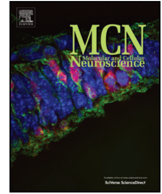
Molecular and Cellular Neuroscience 70 (2016) 22–29

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne



# A subset of interneurons required for *Drosophila* larval locomotion

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ARTICLE INFO

*Article history:*
Received 26 June 2015
Revised 20 November 2015
Accepted 23 November 2015
Available online 24 November 2015

*Keywords:*
Locomotion
Neural circuit
Drosophila
Interneurons

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.

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## 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 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.

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http://dx.doi.org/10.1016/j.mcn.2015.11.008
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## 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, 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 s for the wave to complete its entire posterior-to-anterior cycle (Fig. 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).

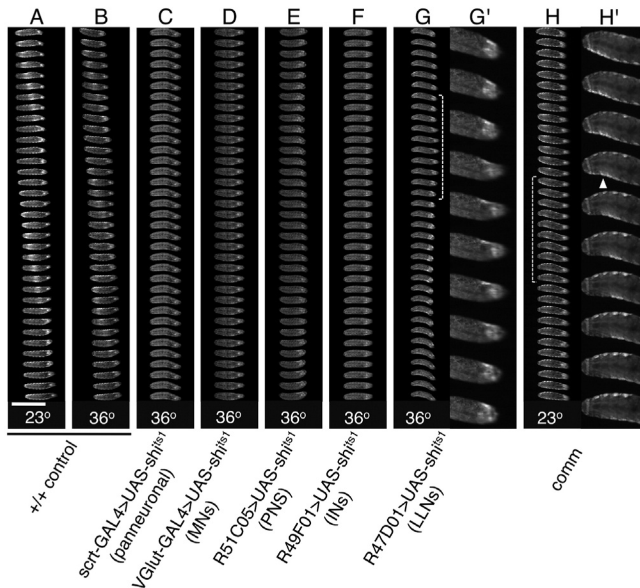


**Fig. 1.** Disruption of larval crawling by synaptically inactivating subsets of neurons with shi$^{ts1}$. Dorsal view of 1st instar larvae carrying the *Mhc-GFP* gene crawling from left to right. Single frames from movies shot at 15 frames/s are shown (movies contained in Supplemental Material). Increasing 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 μm for (G',H').

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 (Fig. 1C; Supplemental Movie 1C; Fig. 2). By contrast, wild type individuals and *UAS shi*$^{ts1}$ control animals show a normal increase in locomotor wave frequency at the higher temperature (Fig. 1B; Supplemental Movie 1B; Fig. 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 (Fig. 1D; Supplemental Movie 1D; Fig. 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 (Fig. 1E; Supplemental Movie 1E; Fig. 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 (Fig. 2D, Table 1). First instar larvae expressing shi$^{ts1}$ with *R49F01-GAL4* show no evidence of locomotion at 36 °C (Fig. 1F; Supplemental Movie 1F; Fig. 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 (Fig. 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 s 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 (Fig. 2E) (Doe, 1992; Wheeler et al., 2006).

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