Sexually dimorphic mechanosensory neurons regulate copulation duration and persistence in male *Drosophila*

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Abstract

Peripheral sensory neurons are the gateway to the environment across species. In *Drosophila*, olfactory and gustatory senses are required to initiate courtship, as well as for the escalation of courtship patterns that lead to copulation. To be successful, copulation must last long enough to ensure the transfer of sperm and seminal fluid that ultimately leads to fertilization. The fly genitalia contain sex-specific bristle hairs innervated by mechanosensory neurons. To date, the role of the sensory information collected by these peripheral neurons in male copulatory behavior is unknown. Here, we employed genetic manipulations that allow driving gene expression in the male genitalia as a tool to uncover the role of these genitalia specific neurons in copulation. We found that the sensory information received by the mechanosensory neurons (MSNs) at the male genitalia plays a key role in copulation duration. We confirmed that these MSNs are cholinergic and co-express both *fru* and *dsx*. Moreover, our results show that the function of these *fru/dsx* cholinergic MSNs is required for copulation persistence, which ensures copulation is undisrupted in the presence of environmental stress before sperm transfer is complete.

Introduction

An animal's decision to perform a certain behavior requires the integration of external stimuli with homeostatic regulation. The nervous system collects environmental sensory information and integrates it with the internal status of the animal to generate motor signals that elicit an appropriate behavioral response. *Drosophila* male courtship is an excellent model to investigate how complex behavior is coordinated by the nervous system. The development of the courtship neural circuit is largely controlled by two sex determination genes, *fruitless (fru)* and *doublesex (dsx)*¹⁻³. The ~2000 *fru*-expressing neurons, comprising of sensory, integration, and motor neurons, control most aspects of male courtship behavior⁴⁻⁹. However, the complete courtship neural circuit also requires *dsx*-expressing neurons, a subset of which co-expresses *fru*¹⁰⁻¹⁴.

Successful courtship requires the processing of sensory information received by peripheral sensory neurons¹⁵. In the antenna, *fru* olfactory receptor neurons (ORNs) receive and transmit volatile olfactory signals to the antennal lobe in the brain; *fru* ORNs expressing the receptor Or67d respond to the male pheromone cVA which inhibits mating behavior in males but promotes mating behaviors in females¹⁶. Gustatory receptor neurons (GRNs) in the front legs receive and transmit non-volatile gustatory signals to the CNS. For example, *fru* GRNs expressing the ion channels *ppk23* and *ppk29* are required to detect inhibitory signals on males and excitatory signals on females during courtship¹⁷. *Dsx* neurons expressing the receptor Gr68a respond to the anti-aphrodisiac pheromone CH503 and inhibit male courtship¹⁸. Acoustic information from the movement of the female detected by the Johnston's organ neurons (JONs) helps to promote courtship initiation in males¹⁹. Finally, the visual contribution to the initiation and maintenance of courtship has also been shown in a treadmill-based courtship assay²⁰.

When the female is receptive to male courtship, she will slow down and spread her wings, allowing the male to mount. The male initiates copulation by bending his abdomen forward to attach the genitalia. The male terminalia is divided in two regions: the periphallallic structures and the intromittent organ. The periphallallic structures form weaker connections to the intromittent organ and can be easily separated by dissection. The periphallallic structures are subdivided into four regions: the epandrial ventral lobe (formerly called: lateral plate) (EVL), the surstylus (formerly called: clasper) (SUR), the epandrial posterior lobe (formerly called posterior lobe) (EPL), and the cercus (formerly called: anal plate) (CER)²¹. Genital coupling, as revealed by a high-resolution electron microscopic time sequence analysis, involves the active movement of these periphallallic structures²². The surstylus bends medially and is hidden from view. Within 10 minutes of copulation, the cercus aligns with the female oviscape and achieves genital coupling. Each periphallallic region contains an array of stereotypic, species and sex-specific bristles, and each bristle is innervated by a bipolar mechanosensory neuron²³. The neural implication of this sensory information collected during copulation is unclear.

Copulation must last long enough to ensure successful sperm transfer, which does not occur until five minutes after copulation starts²⁴. The regulation of copulation time in *Drosophila* is a complicated process that does not simply depend on the volume or the transfer of sperm and seminal fluid, since mutants defective in their synthesis still have normal copulation duration²⁴. Both neuronal and non-neuronal factors can influence mating time. Neuronal regulators include a small cohort of *fru* neurons that co-express the transcription factor *engrailed*²⁵ and a subset of 4-5 *fru* neurons that innervate the male reproductive tissues^{26,27}. The latter neurons control copulation duration in response to sperm transfer²⁶. Non-neuronal factors include mutations in the circadian clock gene *period*, which exhibit longer copulation duration²⁸, and environmental factors such as the gut microbiome²⁹ and environmental stressors²⁴. Copulation persistence describes the maintenance of copulation in the presence of stressful stimuli, which peaks within the first 5 minutes of copulation and decreases over time²⁴. This is an important cost-benefit analysis that ensures species survival. The neural circuit for this cost-benefit analysis includes 8 *dsx*/GABA neurons in the ventral cord that decrease copulation persistence. With opposite action, ventral cord DA neurons increase copulation persistence²⁴. However, the neural input required to initiate copulation persistence is unknown. In this study, we show that sensory information received by the mechanosensory neurons (MSNs) at the male genitalia is a novel neuronal regulator of copulation duration and is critical for the maintenance of copulation in the presence of stressful stimuli before sperm transfer is complete.

