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Genetic and viral approaches to record or manipulate neurons in insects

Herman A Dierick[1], Yehuda Ben-Shahar[2], Baranidharan Raman[3] and Fabrizio Gabbiani[4]

The development of genetically encoded tools to record and manipulate neurons *in vivo* has greatly increased our understanding of how neuronal activity affects behavior. Recent advances enable the use of these tools in species not typically considered genetically tractable. This progress is revolutionizing neuroscience in general, and insect neuroethology in particular. Here we cover the latest innovations and some of their applications in phylogenetically diverse insect species. We discuss the importance and implications of these approaches for both basic and translational research. We focus on genetically encoded and virally encoded tools used for calcium imaging, optogenetics, and synaptic silencing. Finally, we discuss potential future developments of universally applicable, modular, and user-friendly genetic toolkits for neuroethological studies of insect behavior.

Addresses
[1] Dep. of Human and Molecular Genetics, Baylor College of Medicine, United States
[2] Dep. of Biology, Washington University in St. Louis, United States
[3] Dep. of Bioengineering, Washington University in St. Louis, United States
[4] Dep. of Neuroscience, Baylor College of Medicine, United States

Corresponding author: Gabbiani, Fabrizio (gabbiani@bcm.edu)

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Introduction

The last few decades have produced rapid progress in the control of gene expression and manipulation in specific neural tissues and neuron types. The main drivers behind these advances have been traditional genetically tractable animal species such as the vinegar fly *Drosophila melanogaster*, the zebrafish *Danio rerio*, the mouse *Mus musculus*, and the nematode *Caenorhabditis elegans*. Their success largely derived from the fruitful integration of classical genetics and molecular biology. By borrowing components of organisms like bacteria, yeast, algae, fungi, and jelly fish scientists engineered new molecular tools to probe the nervous system with high spatial and temporal resolution [1,2]. Subsequently, these tools have greatly advanced the mapping of neural circuits to behavior.

Independently, neuroethological approaches have advanced our understanding of brain function by integrating behavioral, neurophysiological and computational methods in a comparative and ecological framework. Such studies have focused on a wide range of behaviors in vertebrate and invertebrate species, for example, vocal learning in songbirds [3], auditory sound localization in barn owls [4], the electrical jamming avoidance response of weakly electric fish [5], olfactory processing in several insect species [6], including honeybees [7] and grasshoppers [8], sound discrimination in crickets and grasshoppers [9,10], pheromone scent tracking in silk moths [11,12], and air displacement sensing in the cercal system of crickets [13]. This approach was designed to provide a comprehensive explanation of behavior summarized by four distinct levels of analysis: *(i)* the function of a particular behavior, *(ii)* its phylogenetic origin, and, most importantly, *(iii)* its neurobiological implementation, and *(iv)* development. Tinbergen, one of the founders of modern ethology along with von Frisch and Lorenz, proposed this scheme [14–17]. The merging of these ethological principles and neurobiological methods led to the field of neuroethology that favors the selection of a diverse set of 'specialists' for studying specific behaviors [18].

Extending genetic methods to such species would greatly expand our perspective and usher in a new era of comparative neuroethological research. This type of transgenesis — the process of integrating exogenous or modified genes, that is, transgenes, into the genome of a recipient organism — might have seemed a distant dream only a few years ago. However, it is now starting to be broadly applied based on the ever-decreasing costs of sequencing and the recent development of precise genome editing tools. Here, we review the techniques at play and specific examples of their applications to insect neurons, focusing on work not covered extensively earlier [19].

Approaches to transgenesis

With the exception of *Drosophila* until recently, transgenic manipulations of most insect species completely

depended on the use of modified transposons such as *piggyBac* [20]. However, the rapid development of CRISPR/Cas9-dependent genome editing [21,22], derived from a bacterial antiviral acquired immunity system, is blurring the boundaries between 'genetically tractable' and 'non-genetically tractable' model systems (CRISPR stands for 'clustered regularly interspaced short palindromic repeats', a component of this adaptive immune system, and Cas9 for 'CRISPR associated nuclease, type 9'). Both transposon-mediated and CRISPR/Cas9-mediated genome transformations usually depend on the injection of nucleic acids/proteins into embryos to target the future germline cells thus resulting in heritable transgene integration (alternative delivery routes for CRISPR/Cas9 are described below). The main difference between these techniques is that transposons are typically inserted semi-randomly (see below) while CRISPR/Cas9 can target any specific site in the genome, giving it higher precision and flexibility. A hybrid natural system of CRISPR/Cas9-guided transposition was also recently discovered [23,24].

