Trends in Genetics

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Review

Technological advances in mosquito olfaction neurogenetics

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Gene-editing technologies have revolutionized the field of mosquito sensory biology. These technologies have been used to knock in reporter genes in-frame with neuronal genes and tag specific mosquito neurons to detect their activities using binary expression systems. Despite these advances, novel tools still need to be developed to elucidate the transmission of olfactory signals from the periphery to the brain. Here, we propose the development of a set of tools, including novel driver lines as well as sensors of neuromodulatory activities, which can advance our knowledge of how sensory input triggers behavioral outputs. This information can change our understanding of mosquito neurobiology and lead to the development of strategies for mosquito behavioral manipulation to reduce bites and disease transmission.

Highlights

Gene-editing technologies have been used to mutate multiple sensory receptors, and their roles in mosquito behaviors have been established.

The genomes of mosquitoes have also been modified to encode fluorescent labeling markers and sensors of neuronal activities.

Single cell RNA-sequencing has expanded the repertoire of potential driver lines that can be engineered to investigate mosquito higher brain centers. The establishment of alternative binary expression systems will further resolve the spatiotemporal control of gene expression.

The design and implementation of synaptic sensors can further highlight the details of the neural pathways triggering specific odor-evoked mosquito behaviors.

The establishment of such novel genetic tools can set the stage for the development of strategies of behavioral manipulation to reduce bites and disease transmission.

Current advances in mosquito neurogenetics

Gene-editing technologies and the mosquito sensory system

Gene-editing technologies (see Glossary) have flourished and allow functional genetics studies in many non-model organisms, including mosquitoes [1]. Initial attempts to engineering the genomes of mosquitoes were accomplished using a plasmid encoding a transposable element, such as Hermes, Mariner, and piggyBac, carrying a gene of interest along with a helper plasmid encoding a transposase [2]. Even though multiple genes were inserted in the genomes of mosquitoes using this strategy, the lack of site-specific integration required the development of programmable gene-editing strategies [2].

The zinc finger nuclease (ZFN) gene-editing strategy takes advantage of the zinc finger DNA-binding domains present in multiple transcription factors and the FokI restriction enzyme from *Flavobacterium okeanokoites* [3]. As each zinc finger domain interacts with three nucleotides, the specificity of the FokI-zinc finger domain-fused protein relies upon the presence of multiple zinc finger domains (Figure 1A) [3]. The transcription activator-like element nuclease (TALEN) gene-editing technique relies upon the TALEs of the bacterium *Xanthomonas*, which bind to specific nucleotide sequences based on specific two-amino acid codes [4]. Fusing specific TALEs to the FokI nuclease (Figure 1B) can be used to generate targeted-specific mutations [4].

More recently, a gene-editing technology based on the immune defense system of bacteria against phage invasion was developed: clustered, regularly interspaced, short palindromic repeats (CRISPR) and the Cas9 nuclease [5]. This system comprises an endogenous Cas9 nuclease, a noncoding transactivator RNA (trcRNA), and transcribed RNAs (CRISPR-RNAs or crRNA) from exogenous DNAs (plasmids or bacteriophages) integrated into the CRISPR locus [6]. These three components assemble into the CRISPR-Cas9 complex and perform target-site cleavage of DNA sequences homologous to the crRNAs (Figure 1C) [6,7].

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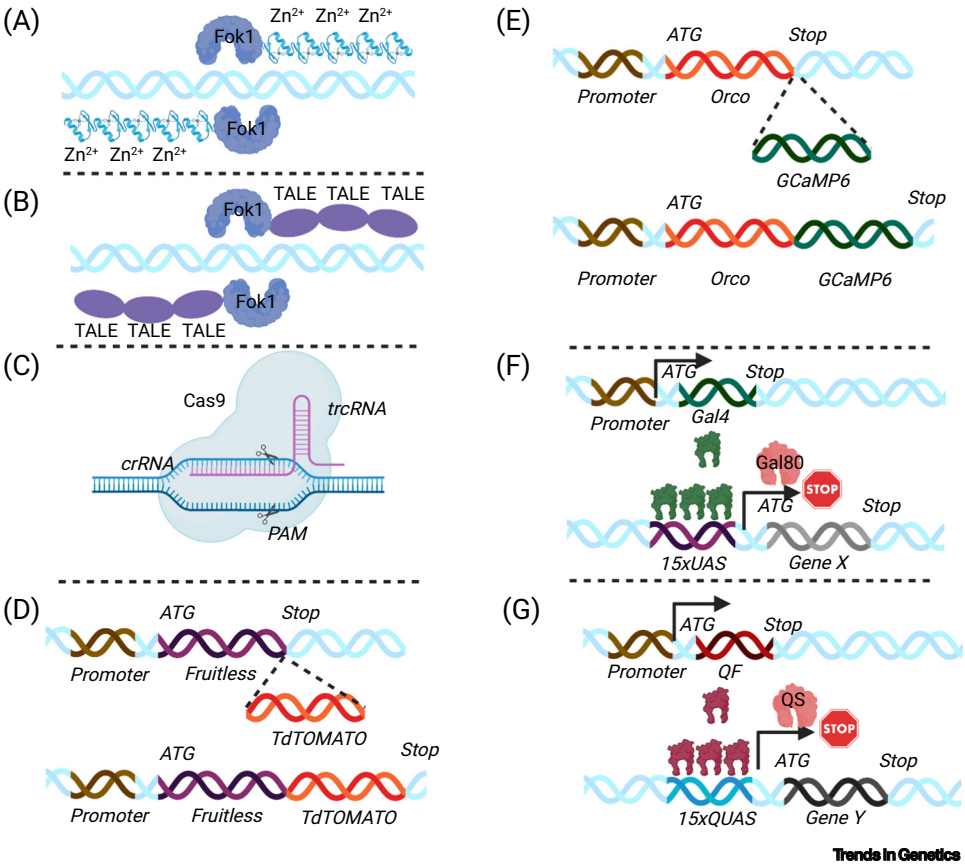

Figure 1. Gene editing in the mosquito olfactory system. (A) Zinc finger nucleases binding to DNA through zinc finger domains and performing double-strand breaks (DSBs) through the nuclease activity of the enzyme FokI. (B) Transcription activator-like element nuclease (TALEN): double-strand DNA breaks mediated by FokI nucleases and DNA binding mediated by the TALE domains. (C) Clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9-mediated DSBs. (D) Construct strategy to express the fluorescent marker TdTOMATO in neurons expressing the fruitless transcription factor. (E) Construct strategy to express the GCaMP6 calcium sensor in neurons housing the Orco co-receptor. (F) Binary Gal4/UAS and (G) Q expression systems. Protein expression is carried out after crossing a mosquito line expressing the transactivator (Gal4 and QF) to another line transformed with the effector sequences (*UAS* and *QUAS*). Abbreviation: PAM, protospacer adjacent domain (*NGG*).