Results

Discovery of *fruitless* (*fru*) neurons that regulate copulation duration

To uncover the neuronal regulators of copulation duration, we utilized an intersectional genetic approach, involving both the GAL4/UAS³⁰ and FLP/FRT⁷ expression systems. The details of the intersectional genetic system and how we generated a FLP enhancer trap screen had been described elsewhere^{27,31,32}. Briefly, the target gene is downstream of a DNA sequence that contains the binding site (UAS) for the transcription factor GAL4, followed by a DNA sequence that contains a stop codon flanked by the FLP recombinase recognition site FRT (UAS-FRT-stop-FRT-target gene). A cell must possess an active promoter for FLP (to remove the stop codon by recombination) and an active promoter for GAL4 (to bind to UAS) to activate expression of the target gene. As an additional tool to restrict gene expression, *tsh*^{GAL80} is used to inhibit GAL4 expression specifically in the ventral cord. From here forward, the genetic nomenclature will list all transgenes that are responsible to drive expression of the target gene (e.g. “X^{GAL4},FLP[#]>target gene” denotes a fly that carries a GAL4 driver controlled by the promoter of X, and the FLP line # from the enhancer trap screen, expressing the target gene). For the relevant neuronal populations, we expressed either GFP (UAS-FRT-stop-FRT-GFP) to visualize the morphology or the neuronal silencer tetanus toxin (UAS-FRT-stop-FRT-TNT) to block neuronal activity. Previously, we identified a cluster of ~5 *fru* 5HT/DA neurons (*fru*-sAbg-1) in the male abdominal ganglion that innervate various male reproductive tissues and regulates copulation duration²⁷. In that study, we observed a reduction in copulation duration when we combined FLP335 with either *fru*-GAL4, that targets most fruitless neurons (Fru^{GAL4},FLP³³⁵,*tsh*^{GAL80}>TNT), with Th-GAL4, that targets dopaminergic neurons (Th^{GAL4},FLP³³⁵,*tsh*^{GAL80}>TNT), or with 5HT-GAL4, that targets serotonergic neurons (5HT^{GAL4},FLP³³⁵,*tsh*^{GAL80}>TNT)²⁷. However, neither silencing the subsets of dopaminergic nor serotonergic neurons can recapitulate the copulation phenotype observed when we targeted the broader fruitless circuit. The result suggested that additional *fru* neurons contributed to the copulation duration phenotype²⁷. Indeed, the expression pattern for Fru^{GAL4},FLP³³⁵,*tsh*^{GAL80}>GFP males extended beyond the accessory glands and seminal vesicles all the way to the genitalia (Figure 1A). Upon further examination, we were able to trace GFP expression to the male genitalia.

To further confirm the genitalia neurons are implicated in copulation duration control, we screened our enhancer trap FLP library (ref?) to search for other FLP lines that affected copulatory behaviors. We identified another FLP line that showed expression at the genitalia and also reduced copulation duration when it is combined with *fru*-GAL4 to express TNT in males (Fru^{GAL4},FLP³⁸⁶,*tsh*^{GAL80}>TNT) (Figure 1D). The experimental males (Fru^{GAL4},FLP³⁸⁶,*tsh*^{GAL80}>GFP) showed similar expression in *fru* ORNs, JONs, and GRNs compared to Fru^{GAL4},FLP³³⁵,*tsh*^{GAL80}>GFP males (Figure 1B). However, the prominent *fru*-sAbg-1 neurons in the abdominal ganglion that innervate the male reproductive tissue were noticeably missing, leaving only the sexually dimorphic arborizations (ANN1) in the abdominal ganglion originating from the MSNs in the genitalia. The expression pattern in the peripheral tissues that extend out from the ventral cord of Fru^{GAL4},FLP³⁸⁶,*tsh*^{GAL80}>GFP males was highly restricted, showing GFP expression only in the male genitalia (Figure 1B). Although we still observed expression for *fru* GRNs, it is less consistent compared to Fru^{GAL4},FLP³³⁵,*tsh*^{GAL80}>GFP. In summary, three common *fru* neurons are consistently labeled by both FLP lines: 1) the sexually dimorphic projections in the glomeruli from *fru* ORNs (DA1,

VA1v, VL2a), 2) projections in the antennal mechanosensory motor complex (AMMC) from *fru* JONs, and 3) projections in the abdominal ganglion (ANN1) from *fru* MSNs in the genitalia (Figure 1C, Table 1). The copulation duration of $Fru^{GAL4}, FLP^{386}, tsh^{GAL80} > TNT$ was shortened by ~26% compared to the TNT inactive control (Figure 1D). This difference is comparable to that observed in $Fru^{GAL4}, FLP^{335}, tsh^{GAL80} > TNT$ males. Of the three groups of *fru* neurons targeted by both FLP lines, we hypothesized that ANN1 from *fru* MSNs in the genitalia were the most likely to contribute to the copulation duration phenotype since we showed previously that silencing *fru* ORNs and GRNs did not affect copulation duration²⁷. Recently, it was discovered that females sing *in copula* and that this song influences the reproductive success of the male³³. However, the study showed that copulation duration was unaffected by the absence of female singing³³.

Characterization of *fru* genitalia mechanosensory neurons (MSNs)

We further characterized the expression pattern in the genitalia terminals using various genetic combinations. The complete *fru* circuit ($fru > GFP$) in males show consistent GFP expression in a subset of mechanosensory neurons in the epandrial ventral lobe (10 ± 4 , ~50% of EVL bristles), the surstylus (16 ± 2 , ~64% of SUR bristles), and the cercus (20 ± 2 , ~61% of CER bristles) (Figure 2B, Table 2). Adding FLP^{335} or FLP^{386} eliminated GFP expression in the epandrial ventral lobe and restricted expression in *fru* MSNs at the surstylus and the cercus (Figure 2C, Table 2). Amongst these two lines, $Fru^{GAL4}, FLP^{386}, tsh^{GAL80} > GFP$ revealed a more restrictive expression pattern with 8 ± 3 *fru* neurons at the surstylus and 4 ± 2 at the cercus (Table 2). As $Fru^{GAL4}, FLP^{386}, tsh^{GAL80} > TNT$ males resulted in the same copulation duration phenotype, we hypothesized that these 12 neurons (*fru*-MSNs, Table 2) at the genitalia are responsible for the shortened copulation duration phenotype. Since most sensory neurons in *Drosophila* are cholinergic, we hypothesized that *fru*-MSNs are the same. Indeed, using $Cha-GAL80$ that expresses the GAL4 inhibitor in cholinergic neurons eliminated ANN1 expression that originates from the genitalia MSNs (Figure 2D).

