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A Sensory Feedback Circuit Coordinates Muscle Activity in *Drosophila*

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Abstract

Drosophila larval crawling is a simple behavior that allows us to dissect the functions of specific neurons in the intact animal and explore the roles of genes in the specification of those neurons. By inhibiting subsets of neurons in the PNS, we have found that two classes of multidendritic neurons play a major role in larval crawling. The bipolar dendrites and class I mds send a feedback signal to the CNS that keeps the contraction wave progressing quickly, allowing smooth forward movement. Genetic manipulation of the sensory neurons suggests that this feedback depends on proper dendritic morphology and axon pathfinding to appropriate synaptic target areas in the CNS. Our data suggest that coordination of muscle activity in larval crawling requires feedback from neurons acting as proprioceptors, sending a “mission accomplished” signal in response to segment contraction, and resulting in rapid relaxation of the segment and propagation of the wave.

Keywords

locomotion; multidendritic; sensory; proprioception; *Drosophila*; larva; peristalsis

Introduction

Locomotion requires the interplay of the central nervous system (CNS), muscles, and the peripheral nervous system (PNS). Central pattern generators create the rhythms underlying locomotion, and sensory feedback from the PNS provides moment-by-moment adjustments to the pattern. However, we do not fully understand how the three systems interact to produce coordinated movement, or how genes encode development of the circuit.

To address this, we analyzed a simple behavioral circuit in a genetic model organism. *Drosophila* larval crawling is based on a repetitive motor pattern. The muscles of each segment contract, then quickly relax and seamlessly propagate the wave to the next anterior segment, creating a peristaltic wave from tail to head. The larva can crawl at different speeds and change direction, but during motivated forward crawling the pattern is remarkably stereotyped and consists of a series of identical contraction waves (Fig. 1A,B; Video 1). This behavior results from a system comprising thirty muscles, thirty-one motoneurons, and forty-three sensory neurons in each abdominal hemisegment, plus an unknown number of interneurons (Fig. 1C, D; Bate, 1993; Landgraf et al., 1997; Bodmer and Jan, 1987). Larval crawling is an appealing

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system because the simplicity of the behavior and anatomy allow us to understand the circuit on the level of individual cells.

Drosophila larval crawling has been studied previously by a number of investigators. From this work we know about the role of specific genes in crawling behavior. For instance, mutations in K⁺ or Na⁺ channel subunits produce dramatic effects on the crawling path, resulting in changes in speed, turning rate, and translocation efficiency (Wang et al, 1997; Wang et al, 2002; Ainsley et al, 2003). We also know that the relative amounts of tyramine and octopamine play an important role in crawling persistence (Saraswati et al, 2003; Fox et al, 2006), and inhibiting dopaminergic cells results in circling behavior (Suster et al, 2003). The relationships between motoneurons and their muscles has been well-mapped (Landgraf et al, 1997), so we understand the motor output, but what generates the pattern of activity? Is there a well-defined central pattern generator? Does sensory feedback play an important role? Previous studies addressing the role of sensory feedback concluded that feedback is not necessary to produce the basic posterior to anterior contraction pattern, however, in each case the pattern was disrupted, suggesting that sensory feedback helps to shape the normal output (Suster and Bate 2002; Fox et al, 2006).

If sensory feedback is important for normal locomotion, which neurons play this role? Work on walking in adult *Drosophila* and other insects has focused largely on the role of the chordotonal neurons and external sensory organs in organizing leg posture and movement (Kernan et al, 1994; Bässler, 1988; Burrows, 1996; Zill et al, 2004). However, at least one study suggests that multidendritic (or “multipolar”) sensory cells may play a role in adult walking (Guthrie, 1967), and a study in *Manduca* demonstrates an important role for certain multidendritic neurons in larval locomotion (Tamarkin and Levine, 1996). In *Drosophila* larvae, the multidendritic neurons form a complex web of specialized dendritic structures under the cuticle (Brewster and Bodmer, 1995; Grueber et al, 2002). Do these cells play a role in crawling behavior?

To answer these questions, we used techniques that allow us to analyze the *Drosophila* crawling circuit at the level of small sets of individual neurons. The Gal4-UAS system allows manipulation of identifiable subsets of neurons with a variety of different Gal4 drivers (Brand and Perrimon, 1993). Responder constructs such as *UAS-shibire^{ts}* can be used to temporarily inhibit release of neurotransmitter in the neurons of an otherwise healthy animal (Kitamoto, 2001; Thum et al., 2006). Lastly, a GFP-tagged myosin heavy chain line (Mhc-GFP) allows us to directly image the activity of the muscles during crawling. This provides a more direct readout of the motor behavior than the traditional technique of analyzing the larval path, and the GFP-marked muscles reveal subtle defects in the motor pattern. A benefit of these approaches is that cells are studied in the intact animal rather than a reduced preparation, so we can study sensory as well as central neurons. In the work presented here, we focus on the role of body wall sensory neurons in crawling.

The body wall sensory neurons have been a favored system for studying cell lineage, identity, and dendritic morphology (Brewster and Bodmer, 1995; Dambly-Chaudiere and Ghysen, 1987; Bodmer and Jan, 1987; Grueber et al., 2003; Grueber et al., 2002). However, the functions of these neurons are still a mystery. Understanding both the development and the function of specific neurons can help us understand how the genome encodes behavioral circuits.

In our analysis, we found an important role for two classes of multidendritic neurons in larval crawling. These neurons appear to act as proprioceptors, sensing muscle contractions, and provide feedback to the CNS in a “mission-accomplished” signal that speeds the wave along. We found that disruption of dendritic morphology and axon pathfinding interferes with the

feedback, suggesting that these neurons sense the contraction wave and pass that information on to central targets.

Materials and Methods

Gal4 driver lines and other *Drosophila* stocks

Gal4 drivers were collected from many sources, including Ulrike Heberlein, the *Drosophila* Genetic Resource Center in the Kyoto Institute of Technology, the Bloomington Stock Center, Yuh-Nung Jan, Jim Skeath, Dan Tracey, and Tadashi Uemura. In addition, we generated 482 new lines in a Gal4 enhancer trap screen, by mobilizing an X-linked P{GawB} onto chromosomes II or III. The *UAS-shi^{ts}*; *UAS-shi^{ts}* flies were acquired from Toshihiro Kitamoto and described in Kitamoto, 2001. The *scratch-Gal4* was generated in an enhancer trap screen (Shingo Yoshikawa and John Thomas, unpublished data). The *UAS-nls-RedStinger* (pRed H-Stinger) construct, which marks nuclei in red, was acquired from the Bloomington Stock Center (Barolo et al, 2004).

Expression analysis

Gal4 drivers were crossed to *UAS-cd8-GFP*, which localizes GFP to the cell membrane and marks the cell body, dendrites and axons. An overnight incubation at 29°C was used to increase Gal4 expression before evaluation as third instar larvae. For screening, the PNS was observed by pressing larvae under a coverslip in PBS on a sylgard plate. The CNS was dissected out in PBS on a sylgard plate for rapid viewing. Images for publication were prepared on a Zeiss confocal microscope. Sensory neurons under the body wall were imaged from third instar larvae under a coverslip in Aqua-Polymount. For confocal imaging the CNS was dissected and mounted on a slide in PBS.

For the analysis in this paper, we chose Gal4 drivers for their expression patterns in subsets of the PNS. For each line, we carefully characterized the expression pattern in both the PNS and the CNS at the third instar larval stage. We avoided using lines that had strong expression patterns in the CNS, and used only lines with none or limited expression in the CNS (see Supplemental Figure 1). This helped ensure that our inhibition phenotypes are due to the effect on peripheral neurons alone.

GFP protein trap

We generated *Myosin heavy chain-GFP^{c110}* in a protein trap screen using flies carrying a Protein Trap Transposon (Morin et al., 2001). Insertion location was identified by inverse PCR.