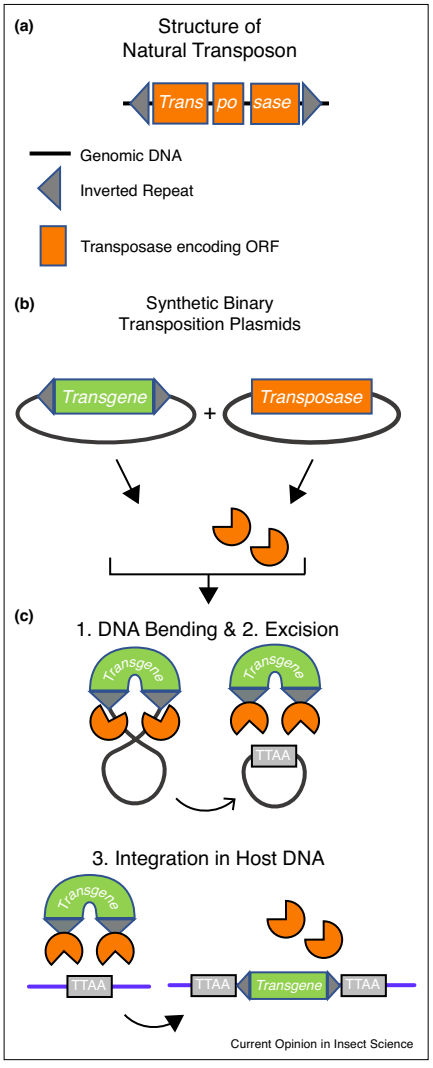
The *piggyBac* transposon

Natural transposons are mobile elements that can extract themselves from their location in the genome and re-insert at another random location. First discovered in maize [25], such sequences exist in many organisms including insects. The *piggyBac* transposon consists of two 13 base pair (bp) long inverted terminal repeat sequences flanking a gene that encodes a transposase enzyme [20]. When the transposase is expressed, it will recognize the inverted terminal repeat sequences, excise the transposon and then re-insert it at a random position in the genome, but preferentially into TTAA sequences. Such a transposon can be used for genetic engineering by replacing the genetic material between the two inverted terminal repeat sequences with a transgene of interest. The transposase enzyme required for inserting the modified transposon into the genome of the host organism is provided separately, for example, by a co-injected helper plasmid or an mRNA that encodes it (Figure 1).

CRISPR/Cas9

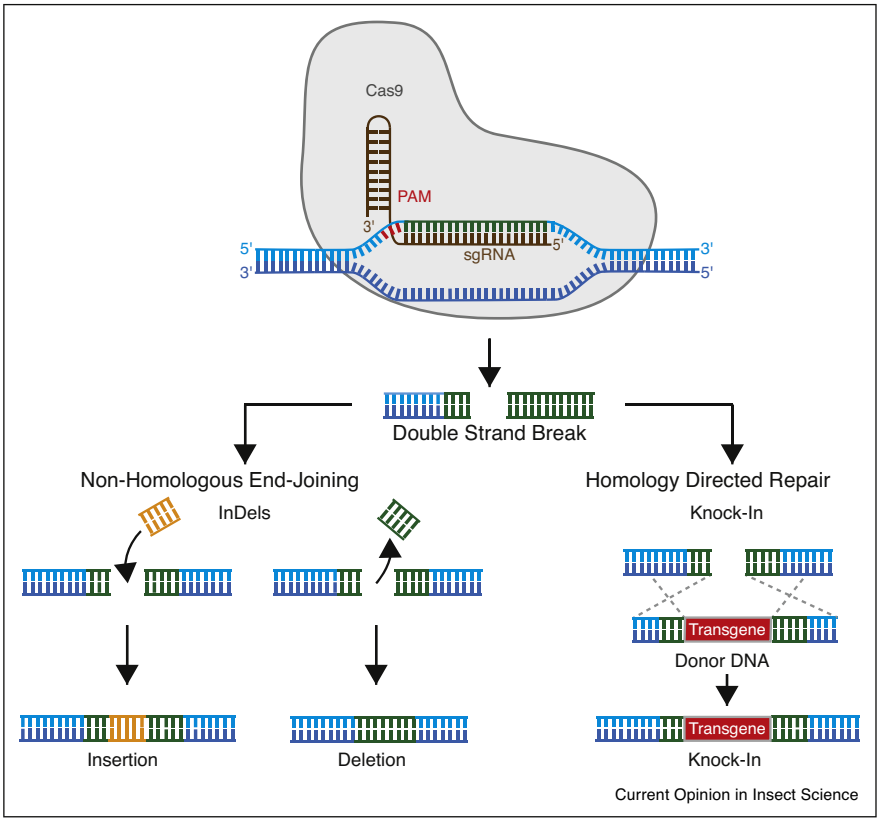
Genome editing with this tool involves an engineered single guide RNA (sgRNA) that recognizes a specific target sequence in the genome, and a nuclease capable of double strand breaks, Cas9 [26]. The combination of sgRNA and Cas9 directs the double strand break to the specific location encoded by the sgRNA sequence, thus allowing to select with high specificity the targeted region for genome editing. The double strand break is then repaired by the cellular machinery through non-homologous end-joining (NHEJ), which will sometimes introduce a small mutation that can inactivate the targeted gene. Alternatively, the double strand break can be mended by homology directed repair (HDR) from a template provided along with the sgRNA/Cas9 enzyme

