A Small Subset of *Fruitless* Subesophageal Neurons Modulate Early Courtship in *Drosophila*

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**Abstract**

We show that a small subset of two to six subesophageal neurons, expressing the male products of the gene *fruitless* (*fru*M), are required in the early stages of the *Drosophila melanogaster* male courtship behavioral program. Loss of *fru*M expression or inhibition of synaptic transmission in these *fru*M(+) neurons results in delayed courtship initiation and a failure to progress to copulation primarily under visually-deficient conditions. We identify a *fru*M-dependent sexually dimorphic arborization in the tritocerebrum made by two of these neurons. Furthermore, these SOG neurons extend descending projections to the thorax and abdominal ganglia. These anatomical and functional characteristics place these neurons in the position to integrate gustatory and higher-order signals in order to properly initiate and progress through early courtship.

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**Introduction**

*Drosophila melanogaster* courtship is a multi-step, progressive series of behaviors established by sex-specific genetic and neurobiological components [1–4]. Work by our lab and others demonstrated that the expression of male products of the gene *fruitless* (*fru*M) is both necessary and sufficient to specify the potential for male courtship behavior. Approximately 2000 neurons in the central nervous system (roughly 2% of the CNS neuronal population) express *Fru*M in clusters scattered throughout the central (CNS) and peripheral nervous systems [5–7]. In the periphery, expression was detected in subsets of primary sensory neurons of the sensory modalities implicated in courtship. Strikingly, *fru*M(+) neurons are dedicated to courtship as inactivating them disrupts courtship behaviors, but has no discernible effect on non-sexual behaviors.

Both *fruitless*Male and another component of the sex determination pathway, *doublesex*Male, are involved in establishment of sexually dimorphic neural circuitry [8]. Activity of *Fru*M is required for the survival of several male-specific neurons or sexually dimorphic projection patterns [9].

Multiple female sensory cues combine to regulate the activation and performance of male courtship behaviors. A feature of these multi-modal sensory inputs is the partial redundancy of some modalities: loss of visual, olfactory, or gustatory perception does not block courtship from occurring *Dros. melanogaster* [10–12]. Instead loss of any one of these three modalities delays the initiation and decreases the quantity of courtship. These functional redundancies suggest a compensatory integration of these multiple pathways in the courtship circuitry.

Several areas of the CNS have been identified as regions of higher-order processing and integration in the courtship circuitry [13–19]. *fru*M(+) projections densely innervate several regions: the lateral protocerebral complex, the mushroom bodies, the mesothoracic triangle in the ventral nerve cord, and the tritocerebral loop. Neurons projecting to the lateral protocerebral complex and mesothoracic triangle induce wing song behavior; subsets of these neurons require *Fru*M and DsxM for survival in males [9]. The mushroom bodies are well-characterized regions controlling memory and learning.

One area of interest is the tritocerebral loop—which lies just ventral to the subesophageal ganglion (SOG)—an area of dense innervations targeted by gustatory, protocerebral/neurosecretory, and stomatogastric inputs [20]. Peripheral gustatory axons, from the mouthparts, subsets of the labellum, and stomatogastric nerves, target the tritocerebrum. The termini of descending tracts from the medial superior protocerebrum—notably the pars intercerebralis, a neurosecretory center—innervate the dorsal tritocerebrum. The higher-order interneurons that process and regulate gustatory inputs have not been fully characterized; the tritocerebral loop innervations likely integrate chemosensory and protocerebral inputs.

Here we targeted subpopulations of *fru*M(+) neurons that regulate chemosensory-dependent courtship initiation. We screened 72 *P(Ga4B) insertions*, driving an RNAi construct targeting *fru*M, *UAS-fru*MR [21], for courtship defects that appear only under conditions where melanogaster is visually deficient [10]. The *P(Ga4B)4-57* line exhibited very limited overlap with *fru*M(+) neurons, *P(Ga4B)4-57* mainly overlapped with two to six *fru*M(+) neurons in the subesophageal ganglion (SOG), two clusters in the ventral nerve cord (VNC), and inconsistently an area just medial to the antennal lobe (mAL). Knockdown of *fru*M or inhibition of synaptic fusion limited to the SOG neurons resulted
in infrared-specific courtship delays, and a failure to progress to copulatory behaviors. Strikingly, the triocerebral projections of these neurons were significantly more extensive in males than in females; this male-specific projection pattern required FruM expression. These fruM\(+\) SOG neurons likely integrate chemosensory inputs in the triocerebral to modulate the initiation and progression of courtship.

