having a synergistic effect during mosquito navigation, the visual and odor modulation is asymmetric and processed by distinct loci of the brain, where olfaction always works preceding to visual selection. But detailed understanding of the molecular and neurophysiological bases of mosquito olfactory behaviors and crucial decision-making events in the brain needs further research.

5.2 Molecular physiology of neuronal signal processing

Mosquitoes are well known for their plasticity in host preference. The selection of host species for blood meal uptake is skewed depending on the availability of the preferred host, the quality of blood meal, and the defensive behavior of the host. Apart from the neuronal firing and neurotransmitter-mediated signal transmission, the molecular factors of the brain are shown to play a crucial role in olfactory learning, neuronal decision-making, and memory formation in insects [73, 74]. The diverse neuromodulators that include neurotransmitters, neuropeptides, neurohormones, and biogenic amines facilitate the nervous system to transduce varied signals and thus enable the insects to manage the complex behavioral events with amazing accuracy [8, 11]. Neurotransmitters are the primary and potent neurochemicals that make synaptic connections between neurons and thus relay information from presynaptic cells to postsynaptic cells. The crucial neurotransmitters in the insect chemosensory system are acetylcholine, gamma-aminobutyric acid (GABA), and nitrous oxide (NO) [17, 75, 76]. Our ongoing study has shown that blood meal intake causes dynamic changes in the neurotransmitter abundance within the brain, suggesting their possible contribution in cognition and food-associated memory formation in adult female mosquitoes [77]. Furthermore, we also showed that the gut of the mosquitoes also can synthesize neurotransmitters and play a crucial role in gut-brain-axis communication during metabolic switch (sugar-fed condition to blood-fed condition) and thus modulate neuronal decision-making process [77].

Neuromodulators include the neuropeptide and biogenic amines, which have an intense effect on mosquito chemosensation, feeding, social behavior, circadian rhythm, and also maintenance of general physiological homeostasis [11, 17, 78–81]. Usually, these neuromodulators are produced by the specialized neurosecretory cells and released into the local vicinity of the brain circuits and in the hemolymph. Both neuropeptides and biogenic amines modulate the response through G protein-coupled receptor signaling pathway [11, 79]. Two important amine neuromodulators are dopamine and serotonin which are found to modulate mosquitoes' learning and memory response. The immunoreactive neurons of serotonin and dopamine innervate all the glomeruli of AL and higher brain regions such as lateral horn and mushroom body, indicating their role in memory formation [73]. Apart from the biogenic amines, 28 neuropeptides have been predicted from the genome

Sl. no.	Peptide hormone name	Function
1.	Adipokinetic hormone	Mobilizes stored carbohydrate
2.	Allatostatin A and C	Regulate juvenile hormone biosynthesis and gut motility
3.	Allatotropin	Stimulates juvenile hormone biosynthesis
4.	CCHamide 1	A nutrient-responsive hormone in *Drosophila* but function not known in mosquitoes
5.	Corazonin	Cardioactive peptide
6.	Diuretic hormone	Myotropic activity, regulation of Malpighian tubule for fluid secretion, osmoregulation, and diuresis
7.	Ecdysis triggering hormone	Trigger ecdysis during larval and pupal molting
8.	Eclosion hormone	Function not known in mosquitoes
9.	FMRFamide	Heart contraction
10.	Insulin-like peptide	Elevate carbohydrate and lipid storage, female reproduction, vitellogenesis, hemocyte differentiation, blood meal digestion
11.	Leukokinin	Diuresis
12.	Neuropeptide F	Inhibition of anterior midgut peristalsis in larval stage
13.	Ovary ecdysteroidogenic hormone	Induces ecdysone production and egg development
14.	Prothoracicotropic hormone	Regulates metamorphosis
15.	Pyrokinin	Regulation of diuresis
16.	Short neuropeptide F	Regulation of host-seeking behavior
17.	Sulfakinin	Function not known in mosquitoes
18.	Tachykinin	Function not known in mosquitoes

Table 1.
List of peptide hormones and their possible functions.

