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A subset of interneurons required for *Drosophila* larval locomotion

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Abstract

Efforts to define the neural circuits generating locomotor behavior have produced an initial understanding of some of the components within the spinal cord, as well as a basic understanding of several invertebrate motor pattern generators. However, how these circuits are assembled during development is poorly understood. We are defining the neural circuit that generates larval locomotion in the genetically tractable fruit fly *Drosophila melanogaster* to study locomotor circuit development. Forward larval locomotion involves a stereotyped posterior-to-anterior segmental translocation of body wall muscle contraction and is generated by a relatively small number of identified muscles, motor and sensory neurons, plus an unknown number of the ~270 bilaterally-paired interneurons per segment of the 1st instar larva. To begin identifying the relevant interneurons, we have conditionally inactivated synaptic transmission of interneuron subsets and assayed for the effects on locomotion. From this screen we have identified a subset of 25 interneurons per hemisegment, called the lateral locomotor neurons (LLNs), that are required for locomotion. Both inactivation and constitutive activation of the LLNs disrupt locomotion, indicating that patterned output of the LLNs is required. By expressing a calcium indicator in the LLNs, we found that they display a posterior-to-anterior wave of activity within the CNS corresponding to the segmental translocation of the muscle contraction wave. Identification of the LLNs represents the first step toward elucidating the circuit generating larval locomotion.

Keywords

Locomotion; Neural circuit; *Drosophila*; Interneurons

1. Introduction

Despite progress in understanding how axons are guided to their final target destinations to eventually synapse with their appropriate target cells, we have little understanding of how

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Competing Interests

The authors declare no financial or non-financial competing interests.

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neural circuits are actually assembled during development. This is true even for well-studied circuits such as those underlying “simpler” rhythmic movements such as locomotion, breathing and digestion (Grossmann et al., 2010; Selverston, 2010). We are developing *Drosophila* larval locomotion as a model system to study circuit function and development.

By virtue of its simplicity, *Drosophila* larval locomotion is an appealing system to understand a neural circuit. Larval locomotion is based on a repetitive motor pattern: muscles within a given segment contract and as they begin to relax the contraction is seamlessly propagated to the next segment, creating a peristaltic wave from tail to head (for forward locomotion), or from head to tail (for backward locomotion). Larvae can crawl at different speeds, both forward and backward, they can pause, head-wave and change direction, but during motivated forward crawling the pattern is remarkably stereotyped and consists of a series of identical contraction waves. This rhythmic locomotor behavior is produced by thoraco-abdominal circuitry, since it is still active when the ventral nerve cord (VNC) is isolated from the brain (Berni et al., 2012).

We confine our studies to 1st (L1) rather than larger 3rd (L3) instar larvae for two reasons: 1) the 1st instar nervous system comprises many fewer neurons, yet it is fully capable of producing the patterned motor output, and 2) the relevant neurons can be traced back to embryogenesis in order to study and manipulate their development. Larval locomotion is generated by a relatively small numbers of identified muscles, motor and sensory neurons, plus an unknown number of the ~270 bilaterally-paired interneurons per segment of the 1st instar larva. As in vertebrates, the identities and relationships between motor neurons and their muscles have been well-mapped, so the rudiments of the motor output is understood, but the identities of the interneurons are unknown. To begin identifying the locomotor interneurons, we expressed a dominant-negative form of *Drosophila* Dynamin, *shibire^{ts1}* (*shi^{ts1}*) (Kitamoto, 2001), to block synaptic release of subsets of interneurons. From this screen, we identified a subset, termed the lateral locomotor neurons (LLNs), that are required for wave progression during locomotion. Either synaptic inactivation or constitutive activation of the LLNs abolishes initiation and progression of the contraction wave, resulting in lack of any locomotion. The LLNs represent the first class of interneurons shown to be necessary for locomotion.

