REVIEW ARTICLE

Neural Circuits Underlying Fly Larval Locomotion

Hiroshi Kohsaka[1],*, Pierre A. Guertin[3] and Akinao Nose[1,2]

[1]*Department of Complexity Science and Engineering, University of Tokyo, Kashiwanoha, Kashiwa, Chiba 277-8561, Japan;* [2]*Department of Physics, Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan;* [3]*Department of Psychiatry & Neurosciences, Laval University, Québec City, QC, Canada*

Abstract: Locomotion is a complex motor behavior that may be expressed in different ways using a variety of strategies depending upon species and pathological or environmental conditions. Quadrupedal or bipedal walking, running, swimming, flying and gliding constitute some of the locomotor modes enabling the body, in all cases, to move from one place to another. Despite these apparent differences in modes of locomotion, both vertebrate and invertebrate species share, at least in part, comparable neural control mechanisms for locomotor rhythm and pattern generation and modulation. Significant advances have been made in recent years in studies of the genetic aspects of these control systems. Findings made specifically using *Drosophila* (fruit fly) models and preparations have contributed to further understanding of the key role of genes in locomotion. This review focuses on some of the main findings made in larval fruit flies while briefly summarizing the basic advantages of using this powerful animal model for studying the neural locomotor system.

1. INTRODUCTION

Invertebrate species have enabled several breakthrough findings on neural circuit functions to be made in many decades; the generation of the action potentials in squid [1], molecular mechanisms of learning and memory [2], the concept of central pattern generators (CPGs) [3,4], identities of neurons composing CPGs [5,6], etc. By virtue of their rich resources of genetic tools, the fruit fly *Drosophila melanogaster* (referred to "*Drosophila*" hereafter) allows us to delve into complex processes in neuroscience [7]. These include: genetic programs for segmentation along the longitudinal body axis [8], neural cell fate determination by Notch signaling [9], specification of neural identity [10], axon guidance [11], synaptogenesis [12], channel gene identification [13,14], learning and memory [15], courtship behavior [16], and circadian rhythm [17].

In recent decades, larval fruit flies have generally been considered a promising model to also examine neural locomotor circuits from a genetics standpoint. Especially, based on accumulated knowledge of developmental and molecular biology of the neurons in this system and accessibility to them with genetic tools, *Drosophila* larval locomotion—essentially characterized by caudal-to-rostral-propagated peristaltic crawling movements of the body—offers a valuable opportunity to link genes to behavior, and sensory inputs to motor outputs in cellular and molecular resolution. Furthermore, models targeted by larval locomotion have expanded to social interaction, neural disease and drug screening. In this review, we will introduce recent advance in studies related to *Drosophila* larval locomotion. Since the literature concerning larval locomotion has been expanding rapidly, we have not tried here to present a comprehensive and complete review of all recent studies. Instead, our intention has been to provide an introduction to *Drosophila* larval locomotion for researchers who do not regularly read about invertebrate locomotor systems.

2. OVERVIEW OF THE STRUCTURE AND DEVELOPMENT OF FLY LARVAE

The development of *Drosophila* larvae into adults is bridged by a holometabolous pupal stage. *Drosophila* goes through three larval stages (instars). Twenty-two hours after the egg is laid, the 1st instar larva hatches. In late stages of the embryonic development, all larval neurons are generated and form synaptic connections [18] (Neurons for adult flies develop during the late larval stage [19]). The 1st instar takes one day, the 2nd instar one day and the 3rd instar takes two days. The late 3rd instar larva forms a pupa, and the adult fly emerges from the pupa during the following five days. Several hours after eclosion, adult flies initiate courtship behavior and the female flies lay eggs. This short life-cycle (~ two weeks), their small body size (less than 5mm), and their omnivorous diet (we rear them with "fly food" containing yeast, sugar and cornmeal) are among the advantages of *Drosophila* as a model organism.

Fly larvae grow in size between every molt. The length of the egg is about 0.5 mm, and after two moltings the 3rd instar larva is 5mm in length (Fig. **1A**). The body wall of the larva is segmented, with three thoracic segments and eight abdominal segments (T1-3 and A1-8 in Fig. **1B**). Each half (hemi)-segment contains about 30 muscles. The muscles can be classified, based on their orientation, as longitudinal, transverse or oblique. Peristaltic locomotion is generated by sequential contraction of muscles from the posterior to anterior segments (in the case of forward locomotion). During the peristaltic wave, contraction of longitudinal muscles precedes that of transverse muscles [20]. The position and orientation of muscles in each segment are almost the same from A1 to A7, and therefore each segment can be regarded as a unit of motor outputs.

The central nervous system (CNS) can be exposed experimentally by cutting the body wall and removing the internal tissues (intestines, a trachea, fat bodies and Malpighian tubes) (Fig. **1C**). The CNS consists of two hemispheres (the brain lobes), the ventral nerve cord (VNC; thoracic and abdominal ganglia), which is analogous to the vertebrate spinal cord, and subesophageal zone (SEZ) in between the brain and VNC (Fig. **1D**). There are a number of neu-