Crawling behavior assay

For the conditional inhibition assay, Gal4 virgins were crossed to *UAS-shi^{ts}*; *Mhc-GFP^{c110}*; *UAS-shi^{ts}* males, and allowed to lay for two days. Constitutive inhibition experiments using tetanus toxin, a toxin which prevents vesicle fusion (*UAS-TeTxLC*; Sweeney et al., 1995) were also performed for all drivers following the same protocol. *UAS-cut* and *UAS-unc5* experiments were performed similarly. However, for the *UAS-cut*, *UAS-unc5* and some *UAS-TeTxLC* experiments, younger larvae were used due to lethality before the third instar stage. *Cut* and *unc5* were used to manipulate the neuronal identity and axon pathfinding of the neurons, respectively (Grueber et al., 2003; Keleman and Dickson, 2001). Larvae were grown at 25°C in uncrowded conditions, with a final incubation at 29°C for 40-44 hours to enhance the Gal4 expression level. The larvae were given a two-hour recovery at room temperature, then scooped into PBS. *Mhc-GFP* females were selected and gently lifted with a paintbrush to a second dish of PBS to rinse off residual food. Larvae were then gently lifted with a paintbrush onto a black agar/ink plate held at 34°C on a peltier heat block. After a 60s acclimation period on the agar

plate, video was recorded for 60s at 15 frames per second. Magnification was typically 12.8X, except in the few experiments where younger larvae were analyzed (*UAS-TeTxLC*, *UAS-cut*, and *UAS-unc5* experiments). During video capture, the moving larva was kept in the field of view by sliding the heat block under the microscope manually. The stock of *clh201-Gal4* also carried *UAS-cd8-GFP*, and as that Gal4 insert is somewhat unstable larvae were confirmed for the presence of *clh201-Gal4* retrospectively by dissection for GFP. The agar/ink plates consisted of 2% agar in water with 1% speedball india ink, topped with white plastic netting and a thin layer of clear 2% agar. The netting provided a faintly-visible white grid in the background that was helpful for aligning video frames. For each experiment, five individual animals were recorded in five 1min videos. *UAS-TeTxLC*, *UAS-cut*, and *UAS-unc5* experiments were also performed on the 34°C heat block to facilitate comparisons.

Video capture and processing

Videos were captured using a CCD camera mounted on the Zeiss Axiophot M2 Bio, with a Scion framegrabber card and Scion Image software on a PC. Scion format videos were exported as a series of TIFF files, then converted using Quicktime Pro on a Macintosh. Videos were compressed and are available as Supplementary Information in Quicktime format. Pseudocolored moviestrips for the figure panels were compiled from the original TIFF images by the following procedure in Adobe Photoshop: 1) 75 frames representing five seconds were copied as layers into a single file and changed to RGB mode, 2) frames were aligned if necessary using the white grid in the background, 3) Autolevels was used to enhance the contrast of a frame containing a mid-point contraction wave, this degree of contrast was mimicked using the brightness/contrast function, and applied identically for all frames, 4) the background of each image was deleted, 5) the Gradient Map function was used to pseudocolor the larva (bright=hot colors, dark=cool colors), and 6) the frames were separated out over vertical space and placed over a black background.

Behavioral quantification

For wave frequency, each of the five videos per experiment was viewed and each contraction wave counted using a hand counter. Number of waves divided by the number of seconds gave “wave frequency”. (Usually the full 60 seconds provided useful data, but when the video included a larva crawling to the edge of the plate only the uninterrupted subset of the video was analyzed.) An average value was determined from the five videos per experiment. For wave duration, the first five forward waves from the five videos were measured for time from tail to head, by marking with the selection tool in Quicktime Pro and converting number of frames to milliseconds. An average was calculated from the five videos per experiment. For *109(2)80-Gal4* × *UAS-shi^{ts}*, wave parameters were calculated from backwards waves, due to the very low number of forward waves. To measure the degree of segment contraction, the longitudinal width of abdominal segment 4 was measured on two sides and averaged, for five waves for five individuals per experiment, and normalized to five interspersed measurements of the maximum (relaxed length), using ImageJ.

Results

A cell-based screen for components of the larval crawling circuit

To identify cells with important roles in crawling behavior, we collected over 1300 Gal4 driver lines from various sources and also generated 482 ourselves. We screened these lines for expression patterns by crossing to *UAS-cd8-GFP*, which targets GFP to the cell membrane and reveals the cell body, axon and dendrites of neurons that express the driver (Lee and Luo, 1999). For further studies, we kept lines that drive expression in small subsets of the CNS or PNS. Of those neuronal drivers, we then screened for function in larval crawling by crossing

to *UAS-shibire^{ts}* (*UAS-shi^{ts}*) to inhibit neurotransmission in the subset of neurons that express the Gal4 (Kitamoto, 2001).

The *UAS-shi^{ts}* construct has been used to study a variety of behaviors in *Drosophila*, including courtship, sleep, memory, olfaction and gustation (Sakai and Kitamoto, 2006; Pitman et al, 2006; Sakai et al, 2004; Suh et al, 2004; Ishimoto et al, 2005). The construct expresses a mutant form of the dynamin protein that inhibits vesicle recycling at the restrictive temperature. At high temperature neurotransmitter release is inhibited, and the behavioral consequences can be observed. Although it is possible that some neurons may not be completely inhibited immediately, a comparative analysis by Thum and colleagues (2006) found that *UAS-shi^{ts}* was the most effective tool for inhibiting neurons in *Drosophila*. (However, while *UAS-shi^{ts}* can effectively inhibit chemical synapses, it is important to remember that it has no effect on electrical synapses.) For our part, we found that a 60 sec acclimation period at 34°C was sufficient to reveal the full behavioral effect using many different drivers. This temperature is somewhat higher than the 29°C restrictive temperature previously described for *shibire^{ts}* (Poodry and Edgar, 1979). However, we empirically determined that this temperature provided the most rapid and consistent inhibition phenotypes (data not shown), without impairing the peristalsis of negative control larvae (Video 1a-c). Our protocol also included a preliminary treatment at 29°C to boost driver expression, which improves reliability (data not shown). Again, our negative controls demonstrate that wildtype larvae are unaffected by this treatment (Video 1a-c). All of our *UAS-shi^{ts}* experiments were also replicated with the non-conditional tetanus toxin construct (*UAS-TeTxLC*) that constitutively blocks synaptic transmission by cleavage of synaptobrevin, thus preventing vesicle fusion (Sweeney et al., 1995). The tetanus experiments produced similar phenotypes in all cases (some examples shown in Video 5).

We found that our regimen for the *UAS-shibire^{ts}* experiments produced full locomotion phenotypes within the first two minutes and that these phenotypes were not enhanced during extended observation periods at 34°C. We used this short treatment to observe the acute effect of removing sensory feedback, avoiding possible complications of indirect behavioral effects that might affect interpretation of the phenotype (see Discussion).

In our experiments, larvae also carried *Mhc-GFP^{C110}*, a GFP protein trap of the myosin heavy chain gene, to facilitate visualization of muscle contraction. We observed the crawling of third instar larvae on a black agar plate at the restrictive temperature to inhibit the neurons. At this warm temperature larvae are strongly motivated to crawl, and wildtype or negative control crawling consists of a series of smooth tail-to-head forward waves in rapid repetition, with occasional pauses and turns (Figure 1A,B; Video 1). We observed a variety of crawling defects in response to inhibiting different neuronal subsets, such as crawling that was too slow, weak, had excessive pausing and turning, excessive backwards waves, postural defects, initiation defects, propagation defects, or discontinuities in the pattern at particular segments. Some driver lines were completely paralyzed when inhibited, while others had no phenotype despite their ability to drive expression in neurons within the CNS. The variety of phenotypes seen with different neuronal subsets assured us that our assay could reveal different types of defects in the circuit, and that the larvae do not simply exhibit a generic response to impairment.

Other researchers have analyzed characteristics of the path taken by crawling larvae to study locomotion (Wang et al., 1997; Osborne et al., 1997; Suster et al., 2003; Ainsley et al., 2003). In our analyses, using larvae with Mhc-GFP marked muscles, we were able to focus directly on the pattern of muscle contractions during crawling, representing aspects of the larval crawling “gait” rather than the path. Gal4 driver lines expressed in restricted subsets of neurons, that caused crawling defects when inhibited, were kept for further analysis. In this report we present our analysis of the role of sensory neurons in the peripheral nervous system, and we focus on a particular propagation defect that results from inhibiting sensory feedback.