2. Results

2.1 Conditional inactivation of locomotion with *shi^{ts1}*

Drosophila larvae have a basic arrangement of 30 muscles in each hemi-segment innervated by ~35 motor neurons (Bate, 1990; Landgraf et al., 1997; Landgraf et al., 2003; Thor et al., 1999). The motor output has been characterized in terms of muscle contraction pattern (Crisp et al., 2008; Heckscher et al., 2012; Hughes and Thomas, 2007; Lahiri et al., 2011; Suster and Bate, 2002) and physiology (Baines, 2006; Barclay et al., 2002; Cattaert and Birman, 2001; Fox et al., 2006; Schaefer et al., 2010). Within a segment there is a stereotyped pattern of muscle activation during forward locomotion. First, a set of longitudinal muscles bilaterally contract, resulting in shortening of the segment from the posterior end. As the muscles begin to relax, the segment elongates in the anterior direction, while the contractile wave translocates to the next anterior segment, resulting in forward

movement. A set of lateral muscles contracts as the wave passes (Heckscher et al., 2012), presumably to help lift the segment from the substrate as it elongates. Translocation of the contraction from a single segment to the next takes 70–100 msec, and thus it takes approximately 1 sec for the wave to complete its entire posterior-to-anterior cycle (Figure 1A; Supplemental Movie 1A).

We first tested whether we could reproducibly abolish 1st instar larval locomotion by inactivating neurons with *shibire^{ts1}* (*shi^{ts1}*) a temperature-sensitive dominant-negative form of the Dynamin GTPase crucial for synaptic vesicle recycling (Newton et al., 2006; van der Bliek and Meyerowitz, 1991). Neurons expressing *shi^{ts1}* in an otherwise wild type background function normally at 23°C, but at temperatures over 30°C synaptic transmission is blocked (Kitamoto, 2001). We empirically determined that 36°C provided the most rapid and consistent inhibition phenotypes, without impairing the locomotion of control larvae (Hughes and Thomas, 2007).

To optimize the effectiveness of *shi^{ts1}*, we constructed flies carrying multiple copies of translationally efficient versions of *shi^{ts1}* (Pfeiffer et al., 2012). We expressed *shi^{ts1}* in sets of neurons using the GAL4/UAS system (Brand and Perrimon, 1993) by which individuals were generated that carry a GAL4 “driver” transgene expressing GAL4 in a defined set of neurons, plus “responder” UAS-*shi^{ts1}* transgenes. To non-invasively monitor motor output to muscles we used a myosin heavy chain-GFP protein fusion (Mhc-GFP) that allowed us to visualize the contraction of muscles during locomotion by the increase in GFP fluorescence during contraction of the actin-myosin complex (Heckscher et al., 2012; Hughes and Thomas, 2007).

When *shi^{ts1}* is expressed panneuronally using the *scrt-GAL4* driver, 1st instar larvae show normal locomotion at 23°C, but are paralyzed within seconds at 36°C, displaying no locomotor behavior (Figure 1C; Supplemental Movie 1C; Figure 2). By contrast, wild type individuals and *UAS shi^{ts1}* control animals show a normal increase in locomotor wave frequency at the higher temperature (Figure 1B; Supplemental Movie 1B; Figure 2). Inactivating all motor neurons using *VGlut-GAL4* (Mahr and Aberle, 2006) to drive *UAS-shi^{ts1}* conditionally abolishes all muscle contraction and locomotion at 36°C, identical to the effect of inactivating all neurons (Figure 1D; Supplemental Movie 1D; Figure 2). Similar to previous studies expressing *shi^{ts1}* in 3rd instar larvae (Hughes and Thomas, 2007; Song et al., 2007), inactivation of PNS neurons with *R51C05-GAL4* (Jenett et al., 2012) severely disrupts locomotion, lengthening the contraction phase of each segment and markedly slowing and eventually stopping the progression of waves (Figure 1E; Supplemental Movie 1E; Figure 2).

2.2 A screen for interneurons required for locomotion

As proof-of-principle for using *shi^{ts1}* in a screen for interneurons controlling patterned locomotor output, we expressed *UAS-shi^{ts1}* with *R49F01-GAL4* (Jenett et al., 2012), a GAL4 line that we found to drive expression in the majority of interneurons, but no motor or sensory neurons (Figure 2D, Table 1). First instar larvae expressing *shi^{ts1}* with *R49F01-GAL4* show no evidence of locomotion at 36°C (Figure 1F; Supplemental Movie 1F; Figure 2I). Similarly, two additional lines, *R42D06-GAL4* and *R28H10-GAL4*, that drive expression

in large subsets of ~150 interneurons, also abolish locomotion (Table 1). Thus, in addition to motor neurons and sensory neurons, synaptic output of at least some interneurons are required for locomotion.