A fused version of the trcRNA and crRNA molecules (guide RNA) has been designed and resulted in a major breakthrough in the advance of gene-editing studies [6]. The fact that the guide RNAs can be designed and easily synthesized allows the use of the CRISPR technology to be programmed and target arguably any DNA sequence, making CRISPR the technology of choice over TALEN and ZFN, which rely upon more laborious protein engineering to target specific sequences.

Even though TALENs have not been used to edit sensory receptor genes, ZFN nucleases were used to induce frame-shift or early stop codons mutations in genes encoding receptors of the mosquito sensory system (Box 1), such as *Orco* [8], *Gr3* [9], and *trpA1* [10], resulting in the expression of nonfunctional receptors. In addition, CRISPR-Cas9 was used to disrupt the coding sequence of multiple receptors of the sensory system as well as a neurotransmitter receptor. These included the genes encoding the dopamine receptor [11], *Ir25a* [12], *Ir8a* [12,13], *Ir76b* [12], the *Gr22-24* CO_2/skin odor **olfactory co-receptors** [14], the *Ir21a* heat receptor [15],

Glossary

Fluorescent markers: proteins that glow specific fluorescent colors when exposed to specific wavelengths of light, such as GFP.
Gene-editing technologies: set of molecular tools that allow genomes to be modified through point mutations, insertions, and deletions.
Neuromodulators: molecules (neuropeptides, hormones, and biogenic amines) that manipulate neuronal response by binding to G-protein-coupled receptors and activating slow-acting metabotropic pathways.
Neuronal response sensors: proteins that emit fluorescence only when neurons are active upon contact with calcium ions, which increase in concentration inside depolarized neurons (e.g., GCaMP).
Olfactory co-receptor: each olfactory receptor pairs with a conserved co-receptor, forming a functional heterocomplex. One to three co-receptors have been described for each family of olfactory receptors.
Olfactory receptor: transmembrane proteins that bind to specific odorant molecules and activate peripheral olfactory neurons. Each neuron exhibits one (or very few) olfactory receptors, allowing the specific detection of very few different odorant molecules.
Optogenetics: use of genes encoding ion channels that allow ion influx by exposure to specific wavelengths of light.
Population replacement: control strategy aiming to replace the target population with another one lacking the undesirable phenotype.
Population suppression: control strategy aiming to reduce the density of the target population.
Rescue lines: line of transgenic organisms in which a functional copy of a gene is inserted into another locus in the genome to rescue the activity of a nonfunctional gene.
Ribosomal skipping sequence: viral sequence, such as T2A, which causes ribosome skipping and allows a single RNA encoding the sequence of two (or multiple) proteins (multicistron sequence) separated by T2A sequence(s) be translated into independent (unlinked) proteins.

Box 1. The mosquito olfactory system in a nutshell

The mosquito sensory system detects stimuli using sensory receptors on the surface of the dendrites of the sensory neurons [67]. In the mosquito olfactory system, sensory neurons or odorant receptor neurons (ORNs) are housed in porous hair-like sensilla decorating the antennae, maxillary palps, and proboscis. Each ORN expresses from one to a few different classes of receptor on their dendrites. The olfactory system takes advantage of three families of olfactory receptors to perceive olfactory stimuli: the odorant, ionotropic, and gustatory receptor families [68]. The odorant receptor gene family encodes seven-pass transmembrane proteins that assemble into a tetramer, perceiving, for the most part, aromatic and heterocyclic odorants in mosquitoes. The ionotropic receptor gene family evolved from the glutamatergic receptor gene family and are fine-tuned for the detection of amines and carboxylic acids. Both families of receptor work by means of the assembly of a tuning receptor along with one or two conserved co-receptors: *Orco* for the odorant receptor gene family and *Ir25a*, *Ir8a*, and/or *Ir76b* for the ionotropic family. Once assembled, the functional receptors are ligand-gated ion channels. Even though the gustatory receptor gene family is regarded as encoding taste receptors, three specific genes have evolved to detect CO_2 and skin volatiles (*Gr1–3*) [68].

Once the olfactory receptors detect specific odor cues, they trigger the activation (depolarization) of ORNs and the transmission of sensory information to specific regions (neuropil) of the antennal lobes, called glomerulus. Each glomerulus receives input information from ORNs expressing the same set of receptors and synapses with GABAergic local interneurons and projection neurons. Whereas the former fine-tunes the sensory input, the latter transmit the sensory information from the antennal lobes to the centers of learning and behavior in the brain: mushroom bodies and lateral horns. For more detailed information about the different aspects of the insect sensory system, refer to [67–69].

the *Ir93a* hygrosensory co-receptor [14], and two opsins involved in vision-guided behavior [16], resulting in nonfunctional receptors.

In addition, CRISPR-Cas9 has been used to knock in genes encoding **fluorescent markers** (*TdTomato* [17]; Figure 1D) and **neuronal response sensors** (*GCaMP* [18]; Figure 1E) to label specific classes of neuron and detect the activities of individual neurons in defined brain regions (neuropils) upon activation by specific olfactory stimuli in mosquitoes.

The main strategies of gene editing applied to our understanding of the mosquito olfactory system are comprehensively described in the following section. The establishment of this technology alongside novel findings obtained with single cell RNA sequencing can pave the way for the development of the next generation of genetic tools to deepen our understanding of mosquito brain connections and how sensory input drives mosquito behavior.