The PNS provides feedback to speed up the crawling pattern

We found 50 *Gal4* lines that drive expression in subsets of the PNS, many of which caused crawling defects when the neurons were inhibited. Of these lines we focused on the drivers that had strong PNS expression in the third instar larva and little or no CNS expression. Expression in central neurons could complicate our analysis by introducing behavioral defects arising from those neurons. To avoid this problem, we fully characterized the expression patterns at the third instar larval stage for each line, and avoided drivers with strong patterns in the CNS (see Supplemental Figure 1).

The line *5-40-Gal4* is expressed throughout the PNS, including strong expression in the multidendritic neurons and the chordotonal neurons, and weaker stochastic expression in the external sensory cells (Fig. 2A,B). The *cd8-GFP* revealed the sensory neuron cell bodies in the body wall plus their axonal projections into the CNS, but no expression by central neurons. The line *NP5092-Gal4* also has similar expression in the PNS, but with expression in a small number of CNS neurons as well.

Inhibiting the PNS with either driver *5-40-Gal4* or *NP5092-Gal4* causes a striking defect in the crawling pattern (Fig. 2D-K; Video 2b-e). The major feature of the PNS-inhibition phenotype is slow progression of the wave with excessively tight muscle contractions. This phenomenon of slow, tight waves was dubbed “toothpasting”, as it evokes the image of someone slowly and firmly pressing all the paste to the front of a toothpaste tube. The slow propagation results in a tenfold decrease in wave frequency, from 2 waves/sec to under 0.2 waves/sec. In addition, the PNS-inhibited larvae have a postural defect, causing them to roll onto their side during crawling. The *5-40-Gal4* × *UAS-shi^{ts}* larvae also have an increased proportion of backwards waves, and excessive curling of the head. We decided to explore the toothpasting defect, since this seems to indicate a role for moment-to-moment sensory feedback at the level of segments of the nervous system.

Multidendritic neurons provide the major component of sensory feedback

Knowing that inhibiting the entire PNS produced a characteristic phenotype, we then asked what crawling defects resulted from inhibiting different types of neurons of the PNS. Three categories of neurons make up the PNS – the multidendritic (md), external sensory (es), and chordotonal (cho) neurons. Which of these cells is responsible for the defects in crawling when the PNS is inhibited? To determine this, we used *Gal4* driver lines expressed in specific categories of sensory neurons.

We isolated a new driver, *clh201-Gal4*, that has strong expression in the md and es neurons but lacks any expression in the chos. We also used the driver *109(2)80-Gal4*, that is expressed in all the md neurons but not in the es or cho neurons (Fig. 3A; Gao et al, 1999). The md neurons have been well-characterized developmentally but not functionally. They have webs of dendritic branches spread under the cuticle, without attachment to any accessory organ or tendon. The appearance of these cells suggests that they may be sensitive to cuticular deformation, but there has been little evidence to support this.

Inhibiting either the *clh201-Gal4* or *109(2)80-Gal4* neurons produced crawling defects similar to inhibiting the entire PNS. The defects included toothpasting (slow, tight waves), rolling, and with *109(2)80-Gal4*, a predominance of backward waves (Fig. 3B,C; Video 3b,c). Inhibiting the neurons unconditionally with tetanus toxin (*UAS-TeTxLC*; Sweeney et al., 1995) produced similar phenotypes (data not shown). Since inhibiting the md-only driver *109(2)80-Gal4* causes strong toothpasting, we can ascribe that phenotype to a lack of md function.

What about the third category of neurons, the chordotonal neurons? The *Gal4* drivers *9-20-Gal4* and *8-73-Gal4* are expressed in all eight of the chordotonal neurons with no expression

in mds or es cells. Crossing these drivers to *UAS-shi^{ts}* revealed only minor defects in crawling, in contrast to the dramatic defects seen with inhibiting the mds (Fig 3D,E; Video 3d,e). Constitutively inhibiting the cho neurons with *UAS-TeTxLC* produced more apparent defects, including weaker contractions and posture problems, but we saw no evidence of the toothpasting phenotype (data not shown). Thus we conclude that the md neurons, but not the chos, are necessary for fast wave propagation. The md neurons appear to play a feedback role that facilitates rapid, smooth contraction waves.

Two classes of md neurons are necessary for fast wave propagation

Inhibiting the md neurons resulted in major crawling defects, including slow, tight wave propagation. But md neurons fall into five different classes with a wide variety of morphologies, from the bipolar dendrite (bd) neurons with a simple linear morphology, to the class IV dendritic arborization (da) neurons with their elaborate tiled webs of dendrites covering large regions of the body wall (Bodmer and Jan, 1987; Grueber et al., 2002). Which classes of md neurons provide the important feedback for crawling behavior?

To narrow it down, we used Gal4 drivers expressing in the bd and class I md neurons (*NP2225-Gal4*; Sugimura et al, 2003), in the class II and III neurons (*1003.3-Gal4*) and in just the class IV neurons (*clh24-Gal4*, *7-33-Gal4*). Inhibiting *NP2225-Gal4* gave the characteristic toothpasting propagation defect (Fig. 4A; Video 4b), while inhibiting the other mds with *1003.3-Gal4*, *clh24-Gal4*, or *7-33-Gal4* resulted in no strong crawling defects (Fig. 4B-D; Video 4c-e). Only three classes of mds are active when *1003.3-Gal4* neurons are inhibited, suggesting that the bds, class I and class IV mds alone are sufficient to support normal crawling. Since *NP2225-Gal4* is expressed in the bd and class I mds, and that driver caused the propagation defect when inhibited, we can ascribe the defect to inhibiting these two classes of neurons.

The bipolar dendrite and class I md neurons share the feedback role

To focus further on specific cells, we used *clh8-Gal4* and *8-113-Gal4* to manipulate the bds, and *2-21-Gal4* to manipulate the class I mds. *clh8-Gal4* is expressed in the longitudinal (dbd, vbd) and lateral (lbd) bipolar dendrites, plus dmd1 (Fig. 5A), while *8-113-Gal4* is expressed in just the longitudinal bipolar dendrites (dbd and vbd), plus dmd1. *2-21-Gal4* is expressed in just the three class I mds (ddaD, ddaE, and vpa), plus the single bipolar dendrite dbd (Fig. 5B; Grueber et al, 2003).

Inhibiting the bd neurons with either *clh8-Gal4* or *8-113-Gal4* produced mild propagation defects (Fig. 5D,E; Video 5b,c). These defects were somewhat more apparent when neurons were inhibited with *UAS-TeTxLC* (Video 5d). In addition, inhibiting the class I mds alone with *2-21-Gal4* also produced mild propagation defects (Fig. 5F; Video 5e,f). When the bd and class I drivers were combined, however, and both classes of neurons were inhibited, much more severe propagation defects, with slow, tight waves, were observed (Fig. 5G,H; Video 5g-i). These combination phenotypes suggest that the bds and class I mds provide similar and partially redundant information – either category working alone can partly but not entirely make up for the loss of the other.

One caveat is that both the bd drivers (*clh8-Gal4* and *8-113-Gal4*) and the “class I md” driver (*2-21-Gal4*) include expression in the dorsal bipolar dendrite dbd neuron (Fig. 5A,B). Could the stronger phenotype be due instead to stronger inhibition of the single dbd neuron? This is possible but seems unlikely, given that the single line *NP2225-Gal4*, with moderate expression in each of the bd and class I md neurons, gives a similar strong phenotype when inhibited (Fig. 4A, Video 4b). It appears that inhibiting the bd and class I md neurons together produces a stronger phenotype than either alone.

Another consideration is that some of our Gal4 drivers include expression in a small number of central neurons as well as the peripheral neurons. However, a careful look at these central expression patterns reveals that there are no central neurons in common to the lines with the toothpasting phenotype (Supplementary Fig. 1). Thus the most parsimonious explanation is that the slow and tight phenotype is due to inhibition of the only neurons in common, namely the biolar dendrite and class I md neurons.