*Address correspondence to this author at the 5-1-5 Kashiwanoha, Kashiwa-shi, Chiba, 277-8561, Japan; Tel/Fax: 81-4-7136-3921;
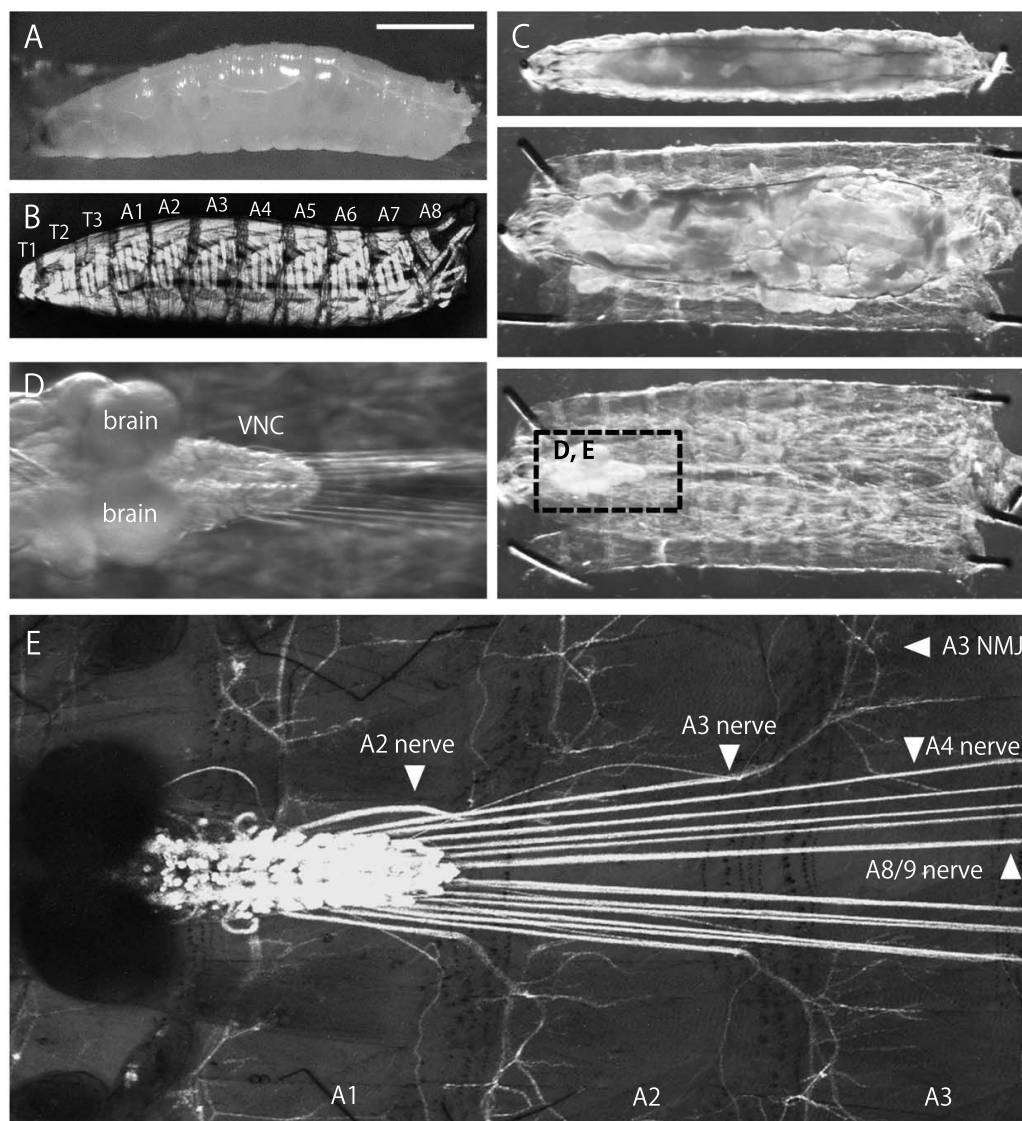E-mail: kohsaka@neuro.k.u-tokyo.ac.jp

Fig. (1). Anatomy of *Drosophila* larvae (3rd instar). (A) A lateral image of *Drosophila* larvae. Scale bar: 1mm. (B) Body wall muscles in larvae visualized by GFP expression. T1-T3, A1-A8 indicate thoracic segment 1-3 and abdominal segment 1-8. (C) Image series of exposure of central nervous system by dissection. Top: the head and tail are pinned down. Middle: The dorsal side of the body wall was cut and body wall was opened to fillet. Bottom: Internal tissues except for nervous system were removed. Dotted rectangle denotes the central nervous system (CNS). (D) Magnified image of the CNS in (C) showing the brain and the VNC (ventral nerve cord). The VNC corresponds to the vertebrate spinal cord. The SEZ (subesophageal zone) locates behind the brains. (E) Motor neurons visualized by GFP expression. Motor neurons in the VNC elongate axons through nerves (A2, A3, A4 and A8/9 nerves marked with arrowheads) to target muscles for forming the neuromuscular junction (NMJ). (A3 NMJ is marked with an arrowhead.)

ronal connections between the brain and VNC [21]. The VNC is segmented into three thoracic neuromeres and eight abdominal neuromeres. Muscles in each body wall segment are innervated by motor neurons in the corresponding neuromeres within the VNC (Fig. **1E**). Motor neurons form neuromuscular junctions on the body wall muscle that are visible in the dissected larvae (Fig. **1E**). Spatiotemporal activity of the motor neurons within the VNC underlies all larval locomotion. Accordingly, motor circuits in the VNC can be considered as a chain of segmental units. Based on this anatomical property, mathematical models are constructed to describe the larval crawling locomotion [22,23].

The larval VNC has been an outstanding model system for studies of neural development (see the review in [24]). We describe some of major discoveries briefly. Neurons in the nerve cord are formed from the neural ectoderm through multiple steps [25]: step 1, segmental and columnar patterning; step 2, neuroblast forma-

tion/specification; step 3, ganglion mother cell (GMC) formation/specification; and step 4, specification of neuron and glia by asymmetric GMC division. In segmental and columnar patterning of the neural ectoderm (step 1), a two-dimensional sheet of the neural ectoderm is divided by gene expression pattern into two orthogonal stripes: segment-polarity genes (*runt*, *wingless* and *gooseberry*) and columnar genes (*vnd*, *ind* and *msh*) [26]. This grid-like expression pattern demarcates compartments to generate specific neural stem cells called neuroblasts (NBs) in each grid. A single cell acquires the NB fate in each compartment through lateral inhibition by Notch-Delta interaction (step 2) [27]. The following specification of NB, GMC and neurons/glia is guided by temporal expression patterns of transcription factors (step 3, 4) [10]. As in vertebrates, *Drosophila* glial cells play essential roles in CNS development and function [28-30]. The number of neurons is regulated by the programmed cell death [31].

The identity of individual motor neurons has been characterized comprehensively, based on the connectivity with body wall muscles, [32,33]. Since inputs to motor neurons are critical determinants for motor outputs, the geometry of motoneuronal dendrites is a key to establishing functional motor circuits. Comprehensive single cell analyses revealed that dendrites of motor neurons form a "myotopic map" [34], with clear correspondence between innervating muscle groups and the dendrite position. This topological connectivity is thought to underlie coordinated control of muscles in the same group. The topology of the dendrites is regulated by guidance molecules at the midline [35], neural activity [36], and steroid hormone [37]. A genetic tool to visualize synaptic contacts [38,39], termed GRASP (GFP reconstitution across synaptic partners) revealed the development of single synapses throughout the larval stages [40].