Fru MSN neurons co-express *doublesex* (*dsx*)

To rule out the contributions of other neuronal populations to the copulation duration phenotype, we investigated other genetic combinations that target genitalia neurons more specifically. Previous research indicated that the genitalia neurons express *dsx*^{11,12,34}. Indeed, we confirmed that the *fru*-MSN neurons are also *dsx* positive (Figure 3A). Replacing *fru*-GAL4 with *dsx*-GAL4 in our genetic combination with FLP^{335} ($Dsx^{GAL4}, FLP^{335} > GFP$) eliminated all expression in the brain (Figure 3C). In the VNC, $Dsx^{GAL4}, FLP^{335} > GFP$ showed consistent expression only in the sexually dimorphic arbors originating

from the foreleg GRNs and the genitalia neurons (Figure 3B, D, E). Using this highly restrictive genetic combination, we silenced these neurons by TNT expression and evaluated the post-copulatory behaviors. Consistent with our hypothesis, copulation duration was still reduced at the same level for $Dsx^{GAL4}, FLP^{335} > TNT$ compared to the control (Figure 3G). These results ruled out the involvement of CNS neurons in the copulation duration phenotype and indicate that the *fru* neurons responsible for the phenotype are also *dsx* positive.

The characteristic male-specific midline crossing of the LAN1 arbors that originated from GRNs on the front legs is regulated by both *fru* and *dsx*³⁵. These GRNs co-express the ion channel *ppk25* that is critical for pheromone detection during early courtship steps¹⁷. To investigate whether the copulation duration phenotype requires female pheromone perception, we paired $Dsx^{GAL4}, FLP^{335} > TNT$ males with females whose pheromone-producing cells were ablated genetically via expression of the pro-apoptotic gene *hid* under an oenocyte-specific promoter³⁶. As expected, the experimental males still exhibited shortened copulation duration compared to the control males, irrespective of whether they were paired with oenocyte-less females (oe-) or their genetic controls (oe+) (Figure 3H). The copulation duration of the control males (TNT-in) is significantly shorter when paired with oe- females compared to control oe+ females (Figure 3H). However, no significant effect was observed in the experimental males (TNT); the lack of significance could be due to the variability of the data from the TNT/oe+ pairs. Taken together, these data suggest that the copulation duration phenotype is driven by the silencing of the *fru/dsx*-MSNs.

Sensory information detected by *fru/dsx* neurons is required for copulation persistence

Next, we asked the biological relevance of a shortened copulation duration as a result of silencing *fru/dsx* MSNs. In a productive copulation pairing, duration must be long enough for the transfer of sperm and accessory gland fluid. In the three genetic combinations presented here, the median copulation duration for the experimental males is 10-14 minutes, which is longer than the minimal time (~8 minutes) necessary for sperm transfer^{25,26}. To evaluate if sperm is successfully transferred from the test male to the target female, we quantified the number of copulation pairings that resulted in fertilization after a single copulation event between a test male and a virgin Canton-S female. Compared to inactive TNT controls, a significantly lower percentage of males where both copulation duration regulating neural clusters (sAbg-1 and MSNs) are silenced ($Fru^{GAL4}, FLP^{335}, tsh^{GAL80} > TNT$ males) could fertilize the virgin females. In contrast, males in which sAbg-1 are not manipulated but MSNs are silenced ($Fru^{GAL4}, FLP^{386}, tsh^{GAL80} > TNT$ and $Dsx^{GAL4}, FLP^{335} > TNT$ males) have relatively normal levels of fertilization rates compared to their respective inactive TNT controls (Figure 4A). Therefore, post-

copulatory fertility is unaffected by the shortened copulation duration due to the silencing of the *fru/dsx*-MSNs.

If fertility is unaffected, we wondered if peak copulation persistence is still possible without sensory input from the genitalia. To evaluate if copulation persistence is affected by the silencing of the *fru/dsx*-MSNs, we applied heat shock as the stress stimulus to copulating flies when copulation persistence is at its peak. We heat-shocked pairs of flies 5 minutes after the onset of copulation and quantified the number of pairs that disengaged. While a large number of pairs in which the *fru/dsx*-MSNs in the male genitalia were silenced by our three genetic combinations ($\text{Fru}^{\text{GAL4}}, \text{FLP}^{335}, \text{tsh}^{\text{GAL80}} > \text{TNT}$; $\text{Fru}^{\text{GAL4}}, \text{FLP}^{386}, \text{tsh}^{\text{GAL80}} > \text{TNT}$; $\text{Dsx}^{\text{GAL4}}, \text{FLP}^{335} > \text{TNT}$) terminated copulation, all pairs with the corresponding control males remained copulating (Figure 4B). These results indicate that the *fru/dsx*-MSNs are important in maintaining copulation persistence in response to environmental stress before sperm transfer is complete.

Discussion

Different neuronal populations have been identified to control three distinct aspects of male copulatory behaviors: genital coupling, copulation duration, and sperm transfer^{26,27,34}. Here, we have characterized the *fru/dsx*-MSNs at the genitalia and identified their novel function in regulating copulation duration and maintaining copulation persistence in the presence of environmental stressors. Although we cannot completely rule out the contribution of pheromone perception in their regulation, the pheromone sensing neurons are not consistently targeted in one of our genetic combination ($\text{Fru}^{\text{GAL4}}, \text{FLP}^{386}, \text{Tsh}^{\text{GAL80}} > \text{GFP}$) (Figure 1B).