We next screened 502 pre-selected lines from a large collection of GAL4 driver lines (Jenett et al., 2012; Manning et al., 2012; Pfeiffer et al., 2008) for reproducibly high, restricted expression in smaller subsets of interneurons. We visualized and charted the projections of the neurons by expressing a *UAS-tau-myc-GFP* reporter (Callahan et al., 1998). We identified 46 lines that show nearly or fully exclusive expression in subsets of interneurons within the CNS of late embryonic/1st instar larvae. These lines were tested behaviorally for their effects on locomotion when used to express *shi^{ts1}* at 36°C (Table 1). Of the 46 lines, 43 lines either had little or no effect or slowed the contraction wave (Figure 2F,G,I; Table 1), but still showed some degree of locomotion, similar to the peristaltic wave slowing seen by inactivation of *period*-expressing neurons (Kohsaka et al., 2014). Two lines slowed the waves to the point where there were less than 1 wave/10 sec interval; these lines showed little locomotion, but displayed clear, albeit very slow, waves. One line completely abolished locomotion. This line, *R47D01-GAL4*, drives expression in a cluster of bilaterally-paired interneurons plus 3 unpaired progeny of the median neuroblast (MNB) situated at the midline (Figure 2E) (Doe, 1992; Wheeler et al., 2006).

2.3 Inactivation with *R47D01-GAL4* abolishes locomotion

Within seconds when placed at 36°C, 1st instar larvae expressing *shi^{ts1}* with *R47D01-GAL4* display no locomotion, either forward or backward (Figure 1G,G'; Supplemental Movie 1G; Figure 2E,I). Interestingly, while the peristaltic waves of muscle contraction are absent, *R47D01-GAL4>UAS-shi^{ts1}* larvae still exhibit head-waving behavior characteristic of larvae in the process of changing direction (Kane et al., 2013; Klein et al., 2015; Luo et al., 2010; Okusawa et al., 2014), in contrast to the complete paralysis seen in larvae panneuronally expressing *shi^{ts1}* at 36°C. This result suggests that locomotion and head waving are mediated by separate neural circuits and that the *R47D01-GAL4* interneurons are involved only in the circuit(s) controlling forward and backward locomotor contraction waves.

To confirm the importance of the *R47D01-GAL4* interneurons in generating locomotion, we ablated them by expressing *UAS-RicinA* (Allen et al., 2002; Hidalgo and Brand, 1997) with *R47D01-GAL4*. *R47D01-GAL4>UAS-RicinA* embryos fail to hatch and show none of the coordinated locomotor-like contraction waves characteristic of late embryos prior to hatching (Crisp et al., 2008; Heckscher et al., 2012). Collectively, these experiments indicate that the neural circuit generating larval locomotion requires the synaptic output of some or all of the *R47D01-GAL4* interneurons.

2.4 Constitutive activation with *R47D01-GAL4* abolishes locomotion

Optogenetically activating the *R47D01-GAL4* interneurons with Channelrhodopsin2 (Gordon and Scott, 2009; Nagel et al., 2003; Petersen and Stowers, 2011) conditionally abolishes locomotion as effectively as inactivating them (Figure 3; Supplemental Movie 2). However, in contrast to *R47D01-GAL4* inactivation which results in lack of any coordinated contraction, constitutive activation during the light-activation pulse results in all segments

contracting simultaneously (Figure 3A, Light On; Supplemental Movie 2). While we don't know precisely what the spiking pattern of the interneurons are upon Channelrhodopsin2 activation (e.g., tonic, longer bursts), this result argues that proper motor output of the locomotor circuit requires specific patterned activity of the *R47D01-GAL4* interneurons and that they directly or indirectly drive motor neuron activity.

2.5 Subclasses of *R47D01-GAL4* interneurons

We charted the *R47D01-GAL4*-expressing neurons using *UAS-tau-myc-GFP*. In late stage embryos, expression is detected in a cluster of 25 dorsolateral interneurons, which we've termed the lateral locomotor neurons (LLNs), per hemisegment lying at the anterior segmental border plus 3 MNB progeny lying at the midline (Doe, 1992; Wheeler et al., 2006) (Figure 4A). This pattern is maintained into 1st instar larvae. To examine the morphologies of smaller subsets of the dorsolateral LLNs, we generated FLP-out clones (Golic and Lindquist, 1989). These clones revealed that the LLNs comprise both ipsilaterally and contralaterally projecting interneurons (Figure 4C,D). The ipsilaterally-projecting neurons remain largely within their segment of origin, while those that project contralaterally cross the midline in either the anterior or posterior commissure and project axons within the adjacent segment. The MNB progeny are interneurons that bifurcate and project axons locally in the anterior commissure (Wheeler et al., 2009). Importantly, the MNB interneurons are unlikely to be required for locomotor output, since another *GAL4* line, *R89D05-GAL4*, drives high levels of expression in unpaired interneurons at the midline, including the MNB progeny, but has little effect on locomotion when used to express *shi^{ts1}* (Figure 2H,I; Table 1). Thus, some or all of the LLNs are required for locomotion.