3. TOOLBOX FOR STUDYING LARVAL MOTOR CIRCUITS

3.1. Genetic Tools

Drosophila affords a major advantage in the genetic approach to neuroscience. The fly genome has been sequenced and found to consist of approximately 13600 genes [41]. About 65% of human disease-causing genes have functional homologs in the fly genome [42], a significant fraction of which is expressed in equivalent tissues in the fly [43]. Several techniques, including chemical mutagens, transposons [44] and genome engineering [45] can be used to mutate genes in the fly genome. The genetic accessibility and phylogenic conservation of genes make flies an ideal model in which to study neural circuits and diseases.

Genetic tools enable us to analyze not only the function of individual genes but also individual neurons. The Gal4-UAS system is a gold standard to express genes in specific cells in *Drosophila* [46]. By combining this with other independent gene expression systems such as the LexA-LexAop system [47] or Q-system [48], one can control the expression of genes in distinct cells. A large arsenal of genetic and molecular probes, including cell membrane markers, synapse markers, calcium imaging probes, voltage imaging probes, optogenetic tools, short hairpin RNAs, neural function activators/silencers and cell death inducers, have been expressed in a variety of cells to study neural networks [49]. In addition, large scale collections of Gal4 lines [50-52], RNA interference lines [53,54] and transposon insertion lines [55] have been intensively generated and made available to researchers.

3.2. Connectomics

In the mid-1980s, the whole nervous system of *C. elegans* was reconstructed based on electron microscopy [56], which has exerted enormous impacts on the following neural circuit studies. The recent growth of connectomics provides a powerful tool to disentangle the neural circuits [57]. A stack of thousands of serial electron micrographs spanning the entire larval CNS was collected [58,59]; using these data, many cells in the larval CNS and the connectivity between them have been traced with the aid of computers [60-62]. Each neuron is assigned a nomenclature based on the cell lineage, namely the origin of a neuroblast the neuron is born from [63]. This assignment is done by comparing the morphology of a neuron based on the EM-reconstruction with that revealed by light-microscopic confocal images (obtained by expressing GFP in single neurons) [52]. For example, interneuron PMSIs (*period*-positive median segmental interneurons—to be described below) [64] belong to lineage 02 in the abdominal neuromere and so are assigned as A02 lineage [59]. Each single neuron in the lineage is named following the scheme A02a neuron, A02b neuron, and so on. Accordingly, every single neuron is named by this nomenclature system. The advent of this invaluable database has been greatly advancing circuit-level researches in the larvae [58,59,65-67].

4. NEURAL CIRCUITS FOR LARVAL BEHAVIOR

4.1. *Drosophila* Larvae in Nature and in the Laboratory

As may be surmised by the name "vinegar fly" or "common fruit fly," *Drosophila* has a clear preference for fermenting fruits [68]. In nature, fly larvae live in rotting fruits, burrowing inside them [68], but in the laboratory, flies are reared in culture bottles containing foods typically consisting of yeast (amino acid source), sugar and corn meal (hydrocarbon source). To observe behavior, larvae are usually placed on an agar plate [69]. Fly larvae show several stereotyped behaviors on the flat agar plate: forward and backward crawling, bending, turning, retreating, rearing [70], hunching [71] and rolling [72]. By combining these behavior components, larvae exhibit more complex behaviors [73], several types of cue-directed locomotion (taxis) [74] and memory-guided behavior [75].

4.2. Basic Locomotion

4.2.1. Intrasegmental Coordination

A major larval behavior is forward locomotion, which is achieved by sequential segmental contractions propagating from the posterior end to the anterior [20]. High-resolution imaging of muscles in freely moving larvae revealed a stereotypic temporal order in muscular contraction within a segment: contraction of longitudinal muscles (whose fiber axis is in the anteroposterior direction) is followed by that of transverse muscles (whose fiber axis is in the dorsoventral direction and perpendicular to the longitudinal muscle fibers). In soft-bodied animals like fly larvae, contraction of longitudinal or transverse muscles leads to shortening or elongation of segments, respectively [20]. The intrasegmental delay in the antagonistic muscles may serve to realize efficient propulsion in larval locomotion. The use of a connectomics approach showed that the intrasegmental phase delay is generated neither by the difference in intrinsic properties of motor neurons nor by time delay in excitatory pre-motor interneurons. Instead, an inhibitory GABAergic neuron iIN1 acts as a delay line to generate the intrasegmental phase difference in the motor neuronal activation (Fig. **2**) [67].

An isolated or semi-intact larval CNS shows neural activity that resembles the pattern of neural activity during larval behavior such as forward locomotion (called "fictive locomotion") [76-78], and enables researchers to study in detail the neural circuit activity underlying locomotion. Surgical ablation experiments in isolated CNS preparations showed that the brain and sensory feedback are dispensable for forward crawling. Thus, the ventral nerve cord is capable of generating forward locomotion autonomously [79]. A comprehensive ablation study showed that even a single neuromere possesses the ability to generate an oscillatory activity pattern [80], and thus likely contains local pattern generating networks.