Results

We identified subpopulations of fruM\(+\) neurons involved in chemosensory-specific pathways via a behavioral screen for proper courtship initiation in visually-deficient conditions. To do this we built on the findings of Meissner et al. [21] who screened a collection of approximately 1000 Gal4 P-element, P(GawB), insertions driving expression of two copies of a fruM RNAi construct, UAS-fruMIR (one insertion on the X and one insertion on the third chromosomes). We screened 65 P(GawB) and 7 candidate Gal4 lines for significant courtship delays to the first unilateral wing extension (courtship latency) in ambient and infrared light (Figure 1A). Due to lower visual resolution in infrared, we could not reliably identify the first instance of orientation/pursuit, the traditional method of measuring courtship latency. Henceforth courtship latency will refer to the average time to first unilateral wing extension.

We targeted lines with infrared-dependent courtship delays and CNS-specific, limited Gal4;fruLexA overlap (Figure 1B) in order to identify candidate fruM\(+\) interneurons that regulate the activation of courtship. fruM\(+\) is a knock-in insertion of the LexA transcriptional activator that drives expression of LexaOp-GFPnls in fruM\(+\) cells [21]. Three lines matched those criteria (Figure 1C).

One line, P(GawB)4-57, drove Gal4 expression that overlapped fruLexA\(+\) neurons in only four fruM\(+\) cell clusters, clusters 1, 7, 8, and 13 in Figure C (Cluster nomenclature from [5]) with extensive overlap in the SOG cluster, cluster 8, in particular. A global principle-components-based regression analysis (Figure 1D)–using the entirety of the behavioral and expression data–correlated courtship latency delays with Gal4;fruLexA\(+\) \(\times\) cluster 8 (Figure 1E), highlighting the importance of the \(P(GawB)\)4-57, fruM\(+\) clusters in courtship.

In addition, previous courtship assays had failed to detect male-male courtship, copulation, or aggression defects in P(GawB)4-57 UAS-fruMIR males [21], suggesting that the P(GawB)4-57, fruM\(+\) neurons primarily functioned to regulate courtship initiation. Furthermore, the basal activity of P(GawB)4-57 UAS-fruMIR males (0.46±0.1 line crossing/min, \(n = 24\)) did not significantly differ (p>0.05) from wild type (0.46±0.1 line crossings/min, \(n = 24\)).

\(P(GawB)\)4-57-driven Tetanus Toxin also Delays Courtship

We also wanted to determine if repression of synaptic transmission in P(GawB)4-57, fru\(+\) neurons recapitulated the fruM RNAi results. Due to extensive non-FruM expression with the P(GawB)4-57 driver, we used an intersectional strategy to inhibit synaptic transmission only in fruM\(+\), P(GawB)4-57 neurons FLP recombinase, under endogenous fruM regulation, limited the expression of tetanus toxin (TNT) to fruM\(+\), P(GawB)4-57\(+\) neurons, which we will denote as fru\(FLP\)4-57\(+\) [13]. TNT-mediated synaptic inhibition in fru\(FLP\)4-57\(+\) males (Figure 2C–D) significantly delayed wing extension behavior (46.55 secs., 95% interval: 31.2–69.4 secs., \(n = 26\)) only under infrared light compared to the inactive TNT\(^{Q4A}\) control (16.7 secs., 95% interval: 10.6–26.3 secs., \(n = 25\), p = 0.001) or the UAS\(>stop\)TNT\(+\); fru\(FLP\)\(+\)/background control (21.1 secs., 95% interval: 13.3–33.4 secs., \(n = 17\), p = 0.03). This behavioral delay was similar to that seen with P(GawB)4-57 -driven UAS-fruMIR expression. Under ambient light, TNT expression in fru\(FLP\)4-57\(+\) cells (\(n = 23\)) did not significantly alter courtship latency compared to the control line (\(n = 21\), Figure 2C).

