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.

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].
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], Grd [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 CO2/skin odor olfactory co-receptors [14], the ir21a heat receptor [15].
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 transactivation 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].
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 QF2w) 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, QF2w 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 QF2w 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 QF2w in brp-expressing neurons [23]. Crossing brp-T2A-QF2w 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 Aedes 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].
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].

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.
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 CO2 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 CO2 receptor neurons in Gr3-knockout mosquitoes (−/− CO2 receptor) using red light (627 nm); thus, this system used light to artificially induce CO2-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 CO2 neurons showed that the CO2-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 CO2 [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]. Gial 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
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]), TgR (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

### Table 1. Transgenic mosquito lines constructed with binary expression systems

<table>
<thead>
<tr>
<th>Knock-in lines</th>
<th>Target gene(s)</th>
<th>Inserted gene(s)</th>
<th>Promoter and transformation marker</th>
<th>Genetic background</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aedes aegypti</td>
<td>fruitlessDM-tdTomato</td>
<td>fruitless (KO) CsChrimson::tdTomato 3xP3-EYFP</td>
<td>Liverpool [17]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PUB-GCaMP6s</td>
<td>PiggyBac insertion site(s) PUB-GCaMP6s OptE-2-DsRed</td>
<td>Liverpool [18]</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Syt1:GCaMP6s</td>
<td>Syt1 (3X)GCaMP6s 3xP3-DsRed</td>
<td>Orlando [23]</td>
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<tr>
<td>brp-T2A-QF2w</td>
<td>brp T2A-QF2w T2A-QF2w 3xP3-DsRed</td>
<td>Orlando [23]</td>
<td></td>
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</tr>
<tr>
<td>Syt1-T2A-QF2-QUAS-GCaMP6s</td>
<td>Syt1 T2A-QF2-QUAS-GCaMP6s 3xP3-DsRed</td>
<td>Orlando [23]</td>
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<tr>
<td>Syt1-T2A-QF2-QUAS-GCaMP6s</td>
<td>Syt1 T2A-QF2-QUAS-GCaMP6s 3xP3-DsRed</td>
<td>Orlando [23]</td>
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<tr>
<td>QUAS-Syt1:tdTomato</td>
<td>PiggyBac insertion site(s) (15X)QUAS-Syt1:tdTomato 3xP3-ECFP</td>
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<td>OrcoQF2Red</td>
<td>Orco (KO) T2A-QF2 3xP3-DsRed2</td>
<td>Liverpool [25]</td>
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<tr>
<td>Ir8aQF2Red</td>
<td>Ir8a (KO) T2A-QF2 3xP3-DsRed2</td>
<td>Liverpool [25]</td>
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<tr>
<td>Gr1QF2Red</td>
<td>Gr1 (KO) T2A-QF2 3xP3-DsRed2</td>
<td>Liverpool [25]</td>
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<tr>
<td>15XQUAS-CaMPAR2</td>
<td>Mariner insertion site(s) (15X)QUAS-CaMPAR2 3xP3-ECFP</td>
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<tr>
<td>15XQUAS-mCD8::GFP</td>
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<tr>
<td>Ir25a-T2A-QF2</td>
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<tr>
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<tr>
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<td>Liverpool [24]</td>
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<tr>
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<td>Orco T2A-Zip::QFDBD 3xP3-DsRed</td>
<td>Liverpool [24]</td>
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<tr>
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<td>Anopheles gambiae</td>
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<td>Ngousso [22]</td>
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<tr>
<td>(15XQUAS-mCD8::GFP</td>
<td>PiggyBac insertion site(s) QUAS-mCD8::GFP 3xP3-ECFP</td>
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</table>

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.
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 mTdTTomato, 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, the glucagon receptor sequence is linked to the binary transcription factor QF by the TEVpcuv under the control of the nSyb pan-neuronal promoter. The fused sequences (hArr::TEVp) 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 (mTdTTomato) 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 Sabethes 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 (PgiSIT [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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