database of *Aedes aegypti* through the bioinformatics approach [82]. Among them, short neuropeptide F (sNPF) was found to play a crucial role in mosquito feeding and inhibition of host-seeking behavior following blood feeding [83, 84]. Recent studies provide contrasting evidence that either sNPF is synthesized from male accessory gland and transferred to the female during mating or female's own sNPF titer is increased in the hemolymph after consumption of blood meal significantly and reduces host-seeking behavior in adult females. While the functions of different neurohormones have been studied in many insects, functional studies in mosquitoes are limited. The wide distribution of peptide hormones throughout the mosquito body from the neurosecretory cells of the brain (corpora allata, corpus cardiacum) to the endocrine cells of the gut enable them to perform diverse function in mosquito physiology such as (1) regulation of metabolism, (2) maintenance of physiological homeostasis, (3) metamorphosis and eclosion, (4) osmoregulation, and (5) regulation of vitellogenesis and gonotropic cycle [11]. **Table 1** summarizes the name of peptide hormones in mosquitoes and their possible functions.

6. The action machinery: salivary gland

A successful feeding event of mosquitoes is regulated by the synchronized action of mosquito navigation and food choice decision which finally tuned with the salivary gland action for successful food uptake. The endocrine system of the salivary gland induces saliva secretion that gets mixed with foods and facilitates the food intake [85]. Mosquitoes have paired salivary glands in their thorax which is flanking in the esophagus. During sugar feeding saliva is mixed with the sugar, and the mixture enters into the crop where digestion commenced. During blood meal ingestion, salivary gland secretions serve in blood vessel localization [86]. The hemostatic and immune factor of the vertebrate host makes the blood meal uptake process challenging for mosquitoes [86]. Thus, salivary glands of mosquitoes are evolved and adapted with a unique ability to serve the leading function during blood meal ingestion by providing secretory salivary factors such as vasodilators, anticoagulant, antihistamines, etc. [86, 87]. Furthermore, salivary gland components not only support mosquitoes to overcome host homeostasis and defense response but also serve as the primary route for parasite transmission and maintenance of disease cycle [86, 88]. Due to the involvement of salivary gland in malaria transmission, most of the previous studies are restricted to the role of salivary gland in blood feeding and pathogen survival [89]. A recent study by Sharma et al. showed that salivary gland has a distinguished ability of gene expression switching to manage the meal-specific (sugar vs. blood meal) molecular responses [90]. But, our understanding of the regulatory mechanism of the neuro-olfactory system modulating salivary gland cocktail composition depending on the type of food is still in infancy.

6.1 Mosquito sialome leads to feeding success

To feed on a vertebrate host, the arthropods are required to overcome a series of obstacles [86]. The saliva produced by the hematophagous insects contains bioactive molecules that counteract host defense [91]. Mosquitoes are reported to feed on arterioles and venules rather than capillaries, and they often probe multiple times at different sites to find a suitable site for feeding [86]. Initiation of feeding induces hemostatic cascade within the host including the platelet aggregation followed by collagen interaction with ADP which supports the blood coagulation pathway [87, 92]. The presence of secretory apyrase enzyme in the salivary gland of blood-feeding arthropods inhibits platelet aggregation by hydrolyzing ATP and ADP into AMP and inorganic phosphate [93]. Vasoconstriction is a common phenomenon following laceration of blood vessels due to insect bite to minimize blood flow and hence loss of blood [87]. The hematophagous insects, including *Aedes aegypti* mosquito saliva, contain sialokinins which act as a vasodilatory molecule by stimulating nitric oxide (NO) production by the endothelial cells via cGMP induction [87, 94, 95]. Except apyrase and sialokinin, salivary specific D7 family proteins have been implicated to function as a scavenger molecule of serotonin, histamine, and norepinephrine and antagonize their vasoconstrictor, platelet-aggregating, and pain-inducing properties [94, 96]. Salivary peroxidases are well known for their potent function as a vasodilator, as it might act as a hydrogen peroxide-dependent destructor of serotonin and noradrenaline [97]. Furthermore, the secretory anophelin protein is reported to inhibit thrombin activity and collagen sequestration and hence delay platelet aggregation [98]. An additional challenge arises from the immune components of the blood meal itself which have been generated during previous exposure of mosquito bites [86]. Thus, successful blood feeding is dependent on the evolution of salivary composition possessing anti-immune molecules to suppress the action of host immune factors. Antitumor necrosis factor in female salivary glands is one of the crucial molecule that may play anti-immune function in hematophagous insects [86].