4.2.2. Intersegmental Activity Propagation

Improvements in genetic tools to drive gene expression in a small number of cells have allowed dissection of premotor neural circuits at a cellular resolution. PMSIs (*period*-positive median segmental interneurons) are identified as *period-Gal4* positive neurons and shown to be premotor inhibitory glutamatergic interneurons (Fig. **2**) [64]. Twenty PMSIs reside in each neuromere and most of them form synaptic contacts with motor neurons. Dual color calcium imaging of PMSIs and motor neurons showed that activation of PMSIs is preceded by that of motor neurons. Optogenetic silencing of PMSIs elongated the motor burst duration. These results indicate a circuit function of PMSIs in shortening the burst duration of motor neurons. Silencing PMSIs slowed down larval crawling, suggesting that PMSIs control the speed of activity propagation within the VNC by shortening the motor burst duration in each segment [64]. Intriguingly, there are similar interneurons in the vertebrate spinal cord. PMSIs share several properties with aIN neurons in the *Xenopus* tadpole [81] and V1 neurons in mice [82]. These neurons are inhibitory, premotor, ipsilateral projection and
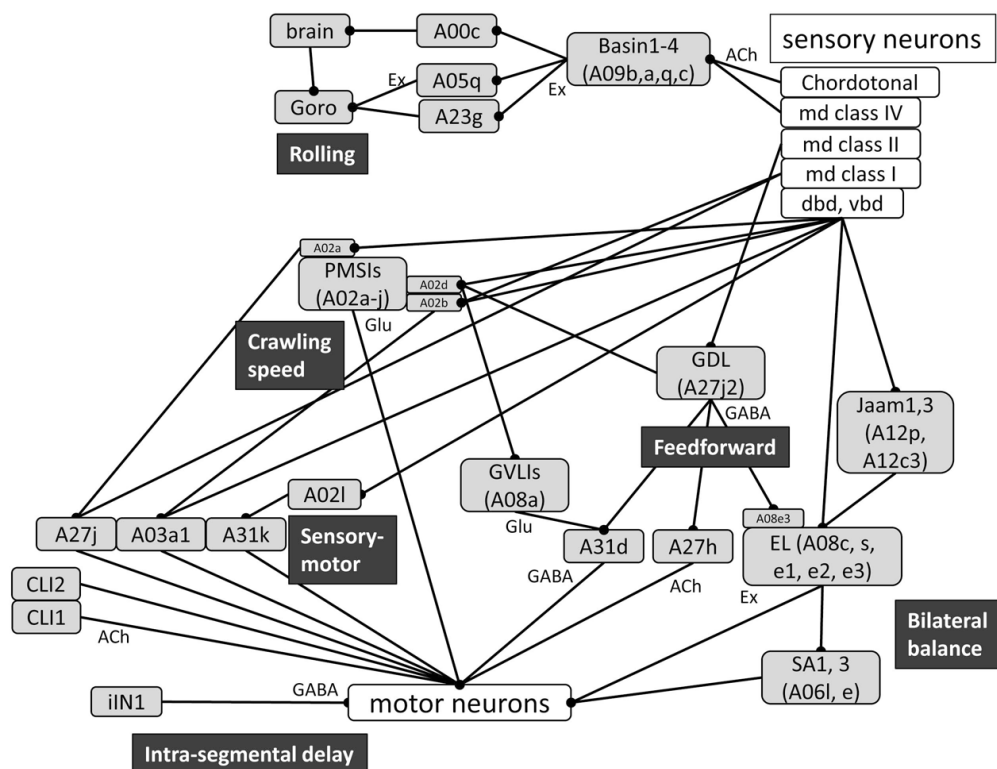
Fig. (2). Motor circuits in *Drosophila* larvae. Neural connectivity in a hemi-neuromere based on [58,59,64-67,83,85]. See text for details. Md: multidendritic neuron, dbd: dorsal bipolar dendritic md neuron, vbd: ventral bipolar dendritic md neuron. For simplicity, motor neurons are shown in a single group, though at least two distinct subsets are important for motor coordination [67].

rhythmically active during locomotion. In addition, blocking these neurons induces slower motor output. This resemblance may reflect a general principle for motor circuit architecture. GVLIs (Glutamatergic Ventro-Lateral Interneurons) are another class of glutamatergic interneurons that is rhythmically active during crawling (Fig. **2**). GVLIs are activated later than PMSIs in the same segment [83], implying the existence of multiple temporally distinct inputs for the termination of motor activity. Another class of interneurons, LLNs (Lateral Locomotor Neurons), located in each segment, shows propagative activity and is required for crawling behavior [84]. Similar wave-like activity was observed in another class of excitatory premotor interneurons CLI1 and CLI2 (Fig. **2**) (Cholinergic Lateral Interneuron 1 and 2) [85].

Genetic dissection of distinct classes of interneurons combined with connectome is also revealing circuit motifs involved in the regulation of crawling. One such motif is composed of A27h neurons, premotor excitatory neurons, and the upstream GDLs (GABAergic Dorso-Lateral interneurons) [65]. A27h appears to excite GDL in the next anterior segment, and GDL in turn appears to inhibit A27h in the same segment. Thus, these neurons form a longitudinal intersegmental feed-forward chain that likely mediates signal propagation during the forward locomotion (Fig. **2**).

Similar to most of the other motor systems, sensory feedback has a great impact on the speed of larval locomotion. Silencing body wall sensory neurons slows down the crawling speed [86-88], indicating sensory feedback acts to increase crawling speed. A putative mechanosensitive TRP (Transient receptor potential) channel, NompC and the TMC (transmembrane channel-like) protein are expressed in the body wall sensory neurons responsible for locomotion and required for normal-speed crawling [89,90]. Pickpocket1 (*ppk1*), a *Drosophila* subunit of the epithelial sodium channel family, is also expressed in the sensory neurons. *ppk1* mutants show faster wave propagation and shorter pausing time and thus faster

locomotion speed [91]. It has been suggested [92] that *ppk1* may form a channel complex with *ppk26* for mechanosensation. Connectomics analysis has clarified a part of neural connectivity from proprioceptive sensory neurons to motor neurons (Fig. **2**) [59], and this information serves as a strong platform for understanding how larvae adjust their locomotion to adapt to a varying environment.

4.2.3. Bilateral Balance

To guide larvae along a straight line, balanced muscular contraction between the left and right side of the body is critical. EL (Eve-positive lateral) neurons are responsible for the balanced activation of bilateral muscle contraction (Fig. **2**) [66], and when they are silenced, the temporal coordination between left and right remains normal, but the contraction power becomes imbalanced. EL neurons (*even-skipped*-positive contralateral innervating neurons) are conserved in vertebrates. Connectomic analysis identified the neural circuit related to the EL neurons (A08c, s, e1, e2 and e3 by lineage-based nomenclature): EL neurons directly innervate motor neurons and indirectly innervate premotor neurons through premotor interneurons SA1, 3 (A06l, e). On the upstream side, EL neurons are directly innervated by proprioceptive sensory neurons dbd and vbd (dorsal and ventral bipolar dendritic multidendritic [md] neurons) and indirectly innervated by these sensory neurons through Jaam1,3 (A12p, A12c3). The connectivity may serve to interpret sensory feedback of muscle contraction amplitudes and balance bilateral muscular contraction. Another frequent behavior component is bending, which occurs during forward crawling or upon pausing. The former is called turning because the combination of bending and forward crawling leads to a change in locomotion direction [70]. Controlling crawling and bending (or "runs" and "turns" respectively) is critical for larval navigation (see the "sense-guided behavior" section, 4-3, below). Turning behavior confers larvae with a chance to change crawling direction and expand their area of exploration. Bilateral asymmetric muscular contraction is

required for turning. The asymmetric neural activity pattern can be observed in isolated larval CNS [93], and genetic analysis of axon guidance molecules showed that commissural connections in thoracic segments are critical for this asymmetric activity. Several neurons in the SEZ region have also been shown to be critical for the control of turning behavior [94].

