



Recent approaches to study the neural bases of complex insect behavior

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Recent advances in biocompatible materials, miniaturized instrumentation, advanced computational algorithms, and genetic tools have enabled the development of novel methods and approaches to quantify the behavior of individuals or groups of animals. In conjunction with technologies that allow simultaneous monitoring of neural responses, quantitative studies of complex behaviors can reveal tighter links between the external sensory cues in the vicinity of the organism and neural responses they elicit, and how internal neural representations finally get mapped onto the behavior generated. In this review, we examine a few approaches that are beginning to be widely exploited for understanding neural-behavioral response transformations.

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Introduction

Classical neuroscience has been built on the back of excellent electrophysiological experiments where scientists were able to probe and manipulate individual or small groups of neurons in simple neuronal circuits [1–4]. While these studies have provided an invaluable understanding of neural computations at the single neuron level, the causal link between neural responses and behavioral outputs often remained tenuous. Recent progress has allowed both monitoring and controlling neurons with high spatial and temporal resolution. Transgenic insects that express optical fluorescent markers or controllable non-native ion channels/pumps in select neurons are widely being used to characterize how these neurons encode information about various sensory cues and drive different motor programs. Combined with clever

manipulations that simultaneously allow insects to ‘behave,’ correlations and sometimes stronger associations between neural and behavioral responses can be probed.

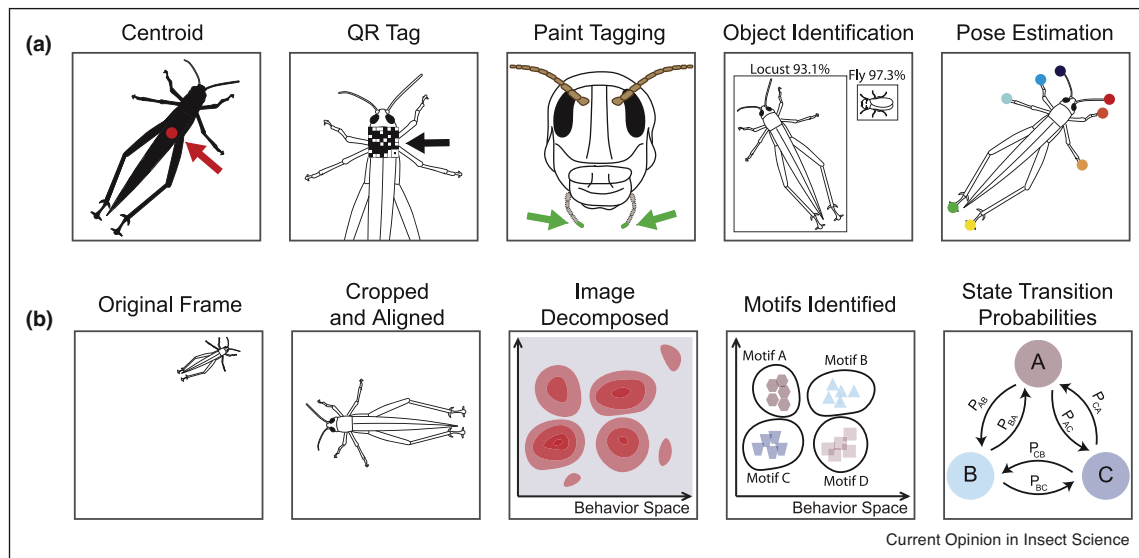
While quantitative methods to understand neural responses are the norm, similar approaches for characterizing behavior are only beginning to be widely adopted. Particularly, behavior in invertebrates can be challenging to quantify due to their smaller scale and alien anatomy compared to mammals. Traditional manual approaches to analyze and quantify insect behavior, while useful, have many limitations. For example, humans can only observe relatively simple behavioral motifs, patterns on short time scales, and are prone to individual biases/inconsistencies. Furthermore, manual identification and classification of behaviors become increasingly challenging as the amount of data generated by experiments continues to grow. With the advent of high-resolution, high-speed cameras and other time-resolved measurements, algorithmic advances in computer vision and machine learning are necessary to automate analysis pipelines. Taking advantage of such advances, much progress has been made to characterize more complicated behaviors with greater speed and certainty.

In this review, we focus on a few recent studies that have taken novel approaches to link neural responses with behavioral response motifs.