In addition to these *R47D01-GAL4* subclasses, there is a cluster of ~15 ventral cells that have no processes in 1st instar larvae. We assume that these cells are premature neurons destined to differentiate later in development and therefore don't play a direct role in the 1st instar larval locomotor circuit.

A subset of interneurons expressing the transcription factor Evenskipped (Eve), the Eve⁺ lateral (EL) interneurons, have recently been found to mediate proper left-right symmetric muscle contraction during locomotion (Heckscher et al., 2015). To determine whether the LLNs overlap with the Eve⁺ interneurons, we generated individuals carrying *R47D01-GAL4* driving nuclear GFP and double-labeled with antibodies to GFP and to Eve. We detected no overlap between the two subsets with the exception of one or two Eve⁺ cells that stochastically express low levels of *R47D01-GAL4*-driven GFP (Figure 4B).

2.6 LLN contralateral projections are not required for normal locomotion

During normal larval locomotion, the left and right sides of each segment contract simultaneously and thus there is coupling between the two sides at some level. In *commissureless (comm)* mutants, which have severely reduced commissures connecting the left and right hemisegments, the wave still translocates from segment to segment with normal cycle periods on each side, but the two sides no longer contract together, resulting in highly defective crawling (Figure 1H,H'; Supplemental Movie 1H). Thus, each

hemisegment is able to independently transmit the contraction wave to the next anterior segment, but coordinated motor output requires coupling between hemisegments mediated by contralaterally-projecting projections.

Drosophila Unc5 acts as a repulsive receptor for Netrins (Keleman and Dickson, 2001; Labrador et al., 2005). By expressing Unc5 in the LLNs using *R47D01-GAL4*, we prevented their contralaterally-projecting axons from crossing the Netrin-expressing midline (Figure 5B). In these larvae, locomotion is normal; the two sides remain coupled during crawling, contraction is symmetrical across segments and the wave progresses normally (Figure 5C). Thus, the contralateral projections of the LLNs are not required for coupling of the hemisegments, a result consistent with the finding that symmetric contraction of the two sides is mediated by a set of interneurons, the Eve⁺ lateral (EL) interneurons, which do not show substantial overlap with the LLNs (Figure 4B, Heckscher et al., 2015). Additionally, the LLN contralateral projections are not required for progression of the wave from segment to segment. Thus, in whatever manner the LLNs are functioning to generate the locomotor pattern, they do so ipsilaterally.

2.7 The LLNs show posterior-to-anterior waves of activity during the contraction wave

To visualize the activity of the LLNs during locomotion we used *R47D01-GAL4* to express the calcium indicator GCaMP (Akerboom et al., 2012; Nakai et al., 2001; Tian et al., 2009). For these experiments we used GCaMP6f, a variant with faster kinetics (Chen et al., 2013). We imaged the CNS through the clear ventral cuticle of intact, behaving 1st instar larvae gently squeezed between a coverslip and an agar surface. Under these conditions, contraction waves are slower than those in larvae crawling unrestricted on the surface, but otherwise appear normal. Within the VNC of such larvae, we detect posterior-to-anterior waves of GCaMP6f activation in the neuropil during waves of muscle contraction (Figure 6). Although it is unknown whether the signal within the LLNs is dendritic, axonal or both, the LLNs show clear rhythmic activity during locomotion.