Figure 1



Basic elements of transposon transgenesis.
(a) Structure of a natural transposon. Transposons have two essential components: inverted terminal repeats (1) that flank a transposase (2) encoding gene. The transposase recognizes the inverted repeats, excises the sequence and transpose the element to a new place in the genome, typically in a recognizable target sequence. ORF: open reading frame.
(b) Binary transposition systems. Scientists have engineered natural transposons into a binary transposition system, where one element has the inverted repeats flanking genetic cargo that replaces the transposase coding sequence to move these genetic components into a genome. These immobile elements can only move in the presence of a transposase-producing element that lacks the inverted repeats and therefore cannot move into a genome itself but can move inverted repeat surrounded cargo elements when they are co-injected into early embryos targeting their future germ line for integration events.
(c) Three step transposition process. A transposase in the form of a helper plasmid or produced by expression from a natural transposon will recognize the inverted terminal repeat sequences, bend the DNA to bring the transposon ends together before excising the element creating double strand breaks that are repaired. The transposase bound to the excised DNA brings the element to a new target site and catalyze an integration event moving the DNA into a new location.

Figure 2



Crispr/Cas9 mediated genome editing.
An engineered single guide RNA (brown sequence) binds to the Cas9 enzyme and recognizes a specific sequence (green sequence) in the target DNA complementary to the guide RNA and immediately adjacent to the PAM sequence (red). The Cas9 enzyme will generate a double strand break four base pairs (bp) from the PAM sequence. The double strand break will be repaired by one of two mechanisms: 1) non-homologous end-joining (NHEJ, left pathway), which can cause a small insertion or deletion (InDel) if repair is incorrect, or 2) homology directed repair (HDR, right pathway) which requires a repair template with homology on both ends immediately adjacent to the cut site. Through homologous recombination anywhere in the flanking sequence the repair template can integrate a transgene sequence (surrounded by the homology arms) into the cut site. This pathway allows knocking DNA into the genome. If two guide RNAs are used, the repair process can replace an existing sequence in the genome with the transgene sequence.

complex and used to introduce a transgene at the targeted location. With two different sgRNAs, a repair template can be used to replace existing sequences with any transgene of interest (Figure 2).

Transgenic payloads

The transgenes delivered by transposon or CRISPR/Cas9 editing fall into three general categories briefly discussed below: reporters, effectors, and components of binary expression systems, which have revolutionized the use of reporters and effectors. An extensive list of these categories and their use in *Drosophila* has been covered elsewhere [2].

Reporter proteins include fluorescent proteins that when expressed under the control of a specific promoter will allow visualization of the cells in which the promoter is active. These include green fluorescent protein (GFP) and its engineered modifications that fluoresce at different wavelengths as well as others such as the red marker tdTomato [27]. In some cases, these fluorescent proteins can be targeted to specific cellular sub compartments such as the nucleus, the lysosome, or the cell membrane [28,29]. Another widely used reporter is the GCaMP family of calcium indicators (e.g. GCaMP6s, where '6' is the generation number, and 's' denotes a slow, high sensitivity version) [30,31]. These indicators change their fluorescence with intracellular calcium concentration and thus allow indirect monitoring of neuronal activity since changes in calcium concentration are typically caused by changes in membrane potential through the opening and closing of calcium channels. Genetically encoded voltage indicators also exist but are more difficult to use [32].

Among effector proteins, channelrhodopsin is a blue light-gated cation channel which enables positive ion

flow into neurons when open [33,34]. This channel and its red-shifted variants make it possible to excite neurons with a light pulse and directly perturb their activity in resolved time windows. Inhibitory versions that target chloride/proton pumps or anion channels exist as well [35–37]. Tetanus toxin light chain (TeTxLC) is another effector protein, which blocks neuronal activity-evoked synaptic transmission (see below) [38].