Quantification of crawling parameters

To aid comparison of the different inhibition experiments, we quantified parameters of the crawling pattern from the video data for each experiment (Fig. 6). Wave duration, wave frequency, and segment contraction tightness all show dramatic differences between larvae with functional bds and class I mds (yellow bars) and larvae with inhibited bds and class I mds (blue bars). Interestingly, when either the bds or class I mds are inhibited separately (with *clh8-Gal4*, *8-113-Gal4* or *2-21-Gal4* drivers), crawling parameters are in the range of wildtype (light blue bars). Inhibiting the bds or the class I mds has a stronger effect when the other class is also inhibited, suggesting that each class can partially compensate for the loss of the other. Also, note that the combination of *8-113-Gal4* plus *2-21-Gal4* (ddaD, ddaE, dmd1, dbd, vbd, and vpda neurons inhibited) has a much stronger effect than *2-21-Gal4* alone (ddaD, ddaE, dbd, and vpda neurons inhibited). This suggests that the neurons vbd and/or dmd1 play a particularly important role. Since contractions of ventral muscles have a slight lead over dorsal muscles (Fox et al., 2006), the ventral longitudinal neuron vbd is in a unique position to sense the contraction wave coming and may provide a major component of the feedback.

Disrupting cell fate and axon guidance disables sensory feedback

Inhibiting neurons is a straightforward way to reveal their role in a behavioral circuit. However, we would like to ask more subtle questions of how neurons carry out a given role by manipulating their development. Transcription factors involved in determining cell identity, axon guidance receptors that help a neuron find its appropriate synaptic target, and channels that give a neuron the appropriate electrical properties, all contribute to a neuron's ability to function in a circuit. If the coding for cell identity, pathfinding, or activity is disrupted, a neuron may not be able to carry out its normal role.

Previous work has shown that the transcription factor Cut plays an important role in determining the identity of different classes of multidendritic neurons. Cells with highly-branched dendritic arbors like the class III and IV mds have high levels of Cut, while less-branched cells like the class I and II mds have absent or low levels of Cut (Grueber et al., 2003). Moreover, manipulating levels of Cut can switch dendritic morphology – removing Cut with a mutation or RNAi transforms class III and IV cells to have fewer branches, while misexpressing Cut in class I mds gives them more branches (Grueber et al., 2003). Cut has also been shown to play a role in specification of axonal projection patterns for some sensory neurons (Merritt et al., 1993). Misexpression of Cut is likely to change neuronal cell fate in a variety of ways, including alteration of dendritic morphology and axonal projections into the CNS.

We increased Cut levels in the mds by driving *UAS-cut* with *clh201-Gal4*. This treatment results in increased dendritic branching, giving a class III-like, highly branched morphology to the class I cells (data not shown; Grueber et al., 2003). Moreover, this treatment caused crawling defects similar to that seen with md inhibition (Fig. 7A-D; Video 6bc).

To directly interfere with the pathfinding of the sensory neurons, we used *UAS-unc5* to misexpress the Netrin receptor Unc5 in the PNS. This receptor causes neurons to experience the Netrin gradient as a repulsive signal, and thus drives axons away from the high Netrin levels

at the CNS midline (Keleman and Dickson, 2001). Accordingly, misexpression of Unc5 throughout the PNS with *5-40-Gal4* resulted in disruptions of sensory input patterns. For this experiment, it was necessary to use the *5-40-Gal4* driver, expressing in a large set of PNS neurons, for the purpose of achieving expression early in development. While the sensory axons projected normally within the peripheral nerves to the CNS, once arrived they failed to grow towards the midline and tended to stall laterally, or in some cases failed to enter the CNS altogether (Fig. 7F, G). Despite the disruption of the sensory input, the overall structure of the CNS appeared normal, although subtle secondary defects would not be detectable (Fig. 7H). Notably, expression of Unc5 phenocopied the effects of inhibiting the neurons, and resulted in propagation defects in the crawling pattern (Fig. 7I-L; Video 6ef). Thus, misexpression of Unc5 appeared to prevent the sensory neurons from reaching their proper central targets, and consequently they were compromised in their ability to properly convey sensory feedback information to the CNS.

Discussion

Sensory feedback promotes wave propagation

To determine the role of the body wall sensory neurons in *Drosophila* larval crawling, we temporarily inhibited the neurons while larvae crawled across an agar surface. This caused major defects in the pattern, due to excessively slow and tight contraction waves. Without sensory feedback, each segment contracted too tightly for too long, and the wave was slow to propagate forward. This suggests that there is normally feedback from the sensory neurons to the central circuit as the wave travels from each segment to the next. Since contractions are exaggerated and propagation is slowed when the neurons are inhibited, the normal active role of the sensory neurons must be to facilitate relaxation of a contracted segment, and rapid forward propagation of the wave. It appears the sensory neurons provide a boost to the much slower pattern inherent to the central pattern generator alone, allowing the larvae to crawl at 2 waves/sec rather than 0.2 waves/sec.

Previous work has emphasized the fact that the central pattern generator still runs without sensory feedback (Suster and Bate, 2002; Fox et al, 2006), however we find the feedback to be a critical component for normal behavior. In their study, Suster and Bate (2002) focused on a locomotor-like behavior in *Drosophila* embryos, which produce peristaltic waves while still in the eggshell. These embryos continued to produce peristaltic waves when the sensory neurons were inhibited with tetanus toxin. However the sensory-inhibited embryos produced only one-third the number of forward waves, and these waves were not organized into bouts. Nevertheless, the peristalsis pattern of a single wave was itself not greatly disrupted. This seems inconsistent with our results that peristalsis is slowed and exaggerated in sensory deprived larvae, but we suggest that the peristalsis behavior in embryos may not rely on sensory feedback. In fact, the long period of peristalsis seen in the embryos (>4.5sec) is consistent with what we find to be the basal peristaltic rate produced by the sensory-deprived larval CPG (2-3sec). When crawling was observed in the sensory-inhibited larvae just after hatching, the slow, disrupted pattern seen is consistent with our results (Suster and Bate, 2002). In the case of the semi-intact preparation used by Fox and colleagues (2006), the normal period of peristalsis averages 6.5 sec, much longer than the 1 sec period they observe in intact larvae (Saraswati et al, 2004). As the authors point out, reduced preparations lacking sensory feedback are well-known to exhibit slowed fictive patterns (Friesen and Cang, 2001). Our results suggest that in the case of *Drosophila* larvae, it is the role of multidendritic neurons in sensory feedback that makes the difference between the period of an intact and a sensory-deprived preparation.

Another study using similar techniques of *UAS-shibire^{ts}*-mediated inhibition of sensory neurons has concluded, similar to us, that the md neurons play an important role in regulating *Drosophila* larval locomotion, and that the cho neurons appear to play little role (Song et al,

2007). These authors found an increase in contraction duration and decrease in frequency associated with all-sensory or all-md inhibition, but not with cho inhibition, consistent with our results. The study used a somewhat different experimental regimen from ours: combining the Gal4 drivers with a single copy of *UAS-shibire^{ts}*, raising the larvae at 18°C, and then subjecting the larvae to a higher non-permissive temperature of 37°C. Under these conditions the full contraction phenotype was not apparent until after 10 minutes of treatment at the non-permissive temperature. This was accompanied by an eventual cessation of peristaltic waves altogether by 20 minutes of treatment, leading to the conclusion that waves cannot be initiated at all in the absence of sensory feedback and therefore there is effectively no independent CPG. However, it is possible that a 20 minute treatment at such high temperature could result in a number of indirect behavioral effects due to the stress of highly contracted, disrupted crawling, and that under such conditions it would be difficult to separate the primary effect of sensory feedback from secondary effects. In support of this notion, we saw no evidence of total paralysis in our sensory experiments. Although we cannot rule out the presence of a low level of residual sensory transmission, we are confident that our regimen produced strong inhibition phenotypes, based on the following evidence: a) We found good concordance between the temperature-sensitive *UAS-shibire^{ts}* experiments and the non-conditional *UAS-TeTxLC* experiments that we performed in parallel (cf. Video 5g and 5i). b) Following the 1 minute acclimation period, we never saw enhancement of the phenotype during longer observation periods at 34°C. In all cases, from subtle locomotion phenotypes to more dramatic paralysis phenotypes seen with pan-neuronal lines, the full phenotypic effect was always seen within the first two minutes. c) The full inhibition phenotypes seen after the first minute, including ten-fold longer wave duration and ten-fold slower frequency, were as strong as those seen after 5-10 minutes under the regimen of Song et al. Thus, we would argue that while the md neurons do play an important role in regulating the locomotor pattern, under conditions of strong inhibition there still remains a weakly active central pattern generator capable of producing slow, overly tight, peristaltic waves.