Labeling specific neuronal groups

To identify specific classes of neuron (expressing a specific odorant receptor), gene-editing technologies have allowed the knock in and expression of fluorescent markers using endogenous promoters specific to those neurons [17,19]. For instance, the red fluorescent protein TdTomato-encoding sequence was knocked in the *fruitless* gene, allowing the identification of neurons expressing the fruitless transcription factor in the brain of both male and female mosquitoes (Figure 1D) [17]. An alternative strategy to express markers in specific neurons is the use of binary expression systems, which are two-part systems encompassing a transcriptional activator and an enhancer. The gene encoded by the transactivator can be expressed under the control of a desired promoter (driver). Once expressed, the transactivator binds to its enhancer and induces the expression of a desired downstream gene (responder) with spatial and temporal resolution [19]. Moreover, each component is expressed by one line of mosquito, allowing the combination of different driver lines (different promoters) with different responder lines (expressing different effector proteins) [19].

The binary expression systems Gal4-UAS (Figure 1F) [20] and Q-transcription activator (Figure 1G) [21] rely upon the transcription activators Gal4 and QF, which bind to the effectors *UAS* and *QUAS*, respectively, inducing transcription of the downstream reporter or effector gene [19]. The

transcription of the downstream genes can be repressed by Gal80 and QS suppressors [19], respectively. As components of these binary expression systems can cause toxicity, precluding more extensive application in different tissues and insect species, removing nonessential domains of the transcriptional activators was vital for the implementation of such a system for the neuronal expression of fluorescent markers in fruit flies and mosquitoes [19]. Two versions of the QF transcription activator lacking the middle domain (QF2 and QF2^{w}) exhibited very low toxicity and similar expression patterns to Gal4 in the *Drosophila melanogaster* neural system [19]. Whereas the QF2 version induces stronger expression of the effector protein in specific neuronal classes, QF2^{w} works best with strong promoters and for expression by multiple neuronal classes (broad expression) [19].

Using binary expression systems to study the mosquito olfactory system has been achieved in *Anopheles gambiae* (M-form or *Anopheles coluzzii*) [22] and *Aedes aegypti* [22]. By crossing a mosquito expressing the transcription activator QF2 driven by the *Orco* promoter with mosquitoes genetically modified with a chimeric construct of the genes encoding the membrane protein mCD8 and GFP, the mosquito offspring displayed GFP labeling in all Orco-expressing odorant receptor neurons (ORNs) (Figure 2A) [19]. With such labeling, the number of ORNs in the antenna and maxillary palps as well as the number of antennal lobe glomeruli in male and female mosquitoes were determined and compared [19]. For *Ae. aegypti*, knocking in the *QF2^{w}* coding sequence along with the *T2A* **ribosomal skipping sequence** at the stop codon of the neuronal gene *brp* (encoding bruchpilot) led to expression of the transcription activator *QF2^{w}* in brp-expressing neurons [23]. Crossing *brp-T2A-QF2^{w}* mosquitoes with a *QUAS-mCD8::GFP* line (Figure 2B) resulted in GFP labeling of neurons in adult and larval brains, as well as antennae, maxillary palps, and labella [23], allowing the expression of potentially any effector gene in all neurons.

In *Ae. aegypti*, the *QF2* transactivator has also been inserted in-frame into the *Orco*, *Ir8a*, *Ir76b*, and *Gr3* odorant co-receptor genes [24,25] and all constructs were capable of driving *QUAS-mCD8::GFP* expression, resulting in strong labeling of the corresponding neurons (Figure 2C) [24,25].For these constructs, the *T2A* sequence was inserted in-frame into the third exon of each target gene, allowing the expression of the *QF2* factor under the control of the natural gene promoters [24,25].

The Q binary expression system has also been used to elegantly show the co-expression of two odorant co-receptors in the same neuron [24]. In these experiments, different domains of the *QF2* coding sequence along with the *T2A* ribosomal skipping sequence were knocked in at the stop codons of *Orco* and *Ir25a* [24]. A functional *QF2* complex capable of binding to *QUAS* and promoting the expression of the fluorescent marker TdTomato (and GCaMP6, see below) was produced only upon expression of both DNA-binding (DBD) and Activation (AD) domains by *Orco* and *Ir25a* promoters, respectively, in the same neuron (Figure 2D), highlighting the neurons co-expressing odorant receptor co-receptors [24].

Detecting neuronal responses

The genetically encoded calcium indicator GCaMP is a green fluorescent reporter that glows upon neuronal response, providing transient labeling of activated neurons [26]. As neurotransmitter release is induced by the opening of voltage-gated calcium channels, the calcium influx is detected by GCaMP, which glows as an indicator of neuronal response [27]. The first demonstration of the use of GCaMP in the mosquito was performed by Bui and colleagues [18], showing that the polyubiquitin promoter drives expression of GCaMP6 in all mosquito tissues [18]. This tool enabled visualization of adult and larval olfactory neural activities upon exposure to odor stimuli [28].