Binary expression systems allow the flexible use of reporters and effectors expressed in a tissue-specific manner. The first of these, GAL4-UAS, was originally developed, and remains ubiquitously used, in *Drosophila* [39]. The yeast *GAL4* gene encodes a transcription activator that binds to the DNA upstream activating sequence (*UAS*) thereby driving expression of genes downstream of *UAS*. Thus, when *GAL4* is under control of a cell-specific or tissue-specific promoter it drives expression of a gene downstream of *UAS* encoding a reporter or effector protein in a cell-specific or tissue-specific manner. The GAL4-UAS system was systematically improved in *Drosophila* by modifying different features of the construct to optimize expression [40,41]. By maintaining independent tissue-specific promoter-GAL4 lines and UAS-reporter/effector lines, a simple cross allows to express any of these reporters or effectors in the desired tissue. The tissue specificity of GAL4 expression depends on the promoter that drives GAL4 and can derive from a specific gene or a cloned promoter or enhancer element [42]. The Q system (QF2-QUAS) is another, independent binary expression system derived from the bread fungus *Neurospora crassa* [43,44]. This system was also developed in *Drosophila* where its use with GAL4/UAS added to the sophistication of genetic manipulations [43]. Just as for GAL4-UAS, the QF2 transcription factor under expression of an endogenous promoter activates genes cloned downstream of a *QUAS* promoter. LexA-LexAop is the third available binary system in *Drosophila*, that originated from the λ phage [45]. For these binary expression systems, methods to further restrict expression to specific neuronal populations or within selected temporal windows sometimes exist. For example, the repressor GAL80 expressed in a tissue-specific manner suppresses the activity of GAL4 [46]. Similarly, QS is a suppressor of the Q system that can be inactivated within a selected temporal window by administration of an exogenous drug [43]. A similar innovation for GAL4 drivers not yet widely adopted is the drug inducible variant, called GeneSwitch, which also allows temporal control of the driver in addition to tissue specificity [47,48].

Transposon-based transgenesis

Transposon-based genetic engineering has a long history in *Drosophila* [49,50] but its most commonly used transposon, the *P-element*, never worked in non-drosophilid insects [51]. The *piggyBac* transposon described above was discovered in the cabbage looper moth and first used to transform the Mediterranean fruit fly [52]. Later, it was shown to work in many other insects [53], including the silk moth *Bombyx mori* [54].

A subsequent powerful application of *piggyBac* transformation in *Bombyx* was its use in adapting the GAL4-UAS expression system [55]. Further improvements similar to those developed in *Drosophila* were exploited in *Bombyx*, leading to reliable expression of GFP, GCaMP, and TeTxLC. These tools helped refine the understanding of how the sex pheromone bombykol impacts male mating behavior, and how ecdysone and insulin-like hormones affect development in a sex-specific manner [56].

CRISPR/Cas9-dependent gene knock-out and knock-in

The semi-random integration pattern of transposons across the genome does not allow targeting specific DNA sequences for modification. Targeted loss-of-function mutations are the bread-and-butter of CRISPR/Cas9. They have been used in a range of insect species to answer questions related to many aspects of their biology, including the role of sensory perception in behavior [19]. One example sheds light on an intriguing phenotype well-known in grasshoppers [57••]. Several grasshopper species possess the ability to transition from their usually solitarious existence to a gregarious one, where they aggregate in swarms that migrate collectively [58,59]. This phenomenon, known as locust phase polyphenism, carries important economical and human implications as locust swarming repeatedly devastates the crops and livelihood of African, Asian, and South American countries and sometimes even large parts of these continents. The mechanisms that regulate the transition from the solitarious to the gregarious phase are not fully understood but rely in part on olfaction. One potential aggregation pheromone is 4-vinylanisole (4VA). Behavioral experiments confirmed that 4VA is attractive to *Locusta migratoria* and emitted by gregarious animals in a density-dependent manner. The olfactory receptor OR35 detects 4VA and its knock-out by CRISPR/Cas9 triggered NHEJ resulted in lost attraction to 4VA in $Or35^{-/-}$ mutants. The attraction of *Locusta migratoria* to 4VA was confirmed in the wild, suggesting that it might be used for locust pest control. Another application of CRISPR/Cas9 mediated gene knock-out revealed the existence of a new pheromone receptor unrelated to similar previously described receptors in moths. This suggests that a class of pheromone receptors may have evolved independently at least twice in this insect family [60].

In mosquitoes, CRISPR/Cas9 gene knock-out was rendered more efficient by endogenous expression of *Cas9* through insertion of this transgene using *piggyBac*. Lines expressing *Cas9* endogenously may allow the development of mosquito population control strategies [61]. One application of gene knock-out in this species has shown

that the mechanisms for ammonia sensing, a component of human sweat, likely differ in vinegar flies and mosquitos [62].