In addition to the tight/slow toothpasting phenotype we observed with multidendritic inhibition, we observed excessive backwards waves when some sets of neurons were inhibited, particularly with the all-md line *109(2)80-Gal4* (Fig. 6D). Backwards crawling is similar to forwards crawling, but with a head-to-tail peristaltic wave. We cannot explain the source of this defect, except to note that backwards waves are a normal behavior that is invoked only occasionally, typically when the larva encounters some aversive stimulus (personal observation; Kernan et al, 1994). As an escape response, backwards crawling may be an indirect consequence of many disruptions, and is difficult to interpret. Because the high level of backwards crawling was not seen as strongly with other lines, we attribute it to neurons uniquely expressing *109(2)80-Gal4*, but did not attempt to localize them further. Instead, we focused on the specific neurons responsible for the feedback necessary to promote rapid relaxation and smooth propagation of the wave.

The bd and class I mds provide feedback for wave propagation

Body wall sensory neurons include a variety of md, es, and cho neurons. By conducting experiments with Gal4 drivers expressed in limited sets of neurons, we were able to assign the sensory feedback role to just two classes of md neurons: the bipolar dendrites and the class I mds.

These neurons are the least elaborately branched of the multidendritic neurons. The bds are linear cells that extend across the width of each segment, while the class I mds have simple dendritic arbors along the longitudinal axis. The simple anatomy of the bds and class I mds make them perfectly suited to sense the variation in longitudinal stretch that accompanies a contraction wave. These two classes of mds were previously found to have unique projection

patterns in the CNS (Merritt and Whittington, 1995). Unlike other sensory neurons, they project to the dorsal neuropil, near the motoneurons. This suggests that they may provide direct feedback onto motor output via monosynaptic connections. These neurons are also unusual in having projections into the CNS that extend over more than one segment (Merritt and Whittington, 1995). This potential to communicate with neighboring neuromeres may allow smooth coordination during wave propagation.

The “mission accomplished” model

The md neurons are thought to be stretch sensitive cells, based on their morphology and data from different insect species (Finlayson and Lowenstein, 1958; Weevers, 1966; Anderson and Finlayson, 1978; Grueber et al., 2001). The *Drosophila* larval mds lie just under the cuticle. When the muscles are relaxed, the sensory neurons are pulled taut. As a contraction wave approaches, added tension is applied to the more posterior segments. When the muscles contract during the peak of the contraction wave, the segment narrows and tension is released. Thus during each peristaltic wave, a segment experiences a cycle of tension and relaxation. We suggest that this information is carried back to the CNS by proprioceptive feedback from the bipolar dendrite and class I md neurons, and that this feedback is critical for coordinating the rapid muscle contractions of a peristaltic wave.

In *Manduca*, a dbd-like neuron called the stretch-receptor sensory neuron responds to stretch by increasing its firing rate (Tamarkin and Levine, 1996). This neuron has both excitatory monosynaptic and inhibitory polysynaptic connections onto motoneurons that overall, appear likely to stabilize the posture of a segment against perturbation (Tamarkin and Levine, 1996). We hypothesize that, like the *Manduca* stretch receptor sensory neuron, the firing frequency of the bipolar dendrite and class I md neurons is an indicator of the degree of longitudinal tension on a segment. This stretch information may excite and inhibit the appropriate motoneurons of the local segment and neighboring segments, facilitating rapid propagation and relaxation. We hypothesize that upon contraction of a segment, the bd and class I md neurons respond by either an increase or decrease in firing frequency, and that this change constitutes a “mission accomplished” signal to the CNS (Fig. 8).

When the signal is missing in the case of the inhibition experiments, the relaxation of the muscles is delayed, they continue tightening excessively, and forward propagation is slowed. In our experiments, the wave nevertheless continues forward at a slower rate, and the muscles belatedly relax. The system has some redundancy – in the absence of sensory feedback the central circuit still has the capacity to push the wave forward, but without feedback the rapid coordination is lost and crawling is awkward and ineffectual. This role for stretch receptors in regulating segment-to-segment propagation may be similar to that seen in leech swimming (Cang and Friesen, 2000) and crayfish swimmeret beating (Heitler 1982; Heitler 1986). In both systems, stretch receptor neurons show activity correlated with the movement pattern, and stimulation of the stretch receptor neurons is sufficient to modify the phase lag between segments.

Since the *Drosophila* bipolar dendrites and class I mds project into the dorsal neuropil of the CNS, they may signal directly to the motoneurons (Merritt and Whittington, 1995). However, since the sensory neurons seem to promote both local relaxation (inhibiting motoneurons) as well as forward propagation (exciting motoneurons), it is likely that one of these roles requires an inhibitory interneuron to change the sign of the signal. This scenario is analogous to the role of muscle spindle fibers in vertebrates. The Ia afferents sense stretch, activating cognate motoneurons via a monosynaptic connection, and inhibiting antagonistic motoneurons via an inhibitory interneuron (Nelson and Mendell, 1978).

The apparent coupling of relaxation and propagation in larval crawling is interesting, because the timing of these events is essential for effective movement. During normal crawling, one segment relaxes just following propagation of the wave to the next segment ahead, forming a seamless peristaltic wave. If relaxation *preceded* forward propagation, the larva would lose the directional push that moves the body forward by hydrostatic pressure. On the other hand, if relaxation were *delayed* relative to propagation, then excessive contraction of segments at the rear could exert a high hydrostatic pressure and rupture the anterior end. The relative timing of relaxation and propagation is crucial, and mechanistically coupling them via the same sensory feedback signal may effectively regulate the pattern.

Further studies are needed to test our model of sensory feedback. It is formally possible that the effect we see on locomotion may be merely due to excitatory drive from the sensory neurons, rather than a cycle-by-cycle feedback. New imaging techniques for observing activity in sets of neurons in *Drosophila* may allow us to see the dynamics of sensory neuron activity during the progression of a peristaltic wave (Wang et al., 2003), addressing the question of whether the neurons are stretch-activated or stretch-inactivated.

Multidendritic neurons and the evolution of the maggot larva

Research on insect walking has put emphasis on the important roles of the chordotonal neurons and the campaniform sensillae in conveying information about leg posture and loading (Bässler, 1988; Burrows, 1996; Zill et al., 2004). Yet in the case of *Drosophila* larvae, we find a major role for the multidendritic neurons instead. Why the discrepancy? One possibility, suggested by Guthrie (1967), is that in legged insects the md neurons do in fact play a role, but their role has been overlooked because of the difficulty of doing electrophysiological recording or because of a lower level of response. Another possibility is that the md neurons have taken on a more important role during the evolution of the soft-bodied larva.

Multidendritic neurons play a variety of roles in legged insects, for respiration, copulation, and feeding (Finlayson and Lowenstein, 1958; Sugawara, 1979; Bowdan and Dethier, 1985). In the cockroach, non-adapting responses from md neurons reflect leg position and might regulate walking (Guthrie, 1967). In *Drosophila*, the bipolar dendrites persist into the adult stage, but their function is unknown (Williams and Shepherd, 1999). Perhaps md neurons play various proprioceptive roles in legged insects, and were co-opted to play specialized locomotor roles in the maggot larva. With a soft body and no limbs, the maggot requires different types of information to judge position, and the md neurons may have been well suited to provide this information. The *Manduca* caterpillar has a dbd-like neuron of the stretch receptor organ that may regulate crawling (Tamarkin and Levine, 1996). The stretch receptor sensory neuron responds to stretch with increased firing, and makes both excitatory monosynaptic and inhibitory polysynaptic connections to motoneurons. The sensory neuron persists in the adult, but is modified to make only excitatory connections, an arrangement which supports the different flexion of the adult abdomen (Tamarkin and Levine, 1996).