When we quantified the courtship index (Figure 2H–I), expression of TNT in fru\(FLP\)4-57\(+\)/P(GawB)4-57\(+\) males also significantly reduced the quantity of courtship in both ambient and infrared light. Under ambient light, TNT expression depressed the courtship index to 0.24±0.01 compared to 0.90±0.05 in the inactive control (p<0.001); under infrared, TNT expression led to a courtship index of 0.23±0.01 compared to 0.43±0.1 in the inactive control (p<0.003) and 0.84±0.5 in the background control (p<0.001).

Another aspect of courtship was also dependent on the proper function of P(GawB)4-57, fru\(M\)\(+\) neurons (Figure 2K–N). Most P(GawB)4-57; UAS-fru\(M\)IR and P(GawB)4-57/UAS\(>stop\); TNT; fru\(FLP\)\(+\) males failed to exhibit any copulatory behaviors when visually deprived (compare Figures 2L–M to 2K). The majority of these males performed wing extension/song behaviors but made no detectable attempts at copulation, measured as at least one instance of pronounced curling of the abdomen (>90° curl from horizontal) and/or mounting attempts.

Under infrared-only lighting (Figure 2L), only 11.5% of P(GawB)4-57; UAS-fru\(M\)IR males (\(n = 30–42\)) progressed to any copulatory behaviors compared to 66.7% and 58.2% of P(GawB)4-57\(+\) and UAS-fru\(M\)IR\/+ controls, respectively (p<0.0001, Fisher’s exact test, \(n = 23–42\)). A similar defect was seen using tetanus toxin in fru\(FLP\)4-57\(+\) males (Figure 2M). Expression of TNT in fru\(FLP\)4-57\(+\) neurons resulted in only 18.2% of males exhibiting any copulation behaviors under infrared light (p<0.0001, n = 44) compared to 66.7% of the inactive TNT controls (\(n = 21\)) or 57.4% (\(n = 21\)) of the background controls. Under ambient light, there was no significant difference between the genotypes for either behavior (Figure 2K).

Noting the effect of silencing synaptic transmission on the fru\(FLP\)4-57\(+\) neurons, we assessed the effect of depolarization via transgenic manipulation. The UAS\(>stop\)TRPA1 insert, encoding a temperature-sensitive cation channel [10,24] allowed us to depolarize the fru\(FLP\)4-57\(+\) neurons by incubation at 28°–32°C (Figure 2E, J, N). Courtship latency, courtship index, and fraction of males performing courtship did not differ significantly from controls. Unilateral wing extension or abdominal curling was not
induced in solitary males. These results suggest that activity from the fruFLP neurons, by itself, may not induce wing extension or copulation behaviors.

Cha-Gal80-mediated Rescue of P[GawB]4-57(UAS-fruMIR) Courtship Defect

Gal4 expression can be further refined using transgenic constructs that drive expression of the Gal4-inhibitor Gal80 [21–23]. Courtship assays using the Cha-Gal80 driver, which expresses in cholinergic neurons, combined with P[GawB]4-57(UAS-fruMIR) transgenes revealed a significant rescue of the courtship defects (Figure 2B, G; green arrows). In P[GawB]4-57(UAS-fruMIR), Cha-Gal80 males, courtship onset, courtship index, and copulation rates were similar to controls. These results should delimit the population of fruM(+)(+) cells responsible for the courtship defects to only those in which the Cha-Gal80 and P[GawB]4-57 drivers were active (Figure 3).

FruM Protein and P[GawB]4-57 show Limited Overlap in the Nervous System

To assess the overlap of P[GawB]4-57 and FruM protein, we first visualized the overlap of anti-FruM fluorescence with either P[GawB]4-57-driven UAS-GFPnls or UAS-mCD8-GFP (Figure 3A–E). Prime and double-prime symbols mark panels showing anti-FruM or merged anti-GFP+anti-FruM fluorescence respectively. Membrane tethered mCD8-GFP allowed us to visualize neuronal projections, while nuclear localized GFPnls allowed a direct comparison with the nuclear expression of FruM.
P[GawB]4-57-driven GFP consistently overlapped with two FruM(+) cells in cluster 7, approximately 6 in cluster 8 in the brain (Figure 3A–C) and two cells each in clusters 17 and 20 in the ventral nerve cord (Figure 3D–E), tabulated in Figure 3L based on 8 samples per genotype. Inconsistent overlap was seen in clusters 3 and 16.