HDR-mediated knock-in of genetic material in generic insects is still rare but that is rapidly changing. One example illustrating the power of this approach, particularly when combined with targeted RNA sequencing (RNAseq) analysis, comes from the mosquito *Aedes aegypti* [63••]. The choice between blood and nectar feeding is well-regulated in female mosquitos but the mechanism of this regulation is poorly understood. Using a combination of tools delivered with *piggyBac* transposons or CRISPR/Cas9, the mechanism of these feeding modalities is now better understood. The *Gr4a* receptor locus in *Ae. aegypti* encodes the gustatory receptor most closely related to sugar-detecting receptors of *Drosophila*. A knock-in of *QF2* in this locus was combined with a *piggyBac* integrated *QUAS-TrpV1* co-expressing a red fluorescent protein (Figure 3). Red fluorescence showed that *Ae. aegypti*'s *Gr4a* is expressed in the labium and the legs, the two major taste appendages of insects. Activation with capsaicin, the chemical trigger of TrpV1, was sufficient to drive the knock-in animals to consume water but not blood, suggesting that nectar feeding is initiated when *Gr4a* expressing neurons are activated. Blood feeding in female mosquitos is done with a syringe-like appendage called the stylet used to pierce the skin of their prey and operated like a straw to suck up blood. The stylet is surrounded by chemosensory neurons that may play a role in blood detection. A knock-in of *QF2* in the pan-neuronal *Brp* locus (see also Ref. [64]) combined with a *piggyBac*-integrated *QUAS-GCaMP6s* (see also Ref. [65]) showed that these neurons respond to blood. They also respond to sugar, salt, sodium bicarbonate ($NaHCO_3$) and ATP, a mixture triggering consumption of blood in females [66]. By exposing each of these components separately and evaluating Ca^{2+} responses, five clusters of neurons were detected. A comparative RNAseq analysis of the female stylet identified two putative ionotropic receptor encoding genes, *Ir7a* and *Ir7f* that may play a role in detecting the chemical components triggering blood engorgement. A *QF2* knock-in targeting these genes showed that they are expressed by a small subset of non-overlapping stylet neurons, and only in females. Ca^{2+} responses showed that *Ir7a* expressing neurons respond to $NaHCO_3$ while *Ir7f* expressing neurons respond to the whole mix, thus likely representing 'integrator' neurons. Interestingly, neither of these neuron clusters responded to nectar-specific sugars.

Another knock-in application of CRISPR/Cas9 in mosquitoes suggests that their olfactory system may be organized unconventionally, with multiple olfactory receptors co-expressed in single olfactory neurons. Further, convergence of olfactory receptor neurons expressing different receptors in the antennal lobe may not be as strictly segregated as documented earlier in such widely divergent species as mice and vinegar flies (Younger *et al.*, bioRxiv doi: 10.1101/2020.11.07.368720). Similar observations have now also been reported in *Drosophila* (Task *et al.*, bioRxiv doi: 10.1101/2020.11.07.355651).