4.2.4. Head Rearing

Rearing behavior, in which larvae raise their anterior end vertically, is rarely observed in larvae crawling on a flat surface [70], but it can be induced by light stimulus or rough surface stimulus [95]. Genetic analysis showed that this behavior is suppressed by 5HT and the 5HT-IB receptor in normal conditions [95]. 5HT-IB positive neurons in the ventral nerve cord express the leucokinin peptide. A receptor for leucokinin is also involved in the regulation of rearing behavior. These data suggest excessive rearing behavior is suppressed by functions of the neural pathway including 5HT, 5HT-IB receptor, leucokinin, and leucokinin receptors. Interestingly, the 5-HT1B receptor, which is well-conserved in mammalian species, has also been found in adult spinal-transected mice to be associated with a different form of rhythmic hindlimb movement—activation of spinal 5-HT1B receptors using agonists induced rhythmic non-forward locomotor movements, unlike 5-HT2A or 5-HT1A receptor activation, which can elicit basic forward stepping [96,97].

4.3. Sensory-Guided Behavior and Neural Circuits

4.3.1. Photon-Guided Behavior

Drosophila show negative phototaxis in most of their larval stages [98], a behavior that is thought to prompt larvae to burrow into fruits, their major nutrition source [98]. Two sets of photosensors are involved in phototaxis of the larvae [99]. The first is the Bolwig's Organ, a set of rhodopsin-expressing single eyes at either side of the head. Each eye is composed of 12 photoreceptor neurons (PRs), which are further divided into two subclasses based on the rhodopsin gene they express: four photoreceptors express the blue-sensitive rhodopsin5 (rh5-PRs) and eight photoreceptors express green-sensitive rhodopsin6 (rh6-PRs) [100]. The second set of photosensors is the non-rhodopsin-expressing class IV multidendritic (md) neurons tiling the larval body wall [101]. Photosensing of class IV neurons depends on Gustatory receptor 28b (Gr28b) and Transient Receptor Potential A1 (TrpA1) cation channel [101] rather than rhodopsins. The two sets of photosensors show distinct light sensitivities: the Bolwig's organ mediates a photophobic response at low light intensity, while class IV md neurons detect stronger light such as direct sunlight [101]. The distinct sensitivities of the two photosensors imply their different function in photo-response behavior: the Bolwig's organ guides phototactic (and circadian, see below) behavior under moderate intensity light, whereas the class IV md neurons sense noxiously strong light to elicit quick escape behavior [99]. Two pairs of neurons in the brain were identified to be involved in phototactic behavior [102]. The neurons express a neuropeptide PTTH (prothoracicotropic hormone), which promotes the light sensitivity of the two photosensors (the Bolwig's organ and class IV md neurons) by its endocrine function [103]. It has been suggested that PTTH enhances negative phototaxis at the end of the larval stage to guide larvae toward a darker site for pupariation [103].

As mentioned in the previous section, larval locomotion involves two basic movements: runs and turns. During runs, the larva locomotes by a series of forward peristalses forming a straight track, and in turns the larva pauses, sweeps its head laterally, then orients the body in a new direction by combining bending and crawling [104]. A spatial change in ambient light intensity affects the frequency of turns [105]. Larvae use head-sweeping to probe the spatial gradient of local luminosity based on temporal processing of sensory inputs, and then pick a darker side for the next run [105].

Neural pathways underlying larval phototaxis have been dissected. The Bolwig's organ consists of two subclasses of photoreceptor neurons, Rh5-PRs and Rh6-PRs [99]. Genetic analysis using rhodopsin mutants showed that only the Rh5-PRs are essential for the negative phototaxis [106]. The Rh5-PRs project their axons to three classes of downstream neurons: optic lobe pioneer neurons, serotonergic neurons and five lateral neurons (LNs) [107]. Of the three targets, the LNs are critical for phototaxis. The five LNs are further subdivided into four PDF (pigment-dispersing factor)-positive cells and one PDF-negative cell (the 5th LN). The PDF-negative cell is suggested to be the major relay neuron for the phototactic response [105]. While blue light induces strong negative phototaxis, green light elicits weaker but significant photo-avoidance [101]. Green-light avoidance is mediated by Rh6-PR and inhibited by GABA [108]. Intriguingly, the concentration of GABA in the brain is regulated by the amount of glutamate in the hemolymph, and the glutamate concentration is in turn regulated by Malpighian tubules, organs analogous to the kidneys in mammals [108].

4.3.2. Temperature-Guided Behavior

The molecular mechanisms of temperature sensing, and neural circuits for temperature-guided behavior have been extensively studied [109,110]. Within a moderate range of temperatures, larvae exhibit thermotaxis behavior [111]. The 1st instar larvae prefer temperatures between 25 and 30 degrees [112,113]. When exposed to temperatures out of this range, larvae show positive (from a lower temperature toward the optimal) or negative (from a higher temperature to the optimal) thermotaxis [111]. As in the case of phototaxis, the thermotaxis behavior includes regulation of running and turning. Quantitative analyses showed that the components of the regulation may be shared between positive and negative thermotaxis [111]. While neural mechanisms for negative thermotaxis remain unclear, recent studies reveal the molecular and cellular underpinnings of positive thermotaxis. One of the three ganglia in the head, known as the dorsal organs, have been shown to be critical for cooler-temperature sensing and positive thermotaxis [114]. The ionotropic channels Ir21a and Ir25a are keys to sensing cooling to enable navigation toward a preferred temperature [115].