Figure 3A shows two GFP(+) FruM(+) cells in cluster 7, also known as mcAl [5] or DT [13]) cells. In the SOG, overlap could be subdivided into three A–P subpopulations: four to six anterior, aSG; two medial, mSG; and two posterior, pSG (Figure 3A–C). Figure 3D–E shows overlap in two cells in cluster 17 at the prothoracic/metathoracic boundary (D) and in cluster 20, the abdominal ganglia (E). No peripheral expression was detected (data not shown).

At this point we will refer to FruM(+) cells by nomenclature given in [13]. The P[GawB]4-57(+) cluster 7 cells appear to be the DT6 neurons described in that previous study. Also in that study, they characterized 8 subpopulations in the SOG (aSG1-8, pSG1-2). Some of the P[GawB]4-57(+) aSG neurons, characterized here, appear to be either the aSG3 or aSG6 cells. The P[GawB]4-57(+) mSG and pSG cells described here do not appear to confirm to either the pSG1 or pSG2 populations based on anatomy. A diagram of the nervous system showing FruM expression and cluster locations is shown in Figure 3K, arrows point to locations of the cells in each row of panels.

Cha-Gal80 Repressed P[GawB]4-57-driven GFP Primarily in DT6 and SOG Neurons

We noted that Cha-Gal80 repressed a significant fraction of the P[GawB]4-57-driven UAS-mCD8GFP expression pattern. In Figure 3F–J, hollow arrowheads represent FruM(+) cells where we failed to detect P[GawB]4-57-driven GFP fluorescence mainly in the two P[GawB]4-57 mSG and two-three of the aSG neurons (Figure 3G–H, hollow arrowheads). Conversely Cha-Gal80 reduced but did not eliminate GFP fluorescence in P[GawB]4-57 DT6 cells (Figure 3F, see subpanels 1–2 for clarity). In pSG neurons (Figure 2H) and ventral nerve cord neurons (Figure 2I–J), GFP fluorescence appeared unaffected. Combined with Cha-Gal80 in behavioral studies, we inferred that some of the aSG and both mSG cells were primarily responsible for the courtship defects seen with P[GawB]4-57-driven constructs. We must note, however, that using Cha-Gal80-mediated repression of GFP may not fully reflect the relationship between P[GawB]4-57 expression and behavioral phenotype. Temporal differences between Cha-Gal80 and P[GawB]4-57 expressions or incomplete repression of GFP are caveats to this inference.
fruLP+/4-57 Neurons Project to the Tritocerebrum and Ventral Nerve Cord

In order to highlight projection patterns only from P[GawB]4-57, we visualized the GFP expression pattern in P[GawB]4-57/UAS-mCD8-GFP, fruLP+/4-57 (Figure 4). Fluorescence in fruLP+/4-57 neurons revealed a smaller set compared to anti-FruM::Gal4 overlap, labeling primarily the SOG neurons, with the mSG of 4-57 population the only consistent expression (Figure 4A–C).

In 3/14 brains (Figure 5A), the aDT6/4-57 neurons presented as a single soma located in the periphery of the median bundle, with projections ramifying in the region proximal to the esophagus (eo), just dorsal to the tritocerebrum (Supplemental Figure 1S). Projections continued along the median bundle to the medial superior protocerebrum.

Most aSG/4-57 GFP cells bodies in the SOG had no detectable projections (Figure 4G, arrowheads), but a single aSG/4-57 neuron was seen, in two brains, with extensive arborizations throughout the SOG with a collateral ramifying the inferior lateral protocerebrum (Supplemental Figure 1S, arrowhead, asterisk). pSG/4-57 neurons sent projections to the ventral nerve cord with no detectable arborizations in the brain.

Two mSG/4-57 somas were found in the ventral medial SOG in 14/14 brains examined (Figure 4A–F, arrow). These exhibited similar bilaterally projection patterns extending towards the tritocerebrum (TC) along with two descending tracts into the ventral nerve cord (Figure 4K–L).

mSG/4-57 Neurons make Descending Projections that Target the VNC

We noted a sexual dimorphism in the mSG projections. In female fruLP/+ brains, fluorescence from the tritocerebral neurites was significantly reduced in extent and intensity compared to male projection patterns (compare Figure 4D–F to 4A–C). This difference was more striking in sagittal reconstructions of the tritocerebral projections (Figure 4G–H, arrows).