Retrograde labeling of various genitalia MSNs have shown that most of these neurons project only to the abdominal ganglion except for one neuron from the surstylus (clasper) that projects all the way to the suboesophageal region of the brain³⁴. Since we did not observe any arborizations in the brain, the copulation phenotype appears to be regulated by a subset of the *fru/dsx*-MSNs at the surstylus (claspers) and epandrial ventral lobe (lateral plate) that project specifically to the abdominal ganglion. In addition, the retrograde labeling experiment showed that the axonal terminals of the genitalia neurons juxtapose the dendrites of the abdominal *dsx* glutamatergic (*dsx/vGlut-Abg*) and GABAergic (*dsx/GABA-Abg*) neurons³⁴. Moreover, artificial mechanical stimulation of the genitalia with a minuten pin activated both *dsx/vGlut-Abg* and *dsx/GABA-Abg* neurons³⁴. Therefore, we can infer that *fru/dsx*-MSNs neurons make functional synaptic connections to both *dsx/vGlut* and *dsx/GABA* neurons in the abdominal ganglion (Figure 5). The shortened copulation duration phenotype when *fru/dsx*-MSNs are silenced is a result of *dsx/vGlut-Abg* and/or *dsx/GABA-Abg* not receiving sensory feedback signals from the genitalia.

dsx/vGlut-Abg are all motor neurons that innervate the phallic and periphallal musculature responsible for genital attachment³⁴. On the other hand, *dsx/GABA-Abg* is comprised of a heterogeneous population of interneurons with different functions. While some of these interneurons inhibit the activity of *dsx/vGlut*-

Abg to terminate copulation³⁴, others have different functional roles, such as regulating copulation persistence²⁴. Therefore, sensory signals received from the *fru/dsx*-MSNs can influence the relative activity level of *dsx/vGlut*-Abg and *dsx/GABA*-Abg neurons with opposing actions and modulate the overall tension of the copulatory muscles. In the absence of sensory information, the baseline muscle strength is sufficient to maintain genital coupling long enough for sperm transfer since males with *fru/dsx*-MSNs silenced has normal fertility (Figure 4A). However, our results show that the sensory information encoding the male's correct engagement in copulation is necessary to achieve peak copulation persistence before sperm is transferred (Figure 4B, 5). Sensory information – provided by *fru/dsx*-MSNs – might be a way to measure the quality of the copulation. Genital coupling will lead to bending of the bristle hairs and activate the *fru/dsx*-MSNs. Which bristle hair gets stimulated and the strength of the stimulation will depend on whether genital coupling is established and the morphology of the female genitalia. For example, an abnormal amount of pressure received by a bristle hair could send less signal to activate the copulatory muscle innervated by *dsx-vGlut*-Abg. Similarly, a wrong set of bristles bent could activate the *dsx-GABA*-Abg neurons that inhibit the *dsx-vGlut*-Abg. A suboptimal activation decreases the total muscular tension during genital coupling. Copulation persistence is a result of the fly's ability to analyze the tradeoff of maintaining copulation during exposure to an environmental stressor. Sensory information that signals the quality of the copulation provides a critical factor in this assessment. The recently published transcriptomic atlas for both the ventral nerve cord³⁷ and the male genitalia³⁸ provides new possibilities to develop specific neural markers that will allow further investigation of the neural mechanism of how sensory information from genital coupling is encoded and processed in the CNS.

Materials And Methods

Fly strains

The following strains were used in this study: *fru-GAL4*⁷, *dsx-GAL4*¹¹, *UAS>stop>TNTin*, *UAS>stop>TNT*⁷, *UAS>stop>mCD8::GFP*⁹, *FLP*³³⁵ and *FLP*³⁸⁶ were generated as described in previous study²⁷, *cha-GAL80*³⁹, *tsh-GAL80* from Julie Simpson, and Canton-S strain from the Bloomington Stock Center, Bloomington, IN. Oenocyte less (oe-) flies and their controls were generated as previously described³⁶.

Immunohistochemistry

Dissection of 3-7 day old adult flies and immunohistochemistry of the adult nervous system were carried out as described previously⁴⁰ with some modifications in primary and secondary antibodies. Dissection of male reproductive organs was performed on Sylgard plate covered with 1xPBS. To obtain male genitalia, the lower half of the abdomen were dissected and fixed in 4% PFA at room temperature for 20-30 mins. After which the PFA was replaced with 1xPBS. The genitalia were cut out with a pair of microscissors to ensure a flat surface for mounting. Dissected samples were fixed and proceeded with immunostaining as described previously⁴⁰. The following primary and secondary antibodies were used in

this study: rat polyclonal anti-mCD8 (1:100; Caltag, Burlingame, CA), rabbit anti-GFP (1:600; Invitrogen), mouse anti-GFP (1:500, Life technologies), mouse anti-nc82 (1:20; Hybridoma Bank)⁴¹, rabbit anti-Dsx^M (1:2000) (kindly supplied by Brian Oliver laboratory, NIH), anti-rat IgG conjugated with Alexa Fluor 488 (1:300; Invitrogen), and anti-rabbit IgG conjugated with Alexa Fluor 594 (1:300; Invitrogen).

Microscopy

Images of the ventral cord, the reproductive tissues, and genitalia were acquired using an Olympus Fluoview FV1000 confocal microscope with a 20X objective. ImageJ was used to stitch the overlapping images together. All other images were acquired using a Zeiss LSM 700 confocal microscope. For the genitalia, lambda stacks from 490-600 nm at 20 nm intervals were acquired. The autofluorescence signals were unmixed from the 488 nm signals using the linear unmixing algorithm in the Zen Black software.

Behavioral assays

Husbandry

Flies were raised on standard cornmeal medium and kept on a 12hr:12hr day:night cycle at 25°C in ambient relative humidity. Each newly eclosed adult was collected and aged for 3-7 days in an isolation vial (16 x 100-mm) supplied with ~2 ml of fly food. Virgin, wildtype Canton-S females were aged in groups of 20-40 for 3-7 days. All behavioral experiments were performed at 25°C with ~50% humidity during the first 3 hrs. after lights on.