Specialization of sensory neurons

As for the chordotonals, external sensory neurons, and other classes of multidendritic neurons, lack of a phenotype in our crawling assay does not indicate lack of function. They may provide subtle contributions to locomotor regulation, or they may act in other sensory modalities, such as local touch, nociception, or temperature sensation (Tracey et al., 2003; Liu et al., 2003).

How might the different classes of md neurons specialize for different kinds of sensory information? We know that the morphology of md neurons is specified by identity genes such as *cut* and *abrupt* (Grueber et al., 2003; Sugimura et al., 2004). When we manipulated *cut* levels in the sensory neurons to disrupt dendritic morphology and possibly axonal projections, it

disrupted the ability of the *bd* and class I mds to perform their feedback function. Misexpression of *unc5* in the sensory neurons disrupted their CNS projection patterns, and also blocked sensory feedback. These experiments illustrate the point that a neuron's ability to function in a circuit is a condition of having its correct identity, morphology, and synaptic connections. Further studies are needed to investigate in more detail how the precise role of a given neuron is programmed by its own unique suite of active genes.

CPGs vs. chain of reflexes – a false dichotomy

Our results reveal that sensory feedback plays an important role in generating appropriate motor output for *Drosophila* larval locomotion. However, we find that even with sensory inhibition, the basic generation of waves continues, albeit in a disrupted fashion. Thus, we conclude that a CPG capable of organizing slow peristaltic waves still functions to some extent in the absence of sensory feedback and that it is not necessary to invoke a chain of reflexes scheme as in Song et al. (2007). Our results further illustrate the fact that animal behaviors require both CPGs and sensory feedback working in tandem to generate normal motor patterns. The limitations of studying either the CPG or sensory system in isolation have become evident in recent years, and researchers are addressing the interactions of the two systems (Kristan et al., 2005; Pearson, 2000). It is now clear that a complete understanding of pattern generation will require an appreciation of both aspects, and that pattern generators should perhaps be considered seamlessly integrated Central/Sensory Pattern Generators (CSPGs).

Conclusion

Proprioception is sometimes called “the sixth sense”, and its importance is seldom appreciated unless it is suddenly taken away (see the case of “The Disembodied Lady” in Sacks, 1970). Yet without sensory feedback, everyday motor patterns that we take for granted are nearly impossible to control. Knowing the position of the body and the status of the muscles is crucial for effective and coordinated movement, even for a simple larva.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Ainsley JA, Pettus JM, Bosenko D, Gerstein CE, Zinkevich N, Anderson MG, Adams CM, Welsh MJ, Johnson WA. Enhanced locomotion caused by loss of the *Drosophila* DEG/ENAC protein Pickpocket1. *Curr. Biol* 2003;13:1557–63. [PubMed: 12956960]
- Anderson M, Finlayson FH. Topography and electrical activity of peripheral neurons in the abdomen of the tsetse fly (*Glossina*) in relation to abdominal distension. *Physiol. Entom* 1978;3:157–167.
- Barolo S, Castro B, Posakony JW. New *Drosophila* transgenic reporters: insulated P-element vectors expressing fast-maturing RFP. *BioTechniques* 2004;36:436–442. [PubMed: 15038159]
- Bässler U. Functional principles of pattern generation for walking movements of stick insect forelegs: the role of the femoral chordotonalorgan afferences. *J. Exp. Biol* 1988;136:125–147.
- Bate, M.; Martinez-Arias, A. *The Development of Drosophila melanogaster*. Cold Spring Harbor Laboratory Press; 1993.

- Bodmer R, Jan YN. Morphological differentiation of the embryonic peripheral neurons in *Drosophila*. Roux's Arch. Dev. Biol 1987;196:69–77.
- Bowdan E, Dethier VG. Coordination of a dual inhibitory system regulating feeding behaviour in the blowfly. J. Comp. Phys. A 1986;158:713–722.
- Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Dev 1993;118:401–415.
- Brewster R, Bodmer R. Origin and specification of type II sensory neurons in *Drosophila*. Dev 1995;121:2923–2936.
- Burrows, M. The Neurobiology of an Insect Brain. Oxford Science Publications; Oxford: 1996.
- Caldwell JC, Miller MM, Wing S, Soll DR, Eberl DF. Dynamic analysis of larval locomotion in *Drosophila* chordotonal mutants. PNAS 2003;100:16053–16058. [PubMed: 14673076]
- Cang J, Friesen WO. Sensory modification of leech swimming: rhythmic activity of ventral stretch receptors can change intersegmental phase relationships. J. Neurosci 2000;20:7822–7829. [PubMed: 11027247]
- Dambly-Chaudiere C, Ghysen A. Independent subpatterns of sense organs require independent genes of the achaete-scute complex in *Drosophila* larvae. Genes & Dev 1987;1:297–306.
- Finlayson LH, Lowenstein FRS. The structure and function of abdominal stretch receptors in insects. Proc. Roy. Soc. Lond. B 1958;148:433–449. [PubMed: 13542636]
- Fox LE, Soll DR, Wu CF. Coordination and modulation of locomotion pattern generators in *Drosophila* larvae: effects of altered biogenic amine levels by the tyramine β hydroxylase mutation. J. Neurosci 2006;26:1486–1498. [PubMed: 16452672]
- Fox, LE.; Ueda, A.; Berke, B.; Peng, IF.; Wu, CF. Movement disorders in *Drosophila* mutants of potassium channels and biogenic amine pathways. In: LeDoux, M., editor. Animal Models of Movement Disorders. Academic Press; San Diego: 2005. p. 487-504.
- Friesen WO, Cang J. Sensory and central mechanisms control intersegmental coordination. Curr. Opin. in Neurobio 2001;11:678–683.
- Gao FB, Brenman JE, Jan LY, Jan YN. Genes regulating dendritic outgrowth, branching, and routing in *Drosophila*. Genes and Dev 1999;13:2549–2561. [PubMed: 10521399]
- Grueber WB, Graubard K, Truman JW. Tiling of the body wall by multidendritic sensory neurons in *Manduca sexta*. J. Comp. Neurol 2001;440:271–283. [PubMed: 11745623]
- Grueber WB, Jan LY, Jan YN. Tiling of the *Drosophila* epidermis by multidendritic sensory neurons. Dev 2002;129:2867–2878.
- Grueber WB, Jan LY, Jan YN. Different levels of the homeodomain protein Cut regulate distinct dendrite branching patterns of *Drosophila* multidendritic neurons. Cell 2003;112:805–818. [PubMed: 12654247]
- Guthrie DM. Multipolar stretch receptors and the insect leg reflex. J. Insect Physiol 1967;13:1637–1644.
- Heitler WJ. Non-spiking stretch-receptors in the crayfish swimmeret system. J. Exp. Bio 1982;96:355–366.
- Heitler WJ. Aspects of sensory integration in the crayfish swimmeret system. J. Exp. Bio 1986;120:387–402.
- Ishimoto H, Takahashi K, Ueda R, Tanimura T. G-protein gamma subunit 1 is required for sugar reception in *Drosophila*. EMBO J 2005;24:3259–65. [PubMed: 16121192]
- Keleman K, Dickson BJ. Short- and long-range repulsion by the *Drosophila* Unc5 Netrin receptor. Neuron 2001;32:605–617. [PubMed: 11719202]
- Kernan M, Cowan D, Zuker C. Genetic dissection of mechanosensory transduction: mechanoreception-defective mutations of *Drosophila*. Neuron 1994;12:1195–1206. [PubMed: 8011334]
- Kitamoto T. Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive *shibire* allele in defined neurons. J. Neurobiol 2001;47:81–92. [PubMed: 11291099]
- Kristan WB Jr, Calabrese RL, Friesen WO. Neuronal control of leech behavior. Prog Neurobiol 2005;76:279–327. [PubMed: 16260077]
- Landgraf M, Bossing T, Technau GM, Bate M. The origin, location, and projections of the embryonic abdominal motoneurons in *Drosophila*. J. Neurosci 1997;17:9642–9655. [PubMed: 9391019]