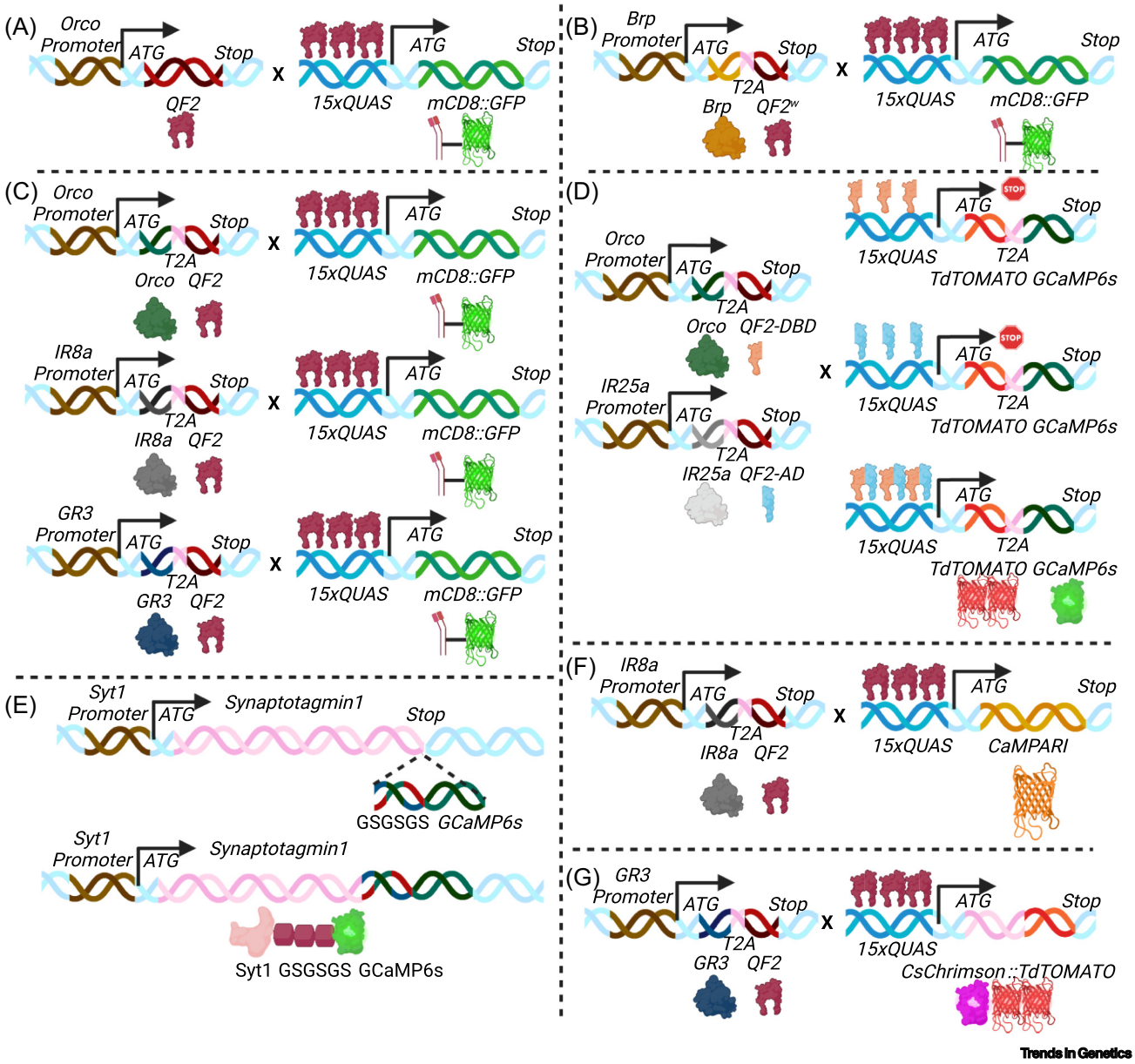

Figure 2. Genetic constructs of the mosquito olfactory system. Using the Q binary system to tag (A) Orco-housing and (B) all neurons with the fluorescent marker GFP fused to the mCD8 protein, which directs the chimera to the synaptic membranes. (C) Using the Q binary system to tag Orco- (top), Ir8a- (middle), and Gr3-housing (bottom) neurons with the fluorescent marker GFP fused to the mCD8 protein. (D) Expressing different QF2 domains, DNA-binding (DBD) and activation (AD) domains by independent promoters to detect genes expressed in the same neurons. (E) Using the promoter of the gene encoding Synaptotagmin1 (*Syt1*) for pan-neuronal expression of the GCaMP6 sensor linked to the native protein by the (3X) flexible linker GS. (F) Using the Q binary system to express CaMPARI2 in Ir8a-expressing neurons. (G) Using the Q binary system to express the CsChrimson-TdTomato fused protein in Gr3-expressing neurons. 15X QUAS sites were present in all the constructs mentioned.

To visualize the activities of Orco-expressing neurons upon odor stimuli, the stop codon of *Orco* was replaced by the coding sequence of GCaMP6 (Figure 1E) [23,29,30]. This strategy allowed the identification of *Ae. aegypti* olfactory glomeruli specifically activated by odors unique to humans or other animals [29]. To extend the expression of GCaMP6 to the whole nervous system, a pan-neuronal driver was designed for *Ae. aegypti* using neuron-specific promoters [23].

By using the CRISPR-Cas9 knock-in technology, the *GCaMP6* coding sequence along with the three (3X) *GS* flexible linkers were inserted in-frame at the stop codon of the gene encoding Synaptotagmin1 (*Syt1*) (Figure 2E) [23]. The fused Syt1-GS-GCaMP6 protein localized in the synapses of all major neuropils, and exposure to single odorants resulted in fluorescence intensity changes. GCaMP6 has also been used to measure the activity of specific neurons in the brain of *An. gambiae* [30,31]. A *QUAS-GCaMP6* line was crossed to an *Orco-QF2*, allowing the identification of specific Orco-expressing neurons detecting specific odorants [31], including known mosquito repellents [30].

In contrast to GCaMP6, the calcium-modulated photoactivatable ratiometric indicator (CaMPARI2) permanently stains neurons upon contact with calcium, allowing post-hoc staining of the tissue targeting other markers [32]. By expressing CaMPARI2 under the control of the *QUAS* effector in one *Ae. aegypti* line and crossing it to the *Ir8a-T2A-QF2* line (Figure 2F), the permanent labeling was achieved of specific antennal lobes upon exposure to an overlay of CO_2 and L-(+)-lactic acid stimuli [25]. This resulted in the identification of olfactory glomeruli not activated by either stimulus alone [25] and unveiled a synergism in odor coding in the primary olfactory center essential to drive attractive behavior [25].

Optogenetics

The ability to trigger action potentials by exposure to a specific light wavelength became possible with the discovery of bacterial opsins that use retinal (a derivative of vitamin A) as cofactors (termed 'rhodopsin') and photobleach following exposure to light [33]. The discovery of a specific group of rhodopsins that encompass ion channels (termed 'channelrhodopsins') and display both ion channel and light-sensing components made it possible to use light and regulate neural activity by expressing a single peptide [33].

In *Ae. aegypti*, the channelrhodopsin protein CsChrimson, which constitutes a red light-activated cation channel, was used to activate CO_2 receptor neurons in *Gr3*-knockout mosquitoes ($^{-/-}$ CO_2 receptor) using red light (627 nm); thus, this system used light to artificially induce CO_2-gated activation and evoke attraction to heat and an artificial (blood mimic) meal [34]. To accomplish this goal, *Gr3-T2A-QF2* mosquitoes were crossed to a *QUAS-CsChrimson* line (Figure 2G). *CsChrimson* was fused with the coding sequence of *TdTomato* for neuronal labeling [34]. Using light to activate the CO_2 neurons showed that the CO_2-gated activation behavior persisted for more than 10 min, allowing females to search and drink the artificial meal up to 14 min after light exposure [34].