3. Discussion

As a first step toward defining which of the ~270 interneurons per hemisegment are involved in the locomotor pattern generator we inhibited synaptic transmission of interneuron subsets with shi^{ts1}. Synaptic inactivation by expression of shi^{ts1} is a highly effective means of inhibiting neurons in *Drosophila* (Kitamoto, 2001; Pauls et al., 2015; Thum et al., 2006) and has been used to study a variety of behaviors, including courtship, circadian behavior, memory, olfaction, 3rd instar larval locomotion, turning behavior and spatial orientation (Akalal et al., 2006; Hughes and Thomas, 2007; Kahsai et al., 2010; Keene et al., 2011; Okusawa et al., 2014; Pitman et al., 2006; Sakai and Kitamoto, 2006; Song et al., 2007; Suh et al., 2004). However, while shi^{ts1} can effectively inhibit chemical synapses, it is important to remember that it has no effect on electrical synapses. Thus, classes of neurons may have been missed in the screen using shi^{ts1} inactivation. In addition, while we pre-selected the GAL4 driver lines for reproducibly high expression levels and used multiple high-expressing shi^{ts1} lines, we may have missed interneuronal subsets whose activities are only partially suppressed due to sub-threshold expression of shi^{ts1}.

Despite the caveats above, we found that when the LLNs are synaptically inactivated, both forward and backward locomotion are abolished. Thus the LLNs are essential for generating both types of locomotion, and while there must be some differences in the circuit(s) underlying forward and backward locomotion, under conditions of LLN inactivation the two behaviors cannot be separated. Interestingly, however, LLN-inactivated larvae still show head-waving behavior normally used to change direction, suggesting that this behavior is mediated by a different circuit not requiring the LLNs. This is consistent with behavioral studies showing that series of alternating motor programs for forward locomotion and head waving underlie direction change during thermotaxis (Klein et al., 2015; Lahiri et al., 2011; Luo et al., 2010). *R47D01-GAL4>UAS-shi^{ts1}* larvae also continue pharyngeal pumping of food at 36°C, consistent with findings that locomotor and food pumping motor programs can be independently modulated (Schoofs et al., 2014).

The LLNs show posterior-to-anterior waves of GCaMP activation during locomotion in intact, behaving larvae. It is important to realize that we are not assaying activity during fictive locomotion in a pinned, filleted preparation, where components such as sensory feedback would be lacking. Instead, we are recording activity during true locomotor behavior that has been slowed by increasing the mechanical load. Since crawling larvae probably encounter such increased loads, the pattern of LLN activation we have observed is likely to represent a normal, albeit slowed, pattern.

Our result showing no effect on locomotion when the LLN contralateral projections are prevented from crossing the midline indicates that the LLNs are functioning ipsilaterally to generate the locomotor output. However, while the contralateral projections are not required, these contralaterally-projecting LLNs may still be involved since they may normally have both ipsilateral and contralateral output, with the ipsilateral output being critical for locomotion. For example, they may drive other contralaterally-projecting interneurons, such as the Eve⁺ EL neurons, to coordinate contraction of the two sides. Similarly, they may synapse onto other intersegmental interneurons that transmit the contraction wave to the next anterior segment.

The next step will be to determine the synaptic connectivity of the LLNs within the pattern-generating circuit. For example, what are the identities of their pre- and post-synaptic partners? Are there other classes of interneurons involved? Given that synchronous, constitutive activation of the LLNs with Channelrhodopsin2 results in uniform muscle contraction, we assume that at least some of the LLNs either directly or indirectly activate motor neurons. While our results with the LLNs represent an important first step toward dissecting the locomotor circuit, the number of cells, at 25 per hemisegment, is still likely too large to uncover the relevant synaptic connections. We anticipate that intersectional approaches combining the GAL4/UAS and LexA/LexAop systems with GAL80 or a split GAL4 (Lai and Lee, 2006; Pfeiffer et al., 2010; Shang et al., 2008) will aid in narrowing down the number of LLNs required for generating locomotion.

4. Materials and Methods

4.1 Fly strains and genetics

5XUAS-shi^{ts1} on the X chromosome (Kitamoto, 2001) was mobilized with 2–3 transposase. The strongest expressing line on the second chromosome was selected and combined with *20XUAS-shi^{ts1}* on the third chromosome (Pfeiffer et al., 2012). *UAS-Unc5* was generated by mobilizing the original line (Keleman and Dickson, 2001) with 2–3 transposase and selecting for the strongest expressing line. Other lines used were *Mhc-GFP^{c100}* (Hughes and Thomas, 2007), *UAS-tau-myc-GFP*, *UAS-GAP-myc-GFP* (Callahan et al., 1998), *hs-FLP*, *UAS-FRT-stop-FRT-mCD8-GFP* (Hong et al., 2009) and the following lines from the Bloomington Stock Center: *UAS-GFP::lacZ.nls*, *UAS-Channelrhodopsin2 (ChR2)::YFP*, *UAS-GCamp6f*, *UAS-RicinA*, Janelia GAL4 lines listed in Table 1.