- Lee T, Luo L. Mosaic analysis with a repressible neurotechnique cell marker for studies of gene function in neuronal morphogenesis. *Neuron* 1999;22:451–461. [PubMed: 10197526]
- Liu L, Yermolaieva O, Johnson WA, Abboud FM, Welsh MJ. Identification and function of thermosensory neurons in *Drosophila* larvae. *Nat. Neurosci* 2003;6:267–273. [PubMed: 12563263]
- Merritt DJ, Whittington PM. Central projections of sensory neurons in the *Drosophila* embryo correlate with sensory modality, soma position, and proneural gene function. *J. Neurosci* 1995;15:1755–1767. [PubMed: 7891133]
- Merritt DJ, Hawken A, Whittington PM. The role of the *cut* gene in the specification of central projections by sensory axons in *Drosophila*. *Neuron* 1993;10:741–752. [PubMed: 8386531]
- Morin X, Daneman R, Zavortink M, Chia W. A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in *Drosophila*. *PNAS* 2001;98:15050–15055. [PubMed: 11742088]
- Nelson SG, Mendell LM. Projection of single knee flexor 1a fibers to homonymous and heteronymous motoneurons. *J. Neurophysiol* 1978;41:778–787. [PubMed: 149185]
- Osborne KA, Robichon A, Burgess E, Butland S, Shaw RA, Coulthard A, Pereira HS, Greenspan RJ, Sokolowski MB. Natural behavior polymorphism due to a cGMP-dependent protein kinase of *Drosophila*. *Science* 1997;277:834–6. [PubMed: 9242616]
- Pearson K. Motor systems. *Current Opinion in Neurobiology* 2000;10:649–654. [PubMed: 11084328]
- Pitman JL, McGill JJ, Keegan KP, Allada R. A dynamic role for the mushroom bodies in promoting sleep in *Drosophila*. *Nature* 2006;441:753–6. [PubMed: 16760979]
- Poodry CA, Edgar L. Reversible alteration in the neuromuscular junctions of *Drosophila melanogaster* bearing a temperature-sensitive mutation, *shibire*. *J. Cell Biol* 1979;81:520–527. [PubMed: 110817]
- Sacks, O. *The Man Who Mistook His Wife for a Hat*. Simon and Schuster; New York: 1970.
- Sakai T, Kitamoto T. Differential roles of two major brain structures, mushroom bodies and central complex, for *Drosophila* male courtship behavior. *J. Neurobiol* 2005;66:821–34. [PubMed: 16673386]
- Sakai T, Tamura T, Kitamoto T, Kidokoro Y. A clock gene, *period*, plays a key role in long-term memory formation in *Drosophila*. *PNAS* 2004;101:16058–63. [PubMed: 15522971]
- Saraswati S, Fox LE, Soll DR, Wu CF. Tyramine and octopamine have opposite effects on the locomotion of *Drosophila* larvae. *J. Neurobiol* 2003;58:425–441. [PubMed: 14978721]
- Song W, Onishi M, Jan LY, Jan YN. Peripheral multidendritic sensory neurons are necessary for rhythmic locomotion behavior in *Drosophila* larvae. *PNAS* 2007;104:5199–204. [PubMed: 17360325]
- Sugawara T. Fine structure of the stretch receptor in the bursa copulatrix of the butterfly, *Pieris rapae crucivora*. *Cell Tissue Res* 1981;217:23–36. [PubMed: 7249045]
- Sugimura K, Yamamoto M, Niwa R, Satoh D, Goto S, Taniguchi M, Hayashi S, Uemura T. Distinct developmental modes and lesion-induced reactions of dendrites of two classes of *Drosophila* sensory neurons. *J. Neurosci* 2003;23:3752–60. [PubMed: 12736346]
- Sugimura K, Satoh D, Estes P, Crews S, Uemura T. Development of morphological diversity of dendrites in *Drosophila* by the BTB-zinc finger protein Abrupt. *Neuron* 2004;43:809–22. [PubMed: 15363392]
- Suh GS, Wong AM, Hergarden AC, Wang JW, Simon AF, Benzer S, Axel R, Anderson DJ. A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature* 2004;431:854–9. [PubMed: 15372051]
- Suster ML, Bate M. Embryonic assembly of a central pattern generator without sensory input. *Nature* 2002;416:174–178. [PubMed: 11894094]
- Suster ML, Martin JR, Sung C, Robinow S. Targeted expression of tetanus toxin reveals sets of neurons involved in larval locomotion in *Drosophila*. *J. Neurobiol* 2003;55:233–46. [PubMed: 12672020]
- Sweeney ST, Broadie K, Keane J, Niemann H, O'Kane CJ. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* 1995;14:341–351. [PubMed: 7857643]
- Tamarkin DA, Levine RB. Synaptic interactions between a muscle-associated proprioceptor and body wall muscle motor neurons in larval and adult *Manduca sexta*. *J. Neurophys* 1996;76:1597–1610.
- Thum AS, Knappek S, Rister J, Dierichs-Schmitt E, Heisenberg M, Tanimoto H. Differential potencies of effector genes in adult *Drosophila*. *J. Comp. Neurol* 2006;498:194–203. [PubMed: 16856137]

- Tracey WD Jr, Wilson RI, Laurent G, Benzer S. Painless, a *Drosophila* gene essential for nociception. *Cell* 2003;113:261–273. [PubMed: 12705873]
- Wang JW, Sylwester AW, Reed D, Wu DA, Soll DR, Wu CF. Morphometric description of the wandering behavior in *Drosophila* larvae: aberrant locomotion in Na⁺ and K⁺ channel mutants revealed by computer-assisted motion analysis. *J. Neurogenet* 1997;11:231–54. [PubMed: 10876655]
- Wang JW, Soll DR, Wu CF. Morphometric description of the wandering behavior in *Drosophila* larvae: a phenotypic analysis of K⁺ channel mutants. *J. Neurogenet* 2002;16:45–63. [PubMed: 12420789]
- Wang JW, Wong AM, Flores J, Vosshall LB, Axel R. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell* 2003;112:271–282. [PubMed: 12553914]
- Weevers R, de G. The physiology of a lepidopteran muscle receptor. *J. Exp. Biol* 1966;44:177–194. [PubMed: 5922735]
- Williams DW, Sheperd D. Persistent larval sensory neurons in adult *Drosophila melanogaster*. *J. Neurobiol* 1999;39:275–286. [PubMed: 10235681]
- Zill S, Schmitz J, Büschges A. Load sensing and control of posture and locomotion. *Arth. Struct. Dev* 2004;33:273–286.

Figure 1. Larval crawling and its three interacting systems

A) Wildtype larva with *Mhc-GFP* labeled muscles, crawling from left to right. In 5 seconds, 11.5 peristaltic waves repeat from tail to head in a stereotyped pattern (red stripes). A single wave (box) is enlarged in B). Images are false-colored: areas of highest muscle contraction and highest GFP-intensity are red, while the most relaxed areas are blue. C) The muscle pattern of a whole larva is shown with *Mhc-GFP*. D) The CNS and PNS are revealed by *scratch-Gal4* driving *UAS-cd8-GFP*. Anterior is to the right for all panels.