Development of rescue lines

As gene-editing technologies have enabled the development of knockout lines of mosquitoes, it is important to confirm that the specific phenotype produced by the gene knockout is due to the knockout itself, rather than to other stochastic changes in the genome of the mutant organism. To reduce the number of steps necessary to develop gene-knockout lines followed by the generation of gene add-back lines (**rescue lines**), the Drug-on, Reporter, Mutant, Rescue (DREaMR) tool was developed for mosquitoes [35]. A DREaMER genetic construct is inserted in the coding sequence of a gene, creating a loss-of-function mutation [35]. As the DREaMER construct contains a transactivator (tTA system induced by tetracycline [36]) that drives the expression of a recoded sequence of the mutated gene, rearing the mosquitoes in tetracycline rescues expression of the protein [35]. Therefore, a single line of mosquito can be used in experiments to demonstrate not only the effects of a gene loss-of-function mutation, but also ideally that reactivating the gene in the same line of mosquitoes rescues the wild-type phenotype.

Single cell and single nucleus RNA-Seq

Single nucleus transcriptomics [snRNA-sequencing (snRNA-Seq); as a proxy of the whole-cell transcriptome, scRNA-Seq) of sensory appendages and nervous tissues has unveiled the transcriptional profiles of specific neural pathways as well as a broader co-expression of **olfactory receptors** among sensory neurons [24,37]. In the olfactory sensory neurons of *Drosophila melanogaster* fruit flies, snRNA-Seq revealed the co-expression of *Orco* and *Ir* co-receptors, such as *Ir76b* (57.9%), *Ir25a* (57.7%), and *Ir8a* (31.6%), as well as in *Gr21a*-cells, which are sensors of CO_2 [37]. In *Ae. aegypti*, extensive overlap *of Ir25a* and *Orco* expression exists, and the former is expressed in neurons that project to almost all antennal lobe glomeruli [24].

snRNA-Seq identification has been performed with brains from male and female *Ae. aegypti* [38]. A median number of 1295 (male) and 1628 (female) genes were detected per nucleus, and 80% of the *Ae. aegypti* protein-coding genes were expressed in these nuclei [38]. Whereas 35 cell clusters expressing specific markers were identified, 15 of which were assigned to known cell types based on the expression of homologs of *Drosophila* neuron-specific markers [38]. Glial cells corresponded to five clusters that expressed the glial marker 'reverse polarity' (*repo*). Even though these cells belong to the nervous system, they do not depolarize [38]. Three clusters encompassed Kenyon cells (KCs), which are the constituents of mushroom bodies [38]. These neurons expressed *eyeless* and *DopR2* as cell-specific markers. Four other clusters expressed the markers *cut* and *acj6*, which are hallmarks of olfactory projection neurons (PNs). One cluster expressed the neuron markers homothorax (*hth*) and brain-specific homeobox (*Bsh*), which are markers of a subset of optical lobe neurons called Mi1 neurons [38]. Neurons of another cluster expressed *hth* and *Lim3* and belonged to the proximal medulla neurons of the optical lobes. The last assigned cluster encompassed monoaminergic neurons and expressed the vesicular transporter *Vmat* [38].

Despite advances in the development of genetic tools using the binary system in mosquitoes to label and neural activity detection (Table 1), the driver lines used thus far usually relied upon the promoters of co-receptors of the peripheral nervous system. Below, we describe available tools in model organisms that could be developed to expand the mosquito genetic toolbox and used to address questions related to the transduction of the olfactory information from the periphery to higher brain centers.

Future technologies to advance mosquito sensory genetics

Novel genetic driver lines

To further explore the transduction of olfactory information from the periphery to the higher brain centers, novel drivers need to be developed that are specific to the local interneurons of the antennal lobes as well as to the projection neurons that connect the antennal lobe to the mushroom bodies and lateral horns, as for the specific cells of these neuropils, such as KCs [39]. Many such lines are available for *Drosophila* and were obtained through the development of enhancer-trap tools [39], which have not been widely explored in mosquitoes [40]. With the advent of single-cell (and single-nucleus) RNA-Seq, multiple neuronal-specific markers have been unveiled [38], allowing the development of new drivers for higher brain center neurons in mosquitoes.

Establishing alternative binary expression systems

Binary expression systems have been used in studies of mosquito gene expression and physiology [41,42]. Despite initial efforts to establish the Gal4/UAS system in *Ae. aegypti* [41,42] and *An. stephensi* [40], the Q system became the binary system of choice because it extended the implementation of binary systems to a mosquito species less amenable to genetic modifications compared with *An. gambiae* [22]. As one of the limitations of current binary