FLP-out clones were generated by crossing *hs-FLP; R47D01-GAL4* to *UAS-FRT-stop-FRT-mCD8-GFP* flies. Eggs were collected for 5 h then heat shocked in a 37°C water bath for 1 h. Late stage embryos and 1st instar larvae were selected for dissection and histological staining.

4.2 Behavior analysis

For the shibire inactivation screen, GAL4 males were crossed to *UAS-shi^{ts1}* females. Embryos were raised at 27°C. After hatching, single L1 larva was placed on a 2.3% agar plate (60 mm diameter) at 36°C. The number of continuous forward peristaltic contractions was counted during a 10 sec period (6 trials per larva). Multiple larvae were examined and averaged to generate a single data point. For the channelrhodopsin assay, parental adult females and their L1 larval progeny were fed yeast containing 100 mM all-trans retinal (Sigma-Aldrich) prior to the behavior assay. Larval locomotion was analyzed as described (Hughes and Thomas, 2007).

4.3 Histology

GAL4 lines were crossed to *UAS-tau-myc-GFP* or *UAS-GAP-myc-GFP* and embryos raised at 27 °C. Immunohistochemistry was performed on late stage embryos and L1 larvae as described (Callahan and Thomas, 1994; Hughes and Thomas, 2007). The following primary antibodies used were used: monoclonal anti-GFP 3E10, rabbit polyclonal anti-GFP (Life Technologies), anti-Eve (Developmental Studies Hybridoma Bank), Cy3-conjugated anti-HRP (Jackson Immuno Research).

4.4 Calcium imaging

A coverslip was placed on L1 larvae crawling on an agar plate. The larvae were gently rolled until oriented ventral side up. The GCaMP6f signal from the ventral nerve cord was captured using a CCD camera running at 15 frames/sec on a Zeiss M2 Bio Quad SV11 stereo microscope. ImageJ software was used for image processing. Signals from segments A4–A7 were measured from each video frame.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Drosophila* larval locomotion is a tractable system for studying neural circuits.
- Neurons required for locomotion are identified by conditional synaptic inactivation.
- A small subset of interneurons, the LLNs, are required for locomotion.
- The LLNs show patterned activity corresponding to locomotor output.

Figure 1.

Disruption of larval crawling by synaptically inactivating subsets of neurons with *shi^{ts1}*. Dorsal view of 1st instar larvae carrying the *Mhc-GFP* gene crawling from left to right. Single frames from movies shot at 15 frames/sec are shown (movies contained in Supplemental Material). Increasing the temperature in controls results in approximately 1.5X increase of locomotor wave speed (**A,B**). When all neurons (**C**), all motor neuron (**D**) or most interneurons (**F**) are inactivated, larvae are paralyzed and show no locomotion. Inactivating all PNS neurons (**E**) severely disrupts larval locomotion, although larvae show some slow uncoordinated movement. Inactivating the LLN neurons (**G**) abolishes locomotion but larvae still exhibit head waving behavior; bracket denotes enlargement shown in (**G'**). *comm* mutant larvae (**H**), which have severe reductions in commissures, display asynchronous contraction waves on the left and right sides, resulting in uncoordinated tail flipping behavior; bracket denotes enlargement shown in **H'** (arrowhead marks a contraction on the right side resulting in tail-flipping behavior). Genotypes: (**A,B**) *Mhc-GFP/+*; (**C**) *Mhc-GFP/UAS-shi^{ts1}; scrt-GAL4/UAS-shi^{ts1}*; (**D**) *Mhc-GFP, VGlut-GAL4/UAS-shi^{ts1}; UAS-shi^{ts1}/+*; (**E**) *Mhc-GFP/UAS-shi^{ts1}; R51C05-GAL4/UAS-shi^{ts1}*; (**F**) *Mhc-GFP/UAS-shi^{ts1}; R49F01-GAL4/UAS-shi^{ts1}*; (**G**) *Mhc-GFP/UAS-shi^{ts1}; R47D01-GAL4/UAS-shi^{ts1}*; (**H**) *Mhc-GFP/+; comm/comm*. Scale bar is 1 mm for (**A-H**), 300 μ m for (**G',H'**).

Figure 2.