Upon sensing an extremely high temperature (>39℃), larvae exhibit a stereotyped escape behavior called rolling, rather than thermotaxis. A TRP channel Painless is critical for this behavior as a heat-sensor [72]. Another TRP channel, TrpA1 is also required for the rolling behavior, although the exact role of the channel remains unclear [116]. Class IV md neurons (see "basic locomotion" section, 4-2, above) are the major sensory neurons involved in the rolling behavior [117]. Class IV md neurons are polymodal nociceptors sensitive to excessive thermal, mechanical or light stimuli. Intriguingly, temporal patterns of calcium influx in class IV neurons possess the information of distinct sensory modality [118]. Recent connectomics and genetic analysis identified downstream neural circuits of the class IV md neuron that mediate the rolling behavior (Fig. **2**). The Goro (Japanese for "rolling") neuron is identified as a command neuron for rolling behavior [58]. There are two major neural pathways from class IV sensory neurons to Goro neurons: one contained within the ventral nerve cord and the other passing through the brain. The direct targets of class IV sensory neurons are the four Basin neurons (Basin1-Basin4), and from these neurons the pathway diverges. Within the ventral nerve cord, some Basins project to the A05q and A23g neurons, which in turn project to the command neuron Goro. On the other path, Basins project to the A00c ascending neurons. The A00c neuron then targets ipsilateral or contralateral neurons in the brain, which in turn project to descending neurons innervating the command neuron Goro. Basin neurons integrate the information conveyed by the nociceptive neurons (class IV md neurons) and the chordotonal neurons, which sense vibration in the air such as that evoked by the wing beating of the fly's natural enemies, the wasps. The vibration sensing enhances the class IV neuron-triggered rolling behavior. Thus, the two

lines of distinct modality information are integrated at the 1st order Basin interneurons, and then are conveyed through multiple pathways to finally converge in the command neuron Goro [58].

4.3.3. Chemotaxis: Chemical-Guided Behavior

Larvae exhibit clear chemotaxis behaviors [119,120]. Neural circuits for larval olfaction have been dissected [75]. The larval olfactory sensing apparatus is the dorsal organ located at the tip of head [121]. A "dome" structure of the dorsal organ is innervated by dendrites of 21 olfactory receptor neurons (ORNs). Each ORN expresses one conventional ligand-binding OR (olfactory receptor) gene and a coreceptor *Or83b*, which is required for targeting of ORs to sensory cilia, where odor is detected [122]. A comprehensive study of responses for OR-odorant pairs showed that olfactory receptors are functionally diverse [123]. Each ORN targets projection neurons (PNs) forming a single glomerulus in the larval antennal lobe (LAL). Accordingly, the connectivity from ORNs to 1st order projection neurons is largely 1: 1. The projection neurons innervate two targets, the mushroom body and the lateral horn [75]. Inhibitory local interneurons within the glomerulus support concentration-invariant odor perception [124].

While the dorsal organs are present at both sides of the animals, a single dorsal organ, or even a single ORN is capable of mediating the chemotaxis behavior [125]. Bilateral sensing has a role in enhancing the accuracy of the chemotaxis [125]. Quantitative studies of animal locomotion during chemotaxis behavior require well-controlled odor administration and high-resolution animal movement detection. Sophisticated methods for the analyses of chemotaxis [74,125-127] have been developed to show that larvae detect spatial odor gradients by sensing via head casting the odor concentration difference between the left and right side of the animal. As is the case with the phototaxis behavior, chemotaxis behavior basically consists of runs and turns. Prior to turns, larvae exhibit head casting, swinging the head sideways to monitor odorant around the head and search the gradient. Based on the gradient, larvae make a decision on the direction to go [126,128,129]. Optogenetic analyses with designed temporal light stimulation have been used to reveal the computation underlying chemotaxis: OSNs function as a slope detector for a positive gradient and as an OFF detector for a negative gradient [130]. Furthermore, the relationship between the sensory inputs and motor outputs can be mathematically described by a linear-nonlinear-Poisson model [131,132].

4.3.4. Mechanical Stimulus-Guided Behavior

Several classes of sensory neurons are present in the body wall including the multidendritic (md) neurons, external sensory neurons and chordotonal neurons [133]. Sensory feedback from these neurons is critical for the regulation of normal peristaltic motion [86-89]. The sensory neurons on the body wall are classified as type I sensilla, including chordotonal organs and external organs, and type II md neurons. The md neurons are divided into the bipolar dendrite neurons, the tracheal dendrite neurons and the dendritic arbor (da) neurons [133]. The 15 da neurons in each abdominal hemisegment are further grouped into four classes based on the complexity of dendritic arbors, from class I with simple arborization to class IV with complex branching [134,135]. Class I neurons are thought to be proprioceptors and their activity is required for normal locomotion [87]. Activation of class II neurons elicits accordion-like body shrinkage [117]. Class II neurons likely function as touch receptors [136]. Class III neurons sense a gentle touch [136] through the nompC receptor [137]. Class IV neurons sense multiple nociceptive stimuli, and activation of class IV neurons induces stereotyped rolling behavior [72,117]. Mechanical nociception is mediated by class IV (and/or class III) neurons expressing a DEG/ENaC protein Ppk1 [138]. The chordotonal organ senses vibration [139]. As described above, integration of nociceptive and vibration inputs enhances the rolling escape behavior [58]. This is reminiscent of findings made in cats, where noxious tail or sexual organ stimulation

(phasic pinching) can trigger per se rhythmic stepping-like movements in the hindlimbs of spinal-transected cats [140,141].

4.3.5. Oxygen-Guided Behavior

Larvae show exploratory behavior by crawling away from food with fewer turns under hyperoxia and hypoxia conditions. In hyperoxia, excess oxygen is monitored by an increase in the level of H_2O_2, the endogenous reactive oxygen species (ROS) metabolized from oxygen. H_2O_2 is detected by DEG/ENaC channel Ppk1 expressed in class IV multidendritic neurons [142]. The level of H_2O_2 is controlled by catalase, which breaks it down into a non-toxic substance in nearby epithelial cells [142]. Hypoxia-induced exploratory behavior is mediated by nitric oxide and the cyclic GMP pathway [143]. A pioneer study reported that there are two variants in larval foraging behavior among the wild-type population: Sitters prefer to stay on food, while Rovers explore around the food [144]. The gene responsible for this difference was identified to code PKG, a cGMP protein-dependent kinase [145]. Since oxygen concentration is low around the food (due to oxygen consumption by yeast), the variation in larval foraging behavior can be explained by a difference in sensitivity to hypoxia caused by the PKG gene variation [143]. Two atypical soluble guanylyl cyclases, which catalyze the synthesis of the intracellular cGMP, are reported to be the oxygen detectors [146]. In addition, H_2O_2 mediates a nociceptive response to harmful ultraviolet radiation [147].

4.4. Memory-Guided Behavior

Larvae are capable of forming associative memory [148]. In a widely-studied association learning paradigm between gustatory inputs (unconditional stimuli) and olfactory inputs (conditional stimuli), 2M fructose is used as a reward and 4M sodium chloride or quinine (0.2% w/w) is used as punishment [75,149]. In the reward association test, odor A is first presented along with the reward. Then, another odor, B, is presented without the reward. After repeating the training sets, larvae are allowed to choose between odor A and odor B on an agar plate (A+/B test) as a test set. To cancel out non-memory factors including innate preference to some odors and adaptation of olfactory sensing, the reciprocal test (A/B+ test) is employed by another group of larvae. By assembling the reciprocal associative data, the memory-related component is extracted [75].