Categorizing and quantifying behavioral responses

Modern advances in data acquisition and processing allow behavioral responses to be monitored at fine temporal and spatial resolutions. Error-prone manual tracking methods have been supplanted by computer vision methods such as centroid/center-of-mass tracking, which allow accurate positional tracking of an organism over time. These algorithms have been further optimized with the use of markers such as RFID tags [5–9], radar transponders [10–12], QR codes [13–17], and non-inhibitive paint [18,19], as well as through the use of denoising and filtering algorithms (Figure 1a). Analysis of animal movements with these approaches is particularly suitable for studying group behavior, and has been used in a wide range of studies, from social interactions in honeybees and ants [20–25], to behavioral variance and modulation by group composition in locusts [26], and social enhancement of light avoidance abilities in cockroaches [27]. Such high-fidelity behavioral tracking combined with genomic and

Figure 1



Methods for tracking and quantifying behavior.

(a) A wide range of tracking tools have been used for quantifying behavioral responses. Centroid/ center-of-mass tracking allows markerless tracking of the position of an insect in a well-defined arena. The addition of markers such as QR codes and colored paints allows more precise tracking of individuals in a group and even specific body parts. Deep learning-based methods allow tracking of different species in a group, untagged tracking of individual body parts, and extraction of behavioral motifs and poses.

(b) A pipeline for extracting behavioral motifs. The organism is identified using an object identification algorithm and its pose is decomposed to a lower dimension. Different behavioral motifs are identified in this low-dimensional space. The identified response motifs and the transition probabilities of moving from one behavioral motif to another can be estimated to develop a probabilistic model for generating a behavioral response.

transcriptomic data could be utilized for probing and understanding the molecular basis for behavioral variations or phenotypic plasticity [28].

More recently, applications of deep neural networks have allowed markerless recognition of individual organisms. Tools such as IDtracker have been successfully utilized to study social interactions during foraging in zebrafish [29], and social modulation of looming response in fruit flies [30]. Among the most noteworthy tools that have recently been developed are DeepPoseKit and DeepLabCut, where the authors trained deep convolutional neural networks (CNNs) to detect organisms by providing a handful of frames labeled with poses of interest [31–33]. Through a process of iterative learning, the CNNs learned to recognize and accurately track the insect with fine temporal (high frame rate) and spatial (few pixels) resolutions. Furthermore, similar approaches allow automated segmentation of complex behavioral responses as sequences of elemental behavioral motifs (Figure 1b) [34,35,36]. These robust networks could be applied to even study insects in their natural environments (field studies), as part of large social groups, and even in the presence of multiple species.

In some scenarios, such as capturing idiosyncratic behaviors (e.g. grooming) or interactions between individuals

in group studies, pose-estimation with 3D tracking might be necessary. In general, 3D pose estimation is a more complex problem and requires the use of multiple synchronized cameras, and calibration of XYZ coordinates across cameras using well-positioned landmarks [37–39], or by custom solutions such as swarm markers [40]. Nevertheless, such approaches have successfully been used to study the physical properties of gnat swarms [40], and the energy dynamics of movement through complex obstacles in cockroaches [41,42]. Computational tools such as DeepLabCut and DeepPoseKit are now enabling estimation of poses in 3D space with minimal equipment requirements [31–33].

Mapping neural activity onto behavioral responses

Precise quantification of behavioral responses can also be exploited to understand the significance of neural activity in different regions and how they evolve. Efforts to understand neural bases for a behavioral response can be roughly categorized into three categories: (i) *the mapping problem*: identifying regions that are activated during a behavior and therefore are potentially important for generating that response, (ii) *the dynamics problem*: understanding how neural responses patterned over time correlates with, and therefore contributes towards, behavioral response changes over time, and (iii) *the idiosyncrasy*

problem: linking neural response variability across organisms with individual behavioral preferences and responses.