Comparing transposon and CRISPR/Cas9 mediated transgenesis

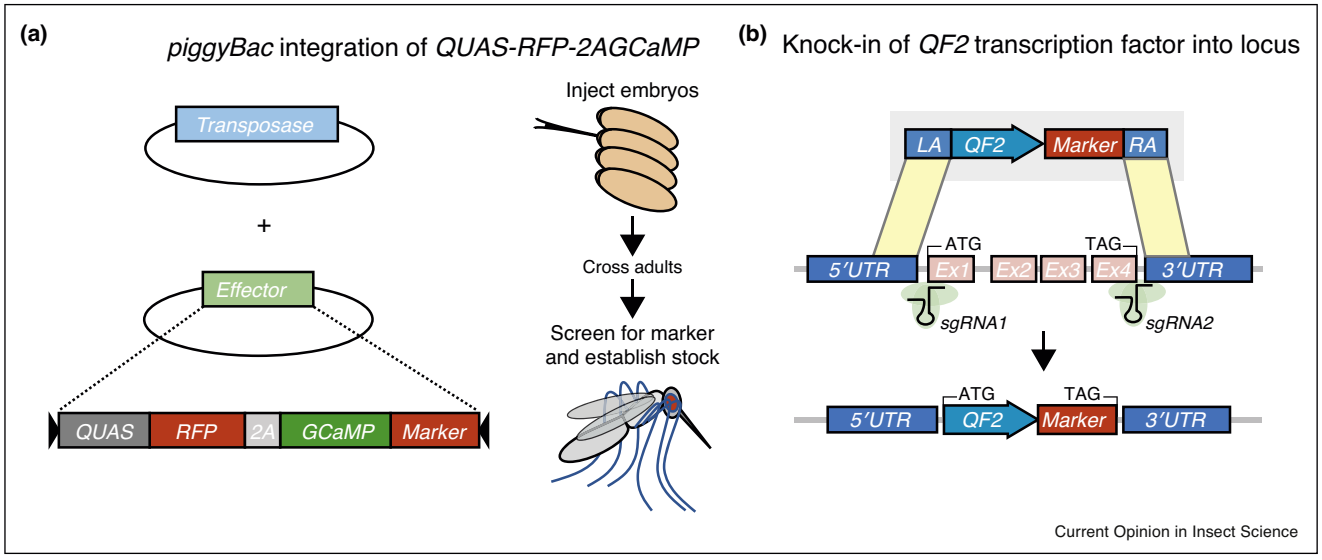
As is clear from the above discussed examples, transposon mediated transgenesis has long been widely used. Its major advantages are broad applicability [53] and relatively high efficiency, even in insects difficult to transform genetically [67]. Its major weakness is that it is not targeted. Most transposons integrate randomly into the genome, with some bias. As a result, transposons can accidentally inactivate a gene causing an unintended phenotype. In *Drosophila* transposons were in fact used early on to mutagenize genes and rapidly identify the resulting mutation [68,69]. In addition to potential mutagenicity, the integrated material is subject to position effects, which can affect expression of the transgene because of the genomic context of the integration site [70]. This makes it hard to compare effectors inserted at different sites. These problems have been bypassed in *Drosophila* by using a viral integration system based on the bacteriophage integrase ϕC31 and its complementary DNA docking sites, *attP* and *attB* [71]. The *attP* docking site is first randomly inserted in the genome through transposition. The different strains generated with specific docking sites [72] can then be used to integrate effectors using ϕC31, a plasmid with the complementary *attB* site, and the cloned payload. Different transgenes integrated in the same site can be compared because they are subject to the same genomic context. This site-specific integration system is now also used in mosquitos [73].

In principle, Crispr/Cas9-based genome-editing addresses all the disadvantages of transposons. However, it can lead to off target effects despite its precision [74]. In addition, the efficiency of HDR mediated integration is still not as high as random or site-directed integration. In the long run, as Crispr/Cas9 transgenesis becomes more efficient and reliable, it may reduce the role of transposition in genome modification or even usurp transposition as in a recently developed hybrid method [23,24].

Other delivery routes for CRISPR/Cas9 genome editing

As mentioned earlier, the molecular components necessary for genome editing are typically injected into an early embryo to target its future germline (but see Ref. [61], discussed above). The editing cocktail is often entirely plasmid-based, but it can be a mixture of gRNA, Cas9 protein or RNA and a donor plasmid for repair. When targeted microinjection is difficult such as for insects with small embryos, an alternate method exploits receptor-mediated endocytosis in the ovaries. In this approach, the

Figure 3



Genetic engineering strategies in non-model system insects.
(a) Transgenesis with *piggyBac*. Embryos are injected with a mixture of helper plasmid, providing the transposase source, and the effector plasmid. The effector plasmid is a transposon that lacks the transposase but still has the inverted repeats that can be recognized by the transposase and therefore integrate in the genome at random locations typically in a TTAA sequence. Injected fertilized eggs will undergo transgenic integrations at low frequency in the cells that will become the future germline of the embryo. Adults that emerge from the injected embryos are crossed to animals of the opposite sex and screened for transformed offspring. The transgenic offspring can be recognized because the effector plasmid that integrates in the genome contains a screenable marker. The screenable marker typically is a fluorescent protein that is expressed with an eye-specific promoter making the eyes of transgenic animals fluorescent [91]. The example effector shown here contains a QUAS response element that will respond to the QF2 transcriptional activator that is generated in a separate animal. The example effector expresses a Ca^{2+} indicator, GCaMP, that will respond with an increase in fluorescence when Ca^{2+} levels in the cell increase [92]. To mark the GCaMP expressing cells, a 2nd fluorescent protein can be expressed from the same QUAS response element (e.g., red fluorescent protein, RFP). Both proteins are produced from the same transcript because they are linked by a viral 2A sequence which leads to bond skipping during translation at the penultimate amino acid of the 2A sequence separating the 2 protein from each other [93].
(b) Crispr/Cas9 mediated knock-in. The cartoon shows a knock-in strategy of the QF2 transcriptional activator in a gene with four exons that will be replaced with the QF2 sequence driving expression of QF2 in the spatio-temporal pattern of the gene that is replaced. To generate the knock-in, embryos are injected as in the *piggyBac* strategy in (a), but instead of a transposase, the Cas9 enzyme is injected with sgRNAs (both can also be provided as plasmids expressing these sequences) that will target the Cas9 enzyme to the two recognition sites cutting the gene out of its location. By providing a repair template plasmid that is co-injected, the gene can be replaced with the QF2 sequence. The replacement happens through HDR via two ~1 kbp flanking homology arms that have identical sequences to those around the sgRNA cut sites in the host DNA (left arm, LA and right arm, RA). To screen the injected embryos for transgenic transformation, surviving adults are crossed as above and screened for the marker that again indicates a successful transgenic event. The event is also molecularly verified to make the sure the integration occurred as intended.