The behavioral defects caused by knockdown of fruM and the dimorphic projection pattern suggested a direct role for FruM in regulating neurite morphology in these neurons. In order to determine whether the sexual dimorphism required fruM expression, we examined the mSG/4-57 projections in fruLP+/fruLP4-57 mutant males (two different genetic backgrounds were used for the fruLP4-57 chromosome). In these fruLP mutant males, the tritocerebral projections were similar to those seen in fruLP+/+ females (Figure 4I, arrow) indicating that fruM expression was required for proper male-specificarbors in the tritocerebrum.
Figure 4. Visualization of fru(+) and P[GawB]4-57 intersection revealed a sexually dimorphic arborization in the tritocerebrum. A–F) Anterior-posterior, G–J) sagittal, and K–L) dorsal-ventral confocal projections. Unless otherwise stated, images are from males. A–I) Z-projections showing GFP fluorescence from male P[GawB]4-57/UAS>stop>mcd8-GFP; fruFLP/+ brains. A–F) Merged images showing anti-NC82 and GFP expressions in male and female brains. In all brains two GFP(+) cell bodies, in the ventral medial SOG (mSG, arrows) project to and make extensive
arborizations in the tritocerebrum. C) In 7/14 brains, 3 cell bodies (aSG, arrowheads) fluoresced at depths of 17–20 μm without detectable neurites. D–F versus A–C) Z-projection showing the weaker tritocerebral arborizations from the two mSG neurons in female P[GawB]4-57/UAS>stop>mCD8-GFP; fru^{FLP}/+ brains. F) pSG marks one posterior GFP (+) neuron. G–I), sagittal reconstructions of mSG projections in a G) P[GawB]4-57/UAS>stop>mCD8-GFP; fru^{FLP}/fru^{ loss} male mutant brain. Dashed lines mark the path of the esophagus. In fru+/fru- males the tritocerebral arbors are significantly larger and fluoresce brighter compared to fru+/fru females or fru mutant males (arrows). J) Expression of the dendritic marker UAS>stop>Dscam17-1-GFP in mSG\(\wedge\)4-57 neurons colocalized with the tritocerebral arbors and anterior to medial tracts. L) Presynaptic marker, UAS>stop>nsyb-GFP was expressed mainly in prothoracic/metathoracic boundary. K) The presence of tsh-Gal80 repressed expression from fru^{M(+)} ventral nerve highlighting the descending projections from mSG and pSG cells. Scale bars = 50 μm. eo = esophageal foramen, TC = tritocerebrum, and SOG = subesophageal ganglion.

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the neuronal polarity of these mSG\(\wedge\)4-57 neurons (Figure 4J, L). GFP fluorescence in P[GawB]4-57/UAS>stop>Dscam17-1-GFP; fru^{FLP} brains were detected in the tritocerebral and anterior SOG tracts (Figure 4J). We detected expression of nsyb-GFP in P[GawB]4-57/UAS>stop>nsyb-GFP; fru^{FLP}/+ brains in the prothoracic/mesothoracic boundary proximal to the likely positions of central pattern generators controlling wing song (Figure 4L). These data are consistent with a role for the mSG\(\wedge\)4-57 neurons in relaying signals targeting the tritocerebrum to the VNC and modulating targets within the ventral tritocerebrum. These were confirmed to be descending termini using the tsh-Gal80 transgene [13] to repress Gal4 activity in VNC fru^{M} (Figure 4K).