6.2 Neurological control over salivation

The experimental evidence about the classical conditioning of salivation in dogs was demonstrated by Pavlov in the early nineteenth century [99, 100]. By definition, classical conditioning refers to the learning procedure where a conditioned stimulus (CS), for example, the sound of a bell, is paired with an unconditioned stimulus (US), such as food which eventually triggers salivation [100], although secretion of saliva is obligatory to facilitate feeding for majority of animals from invertebrates to vertebrates. However, the knowledge of classical conditioning of salivation is restricted to mammals and invertebrate cockroaches [99]. The salivary gland and the saliva make the bridge that joins the mosquito vectors, parasite, and the host together by facilitating blood meal uptake and parasite transmission [86]. But, the cellular and molecular mechanisms underlying the classical conditioning of salivation in mosquitoes remain unknown. Considering the finicky host-seeking behavior of mosquitoes and their preference towards a certain host [47], it can be hypothesized that mosquitoes can learn during the repeated exposure of conditioned stimulus such as host odor and unconditioned stimulus, which is a reward of blood meal [47]. Reward may be appetitive when mosquitoes get benefited from the blood meal or aversive if mosquitoes experience any kind of host defensive behavior [47]. Thus, it can be speculated that mosquitoes should exhibit classical conditioning of salivation, i.e., increase saliva secretion which is tightly regulated by the neuro-olfactory system (**Figure 5**).

Our knowledge about control of insects' salivary secretion is limited to cockroaches, locusts, and blowflies, where neuronal innervation of the salivary gland or neuro-hormonal regulation was reported to play a significant role in salivation [101, 102]. Insects' salivary glands are innervated with nerves that are originated from different sources of the central nervous system [102]. Stomatogastric nervous system projects its nerves in the salivary gland of *Manduca sexta* [103]. The salivary gland of cockroaches (*Periplaneta americana*) is innervated with nerves that are projected from both the stomatogastric system and the subesophageal ganglion [102, 104, 105]. An exception to that is that the blowfly salivary glands are not innervated, but the salivary secretion is regulated by the secretion of the biogenic amine serotonin [102, 106]. Gustatory stimulation leads to the release of

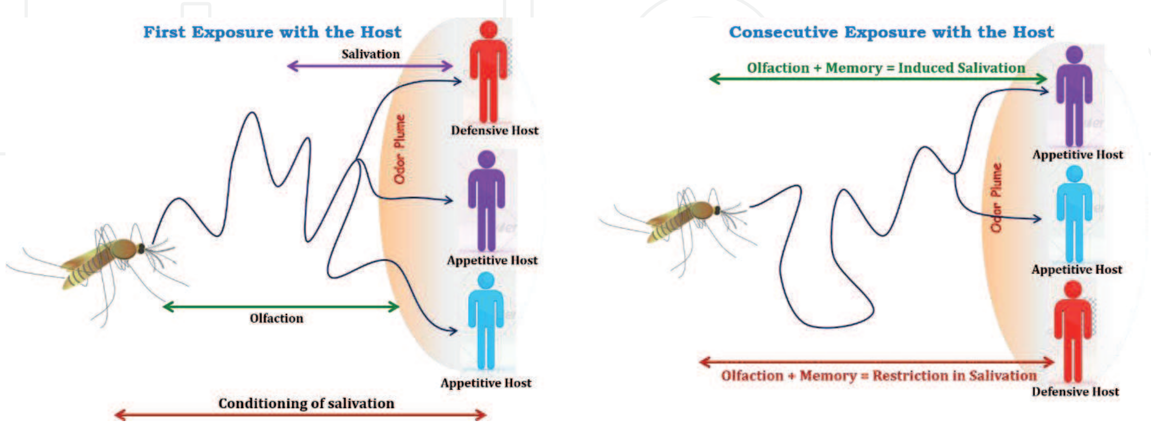

Figure 5.
Graphical illustration of conditioning of salivation in mosquitoes. Mosquitoes navigate towards vertebrate host through olfaction when they sense the odor plume emanating from the host (both appetitive and defensive). Olfaction also induces the salivary secretion (conditioning of salivation) with the aim to facilitate blood meal uptake. But the host's defensive behavior interrupts successful encountering of the mosquito with the host (red-colored human), which mosquitoes can memorize, and during consecutive exposure they probably restrict the salivation process to avoid the respective host, whereas mosquitoes get a reward from the appetitive host through successful blood-feeding without any interference. This positive memory along with olfaction further empowers the navigation process by induction of salivation.