Expression patterns of the GAL4 drivers and quantification of the effects on larval locomotion of expressing *shi^{ts1}*. (A–H) Late stage embryos immunostained for tau-myc-GFP (A–G) and GAP-myc-GFP (H) reporters expressed by GAL4 drivers used in the study. MNs, motor neurons; PNS, peripheral nervous system; INs interneurons; LLNs, lateral locomotor neurons; midline INs, interneurons that include the MNB progeny. (I) Quantification of disruption of 1st instar larval crawling when these GAL4 lines are used to express *shi^{ts1}* to inactivate neurons (N=12). Genotypes: (A) *UAS-tau-myc-GFP/+; scrt-GAL4/+*; (B) *UAS-tau-myc-GFP/VGlut-GAL4*; (C) *UAS-tau-myc-GFP/+; R51C05-GAL4/+*; (D) *UAS-tau-myc-GFP/+; R49F01-GAL4/+*; (E) *UAS-tau-myc-GFP/+; R47D01-GAL4/+*; (F) *UAS-tau-myc-GFP/+; R35B02-GAL4/+*; (G) *UAS-tau-myc-GFP/+; R12C02-GAL4/+*; (H) *UAS-GAP-myc-GFP/R89D05-GAL4*; (I) wild-type: *Mhc-GFP/+*, *UAS-shi^{ts1}*; *Mhc-GFP/UAS-shi^{ts1}*; *UAS-shi^{ts1}/+*, *scrt-GAL4>UAS-shi^{ts1}*; *Mhc-GFP/UAS-shi^{ts1}*; *scrt-GAL4/UAS-shi^{ts1}*, *VGlut-GAL4>UAS-shi^{ts1}*; *Mhc-GFP*, *VGlut-GAL4/UAS-shi^{ts1}*; *UAS-shi^{ts1}/+*, *R51C05>UAS-shi^{ts1}*; *Mhc-GFP/UAS-shi^{ts1}*; *R51C05-GAL4/UAS-shi^{ts1}*, *R49F01>UAS-shi^{ts1}*; *Mhc-GFP/UAS-shi^{ts1}*; *R49F01-GAL4/UAS-shi^{ts1}*, *R47D01>UAS-shi^{ts1}*; *Mhc-GFP/UAS-shi^{ts1}*; *R47D01-GAL4/UAS-shi^{ts1}*, *R35B02>UAS-shi^{ts1}*; *UAS-shi^{ts1}/+*; *R35B02-GAL4/UAS-shi^{ts1}*, *R12C02>UAS-shi^{ts1}*; *UAS-shi^{ts1}/+*; *R12C02-GAL4/UAS-shi^{ts1}*, *R89D05>UAS-shi^{ts1}*; *Mhc-GFP/UAS-shi^{ts1}*; *R89D05-GAL4/UAS-shi^{ts1}*. Scale bar is 50 mm. Error bars are SEM.

Figure 3.

Constitutive activation of *R47D01-GAL4* interneurons by Channelrhodopsin 2 abolishes larval locomotion. (A) Side view of a 1st instar larva that expresses Channelrhodopsin 2 in the *R47D01-GAL4* interneurons crawling from left to right before and after illumination with 470 nm light to activate Channelrhodopsin 2. Every other frame from a movie shot at 15 frames/sec is shown. Arrowheads mark the same segment boundary in each frame. Scale bar is 1 mm. (B) Quantification of the crawling behavior when the *R47D01-GAL4* interneurons are constitutively activated (Light-on) compared to inactivated (Light-off) (N=10). Genotype: *UAS-ChR2::YFP; R47D01-GAL4*. Error bar is SEM.

Figure 4.

Characterization of LLNs. (A) *R47D01-GAL4* interneurons labeled by expression of tau-myc-GFP reporter. Reporter expression is detected in a cluster of 25 LLNs (arrows) plus 3 of the MNB progeny (arrowhead). (B) Double labeling of *R47D01-GAL4* interneurons (nuclei labeled in green) and *Eve*⁺ neurons (nuclei labeled in magenta); The *R47D01-GAL4* interneurons and *Eve*⁺ neurons are largely non-overlapping with only one or two cells per hemisegment showing stochastic co-expression (arrowhead). (C) Heat shock-induced FLP-out clonal subsets of LLNs labeled by mCD8-GFP; one subtype projects contralaterally (top), the other projects ipsilaterally (bottom). (D) Schematic illustration of 3 types of LLNs and the MNB progeny; contralaterally-projecting anterior and posterior commissure LLNs (green and orange, respectively), ipsilaterally projecting LLNs (blue), and MNB interneuron progeny (purple). Genotypes: (A) *UAS-tau-myc-GFP/+; R47D01-GAL4/+*; (B) *UAS-GFP::lacZ.nls/R47D01-GAL4*; (C) *hs-flp; UAS-FRT-stop-FRT-mCD8-GFP/+; R47D01-GAL4/+*. Scale bar is 50 μ m.