Several lines of evidence combined to point to the significant role the subesophageal P[GawB]4-57, Fru^{M(+)} likely play in regulating courtship initiation (Figure 5). One, rescue of the arborizations in the tritocerebrum. C) In 7/14 brains, 3 cell bodies (aSG, arrowheads) fluoresced at depths of 17–20 μm without detectable neurites. D–F versus A–C) Z-projection showing the weaker tritocerebral arborizations from the two mSG neurons in female P[GawB]4-57/UAS>stop>mCD8-GFP; fru^{FLP}/+ brains. F) pSG marks one posterior GFP (+) neuron. G–I), sagittal reconstructions of mSG projections in a G) P[GawB]4-57/UAS>stop>mCD8-GFP; fru^{FLP}/fru^{ loss} male mutant brain. Dashed lines mark the path of the esophagus. In fru+/fru- males the tritocerebral arbors are significantly larger and fluoresce brighter compared to fru+/fru females or fru mutant males (arrows). J) Expression of the dendritic marker UAS>stop>Dscam17-1-GFP in mSG\(\wedge\)4-57 neurons colocalized with the tritocerebral arbors and anterior to medial tracts. L) Presynaptic marker, UAS>stop>nsyb-GFP was expressed mainly in prothoracic/metathoracic boundary. K) The presence of tsh-Gal80 repressed expression from fru^{M(+)} ventral nerve highlighting the descending projections from mSG and pSG cells. Scale bars = 50 μm. eo = esophageal foramen, TC = tritocerebrum, and SOG = subesophageal ganglion.

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Figure 5. mSG\(\wedge\)4-57 expression correlated with behavioral defects. A) Proportion of brains exhibiting male fru^{FLP}\(\wedge\)4-57 GFP expression (n = 14) and locations of GFP (+) neurons in fru^{M} clusters (blue dots). B) Normalized (see methods) dimensions of the tritocerebral arbors measured in the lateral, D–V, and A–P axes for male fru^{M}+/2, female fru^{M}+/2, male fru^{M}–/– and female fru^{M}–/– mutant brains (n = 13, 8, 6). The tritocerebral arbors male fru^{M} heterozygous brains were significantly larger than either male null fru^{M} mutants or female fru^{M} heterozygotes (p<.001). Diagram and table comparing P[GawB]4-57:Fru^{FLP} overlap, C) Cha-Gal80-delimited P[GawB]4-57:Fru^{FLP} overlap, fru^{M}\(\wedge\)4-57 GFP expression, to RNAi-mediated or TNT-mediated behavioral phenotypes.

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behavioral defects due to Cha-Gal80 repression mainly in the mSG neurons, (Figure 2B). Two, strong, consistent intersectional expression in mSGr4-57 cells (Figure 5A). Three, sexual dimorphism of the tritocerebral arbors from the mSGr4-57 cells (Figure 5B). Four, courtship and copulation defects seen upon fruM4-57 tetanus toxin expression (Figure 5C). Five, the behavioral defects were primarily seen under visually-deficient conditions (infrared) where male depend more on chemosensory cues, consistent with the putative function of tritocerebral dendritic projections of the mSGr4-57 cells.

Discussion

Initiation of unilateral wing extension is heavily dependent on visual, olfactory, and gustatory cues. By forcing males to depend on non-visual pathways for courtship and co-expressing tissue-specific fruM RNAi, we screened for fruM(+)-neurons that likely regulate chemosensory-dependent processes in courtship, which manifested as infrared-specific courtship latency defects. The P(GasB)4-57 line driving UAS-fruMIR possessed normal courtship latency in ambient light and significant infrared-specific delays. Notably fruM overlap was strongest in the SOG, while lacking any detectable peripheral expression. Behavioral and anatomical studies using Cha-Gal80, to subdivide the P(GasB)4-57 expression pattern, highlighted a small subpopulation of fruM(+) neurons in the SOG, two-four anterior SGr4-57 neurons and two medial SGr4-57 neurons as responsible for the courtship defects.

mSGr4-57 Neuronal Anatomy Suggests a Direct Role in Regulating Wing Extension and Copulatory Behavior Initiation

Several lines of evidence suggest a direct role for the mSGr4-57 neurons in regulating the initiation of wing extension and copulatory behaviors. First, we detected expression of fluorescent markers in the mSGr4-57 neurons driven by P(GasB)4-57 in all brains, whereas fluorescence was only detected in a subset of animals for the other fruM4-57 subpopulations. The mSGr4-57 neurons made sexually dimorphic arbors in the tritocerebrum (Figure 6A), where male arbors were significantly larger than in wild type female and fru mutant male brains. The mSGr4-57 neuronal tracts extended into the VNC where presynaptic innervation of the mesothoracic triangle was seen (Figure 6B). The mesothoracic triangle is a target of descending command neurons that control wing song [15–17]. Faint projections were detected in the posterior metathoracic/anterior abdominal ganglia, which suggest possible regulation of motor circuitry needed for abdominal curling during copulatory behaviors.