Table 1. Transgenic mosquito lines constructed with binary expression systems

Knock-in lines	Target gene(s)	Inserted gene(s)	Promoter and transformation marker	Genetic background	Refs
Aedes aegypti					
fruitless$^{\Delta M\text{-}tdTomato}$	*fruitless* (KO)	*CsChrimson::tdTomato*	*3xP3-EYFP*	Liverpool	[17]
PUb-GCaMP6s	*PiggyBac* insertion site(s)	*PUb-GCaMP6s*	*OpIE-2-DsRed*	Liverpool	[18]
Syt1:GCaMP6s	*Syt1*	*(3X)GS-GCaMP6s*	*3xP3-DsRed*	Orlando	[23]
brp-T2A-QF2w	*brp*	*T2A-QF2w*	*3xP3-DsRed*	Orlando	[23]
Syt1-T2A-3XGCaMP6s	*Syt1*	*(3x)T2A-GCaMP6s*	*3xP3-DsRed*	Orlando	[23]
Syt1-T2A-QF2-QUAS-GCaMP6s	*Syt1*	*T2A-QF2-QUAS-GCaMP6s*	*3xP3-DsRed*	Orlando	[23]
Syt1-T2A-GAL4d-UAS-GCaMP6s	*Syt1*	*T2A-GAL4d-UAS-GCaMP6s*	*3xP3-DsRed*	Orlando	[23]
QUAS-Syt1:tdTomato	*PiggyBac* insertion site(s)	*(15X)QUAS-Syt1:TdTOMATO*	*3xP3-ECFP*	Orlando	[23]
Orco^{QF2Red}	*Orco* (KO)	*T2A-QF2*	*3xP3-DsRed2*	Liverpool	[25]
Ir8a^{QF2Red}	*Ir8a* (KO)	*T2A-QF2*	*3xP3-DsRed2*	Liverpool	[25]
Gr1^{QF2Red}	*Gr1* (KO)	*T2A-QF2*	*3xP3-DsRed2*	Liverpool	[25]
15XQUAS-CaMPARI2	*Mariner* insertion site(s)	*(15X)QUAS-CaMPARI2*	*3xP3-ECFP*	Liverpool	[25]
15XQUAS-mCD8::GFP	*Mariner* insertion site(s)	*(15X)QUAS-mCD8::GFP*	*3xP3-ECFP*	Liverpool	[25]
Ir25a-T2A-QF2	*Ir25a*	*T2A-QF2*	*3xP3-DsRed*	Liverpool	[24]
Ir76b-T2A-QF2	*Ir76b*	*T2A-QF2*	*3xP3-DsRed*	Liverpool	[24]
Ir8a-T2A-QF2	*Ir8a*	*T2A-QF2*	*3xP3-DsRed*	Liverpool	[24]
Gr3-T2A-QF2	*Gr3*	*T2A-QF2*	*3xP3-DsRed*	Liverpool	[24]
Orco-T2A-QF2	*Orco*	*T2A-QF2*	*3xP3-DsRed*	Liverpool	[24]
Ir25a-T2A-QF2-AD	*Ir25a*	*T2A-QFAD::Zip+*	*3xP3-EYFP*	Liverpool	[24]
Orco-T2A-QF2-DBD	*Orco*	*T2A-Zip::QFDBD*	*3xP3-DsRed*	Liverpool	[24]
orco-T2A-QF2-QUAS-GCaMP6f	*Orco*	*T2A-QF2-QUAS-GCaMP6f*	*3xP3-DsRed*	Orlando	[29]
QUAS- GCaMP7s	*PiggyBac* insertion site(s)	*QUAS-jGCaMP7s-T2A-tdTomato*	*3xP3-ECFP*	Orlando	[29]
QUAS-CsChrimson-tdTomato	*PiggyBac* insertion site(s)	*QUAS-CsChrimson::tdTomato*	*3xP3-DsRed*	Liverpool	[34]
Anopheles gambiae					
Orco-QF2	*PiggyBac* insertion site(s)	*Orco promoter-QF2*	*3xP3-dsRed*	Ngousso	[22]
(15X)QUAS-mCD8-GFP	*PiggyBac* insertion site(s)	*QUAS-mCD8::GFP*	*3xP3-ECFP*	Ngousso	[22]
QUAS-GCaMP6	*PiggyBac* insertion site(s)	*15xQUAS-Gcamp6f*	*3xP3-ECFP*	Ngousso	[30]

expression systems established in mosquitoes is related to their toxicity [19,23], which has been reduced by further engineering [19], efforts to test and implement alternative binary systems mosquitoes should be undertaken. Expanding the binary system toolbox may allow the establishment of spatiotemporal control of gene expression in other mosquito vectors.

Multiple bacterial binary systems have been recently tested in fruit flies [43], which might alternatively be functional in mosquitoes. The *p-CymR* (*Pseudomonas putida* [44]), *PipR* (*Streptomyces coelicolor* [45]), *TtgR* (*Pseudomonas putida* [46]), *VanR* (*Caulobacter crescentus* [46]), alongside the well-characterized (and mosquito-established [35,47]) *tTA* system (*Escherichia coli* [36]) showed strong tissue-specific expression under the control of a flight muscle promoter [43]. For all these systems as well as the Q systems, small molecules can be used to repress the expression of the system, adding another dimensionality of gene expression control. When quinic acid binds to the QS repressor (Figure 1G), it releases the QF transactivator and allows

expression of the gene(s) downstream of the *QUAS* operator [21]. For the alternative systems described above, the small molecules bind directly to the transactivators, inhibiting the expression of the genes downstream of the operators [43] without the need for an extra repressor, such as QS [21]. The *p-CymR*, *PipR*, *TtgR*, *VanR*, and *tTA* binary systems are repressed by cumate, virginiamycin M1, phloretin, vanillic acid, and doxycycline/tetracycline, respectively.

As mentioned in the following section, other binary expression systems, such as *LexA/LexAop* [48] and *CRISPR/dCas9-VPR*-based transactivators, have been used in fruit flies [48–50] and recently in mosquitoes [51], alone or in combination with the *Gal4/UAS* and the Q systems. Combining the binary system in mosquitoes will allow the development of sensors of neuronal response and strengthen our understanding of the transmission of sensory input to higher brain centers.

Sensors for neuromodulatory activities

All the neural circuits discussed thus far are genetically hardwired and evolved to trigger innate behaviors via their fast neurotransmitters and ion-gated receptors [52]. Nonetheless, context and internal state can modify such innate behaviors upon the action of **neuromodulators** [53]. For instance, dehydration can improve the innate blood-feeding behavior of mosquitoes [54].