Figure 2. Inhibiting the PNS disrupts larval crawling

A, B) 5-40-Gal4 driving *UAS-cd8-GFP* and *UAS-nls-RedStinger* reveals the sensory neuron nuclei (yellow) and cell membranes with elaborate arborizations (green). Expression includes md neurons (blue labels), es neurons (green labels) and cho neurons (red labels). The diagram shows Gal4 driver expression patterns, with blue, green, and red boxes corresponding to the five classes of md neurons, the es neurons as a whole, and the four sets of cho neurons. Colored boxes represent expression, gray boxes represent lack of expression. C) The negative control larva (*w/UAS-shi^{ts}; Mhc-GFP/+ ; UAS-shi^{ts}/+*) shows a typical wildtype crawling pattern of about 2 waves/sec (5 sec shown here and in all moviestrips). D) Inhibiting the PNS with 5-40-Gal4 driving *UAS-shi^{ts}* causes a major disruption to the crawling pattern, here showing 5 sec with only a single very slow, tight forward wave. E) Another 5-40-Gal4 inhibition experiment shows two slow, tight backwards waves and head curls. F, G) NP5092-Gal4 driving *UAS-shi^{ts}* shows similar defects. H-K) Enlarged views of 12 frame segments marked with white lines in D-G. L) The sensory neurons of a single abdominal hemisegment are represented with mds (blue), chos (red) and es neurons (green), shown with anterior to the left. M) Expression patterns of the Gal4 drivers in third instar larvae are diagrammed.

Figure 3. Multidendritic neurons provide the major component of sensory feedback

A) *109(2)80-Gal4* driving *UAS-cd8-GFP* and *UAS-nls-RedStinger* reveals the nuclei (yellow) and arborizations (green) of the multidendritic neurons (blue labels). B) Inhibiting the md and es neurons with *clh201-Gal4* driving *UAS-shi^{ts}* disrupts the crawling pattern, here showing only 1.5 waves in 5 sec. C) Inhibiting only the mds with *109(2)80-Gal4* driving *UAS-shi^{ts}* produces a similar defect. D, E) In contrast, inhibiting the chordotonal neurons with *9-20-Gal4* or *8-73-Gal4* driving *UAS-shi^{ts}* does not disrupt crawling. F-I) Enlarged views of 12 frame segments marked with white lines in B-E.

Figure 4. Only a subset of the md neurons is necessary for proper crawling

A) Inhibiting just the bipolar dendrite and class I mds with *NP2225-Gal4* driving *UAS-shi^{ts}* produces defective crawling with slow, tight waves. B) Inhibiting the class II and III neurons with *1003.3-Gal4* driving *UAS-shi^{ts}* does not interfere with normal crawling. C, D) Inhibiting the class IV mds with *clh24-Gal4* or *7-33-Gal4* does not interfere with normal crawling. E-H) Enlarged views of 12 frame segments marked with white lines in A-D.

Figure 5. The bipolar dendrites and class I mds are partially redundant

A) *clh8-Gal4* driving *UAS-cd8-GFP* and *UAS-nls-RedStinger* shows expression in the three bipolar dendrite neurons (dbd, lbd, and vbd) as well as dmd1. B) *2-21-Gal4* driving *UAS-cd8-GFP* shows the three class I neurons (ddaD, ddaE, and vpda) plus dbd. C) Combining the two Gal4 drivers gives expression in all bipolar dendrites and class I mds, plus dmd1. D, E) Inhibiting the bipolar dendrites with *clh8-Gal4* or *8-113-Gal4* driving *UAS-shi^{ts}* causes mild defects in crawling, seen here as slower, tighter waves. F) Inhibiting the class I mds with *2-21-Gal4* also causes mild crawling defects. G, H) Inhibiting all bipolar dendrite and class I mds simultaneously with combinations of the Gal4 drivers causes severe disruptions in the crawling pattern. I-L) Enlarged views of 12 frame segments marked with white lines in E-H.

Figure 6. The bipolar dendrites and class I mds are necessary for proper wave speed and tightness of contraction

Parameters of crawling behavior were quantified from five individuals for each experiment. Gal4 drivers that inhibit the bipolar dendrite and class I mds are represented by blue bars, drivers inhibiting either the bipolar dendrites or the class I mds have light blue bars, and drivers that do not inhibit the bipolar dendrites and class I mds have yellow bars. Negative controls A and B refer to experiments with the *UAS-shi^{ts}* responder alone and the *5-40-Gal4* driver alone, respectively, while wt larvae carry only the Mhc-GFP insert. A) Duration of the peristaltic wave is significantly longer when the bipolar dendrites and class I mds are inhibited. Wave duration is the time from the contraction at the tail to contraction at the head, and excludes the between-wave pause interval. Wildtype waves have a short duration of <500ms (yellow bars), but when the bd and class I md neurons are inhibited the wave duration increases up to 2000-3000ms (blue bars). When only the bds or class I mds are inhibited separately, the wave duration is near the wildtype range (light blue bars). Because *109(2)80-Gal4* × *UAS-shi^{ts}* crawling was dominated almost entirely by backwards waves, the wave duration was calculated from backwards waves. B) Wave frequency is decreased when the bipolar dendrites and class I mds are inhibited. Wave frequency is about 1.5-2.0 waves/sec for negative controls and for all the non-bd/class I experiments (yellow bars), but waves are much less frequent (<0.5 waves/sec) when the bd and class I mds are inhibited (blue bars). When only the bds or class I mds are inhibited alone, the wave frequency is within the normal range (~1.5 waves/sec; light blue bars). C) Segments contract more tightly when the bd and class I mds are inhibited. Bars show the segment width for segment A4 at the maximum contraction, as a fraction of the relaxed segment width between contractions, calculated from 5 waves from 5 individuals per experiment. D) The fraction of backwards waves is increased strongly in the *109(2)80-Gal4* line, and weakly with some of the other sensory inhibited lines. Bars are slightly offset to clarify the several data points with zero backwards waves. Error bars represent standard deviation.

Figure 7. Altering sensory neuron morphology or axon projections disrupts sensory feedback
 Misexpression of the identity gene *cut* is known to result in defective dendritic arborizations in md neurons (Grueber et al., 2003) and possibly projection defects as well (Merritt et al., 1993). Misexpression of *unc5* is known to cause repulsion of neurons from the CNS midline (Keleman and Dickson 2001). Expression of either gene in sensory neurons interferes with their sensory feedback function and disrupts crawling behavior. A, B) Misexpression of *cut* in the md and es neurons with *clh201-Gal4* driving *UAS-cut* results in defective crawling consisting of slow tight waves, similar to the result of inhibited the neurons. C, D) Enlarged views of frames marked with white lines in A,B). E) Normal pattern of sensory projections within the CNS neuropile in the negative control (*w 5-40-Gal4/w; UAS-cd8-GFP Mhc-GFP/+*). Top, whole CNS; bottom, boxed region enlarged. F,G) When *Unc5* is misexpressed, the sensory projections fail to project normally within the CNS, and either stall in the lateral region (asterisk) or fail to enter the CNS at all (arrow; genotype is *w 5-40-Gal4/w; UAS-cd8-GFP Mhc-GFP/GS-unc5; +/UAS-Unc5*). The CNS shown in (F,G) belonged to the larva whose crawling is shown in (I). H) FasII staining (red) reveals the normal pattern of axon bundles (arrow), showing that the overall structure of the CNS is not disrupted despite misrouting of the sensory neurons (green). Anterior to left for (E-H). I, J) Misexpression of *Unc5* in the PNS with *5-40-Gal4* and *UAS-unc5* causes crawling defects similar to the inhibition experiments. K, L) Enlarged views of frames marked with white lines in G,H). For all *Cut* and *Unc5* experiments, first instar larvae are shown because the crawling defects causes lethality at later stages.

Figure 8. A “mission accomplished” model for sensory feedback

Crawling requires the integration of motorneuron activity, muscle contractions, and sensory feedback. We suggest that the main function of the bd/class I md sensory feedback is to report a “mission accomplished” signal to the CNS after a successful contraction, and thereby induce muscle relaxation and forward propagation of the wave. Larval musculature is shown at top, with abdominal segments A3-A5 marked (blue box). An enlargement of the CNS shows the corresponding neuromeres A3-A5 (orange box). In A-C, approximately 100 msec are shown as the contraction wave passes through A5, A4, and A3 (blue boxes). The corresponding activity in the neuromeres are shown (orange boxes) with active motorneurons (orange cells) and the subsequent sensory input (blue arrows). Focusing on the contraction in A4 (B), we suggest a four part mechanism: 1) the motorneurons fire, 2) muscles contract, 3) the bd and class I mds respond to the change in tension, 4) sending a “successful contraction” message to the CNS which causes muscle relaxation in A4 and a propagation signal to A3.