Copulation Assay

Copulation assays were performed in 12-well plates (Thermo Scientific BioLite Multidish). A square glass plate covering four wells was used as the lid. Assays were performed in 4 wells with ~5 ml standard fly food to maintain humidity in each well. An experimental male was paired with a virgin female and the courtship behavior was videotaped for at least an hour. If a pair did not copulate within an hour, it was considered unsuccessful. Copulation duration was calculated from the beginning of genital coupling until the male was dismounted from the female.

Fertility Assay

Freshly hatched males were isolated into a glass tube (16x100mm) with 2 ml of food and kept for 4-5 days at standard conditions (25°C, 50% RH). A 6-7 day old CS virgin female was introduced into each tube using an aspirator. After transferring the vials back to standard conditions, the flies were allowed to interact for 1h and observed every 10 mins for successful copulation. Tubes with pairs that did not copulate were discarded. For the rest of the tubes, males were removed by quick anaesthetization and females were allowed to lay eggs for 24h and then discarded. Fertility was recorded after 7 days by observing the presence of progeny.

Persistence Assay

A single experimental male was paired with a wildtype CS virgin female in a glass tube (16x100 mm) with or without food. If copulated in a vial with food, the pairs were transferred to an empty vial before heat shock to ensure efficient heat transfer. Each pair that successfully copulated (within 5 mins) was subjected to heat stress by submersing the empty glass vial (16x100 mm) in a 37°C water bath for 30 seconds. Frequency of copulation termination was recorded.

References

1. Burtis, K. C. & Baker, B. S. *Drosophila* doublesex gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides., **56**, 997–1010 [https://doi.org/doi:10.1016/0092-8674\(89\)90633-8](https://doi.org/doi:10.1016/0092-8674(89)90633-8) (1989).
2. Ito, H. *et al.* Sexual orientation in *Drosophila* is altered by the satori mutation in the sex-determination gene fruitless that encodes a zinc finger protein with a BTB domain. *Proc Natl Acad Sci U S A*, **93**, 9687–9692 (1996).
3. Ryner, L. C. *et al.* Control of male sexual behavior and sexual orientation in *Drosophila* by the fruitless gene., **87**, 1079–1089 (1996). doi:S0092-8674(00)81802-4 [pii]
4. Lee, G. *et al.* Spatial, temporal, and sexually dimorphic expression patterns of the fruitless gene in the *Drosophila* central nervous system. *J Neurobiol*, **43**, 404–426 [https://doi.org/doi:10.1002/1097-4695\(20000615\)43:4<404::AID-NEU8>3](https://doi.org/doi:10.1002/1097-4695(20000615)43:4<404::AID-NEU8>3) (2000). 0.CO;2-D [pii]
5. Demir, E. & Dickson, B. J. fruitless splicing specifies male courtship behavior in *Drosophila*. *Cell* **121**, 785-794, doi:S0092-8674(05)00407-1 [pii]10.1016/j.cell.2005.04.027 (2005)
6. Manoli, D. S. *et al.* Male-specific fruitless specifies the neural substrates of *Drosophila* courtship behaviour., **436**, 395–400, doi:nature03859 (2005). [pii]10.1038/nature03859
7. Stockinger, P., Kvitsiani, D., Rotkopf, S., Tirian, L. & Dickson, B. J. Neural circuitry that governs *Drosophila* male courtship behavior. *Cell* **121**, 795-807, doi:S0092-8674(05)00406-X [pii]10.1016/j.cell. 2005.04.026 (2005)
8. Cachero, S., Ostrovsky, A. D., Yu, J. Y., Dickson, B. J. & Jefferis, G. S. Sexual Dimorphism in the Fly Brain. *Curr Biol* **20**, 1589-1601, doi:S0960-9822(10)00947-4 [pii]10.1016/j.cub. 2010.07.045 (2010)
9. Yu, J. Y., Kanai, M. I., Demir, E., Jefferis, G. S. & Dickson, B. J. Cellular Organization of the Neural Circuit that Drives *Drosophila* Courtship Behavior. *Curr Biol* **20**, 1602-1614, doi:S0960-9822(10)01015-8 [pii]10.1016/j.cub. 2010.08.025 (2010)
10. Rideout, E. J., Billeter, J. C. & Goodwin, S. F. The sex-determination genes fruitless and doublesex specify a neural substrate required for courtship song. *Curr Biol*, **17**, 1473–1478 <https://doi.org/doi:10.1016/j.cub.2007.07.047> (2007).
11. Rideout, E. J., Dornan, A. J., Neville, M. C., Eadie, S. & Goodwin, S. F. Control of sexual differentiation and behavior by the doublesex gene in *Drosophila melanogaster*. *Nat Neurosci* **13**, 458-466, doi:nn.2515 [pii]10.1038/nn.2515 (2010)