gene-editing cargo is combined with a ligand derived from yolk protein precursors. The molecular cargo is then delivered to the ovaries of females, so it can be deposited in oocytes during yolk formation (vitellogenesis). This technique referred to as Receptor-Mediated Ovary Transduction of Cargo (ReMOT Control) has been demonstrated in several insects including jewel wasps (*Nasonia vitripennis* [75•]), mosquitos [76] and whiteflies (*Bemisia tabaci* [77]). An adaptation of the ReMOT control approach was also proposed in insect models where yolk protein precursors are not known or are difficult to synthesize. In this approach, delivery to ovaries is achieved using nanosphere peptides that encapsulate ribonucleoproteins [75•].

Optogenetics through viral transfection in generic insects

Transient transfection of insect neurons using recombinant viruses represents an alternative to germline transgenic transformation. Although not yet implemented in insects, this method is often combined with complex transgenesis in mice [78]. Its advantage is that it does not involve genome transformation, making it simpler to implement. The disadvantage is that it is limited in its specificity and reproducibility, which are mainly determined by the injection site and the number of viral particles injected into the animal. The Sindbis virus has been used to study development of butterfly wings and of beetle embryos [79]. Recently, the Semliki forest

virus, an alpha virus closely related to Sindbis, was used in grasshoppers to deliver channelrhodopsin [80•]. This technique allowed channelrhodopsin expression in excitatory neurons presynaptic to an identified collision-detecting visual neuron of the grasshopper optic lobe, the lobula giant movement detector (LGMD) [81]. Subsequent stimulation through pulses of laser light elicited vigorous spiking in the LGMD, thus demonstrating that optogenetics can be implemented in non-genetic model insects using virus-mediated delivery (Figure 4).

Conclusions and outlook

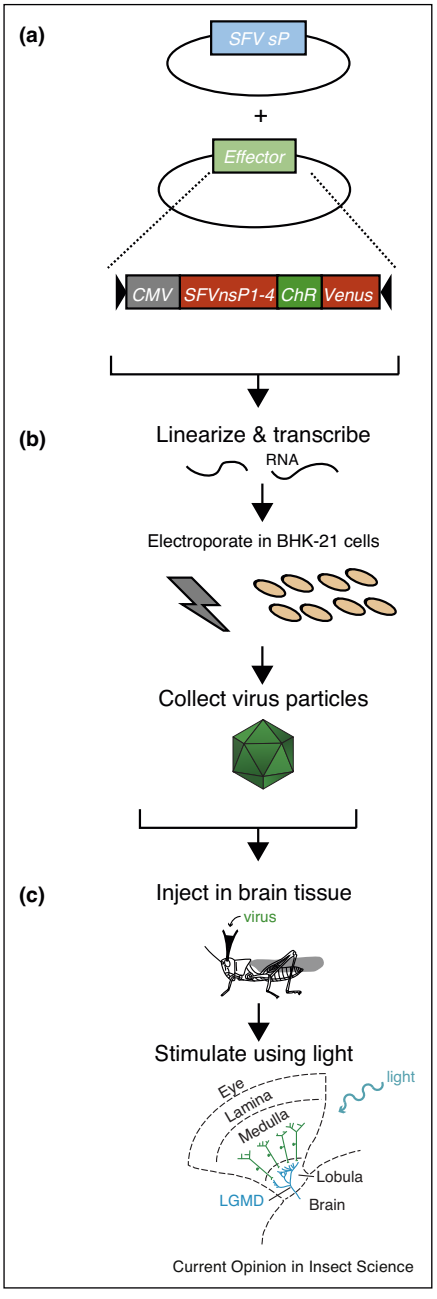
The combination of high throughput genomic approaches with genome-editing technologies is allowing unprecedented genetic manipulation in generic insects. Tools developed in traditional genetic models are now adapted to these non-traditional insect models. We highlighted examples where this approach revealed novel mechanisms previously poorly understood. These approaches will likely expand into more animals, exploiting their unique biological features to investigate novel aspects of brain function and behavior.