The sexually dimorphic projections of the mSGr4-57 suggest sex-specific roles in receiving tritocerebral signals in males. In males, fruM knockdown and silencing of fruMIR4-57 neurons resulted in a failure to progress to copulation, a behavior that follows proboscis contact with a female (“licking”). The internal mouthparts house gustatory sensilla that likely detect contact female pheromones accessed via licking behavior.

We cannot rule out functions for the non-mSGr4-57 neurons, particularly the DT6r4-57 (aSG) neurons (Figure 6C) in regulating courtship initiation, however. Our approach infers, but does not conclusively demonstrate that the mSGr4-57 neurons are responsible for the courtship initiation and copulation defects. Further studies are required to conclusively identify the neurons responsible for each behavioral phenotype and their exact roles.

A Comparison to fruM(+) SOG Neuron in other Studies

Several studies have examined the projections of fruM(+) neurons in the SOG. Antibody staining using anti-Fruitless identified 12±2 total FruM(+) nuclei in the SOG in the 2-day pupal brain [5]. An intersectional study, using 131 Gal4 lines with sparse overlap with fruM(+) identified 8 fruM(+) SOG neuronal classes divided into six anterior, aSG1-6, and two posterior neuronal types, pSG1-2 [13]. At least one aSG4-57 neuron’s projection pattern, identified here, is consistent with the aSG5 class identified in that larger-scale study. Cachero et al. [14] used mosaic analyses of fruGal4 to identify larval neuroblast clonal populations of fruGal4(+) neurons. Cachero et al. identified six clones in SOG, however, none appear to correspond to neurons identified here. It appears that these broad mapping studies, while extensive, have not exhaustively identified fru-expressing neurons in the SOG.

Using tdc2-Gal4, three studies [24–26] characterized three octopaminergic FruM(+) neurons in the SOG; designated VPM1 and VPM2 (ventral paired median) and one VUM1 (ventral unpaired median) neuron. Expression of tdc2-Gal4-driven UAS-fruMIR leads to courtship latency delays but no copulation defect. The VUM1 neuron tritocerebral projections appear similar to the mSGr4-57 projections, however, no descending tracts to the VNC were reported. The VPM1 and VPM2 appear to correspond to the DT8 neurons Repression of fruM using tdc2-Gal4 appeared to primarily disrupt male-female discrimination, resulting in significant male-male courtship, whereas we detected no significant male-male courtship using P(GasB)4-57.

The Tritocerebrum is a Major Site of Signal Convergence for fruM Circuitry

Given the extensive projections of fruM(+) innervations, the tritocerebrum appears to be a site of gustatory integration with higher-order information in male courtship. The extensive, sexually dimorphic arbors from the mSGr4-57 receive signals in the tritocerebrum that serve to regulate the progression to copulation in males and the performance of courtship. The tritocerebrum is targeted directly by gustatory afferents from the mouthparts via the pharyngeal nerves, indirectly via the SOG interneurons, which could relay signals from proboscis gustatory afferents entering via the labial nerve, and by descending tracts from the par intercerebralis of the superior medial protocerebrum (SMPR in Figure 6), which contains many neurosecretory cells [27–28]. These mSGr4-57 cells could then relay signals to circuitry controlling wing extension/song in the metathoracic triangle and copulation/abdominal curling in the anterior abdominal ganglia.

The decision to perform courtship by males likely weighs the receptivity of the female versus the cost of female rejection via escape, with greater costs associated with later steps in the ritual, i.e. copulation. In open environs, escape behaviors exhibited by males, with greater costs associated with later steps in the ritual, i.e. copulation. In open environs, escape behaviors exhibited by rejecting females likely results in the cessation of the courtship unless the male correctly gauges receptivity. We propose that the fruM(+) SOG neurons identified here play a vital link between detection of female receptivity cues and integration of higher-order signals in order to appropriate initiate wing extension and copulatory behaviors.

Experimental Procedures

Behavioral Assays

Courtship assays. Courtship assays were conducted according to to established protocols [29]. Males were entrained in isolation for 3–5 days post-eclosion and then single males were presented with a 1–2-day-old Canton S virgin female. Single male and
female pairs were placed into custom-made plexiglass chambers with hollowed circular chambers, 10 mm in diameter and 6 mm in height, separated by plastic transparencies. Contact between courtship pairs was initiated by removal of the transparencies. Courtship behaviors assayed in ambient fluorescent light and in infrared light, recorded for 10–15 minutes, and logged using the LifesongX annotation program. Initial screening of P[GawB] lines involved 9–12 males, while testing of candidate lines involved 20–40 males.