12. Robinett, C. C., Vaughan, A. G., Knapp, J. M. & Baker, B. S. Sex and the single cell. II. There is a time and place for sex. *PLoS Biol*, **8**, e1000365 <https://doi.org/doi:10.1371/journal.pbio.1000365> (2010).
13. Villella, A. & Hall, J. C. Courtship anomalies caused by doublesex mutations in *Drosophila melanogaster*, **143**, 331–344 (1996).
14. Pan, Y. & Baker, B. S. Genetic identification and separation of innate and experience-dependent courtship behaviors in *Drosophila*, **156**, 236–248 <https://doi.org/doi:10.1016/j.cell.2013.11.041> (2014).
15. Yamamoto, D. & Koganezawa, M. Genes and circuits of courtship behaviour in *Drosophila* males. *Nat Rev Neurosci*, **14**, 681–692 <https://doi.org/doi:10.1038/nrn3567> (2013).
16. Kurtovic, A., Widmer, A. & Dickson, B. J. A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. *Nature* **446**, 542–546, doi:nature05672 [pii]10.1038/nature05672 (2007)
17. Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L. & Scott, K. Contact Chemoreceptors Mediate Male-Male Repulsion and Male-Female Attraction during *Drosophila* Courtship. *Cell* **149**, 1140–1151, doi:S0092-8674(12)00523-5 [pii]10.1016/j.cell.2012.03.045 (2012)
18. Shankar, S. *et al.* The neuropeptide tachykinin is essential for pheromone detection in a gustatory neural circuit. *eLife*, **4**, e06914 <https://doi.org/doi:10.7554/eLife.06914> (2015).
19. Ejima, A. & Griffith, L. C. Courtship initiation is stimulated by acoustic signals in *Drosophila melanogaster*. *PLoS One*, **3**, e3246 <https://doi.org/doi:10.1371/journal.pone.0003246> (2008).
20. Kohatsu, S. & Yamamoto, D. Visually induced initiation of *Drosophila* innate courtship-like following pursuit is mediated by central excitatory state. *Nat Commun*, **6**, 6457 <https://doi.org/doi:10.1038/ncomms7457> (2015).
21. Rice, G. *et al.* A standardized nomenclature and atlas of the male terminalia of *Drosophila melanogaster*, **13**, 51–64 <https://doi.org/doi:10.1080/19336934.2019.1653733> (2019).
22. Jagadeeshan, S. & Singh, R. S. A time-sequence functional analysis of mating behaviour and genital coupling in *Drosophila*: role of cryptic female choice and male sex-drive in the evolution of male genitalia. *J Evol Biol* **19**, 1058–1070, doi:JEB1099 [pii]10.1111/j.1420-9101.2006.01099.x (2006)
23. Taylor, B. J. Sexually dimorphic neurons of the terminalia of *Drosophila melanogaster*: II. Sex-specific axonal arborizations in the central nervous system. *J Neurogenet*, **5**, 193–213 (1989).
24. Crickmore, M. A. & Vosshall, L. B. Opposing dopaminergic and GABAergic neurons control the duration and persistence of copulation in *Drosophila*, **155**, 881–893 <https://doi.org/doi:10.1016/j.cell.2013.09.055> (2013).
25. Latham, K. L., Liu, Y. S. & Taylor, B. J. A small cohort of FRU(M) and Engrailed-expressing neurons mediate successful copulation in *Drosophila melanogaster*. *BMC Neurosci*, **14**, 57 <https://doi.org/doi:10.1186/1471-2202-14-57> (2013).
26. Tayler, T. D., Pacheco, D. A., Hergarden, A. C., Murthy, M. & Anderson, D. J. A neuropeptide circuit that coordinates sperm transfer and copulation duration in *Drosophila*. *Proc Natl Acad Sci U S A*, **109**, 20697–20702 <https://doi.org/doi:10.1073/pnas.1218246109> (2012).

27. Jois, S., Chan, Y. B., Fernandez, M. P. & Leung, A. K. Characterization of the Sexually Dimorphic fruitless Neurons That Regulate Copulation Duration. *Front Physiol*, **9**, 780 <https://doi.org/doi:10.3389/fphys.2018.00780> (2018).
28. Beaver, L. M. & Giebultowicz, J. M. Regulation of copulation duration by period and timeless in *Drosophila melanogaster*. *Curr Biol*, **14**, 1492–1497 <https://doi.org/doi:10.1016/j.cub.2004.08.022> S0960982204006050 (2004). [pii]
29. Lewis, Z., Heys, C., Prescott, M. & Lize, A. You are what you eat., **5**, 541–543 <https://doi.org/doi:10.4161/gmic.29153> (2014).
30. Brand, A. H. & Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, **118**, 401–415 (1993).
31. Alekseyenko, O. V. *et al.* Single serotonergic neurons that modulate aggression in *Drosophila*. *Curr Biol*, **24**, 2700–2707 <https://doi.org/doi:10.1016/j.cub.2014.09.051> (2014).
32. Alekseyenko, O. V., Chan, Y. B., Li, R. & Kravitz, E. A. Single dopaminergic neurons that modulate aggression in *Drosophila*. *Proc Natl Acad Sci U S A* **110**, 6151–6156, doi:1303446110 [pii]10.1073/pnas.1303446110 (2013)
33. Kerwin, P., Yuan, J. & von Philipsborn, A. C. Female copulation song is modulated by seminal fluid. *Nat Commun*, **11**, 1430 <https://doi.org/doi:10.1038/s41467-020-15260-6> (2020).
34. Pavlou, H. J. *et al.* Neural circuitry coordinating male copulation. *eLife*, **5**, <https://doi.org/doi:10.7554/eLife.20713> (2016).
35. Mellert, D. J., Knapp, J. M., Manoli, D. S., Meissner, G. W. & Baker, B. S. Midline crossing by gustatory receptor neuron axons is regulated by fruitless, doublesex and the Roundabout receptors. *Development* **137**, 323–332, doi:137/2/323 [pii]10.1242/dev.045047 (2010)
36. Billeter, J. C., Atallah, J., Krupp, J. J., Millar, J. G. & Levine, J. D. Specialized cells tag sexual and species identity in *Drosophila melanogaster*., **461**, 987–991 <https://doi.org/doi:10.1038/nature08495> (2009).
37. Allen, A. M. *et al.* A single-cell transcriptomic atlas of the adult *Drosophila* ventral nerve cord. *eLife*, **9**, <https://doi.org/doi:10.7554/eLife.54074> (2020).
38. Vincent, B. J. *et al.* An Atlas of Transcription Factors Expressed in Male Pupal Terminalia of *Drosophila melanogaster*. *G3* **9**, 3961–3972, doi:10.1534/g3.119.400788 (2019)
39. Kitamoto, T. Conditional disruption of synaptic transmission induces male-male courtship behavior in *Drosophila*. *Proc Natl Acad Sci U S A*, **99**, 13232–13237 <https://doi.org/doi:10.1073/pnas.202489099> (2002).
40. Mundiyanapurath, S., Chan, Y. B., Leung, A. K. & Kravitz, E. A. Feminizing cholinergic neurons in a male *Drosophila* nervous system enhances aggression. *Fly (Austin)*, **3**, 179–184, doi:8989 (2009). [pii]
41. Hofbauer, A. *et al.* The Wuerzburg hybridoma library against *Drosophila* brain. *J Neurogenet*, **23**, 78–91, doi:907468467 (2009). [pii]10.1080/01677060802471627

Declarations

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Author Contributions

AL, YC, and MF conceived and designed the experiments. AL, YC, SJ, NP carried out the experiments. AL led the project. AL wrote the manuscript. LJ helped with revision. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Tables

Table 1. Summary of *fru* expression restricted by FLP³³⁵ or FLP³⁸⁶ in combination with *fru*-GAL4 and *tsh*-GAL80.