Figure 5.

Contralateral projections of the LLNs are not required for normal locomotion. (A,B) LLNs labeled with tau-myc-GFP expressed by *R47D01-GAL4*. In individuals misexpressing Unc5 (B), contralaterally-projecting LLNs are prevented from crossing the midline. Scale bar is 50 μ m. (C) Quantification of crawling behavior of larvae with normal LLNs and larvae with LLNs lacking contralateral projections (N=10). Genotypes: (A) *UAS-tau-myc-GFP/+; R47D01-GAL4/+*; (B) *UAS-tau-myc-GFP/UAS-Unc5; R47D01-GAL4/+*; (C) *R47D01: Mhc-GFP/+; R47D01-GAL4/+*, *R47D01>UAS-Unc5: Mhc-GFP/UAS-Unc5; R47D01-GAL4/+*. Error bars are SEM.

Figure 6.

Calcium imaging of LLNs during larval locomotion. **(A)** Ventral view of the VNC of a crawling 1st instar larva that expresses the calcium indicator GCaMP6f in the LLNs. Each panel is a single frame from a movie shot at 15 frames/sec; the relative time for each frame is denoted below each panel. Arrows indicate the position of the abdominal segments A8 to A4. Scale bar is 50 μm . **(B)** Quantification of the relative strength of fluorescence signals of the LLNs in segments A7 through A4. Genotype: *UAS-GCaMP6f; R47D01-GAL4*.

Table 1

GAL4 lines tested for effects on locomotion

GAL4 line	Interneuron Subset Size ^I	Average number of waves/10s ±S.D. (N=12)
UAS-shi ^{ts1} control	-	15.30±2.28
R49F01	L	0.00±0.00
R42D06	L	0.00±0.00
R28H10	L	0.00±0.00
R47D01 (LLNs)	M	0.00±0.00
R49H08	M	0.61±0.61
R69C07	M	0.83±1.03
R13E03	M	2.33±0.52
R53F11	M	2.50±2.35
R21C02	M	3.33±2.25
R51E02	M	4.00±0.00
R38C03	M	4.00±1.00
R60G02	M	4.33±2.74
R76G04	M	4.50±1.76
R47A04	M	4.67±2.55
R50B09	M	4.75±1.48
R89A05	M	5.17±1.47
R47A12	M	5.33±1.21
R48A07	M	5.60±0.68
R33B07	M	5.67±1.63
R69E07	M	5.75±1.29
R65G04	M	6.33±1.37
R53F03	M	6.50±3.00
R20E12	M	6.58±2.31
R12F08	S	6.83±1.17
R65B04	S	6.83±3.13
R65C02	S	7.00±2.83
R23E11	S	7.17±1.17
R69E09	S	7.33±1.15
R36C07	M	7.50±3.27
R40C10	S	8.60±1.67
R50E03	S	9.00±2.45
R51C08	M	9.00±2.65
R73G11	S	9.00±2.71
R16E01	S	9.33±1.15
R72H05	S	9.33±1.53
R34B10	S	9.67±1.63
R27C08	M	9.83±2.04
R29A03	S	10.00±1.00

GAL4 line	Interneuron Subset Size ^L	Average number of waves/10s ±S.D. (N=12)
R38B07	M	10.00±1.00
R33G08	M	10.00±1.73
R47A11	S	10.50±0.84
R56E11	S	10.67±1.53
R35B02	M	10.67±2.42
R29A11	M	11.00±1.73
R11H12	S	11.67±1.53
R46H04	M	11.83±3.49
R95H11	M	12.33±1.75
R89D05 (midline INs)	S	12.56±1.51
R12C02	S	14.33±1.53

^L L: >150 interneurons, M: 25–50 interneurons, S: <25 interneurons, LLN: lateral locomotor neurons, INs: interneurons.