To map the circuits that specific neuromodulators act upon in mosquitoes, genetic tools need to be developed to detect the release of endogenous neuromodulators [55,56]. The Transcriptional Activation following Arrestin Translocation (Tango) system is a sensor of neuromodulatory activities [55,56] that has been adapted to *D. melanogaster* to sense transient interactions between neuromodulators and receptors and record them by expression of a reporter molecule [55,56]. In fruit flies, the Tango system was built to detect the activity of dopamine in taste neurons. To do so, a transgenic fly line was constructed with a *UAS* binding site controlling the expression of an exogenous dopamine receptor tagged to a TEV protease cleavage site, the *lexA* transcription factor (tagged by the HA epitope), the *2A* ribosomal skipping sequence, and the *arrestin1* gene linked to the *TEV protease* sequence (Figure 3A). The *2A* sequence allows the generation of a bicistronic transcript that is translated into two polypeptides: (i) the dopamine receptor linked to the lexA transcription factor by the TEV protease cleavage site (TEVp cv); and (ii) arrestin1 linked to the TEV protease. The fly line also has a lexA operator (*lexAop*) controlling expression of the reporter fluorescent protein-encoding gene (Figure 3A). Binding of dopamine to its receptor triggers the recruitment of the arrestin1-TEV protease to the cell membrane and leads to cleavage of the TEV protease cleavage site and release of the lexA transcription factor. The latter translocates to the nucleus and binds to *lexAop*, inducing the expression of GFP (mCD2::GFP) (Figure 3A) [38,39]. In this system, expression of the *UAS* components was driven by a Gal4-GeneSwitch construct under the control of a pan-neuronal promoter (*elav*), only induced by the presence of an exogenous hormone (RU486) [56]. With this Tango fly line, it was shown that hunger increases behavioral sensitivity to sugar by the release of dopamine onto primary taste neurons [56].

The next generation of Tango design replaced the artificial transcription factor (*LexA*) with a deactivated version of the Cas9 nuclease (dCas9) (Figure 3B), which allows programmed control of the expression of multiple endogenous genes in response to specific extracellular signaling molecules [57]. In this new system (CRISPR ChaCha), dCas9 is linked to beta-arrestin-2 (ARRB2) by the TEVp cv. dCas9 is also fused to transcription activators to regulate the transcription of target genes (Figure 3B). The TEV protease is then linked to the C termini of the G-protein-coupled receptor (GPCR) via the V2 tail of the arginine vasopressin receptor 2. Upon ligand binding to the GPCR, arrestin-TCS-dCas9 is recruited and cleaved by the TEV protease, releasing dCas9, which translocates to the nucleus and activates the expression of the reporter gene (Figure 3B) [57].

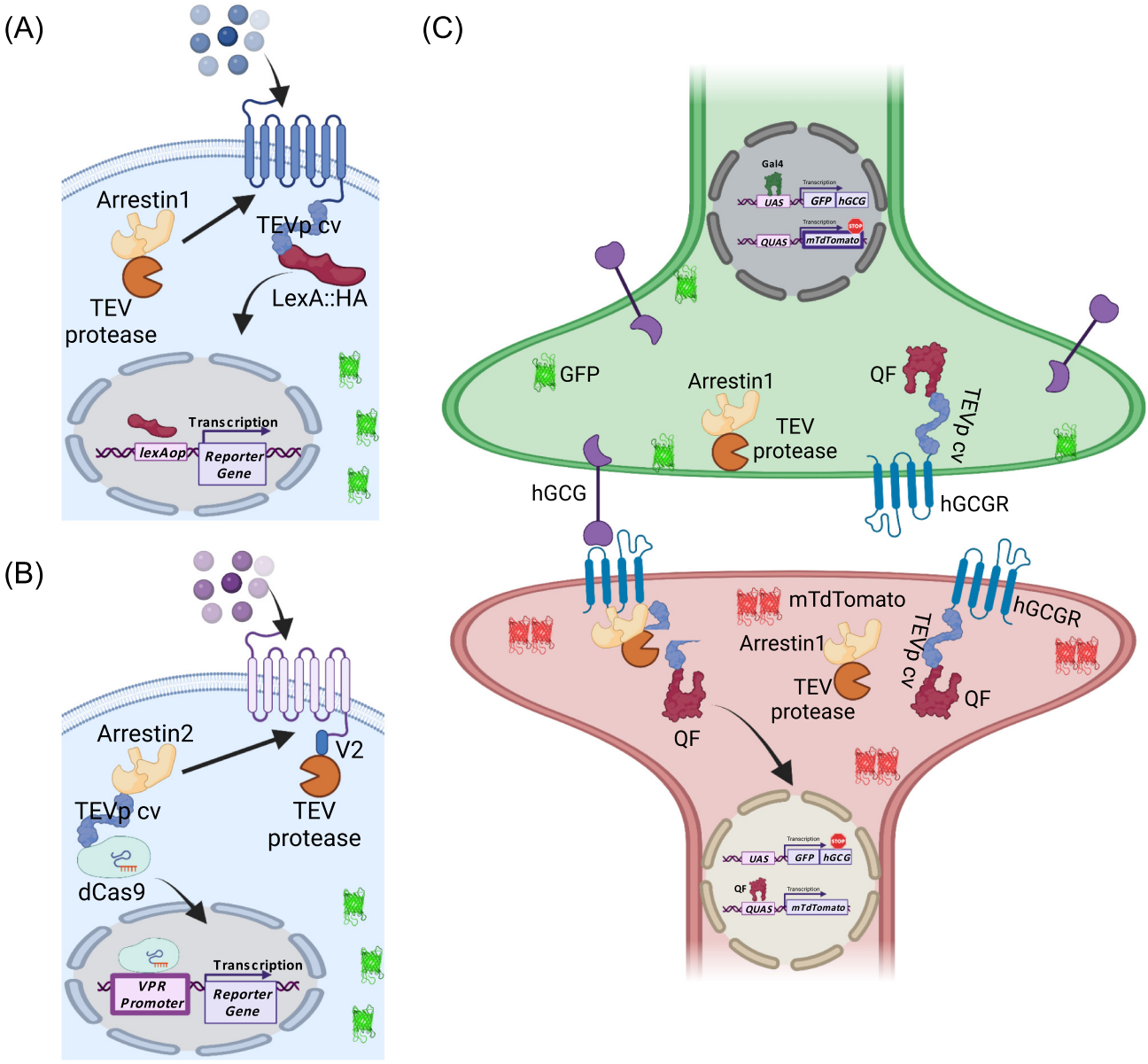

Figure 3. Potential new technologies for the study of the mosquito olfactory system. (A) The Transcriptional Activation following Arrestin Translocation (Tango) system. Upon dopamine binding to dopamine receptors, the arrestin1::TEV protease complex is recruited and cleaves the TEV cleavage site (TEVp cv), releasing the LexA transcription factor. LexA is translocated to the nucleus, binds to the LexA operator (*lexAop*), and induces expression of the reporter (mCD2-GFP) gene. (B) The Tango ChaCha system. The binding of the ligand to the G-protein-coupled receptor (GPCR) induces the recruitment of arrestin2 (linked to a dCas9 complex), inducing the TEV protease, linked to the GPCR in this system, to cleave the TEVp cv and release the dCas9 complex. The latter is translocated to the nucleus, where it induces the expression of a reporter gene. (C) Trans-synaptic Tango system. A specific Gal4 line (not shown) induces GFP expression and labeling of specific sensory neurons and also the expression of human glucagon (hGCG). Pan-neuronal drivers (not shown) drive the expression of the arrestin:TEV protease complex as well as the glucagon receptor linked to the QF transcription factor (hGCGR-QF). The binding of hGCG to hGCGR leads to the recruitment of arrestin:TEV protease and release of the QF transcription factor in the postsynaptic neuron. QF translocates to the nucleus, binds to the QUAS site, and induces the expression of a reporter mTdTomato, labeling the postsynaptic neurons with a red fluorescence.