Neurons	Arbors	FLP335	FLP386
ORNs (Peripheral)	DA1 (Brain)	+	+
	VA1v (Brain)	+	+
	VL2a (Brain)	+	+
JONs (Peripheral)	AMMC (Brain)	+	+
MSNs (Peripheral)	ANN1 (VNC)	+	+
GRNs (Peripheral)	LAN1 (VNC)	+	+
~5 5HT/DA Abg-1 (VNC)	Reproductive tissues (Peripheral)	+	
~12 AbgNs uncharacterized (VNC)		+	
~8 AbgNs uncharacterized (VNC)			+

In brackets is the anatomical location of the neurons or arbors. ORN, olfactory receptor neuron; JON, Johnston's organ neuron; MSN, mechanosensory neuron; GRN, gustatory receptor neuron; AbgN, abdominal neuron; 5HT, serotonergic neuron; DA, dopaminergic neuron; VNC, ventral nerve cord.

Table 2. Expression Summary of the Male Genitalia of the Different Genetic Combinations.

Transgene Present			Epandrial Ventral Lobe (EVL)		Surstylus (SUR)		Cercus (CER)		n
FLP	<i>fru</i> -Gal4	Tsh-Gal80	Percent Sample Showing Expression (%)	Average Number of Cells	Percent Sample Showing Expression (%)	Average Number of Cells	Percent Sample Showing Expression (%)	Average Number of Cells	
-	-	-	-	20*±0.34		25*±0.63		32.7*±1.56	-
-	+	-	100	10±4	100	16±2	100	20±2	12
335	+	+	0	-	100	10±3	100	7±2	10
386	+	+	0	-	94	8±3	88	4±2	16

*Based on bristle count published in Taylor 1998.

Figures

Figure 1

Different groups of fru neurons regulate copulation duration. A, B) Comparison of fru neuronal expression restricted by two different FLP lines. Expression profile of Fru^{GAL4},FLP³³⁵,tsh^{GAL80}>GFP (A) is compared to that of Fru^{GAL4},FLP³⁸⁶,tsh^{GAL80}>GFP (B). CNS tissues were stained with anti-mCD8 (green) and nc82 (magenta) and peripheral tissues were stained with anti-mCD8 (green) only. Expression in the reproductive tissues was notably eliminated via FLP³⁸⁶. Scale Bar = 50 μ m. C) Venn diagrams

highlighting the common fru neurons targeted by both FLP lines as shown in A) and B). Names of the arbors are in brackets followed by the names of the neuronal cluster where they are originated. Three common fru neurons were consistently labeled by both FLP lines: 1) the sexually dimorphic projections in the glomeruli from fru ORNs (DA1, VA1v, VL2a), 2) projections in the antennal mechanosensory motor complex (AMMC) from fru JONs, 3) projections in the abdominal ganglion (ANN1) from fru MSNs in the genitalia. See also Table 1 in text. (D) Effect of silencing FLP335 and FLP386 restricted labeling of fru-GAL4. A shortened copulation duration was observed in both genetic combinations, therefore, the common neuronal clusters targeted by both FLP lines (ORNs, JONs, MSNs) were responsible for the copulation phenotype. Black dots are individual values. Red lines indicate the median with interquartile range (n=22-31). **p<0.0034, ****p<0.0001 by Mann Whitney test.

Figure 2

Characterization of *fru* neurons in the male genitalia. A) Light microscope image of a male genitalia adapted from a previous work 21. The male genitalia are comprised of the periphallic structures: cercus (CER), surstylus (SUR), epandrial ventral lobe (EVL) and the phallic structures: aedeagus (A). The phallic structures are attached to copulatory muscles (Figure 5) and located just anterior to the periphallic structures. Stereotypical bristles on the surface of these cuticular plates are innervated by

mechanosensory neurons (MSNs)²³. B) fru MSNs at the male genitalia. Expression profile of fru-GAL4 driving UASmCD8::GFP in the male genitalia. fru expression was observed in ~50% of MSNs at the epandrial ventral lobe, ~64% of MSNs at the surstylus, and ~61% of MSNs at the cercus (Table 2). C) fru MSNs restricted by FLP335. Expression profile of fru-GAL4, UAS>stop>mCD8::GFP, FLP335, tsh-GAL80 in the male genitalia. Adding FLP335 eliminated GFP expression in the epandrial ventral lobe and restricted expression at the surstylus and the cercus (Table 2). The autofluorescence and 488 signals were unmixed as described in Methods. D) fru MSNs are cholinergic. Expression profile of FruGAL4,FLP335,ChaGAL80>GFP in the male ventral nerve cord. Adding the transgene cha-Gal80, which inhibits GAL4 in cholinergic neurons, eliminated ANN1 expression that originated from the genitalia MSNs (compare to Figure 1A, B). Only fru-Abg-1 and other neurons in the abdominal ganglion showed expression. Scale Bar = 50 μ m.