A comprehensive understanding of behavior requires integration at many levels. First, the neurons and circuits that control behavior must be identified. Then, we must understand how activity in these circuits affects behavior and identify the genes required for circuit development or function. Tissue-specific transcriptome data, temporal expression profiles, and single cell atlases will help find genes that could be targets for editing and functional studies. A reference genome is also important to make the best use of such profiling data.

Many of these future developments will have translational and societal benefits. For example, mosquitos are vectors for a range of diseases [82]. Manipulating their genomes and learning about their behaviors will help control the spread of diseases associated with them. Honey bees are critical for agriculture and understanding their biology could potentially save entire industries [83]. Locusts cause devastating plagues that regularly destroy the food supply of entire countries, often underdeveloped [84]. Understanding the mechanisms switching on gregarious swarming will only be possible through an integrated approach dependent on all the techniques discussed above.

What are some of the challenges we need to overcome to make these new approaches usable in a broad range of species [73]? One problem not yet fully resolved is tissue-specific expression. In *Drosophila* many thousands of *GAL4* lines with different promoters and enhancers are available [41] to drive the expression of effectors allowing to image neurons or manipulate their function. Such large collections, maintained independently by entities like the Bloomington Drosophila Stock Center (https://bdsc.

Figure 4



Viral transfection of insect neurons using Semliki Forest Virus (SFV).
(a) The main effector plasmid codes for the non-structural SFV proteins one to four (SFVnsP1-4), for an effector protein such as channelrhodopsin (ChR) and a fluorescent marker (Venus) under control of a ubiquitous promoter (CMV). Additionally, the SFV structural proteins are provided in a distinct helper plasmid so as not to be packaged during viral production, resulting in the fabrication of replication-deficient virions.
(b) The plasmids are linearized and transcribed before being electroporated in BHK-21 cells. The assembled SFV particles are collected for subsequent *in vivo* experiments.
(c) The virus is injected through the eye into the brain of live grasshoppers fed with retinal. After a few days the animals are used to image stained neurons and stimulate them using cyan light. This allows trans-synaptic stimulation of the LGMD neuron.

indiana.edu/), simply do not exist for other insects. Identifying and testing similar promoters in other species will need to be optimized. For this purpose, genome sequences and cell-specific expression atlases will be necessary. A virally mediated approach may help test putative promoters before making the effort of modifying the genome of an animal. Some genes, for example the rate limiting enzymes in the synthesis of serotonin and dopamine, have narrow expression patterns so a knock-in in their locus may be one of the easier expression tools to generate (Sects. 5.9 and 5.10 of Ref. [85]). Neuropeptides are also expressed in limited neuronal populations (Sects. 5.13–5.27 of Ref. [85]) and could be attractive targets since they play key roles in regulating basic biological functions and behavior, such as feeding and reproduction.

Another problem is the efficiency of CRISPR/Cas9 transgenesis. In mosquitos for example, thousands of embryos must be injected to find a successful CRISPR/Cas9-mediated transgenic HDR event, in contrast to *piggyBac*-mediated transgenesis. One solution is to use a selection rather than a screening approach. By coupling a selection marker to the transgene, only transformed animals will survive, eliminating the need to examine all offspring for the presence of the screening marker, often a fluorescent protein expressed in the eye. This approach was recently developed in *Drosophila* and reduces the pool of animals screened from several hundreds to just a few [86••]. The 3xP3 enhancer widely used for screening works well across many insects [87], showing robust expression in the eyes, but few other marker enhancers exist. For selection, an enhancer that is expressed early in development is necessary and several promoters, either viral [88] or constitutive [89], could work for this purpose. Developing new enhancers for marker expression would nevertheless also be useful. Another solution to low transgenic efficiency may be to simplify the delivery cocktail. Adding all the required components into a single plasmid increased the rate of transgenesis in microorganisms [90] and may work similarly in *Drosophila* (Dierick, unpublished results).

While still challenging, advances in insect transgenesis will likely transform both basic and applied insect research, including studies of neural function and behavior. They will finally enable us to comprehensively address Tinbergen's four questions and provide new avenues for understanding brain function and behavior from a comparative, evolutionary perspective. As illustrated by the examples discussed above, these advances can shed light on all of Tinbergen's questions when combined with techniques such as behavior, electrophysiology, imaging, or phylogenetic and RNAseq analysis. This new road is promising to further blur the lines between traditional genetic model systems and those that were largely refractory to genetic analysis.

Conflict of interest statement

Nothing declared.

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- • of special interest
- •• of outstanding interest

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