**Activity monitoring.** Recording of basal activity was done according to established protocols [30]. Individual males, entrained and aged in the same method for courtship assays, were placed into glass tubes, sealed at one end and plugged with Drosophila media at the other end. The average number of line crossings, measured by an infrared laser, were recorded over a 24 hour period for 16–32 males using the Drosophila Activity Monitoring System I (TriKinetics).

**Fly Strains**

UAS-mCD8GFP were obtained from the Bloomington Stock Center. UAS-fruIR, fruP1stop and fruP1-LexA were constructed by D. Manoli [19,21]. fruP1-LexA was backcrossed with 5 generations into the white Berlin background both the original stock and backcrossed strain was used. UAS>stop>mCD8-GFP, UAS>stop>TNT, UAS>stop>TNTQ4A, and fruP1stop lines are described in [13]. P[GawB] enhancer trap lines was obtained from Ulrike Heberlein [21].

**Constructs**

LexAop-GFPPls: A BglII-SphI fragment containing a LexAop response element (a gift from D. Manoli) was swapped into pStinger [31] replacing the UAS element.

**Immunofluorescence**

CNS and peripheral tissue were dissected and fixed using standard techniques [32]. Rat anti-Fru antibody was used at 1:100 (Lee et al. 2000). Rabbit anti-GFP was used at 1:500 (Invitrogen). CY3-conjugated goat anti-rat and FITC-conjugated goat anti-rabbit secondary antibodies were used at 1:1000 (Jackson Immuno-research).

**Confocal Microscopy**

Tissues were analyzed using a Zeiss LSM510 with a 40X oil objective. Images were taken at 1024×1024 pixels with slices at 1.0 to 1.5 μm intervals.

**Imaging Analyses**

Image analyses were conducted using ImageJ and Fiji. The fluorescence density of the neurites was analyzed by sampling the integrated density of the target area and correcting for background by subtracting the fluorescence from three neighboring regions with no detectable labeled neurites. Normalization of tritocerebral arbors was achieved by establishing the average male, female, and fru mutant male brain size. Measurements were adjusted by a normalization factor per axis (sample brain axis/average brain axis).

**Statistical Analyses**

JMP10 (SAS software) and R (http://www.r-project.org/) were used for statistical analyses.

**Partial least squares.** PLS does an iterative extraction on both the predictor (P[GawB]:fruP1-LexA overlap) and response (courtship latency) data sets to derive latent variables with the constraint that these latent variables explain the covariance between the data sets [33]. Significance testing was done using random resampling without replacement of the original overlap
data set. 500 resampled matrices were constructed and analyzed with PLS to estimate confidence intervals.

Behavior analyses. ANOVA was conducted with a Tukey HSD post-test to determine significance of differences in courtship log-transformed latency means. For graphs, latency was back-transformed. Correlation of activity with latency was tested by Pearson’s correlation. Significance tests for courtship index and courtship/copulation percentages were done using Fisher’s exact test. Confidence intervals for proportions were estimated using the Clopper-Pearson interval.

Supporting Information

Figure S1 aSG4-57 projections. Frontal confocal projections from P(GaelB)4-57/UAS>stop>mCD8-GFP, fru42T/+ animals. In this brain, a DT6/n4-57, aSG4-57, two mSG/n4-57, and one pSG/n4-57 neuron are visible. The DT6/n4-57 neuron projects to the superior medial protocerebrum (smpr). Extensive, fine arbors from the aSG/n4-57 neuron project bilaterally throughout the SOG. A collateral extends to the inferior lateral protocerebrum (ilpr). Not visible in this section the pSG/n4-57 neuron extends descending into the cervical connective. Scale bar = 50 µm. (TIF)

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Author Contributions

Conceived and designed the experiments: DHT. Performed the experiments: DHT. Analyzed the data: DHT BSB. Contributed reagents/materials/analysis tools: DHT GWM RLF BSB. Wrote the paper: DHT.

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