Neural connectivity of chemosensory circuits has been identified [121] (see the "chemotaxis" section above). The 21 olfactory receptor neurons in each of the dorsal organs target the larval antenna lobe (LAL). Projection neurons in LAL target the mushroom body (MB), which possesses up to 34 stereotypic calyx glomeruli [150]. The cell number of the MB neurons is estimated to be about 600, outnumbering 34 projections from LAL, suggesting this is a site of network divergence [150,151]. Gustatory sensory neurons locate at two distinct sites: external organs including the terminal organ, the ventral organ and the dorsal organ, and internal chemosensory organs comprising the dorsal, ventral and posterior pharyngeal sense organs [152]. The number of gustatory neurons in these organs is about 80 [153]. Four major target regions for the gustatory sensory neurons are located in the larval SEG (Subesophageal ganglion) [152]. Potential target neurons in the SEG are Hugin peptide positive neurons, which are responsible for feeding behavior [154].

As in the case of adult flies [155], the mushroom body is critical for learning in *Drosophila* larvae [156]. Octopamine neurons are reported to transmit the reward signal [156,157], and a subset of dopamine neurons sends punishment signals [158]. Recently, another subset of dopamine neurons was shown to transmit a reward signal [159]. These evaluating (valence) signals, initially driven by the gustatory stimuli and conveyed to the mushroom body by the octopamine/dopamine neurons, are thought to mediate associative learning. PNs (projection neurons) in LAL target not only the mushroom body but also the lateral horn. Premotor circuits con-

ducting the odor-triggered behavior may receive the sensory information through the lateral horn during innate behavior and through the mushroom body for learned behavior [148].

Other learning paradigms have been developed. Larvae show an innate preference to darkness, and by harnessing this property, visual inputs can be used as unconditional stimuli [160]. Electric shock can also be used as punishment [161]. Using electric shock, relief-associated learning can be established [162].

4.5. Circadian Behavior

Whereas the 3rd instar stage lasts for only two days, larvae possess a circadian rhythm, and larval photophobic behavior shows a circadian rhythm [163]. The circadian rhythm requires clock neurons (LNs, lateral neurons) and so-called clock genes: mutations in the *cycle* or *clock* gene enhance photophobic behavior, whereas mutations in *period* or *timeless* weaken photophobic behavior [163]. The photophobic response requires the visual system (the Bolwig's organ) and the clock neurons. Activation of the Bolwig's organ increases neuronal activity of the PDF (Pigment Dispersing Factor peptide)-expressing ventral lateral neurons (LNv) [164]. LNv neurons promote larval light avoidance, whereas other clock neurons DN1s (dorsal clock neurons) inhibit the behavior [165]. The PDF receptor and mGluRA (metabotropic glutamate receptor A) cooperate to maintain LNv synchrony and promote strong oscillation of the clock protein Timeless, which suggests that the master pacemaker LNv neurons require extracellular inputs to generate normal oscillation [166]. Inward rectifier K channel is expressed at dusk, which is crucial for larval light avoidance [167].

4.6. Feeding Behavior

As in vertebrates, insulin signaling is critical for feeding behavior. Hyperactivation of insulin signaling reduces feeding behavior [168]. In starved conditions, larvae feed even on noxious foods. Upregulation of insulin signaling in NPF (neuropeptide F) receptor (a homolog of the mammalian neuropeptide Y) positive neurons suppresses this risky feeding behavior [169]. Insulin signaling in the mushroom body is critical for feeding behavior [170] and motivation to feed on preferred foods is regulated by octopamine neurons [171].

Feeding behavior requires coordinated muscle contractions in the mouth. Motor neurons regulating feeding behavior have been characterized [172]. The hugin neuropeptide is reported as a key in regulating feeding behavior [173]. Hugin neurons are innervated by gustatory sensory organs and target their axons to the pharyngeal motor apparatus, to the protocerebrum and to the neuroendocrine system [154].

4.7. Social Behavior

Larvae tend to group on a substrate. They are attracted to a patch of food previously occupied by other larvae, suggesting some substance produced by other larvae induces the aggregation behavior [174], which may be conspecific [175]. One benefit of larval aggregation is predicted to be the improvement of digging and burrowing ability into hard food substrate [176], and it can also affect the density of palatable yeast on fruits [177]. However, overcrowding leads to overproduction of toxic wastes such as ammonia [178]. Two long-chain fatty acid cuticular hydrocarbons were identified as signals mediating social interaction [179]. These signals are received by a single chemosensory neuron expressing DEG/ENaC channel subunits, Ppk23 and Ppk29 [179]. Social attraction is also regulated by the larval microbiome [180]. Larvae show cooperative digging through agar to search for food-free sites suitable for pupation [181], a behavior controlled during development by neuropeptide F, the homolog of mammalian neuropeptide Y [181].

Visual cues are also critical for social interaction in fly larvae. Larvae can identify other larvae by using visual information [182], and can discriminate the morphology of other larvae, including the

difference between wild-type and *tubby* mutant (which have altered morphology) larvae [183]. Interestingly, this recognition is established in a specific critical period, L2 to early L3 [183]. However, in the context of olfactory learning, larvae are not affected by the presence of other larvae [184].

5. DISEASE MODELS AND DRUG DISCOVERY

5.1. Fly Larvae as Disease Models

Genetic accessibility to motor circuits enables us to tackle molecular mechanisms in neural disease. Spinal muscular atrophy (SMA) is a lethal human disease characterized by motor neuron functional alterations and muscle deterioration, and caused by low expression levels of the survival motor neuron (*smn*) gene [185]. *Drosophila smn* mutant larvae show phenotypes similar to the SMA patient: muscle, motorneuron (glutamatergic neurotransmission in *Drosophila*) and locomotion defects [186]. Surprisingly, these phenotypes are not rescued by restoration of *smn* expression in either muscles or motor neurons. Instead, *smn* expression in cholinergic interneurons and proprioceptive neurons is required to rescue the neuromuscular junction (NMJ) and locomotion phenotypes, showing that deficits in sensory-motor circuits should affect the function of NMJ non-autonomously. This discovery suggests that activation of the motor neural network could ameliorate SMA disease [186] (but see also [187]). The *smn* protein is a component of the RNA splicing machinery. A transmembrane gene *stasimon* was identified as the target of *smn* and loss of *stasimon* induces similar phenotypes to *smn* mutant larvae in motor circuits [188].