serotonin from the neurons into the hemolymph which acts as a neurohormone and alters the cytosolic calcium (Ca^{2+}) and adenosine cyclic monophosphate (cAMP) concentration within the secretory cells of the salivary gland [107]. The increased calcium level consequently facilitates the movement of chloride (Cl^-) ions from the hemolymph side into the lumen of the gland. On the contrary, cAMP was found to stimulate potassium (K^+) transport towards the luminal side of the salivary gland. The simultaneous induction of two different pathways leads to the activation of either phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP3)/diacylglycerol/Ca^{2+} signaling pathway or cyclic AMP/adenylyl cyclase signaling cascade which

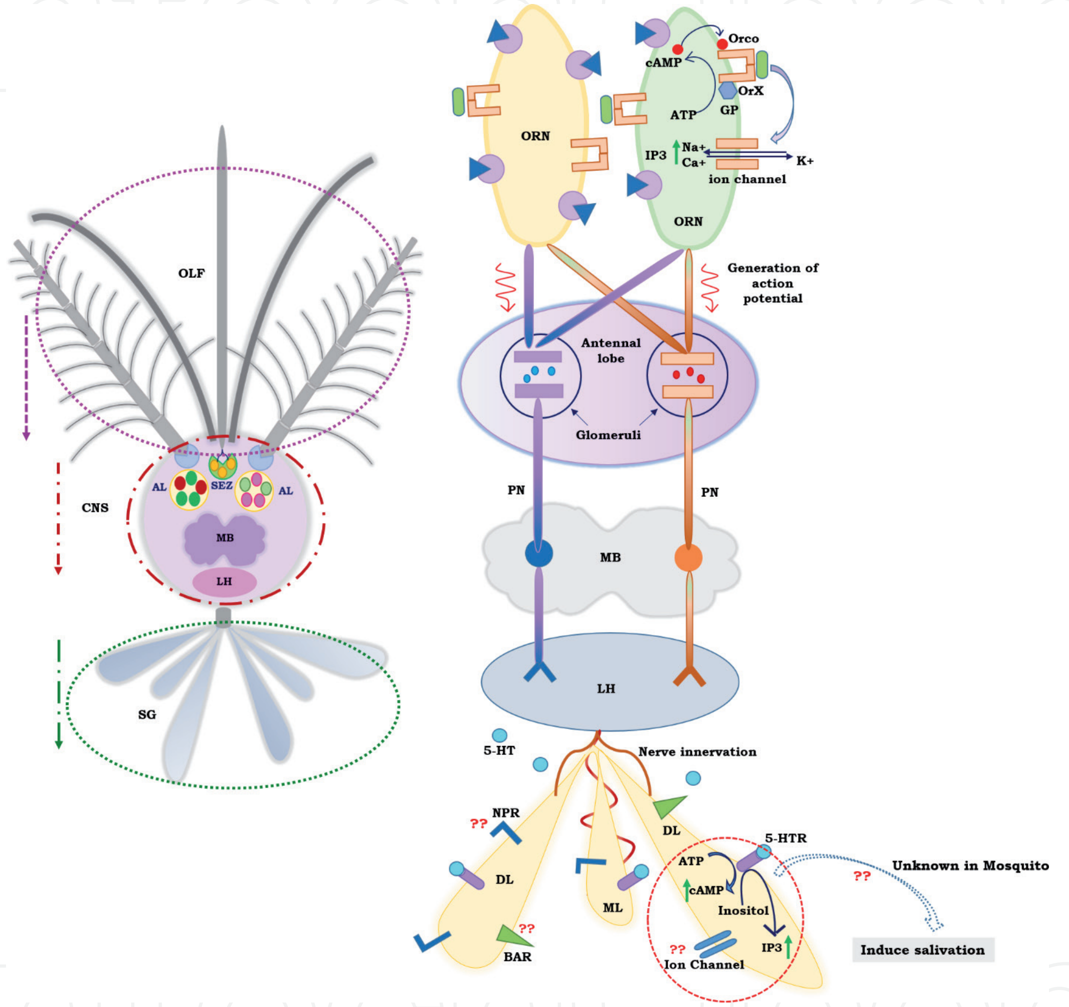

Figure 6.
The tripartite communication of three tissues [olfactory tissue (OLF), central nervous system (CNS)/brain, and salivary gland (SG)] for successful feeding. The left picture showed the flow of signal from odor response to salivary action, which is indicated by the downward arrows. The right picture is the detailed representation of the left one. Primarily, odorants bind with their cognate receptors, present on the dendritic membrane of the olfactory receptor neurons (ORNs). Odor binding initiates the downstream signal transduction procedure, which includes the synthesis of either the second messengers (cAMP, IP3) or change in the membrane ion channel conformation which then allows the flow of ions (Na^+, Ca^+, and K^+) and facilitates the change in membrane potential and consequently generates the action potential. This action potential rapidly moves through the axons towards the CNS (indicated as red arrow). The antennal lobe (AL) is the primary site for odor perception in mosquitoes. The axons of the ORNs expressing the same receptors which bind to a particular odor molecule merge in a single AL (indicated by orange and blue rods). From the AL, the odor signal then transmitted to higher brain centers [mushroom body (MB), and lateral horn (LH)] through projection neurons (PNs). Along with the neuromodulator-mediated regulation, nerve innervation (originating from the higher brain region) also regulates salivation of the salivary gland in insects (indicated by red zigzag lines over the salivary gland). One of the biogenic amines, the 5-hydroxy tryptamine (5-HT), and its cognate receptor (highlighted in sky blue circle and purple rods) facilitate salivation. But this receptor-mediated downstream signal transduction events and the resultant change in salivary gland membrane potential is not known in the case of mosquitoes (highlighted in red circle). The involvement of other biogenic amine receptors (BAR) and neuropeptide receptors (NPR) in saliva regulation is also yet to be explored. DL, distant lobe; ML, medial lobe of the salivary gland.