Sensors of neurotransmitter release

As an alternative to GCaMP6 and CaMPARI2, sensors of neurotransmitter release (spH) can also be used to detect neuronal activities upon stimulation [58,59]. Amino acid-specific substitutions in the GFP molecule led to the development of a pH sensor (pHluorins) that fluoresces with higher intensity at a neutral pH and shows low excitation at acidic pHs [58]. The neurotransmitter molecules are stored in endosomal vesicles under low pH and are released through exocytosis at the synapse where the pH is around neutral (7.4). Then, pHluorins are tagged with an endosomal protein so that the GFP fluorescence is only observed at the synapsis (not in the somata) after neurons are depolarized [58].

The genetically encoded spH under control of the *D. melanogaster Or83b* (*Orco*) promoter showed odorant-induced neurotransmitter release in the olfactory glomeruli DM3 and VA2 as well as lack of activity in the DM2 glomerulus of the *Δhalo* mutant, which lacks sensory input by Or22 sensory neurons [59]. Driving the expression of spH in a broad subset of projection neurons using the GH146-Gal4 line also allowed visualization of neurotransmitter release by PNs upon odor stimulation [59].

Trans-synaptic sensors

Individual neurons are connected through their synapses forming the neural circuits. Understanding how such circuits work relies upon mapping such connected neurons. As discussed in Box 1, ORNs perceive environmental odorants and transmit the information through action potentials to specific antennal lobe glomeruli and synapses with lateral interneurons and/or projection neurons. The latter transmit the input information to neurons in the higher brain centers involved with learned and innate behaviors (Box 1). Mapping the trajectories of such circuits (connectome) can shed light on the roles of specific ORNs in insect behavior [60].

To map the connections between neurons, the Trans-Tango method for anterograde trans-synaptic tracing has been developed for fruit flies, which takes advantage of an exogenous ligand–receptor pair for circuit labeling that can detect connectivities in any neural circuits regardless of the neurotransmitter governing the synaptic interaction [61]. Using glucagon and its receptor of humans, the *glucagon receptor* sequence is linked to the binary transcription factor *QF* by the TEVp cv under the control of the *nSyb* pan-neuronal promoter. The fused sequences (*hArr::TEV*) of the human genes encoding beta-arrestin and TEV protease were placed under the control of another pan-neuronal promoter (*Elav*) (Figure 3C). To express glucagon at the synaptic space, the sequence encompassing the cytosolic and transmembrane domains of *Drosophila Neuroxin1* was fused to the sequence of the extracellular domain of the human cell-adhesion protein *ICAM1*, a *myc-tag* sequence, and a variant sequence of the *glucagon neuropeptide* that induces strong receptor activation (Figure 3C). Another *D. melanogaster* line was developed with the sequence of a membrane version of postsynaptic reporter (mTdTomato) inserted downstream of QF binding sites (*QUAS*), and the membrane-bound presynaptic marker *mycGFP* sequence was inserted downstream of Gal4-binding sites (*UAS*) (Figure 3C). By genetically crossing the line with the constructs under the control of pan-neuronal promoters with a specific *Gal4* driver line, the desired sensory neurons are labeled with GFP, whereas the interneurons in the antennal lobes and projection neurons to the lateral horn and mushroom bodies are labeled with tdTomato, allowing tracing of the transmission of an external signal from the periphery to the higher brain centers [61].

Concluding remarks

Mosquito neurogenetics has advanced substantially over the past decade with the implementation of the Q binary expression system to drive the expression of morphological (mCD8-GFP and

Outstanding questions

Can gene editing be expanded to modify the genomes of other mosquito vectors, such as *Culex*, *Haemagogus*, and *Sabbetes* mosquitoes?

Will alternative binary systems be functional in alternative mosquito species and increase the resolution of gene expression control in *Aedes* and *Anopheles* mosquitoes?

Can multiple genetic constructs be stably maintained in a mosquito genome without the help of balancer chromosomes?

Can neuron switches be designed to control the activities of neural circuits governing the innate behaviors of mosquitoes?

TdTOMATO) and activity (GCaMP and CaMPARI) sensors as well as the use of **optogenetics** (CsChrimson) to activate specific neurons. Developing tools to trace the transmission of sensory information from the peripheral sensory system to the higher brain centers can not only lead to a better understanding of how sensory input can trigger mosquito behavioral output (see Outstanding questions), but also open the door for the development of novel ways to manipulate mosquito behaviors. As the mosquito peripheral olfactory system is very diverse in receptor number and arrangement [24], explaining why olfactory and thermoreceptor-knockout mosquito lines still find humans [9,15], turning off neurons that drive mosquito anthropophilic behavior in the higher brain centers might be a way to divert mosquitoes to feed on other animals. Such a mechanism could be spread out into a population via a **population replacement** approach [62,63], reducing the local human biting rate. Developing genetic strategies for mosquito behavioral control applied alone or in combination with other effective **population suppression** strategies (*Wolbachia* [64] and the targeted Sterile Insect Techniques (SITs), such as the Improved SIT [65] and the Precision Guided SIT (PgSIT [66])) might pave the way for the deployment of effective vector-based control strategies against mosquito-borne diseases.

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Declaration of interests

O.S.A. is a founder of Agragene, Inc. and Synvect, Inc. with an equity interest. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. The remaining author declares no competing interests.

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