As the studies above show, *Drosophila* larval motor circuits, especially the larval neuromuscular junction, serve as a powerful system to gain insight into the molecular mechanisms of neural disease and to help in the search for therapeutic tools. In the following part, we briefly introduce several recent studies on neural diseases using *Drosophila* motor system.

Charcot-Marie-Tooth disease type 4J is an inherited human genetic disorder affecting the peripheral nervous system (PNS) and in which FIG 4 is mutated. The *Drosophila* genome possesses a FIG homolog, dFIG4. Knockdown of dFIG in motor neurons shortens the size of NMJ synapses, indicating a requirement of FIG for formation and/or maintenance of the presynaptic terminals [189]. Charcot-Marie-Tooth disease type 2B (CMT2B) is caused by mutations of a small GTPase, Rab7. Expression of a mutated form of Rab7 is established as a *Drosophila* CMT2B model. The studies of this model showed that deficits in vesicle transport might be responsible for the pathology of CMT2B [190].

Alpha-Synuclein (alpha-Syn) is one of the key factors for Lewy bodies, which are proteinaceous depositions appearing in Parkinson's disease (PD). Genetic analysis using larval neuromuscular junctions showed Rab11 is capable of ameliorating defects in larval locomotion induced by alpha-Syn [191]. Expression of a mutated form of alpha-Syn in dopaminergic neurons in the larvae showed stage-dependent motor defects accompanied by loss of DA neurons. Chronically exposed to a pesticide rotenone, these larvae showed more severe defects, suggesting that this model can be used to explore potential therapies for PD treatment [192].

In amyotrophic lateral sclerosis, TDP-43 shows cytoplasmic accumulation and nuclear clearance. Loss of function of *Drosophila* TDP-43, TAR DNA Binding Protein Homolog (TBPH), affects larval locomotion [193]. Both loss and gain of function of TDP-43 affect excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2). Muscle-specific loss of function of TDP-43 affected locomotion [194].

App, an amyloid precursor protein, is involved in Alzheimer's disease (AD). Loss of function of App is associated with axonal transport defects. Increasing histone acetyltranferase Tip60 (HAT) rescued the App-induced axonal transport defects and locomotion deficits, suggesting HAT modulators can be used for treatment of

cognitive disorders, including AD [195]. A disease model of AD in *Drosophila* was established by expressing the human amyloid precursor protein and beta-site App-cleaving enzyme (BACE) in fly neurons [196]. Over-expression of these two genes in neurons causes alterations in NMJ morphology in decreases in presynaptic terminal size and postsynaptic protein levels. These phenotypes are suppressed by gamma-secretase inhibitor, suggesting the larval model can be used to test AD therapeutics [197].

The abnormal expansion of the polyglutamine tract in the human Huntingtin protein (polyQ-hHtt) leads to Huntington's disease. It is known that wild-type Huntingtin provides a protective role for the polyQ-hHtt induced defects, and by using larval motor neurons as a model, a 23 amino acid-long hHtt peptide was shown to play a protective role for the polyQ-hHtt aggregation and the accompanying locomotor dysfunction [198].

Misfolding of the Prion protein, leading to a neurotoxic PrP-scrapie form, induces the development of neurodegenerative conditions but the physiological roles of wild-type PrP remain elusive. Expression of wild-type PrP in larval motor neurons showed that PrP enhances synaptic release probability and increases the locomotor activities, which raise the possibility that prion pathogenesis is caused not only by a gain of the neurotoxic PrP-scrapie form but also by a lack of functional wild-type PrP [199].

Autosomal-dominant hereditary spastic paraplegia (AD-HSP) is modeled by a loss-of-function mutation of *spastin*, the gene encoding microtubule-severing AAA ATPase. Rearing larvae at low temperature (18°C) ameliorates larval synaptic defects caused by the *spastin* mutation, which suggests mild hypothermia can be used as a therapeutic approach for AD-HSP [200].

Cognitive impairments in Williams syndrome are caused by LIMK1 hemizygosity. Loss of function mutation of the *agnostic* gene encoding *Drosophila limk1* affected the larval locomotion, which suggests the usefulness of this mutant for studying molecular mechanisms for Williams syndrome [201].

5.2. Fly Larvae for Drug Discovery

Drosophila larvae have also been a useful model for drug discovery. Calcium imaging of an isolated nervous system of epilepsy model larvae provides a rapid method to screen antiepileptic drugs [202]. Since a wide range of nanoparticles has been developed, efficient testing of their safety and risks for the human health and environment is required, and fly larvae are one of the promising model systems for toxicology [203]. Psychostimulant amphetamine increases extracellular dopamine by eliciting dopamine efflux mediated by dopamine transporter. Amphetamine-induced hyper-locomotion of larvae was used to identify molecular mechanisms for dopamine efflux by amphetamine [204].

The interaction between the nervous systems and other tissues can be examined using fly larvae. Obesity, cardiovascular disease and type 2 diabetes have all been demonstrated to be associated with the prenatal nutritional environment. Excess maternal calories alters the body composition of the larval offspring for at least two generations, which indicates larvae can be used to model transgenerational metabolic processes, to study the underlying molecular mechanisms, and to search for therapeutic drugs [205]. Deletion of the tumor suppressor gene, *lethal(2) giant larvae* causes brain tumor in larvae. Treatment of potential antitumor drugs rescued the brain tumor phenotype and larval locomotion, suggesting larvae can be used to screen antitumor drugs [206].

6. CONCLUDING REMARKS

The *Drosophila* larvae genome is composed of about the order of 10^4 genes, and around 10^4 neurons form neural circuits in the central nervous system of fly larvae. As of today, despite significant advances made possible using fruit flies as a research model, we have to conclude that most of these genes and neurons have roles in locomotor rhythm and pattern generation that remain incompletely understood. However, as summarized briefly in this review, clear breakthrough findings have been made in recent years using the many powerful genetic tools applicable to research in *Drosophila*. The wide variety of locomotor behaviors, accessibility to single genes and neurons, highly conserved neural circuits and gene pathways, are only some of the unique advantages of this animal model, that can still be considered as a promising tool for many additional studies aimed at further dissecting genetically the basic central circuits involved in the control of locomotion.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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