are the potent secondary messengers found to play a significant role in insect salivation [107, 108]. Studies in blood-feeding insects are limited to *Aedes aegypti* mosquito and on tsetse flies *Glossina pallidipes*, depicting the presence of serotonergic innervation in their salivary glands [109, 110]. Together it can be stated that both the biogenic amines and neuropeptides play a crucial role in insect salivation by modulating the salivary glands' ability to alter second messenger level and ion channel conformation. Furthermore, olfactory conditioning of salivation is directly linked to long-term memory formation which is accomplished by the active involvement of NO signaling for the induction of protein synthesis required for memory signature [111]. Salivary conditioning is also suitable to monitor the activity pattern of salivary neurons located in specific regions of the brain; thus this conditioning system will be suitable for the study of molecular mechanisms of learning and memory formation in mosquitoes' brains (**Figure 6**).

7. Conclusion and future direction

Evolution and adaptation to blood-feeding behavior in adult female mosquitoes provided a natural mechanism for their reproductive success. Here, we propose a system biology approach which defines the harmonious actions of the olfactory, the brain, and salivary glands, regulating the complex feeding behavior of mosquitoes. However, deciphering the molecular basis on how mosquitoes meet and manage the conflicting demands of sugar feeding vs. blood-feeding and how olfactory conditioning of salivation commenced may lead to the identification of crucial molecular targets including different neurohormones, biogenic amines, neuropeptides, and their receptors for genetic manipulation. Functional genomics and the advancement of electrophysiological techniques illuminate our understanding of mosquitoes' sensory systems. Although it is challenging to identify the species-specific potential olfactory factors that play a pivotal role in mosquitoes' host-seeking and blood-feeding behavior, it will be very effective for the development of novel approaches to control different mosquito populations. The efficacy of emerging genetic tools such as CRISPR/Cas9, a gene drive technology in mosquitoes, can facilitate the molecular understanding of neuronal mechanism of olfactory selection and differential learning and memory formation across different mosquito species which can be manipulated for more effective disruption of host-seeking behavior. Furthermore, unraveling the microbiome-gut-brain-axis communication mechanism during metabolic switch in mosquitoes may enlighten the innovative idea of microbiome-mediated alteration of mosquitoes' olfactory perception.

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