Figure 3

fru/dsx MSNs regulates copulation duration. A) *fru* MSNs co-express *dsx*. Expression profile of *Fru*^{GAL4,FLP335>FLP} in the male genitalia. Double staining with anti-mCD8 (green) and anti-*dsx* (magenta) showed coexpression of *dsx* in *fru*-MSNs at the surstylus and the cercus of the genitalia. Scale Bar = 50 μ m. B) Venn diagram summarizing *fru* and *dsx* expression pattern restricted by *FLP335*. The sexually dimorphic LAN1 and ANN1 arbors originated from the GRNs on the front legs and MSNs of the

genitalia were the only common expression observed in both GAL4 lines. C-F) Expression profile of DsxGAL4,FLP335>GFP. The CNS tissues were stained with anti-mCD8 (green) and anti-nc82 (magenta). No expression was observed in the brain (C), and the ventral nerve cord showed expression for the sexually dimorphic LAN1 arbors that originated from the GRNs in the front legs and the sexually dimorphic ANN1 arbors that originated from the MSNs in the genitalia (D). E) The genitalia were stained with anti-mCD8 (green), autofluorescence (magenta). Expression was observed in the MSNs located on CER, SUR, and EVL of the genitalia. Scale Bar = 50 μ m. F) GFP expression on the genitalia overlaid with a DIC image of the cuticle. F) Effect of silencing FLP335 restricted labeling of dsx-GAL4. The copulation duration was shortened by ~1.6 fold in the experimental line compared to the control (DsxGAL4,FLP335>TNTin: n=22; DsxGAL4,FLP335 >TNT: n=24; black dots are individual values; red lines indicate the median with interquartile range; **p<0.0029, ***p<0.0001 by Mann Whitney test). G) Pheromone requirement in the regulation of copulation duration. Mating pairs were set up between males expressing TNT in FLP335 restricted dsx neurons (TNT and TNT-in=inactive control) and either females with ablated oenocytes (oe-) or their genetic controls (oe+). When paired with control females, the copulation duration was shortened by 1.9-fold for the experimental line (active TNT) compared to the control line with an inactive TNT expressed in the subset of dsx neurons restricted by FLP335 (FLP335, dsx>TNTin: n=33; FLP335, dsx>TNT: n=40). Black dots indicate individual values; red lines indicate the median with interquartile range. One-way ANOVA Kruskal-Wallis, adjusted p value at <0.0001 using Dunn's multiple comparisons test). When paired with oe- females, copulation duration was shortened by 1.2-fold in the experimental males compared to the control males (DsxGAL4,FLP335>TNTin: n=37; DsxGAL4,FLP335>TNT: n=28; One-way ANOVA Kruskal-Wallis, adjusted p value at <0.0289 using Dunn's multiple comparisons test). When paired with control males (DsxGAL4,FLP335>TNTin), oe- females led to a small but significant reduction of 1.1-fold in copulation duration compared to the oe+ control (DsxGAL4,FLP335>TNTin vs oe+: n=33, DsxGAL4,FLP335>TNTin vs. oe-: n=37; One-way ANOVA Kruskal-Wallis, adjusted p value at <0.0088 using Dunn's multiple comparisons test). When paired with experimental males (DsxGAL4,FLP335>TNT), no significant difference was observed between oe+ and oe- females (DsxGAL4,FLP335>TNT vs oe+: n=40, DsxGAL4,FLP335>TNT vs. oe-: n=28; One-way ANOVA Kruskal-Wallis, adjusted p value at >0.9999, Dunn's multiple comparisons test).

Figure 4

Sensory information detected by fru/dsx-MSNs is necessary to maintain copulation persistence. Mating pairs were set up between males expressing TNT in either FLP335 or FLP386 restricted dsx or fru neurons and CS females. A) The fertility of the female after a single copulation event was assessed. Percentage of females that produced progeny (FruGAL4,FLP335,tshGAL80>TNTin: n=15; FruGAL4,FLP335,tshGAL80>TNT: n=15; FruGAL4,FLP386,tshGAL80>TNTin: n=19; FruGAL4,FLP386,tshGAL80>TNT: n=16; DsxGAL4,FLP335>TNTin: n=27; DsxGAL4,FLP335>TNT: n=26) n.s.=not significant, **p = 0.0063 by Fisher's exact test. Females mated with males of FruGAL4,FLP335,tshGAL80>TNT, which included both copulation duration regulating neural clusters (fru-sAbg-1 and fru/dsx-MSNs) were less likely to produce progeny compared to the TNT-in controls. On the other hand, females mated with males of FruGAL4,FLP386,tshGAL80>TNT or dsxGAL4,FLP335>TNT, both of which include only one copulation duration regulating neural cluster (fru/dsx-MSNs) had normal fertility. B) Copulation persistence was assessed by heat shocking copulating pairs within the first 5 mins of copulation initiation. Percentage of mating pairs that remained copulated during heat shock (FruGAL4,FLP335,tshGAL80>TNTin: n=13; FruGAL4,FLP335,tshGAL80>TNT: n=10; FruGAL4,FLP386,tshGAL80>TNTin: n=18; FruGAL4,FLP386,tshGAL80>TNT: n=15; DsxGAL4,FLP335>TNTin: n=30; DsxGAL4,FLP335>TNT: n=22) ****p<0.0001, **p=0.0045, by Fisher's exact test. TNT-silencing of neurons by the three genetic combinations all resulted in higher percentage of males that terminated copulation upon heat shock compared to their respective TNT-in controls.

Figure 5

Proposed model of how sensory information from the MSNs is incorporated into the neural circuit that governs copulation persistence. Genital coupling requires the activation of the dsx/vGlut motor neurons that innervate the copulatory muscles. The resulting muscular tension is proportional to the activity level of the dsx/vGlut motor neurons, which are antagonistically regulated by dsx/GABA interneurons. These copulation regulating neurons are functionally connected to the axonal terminals of sensory neurons

from the genitalia bristles³⁴. We propose that a suboptimal sensory input will reduce the total muscular tension due to less activation of the dsx/vGlut motor neurons or more activation of the dsx/GABA interneurons. Copulation persistence ensures the maintenance of copulation before sperm transfer in the presence of stressful stimulus and is regulated by opposing actions of dsx/GABA and the dopaminergic system in the ventral cord²⁴. This persistence requires optimal sensory input from the genitalia bristles.