The mapping problem

The first step in understanding neural mechanisms that mediate a behavioral response is to determine which neurons and neural circuits are important for (i) processing the sensory cue that triggers the behavior, and (ii) executing the motor programs that carry out the voluntary movement. Since monitoring responses of all neurons in the insect brain during the execution of the behavioral response is not feasible yet, one remedying step would be to identify all neurons that were active and therefore potentially important for generating that behavioral response. The immediate early genes (IEGs) that are rapidly and transiently expressed in neurons following activation are one family of candidate markers that could be used for labeling active neurons. In two recent studies, a specific IEG was used to identify a few neural loci important for courtship behavior in male silkmoths (*Bombyx mori*) [43] and fruit flies (*Drosophila melanogaster*) [44]. In both these insects, exposure to female pheromone increased neural responses and therefore elevated *hr38* expression in activated neurons throughout the brain. The labeled activity maps partially overlapped with neurons that are known to be part of sexually dimorphic circuits expressing *fruitless* (*fru*) and/or *doublesex* (*dsx*). Notably, in the fruit fly system, activity-dependent expression of light-activated ion channels (*CsChrimson*) in *fru* or *dsx* neurons allowed reactivation of the neural circuits with red light and thereby regenerate the abdomen bending behavior observed in males during courtship [44]. Since IEGs, such as *hr38*, are also found in other insects such as honeybees [45], whether this strategy for labeling neurons important for a specific behavior can be widely adopted remains to be determined.

Alternately, using the genetic tool kit in fruit flies (*D. melanogaster*), a recent *tour de force* study combined genetic perturbations and machine vision analysis of behavior to identify the neural populations in the fly brain that might contribute to a wide variety of motor responses [46^{••}]. Analyzing videos from an incredible 400 000 flies, behavioral motifs were identified in a semi-supervised fashion (i.e. part human and part algorithmic labeling). In parallel, expression patterns of ~2200 genes and the effect of neural activation on different behavioral motifs were analyzed to generate ‘brain-behavioral correlation maps (Figure 2a).’ As can be noted, this high-throughput approach allows the identification of large ensembles of neurons that contribute to a mean behavioral response (i.e. averaged over time and individuals).

The dynamics problem

The behavioral responses elicited by most stimuli are often temporally structured (i.e. dynamic). Can the

behavioral responses at different time points, and the transitions between them be predicted from the neural activity? This issue was investigated in the context of olfaction and odor-driven behavior in locusts (*Schistocerca americana*) [19]. The authors showed that most odorants activate two distinct ensembles of neurons, one during the stimulus presentations (ON ensemble), and the second after stimulus termination (OFF ensemble). The authors found that the ON neural response was sufficient to predict whether an odorant would evoke a behavioral response and the speed of response initiation (i.e. the opening of sensory appendages close to the mouth upon conditioned stimulus presentations in anticipation of the reward). However, the OFF ensemble responses were more reliable predictors of when the behavioral response to the conditioned stimulus was terminated.

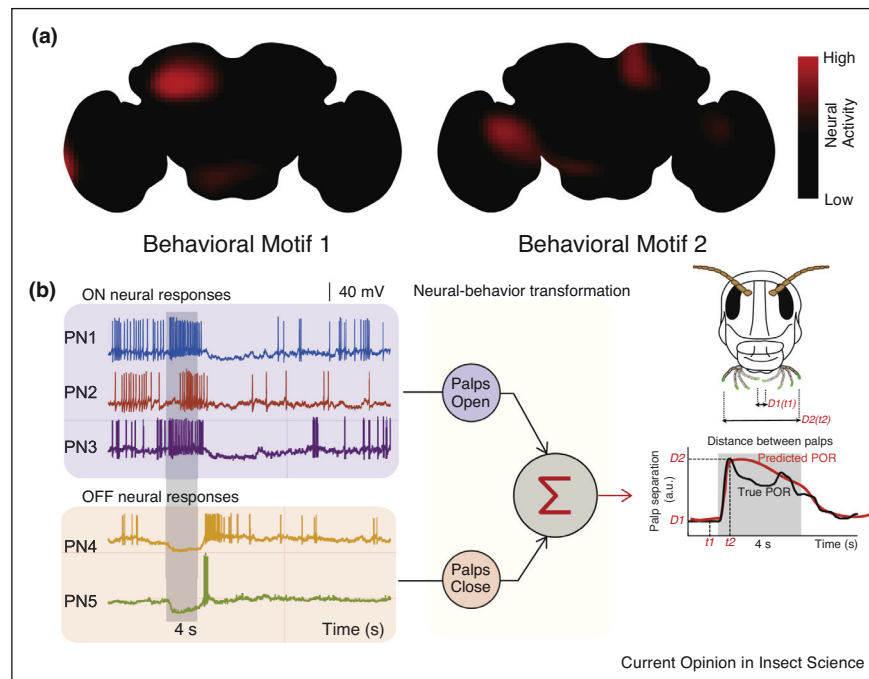
In a series of follow-up studies, the authors found neural responses evoked by the same odorant varied when perturbed by altering background odors, ambient conditions, and stimulus history. Yet, the behavioral responses were robust to such perturbations, and the locusts trained to recognize an odorant could do so and open their palps in an invariant fashion [19,47,48,49[•]]. To resolve this apparent confound, the authors demonstrated a simple logical classifier (OR-of-ANDs) where a flexible combination of active ON and OFF neurons was sufficient to successfully predict the invariant behavioral response observed (Figure 2b) 49[•]].

In sum, these results indicate how activating different neural ensembles that are part of the same circuit over time could help orchestrate predictable temporally patterned changes observed in behavior, and that the neural-behavioral mapping could be highly robust to extrinsic perturbations.

The idiosyncrasy problem

Does a sensory stimulus drive stereotyped neural responses in different individuals, or do the neural responses vary and thereby could underlie the generation of idiosyncratic behaviors among individuals? A recent study in flies characterized the variability of innate odor preferences amongst flies from the same inbred lab wild-type colony [50]. Using a two-choice odor assay, the authors found that the distribution of odor preference scores across flies varied when compared to the null distribution (i.e. no odor preference). The odor preferences remained stable across days. Notably, imaging of odor-evoked responses in transgenic flies expressing genetically encoded calcium indicators in the antennal lobe’s principal neurons revealed gross stereotyped responses that were embellished with several fly specific response variations. The authors found that odor preference variability could be reduced through endogenous mechanisms such as serotonin synthesis inhibition (by feeding flies with alpha-methyltryptophan), carrying

Figure 2



Neural-behavioral response transformations.

(a) Genetic tools in the *Drosophila* model can be exploited to determine which brain region contributed to generating a behavioral response. A cartoon version of the 'brain-behavior maps' that can be expected from this approach is shown.

(b) A model for mapping neural responses and how they change over time onto behavioral response dynamics in locusts is shown. Different sets of projection neurons were activated during (ON response), and after the termination of a stimulus (OFF response). Responses from the ensemble of ON neurons were sufficient to predict the onset of the behavioral response, whereas OFF neuronal responses were better indicators of when and how the behavioral responses terminated [47].

mutant dopamine receptor gene (*Dop1R1*), or by activating different subtypes of local neurons in the antennal lobe. On the other hand, exogenous variables such as changes in weather (higher variability observed during winter) and diet (switch from normal cornmeal/dextrose to commercial flake food) led to increases in behavioral variability.

Neural recordings during the execution of a behavioral response

Compared to the studies discussed so far, a more direct approach would involve monitoring neural responses in a behaving insect. Recently, the use of virtual reality setups and electrophysiological recordings in ambulatory preparations is being explored to go beyond neural-behavioral correlational maps.

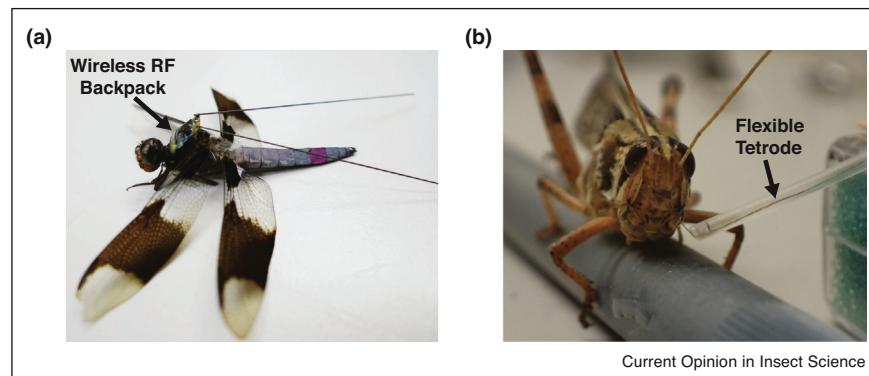
Virtual reality setups

Virtual reality (VR) setups have become increasingly popular for simultaneous recordings of neural responses while the external stimuli are presented in a controlled manner to affect behaviors. A majority of the VR setups have used tethered flight or head-fixed walking on a floating ball while visual, olfactory, or mechanosensory

inputs are systematically varied. Using such a VR setup, a recent study had explored how different flying insects (fruit flies, mosquitos, hoverflies, and crane flies) navigate in complex environments and perform long-range search behavior as they forage for food [51^{••}]. Manipulating visual, olfactory, and mechanosensory cues the authors found that flying insects could use computations such as object segmentation, perspective and motion parallax, and multimodal integration in real-time as they localized and moved towards a target.

In addition to investigating behavioral algorithms exploited by insects, the VR setups have been used to examine the neural representation of a behavioral response. In a recent study in fruit flies (*D. melanogaster*), a walking setup directly positioned under a 2-photon microscope was used to investigate how the head direction is encoded in the ellipsoid body of the fly's central complex [52^{••}]. To understand the structure of the recurrent ellipsoid body neuronal network, the authors used a combination of focused ion beam electron microscopy to reveal synaptic connections between circuit elements. Next, using RNA-sequencing and fluorescence *in-situ* hybridization they characterized receptors and

Figure 3



(a) Photo of a dragonfly with a mounted wireless recording backpack, capable of recording EMG and nerve cord signals in a mobile preparation, using RF signals to power the electronics. (Image adapted from Thomas *et al.* provided by Dr Anthony Leonardo.) [59].

(b) Photo of a tethered ambulatory locust preparation with a flexible electrode surgically implanted in the antennal lobe for recording olfactory signals during behavioral assays [62].

neurotransmitters at various synaptic connections and therefore the type of interactions (excitatory versus inhibitory) at various parts of the network. Finally, imaging calcium signals from ellipsoid body neurons in head-fixed walking flies, the authors dissected how the different structural motifs of this network facilitate its functioning as a ring attractor (a compass-like neural network where neurons tuned to the same head direction excite each other, but neurons encoding different head directions inhibit one another).

VR techniques have also been successfully used to study the neural bases for a wide range of behaviors including, looming objects and motion parallax in locusts [53], stereopsis in the praying mantis [54], sensorimotor flight control in *Drosophila* [55], and selective attention in honeybees [28]. While there are some restrictions imposed on the mobility of the organism, this approach offers a great alternative when outdoor studies can be too expensive or infeasible (e.g. due to weather conditions). Combining markerless tracking with dynamically adjusting arenas such as treadmills [56] and wind tunnels has the potential to allow more unrestricted behaviors, but further work remains to be performed in terms of accurate tracking, precise closed-loop control, arena design, and stimulus delivery.

Neural recordings in ambulatory preparations

To achieve simultaneous neural and behavior monitoring, fast and accurate tracking of animal behavior is, however, only half the story. The scale of insects has posed a significant engineering challenge in the fabrication of recording probes that can allow relatively unrestricted movement. Recent advances in microfabrication techniques have led to the development of miniature electrode arrays which have the potential for use in many insect

models. However, the commercial availability of suitable probes remains limited for mobile experiments, and preparing custom in-house electrodes is often an easier solution [57].

Traditionally, mobile preparations have required balancing the increase in noise with electrode length against the mobility offered to the animal. While relatively long electrodes that do not hinder the insect's movement have been used successfully [58], miniaturization of amplifier technology has increasingly made it possible to mount the pre-amplifiers and digitizers for data transmission directly on the animal, minimizing noise while enabling free-roaming movement [59–61]. This can optionally be combined with small wireless transmitters to provide nearly unfettered mobility to the animal being recorded (Figure 3a). Such an approach has been combined with custom electrodes to localize odor sources and study neural underpinnings of behavioral responses in locusts (Figure 3b) [53,62], and movement in cockroaches [58].

Conclusions

The last decade has seen several advances made towards developing genetic, electrophysiological, computational, and instrumentation toolkits to understand molecular and neural bases for behavioral responses in insects. In particular, the ability to monitor and quantify behavioral responses has provided key constraints in interpreting and understanding the functional relevance of neural responses observed in different brain regions and over time. The current set of studies, however, have only begun to probe and understand the rules that govern how variable and complex neural activity underlie behavioral responses. A more complete understanding of these rules would require, at minimum, technical advances to address the following challenges:

- Can we monitor neural and behavioral responses from a single insect over extended periods (days to months to entire lifetime)?
- Can we create a wider library of behavioral responses that can be monitored and quantified?
- Can we monitor several insects simultaneously and determine how they interact?

Notwithstanding the progress made, current approaches have been designed for noiseless settings inside a laboratory, and therefore limit the complexity of experiments that can be performed. Thus, there is still a need for designing solutions to the problem of monitoring and quantifying neural/behavioral responses in complex and eventually in open field settings or their natural habitats.

Conflict of interest statement

The authors have no conflict of interest to declare.

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