Sex-specific regulation of Lgr3 in Drosophila neurons

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The development of sexually dimorphic morphology and the potential for sexually dimorphic behavior in Drosophila are regulated by the fruitless (Fru) and doublesex (Dsx) transcription factors. Several direct targets of Dsx have been identified, but direct Fru targets have not been definitively identified. We show that Drosophila leucine-rich repeat G protein–coupled receptor 3 (Lgr3) is regulated by Fru and Dsx in separate populations of neurons. Lgr3 is a member of the relaxin-receptor family and a receptor for Dllp8, necessary for control of organ growth. Lgr3 expression in the anterior central brain of males is inhibited by the B isoform of Fru, whose DNA binding domain interacts with a short region of an Lgr3 intron. Fru A and C isoform mutants had no observed effect on Lgr3 expression. The female form of Dsx (DsxF) separately up- and down-regulates Lgr3 expression in distinct neurons in the abdominal ganglion through female- and male-specific Lgr3 enhancers. Excitation of neural activity in the DsxF–up-regulated abdominal ganglion neurons inhibits female receptivity, indicating the importance of these neurons for sexual behavior. Coordinated regulation of Lgr3 by Fru and Dsx marks a point of convergence of the two branches of the sex-determination hierarchy.

Lgr3 | fruitless | doublesex | Drosophila | enhancer

Most animal species are comprised of female and male individuals, in which sex differences in form and behavior are specified by their genetic makeup. The developmental processes by which genes build sex-specific differences into the nervous system, and hence encode the potential for sex-specific behavior, have long been of interest (1).

In Drosophila melanogaster the assessment of the number of X chromosomes leads to sex-differential splicing of transcripts from genes making up the sex-determination hierarchy, in particular the terminal genes of that hierarchy, fruitless (fru) and doublesex [dsx (2), reviewed in ref. 3]. fru and dsx encode sex-specific Zn-finger transcription factors that alter, either directly or indirectly, the expression of downstream genes to produce the sexually dimorphic elements of flies. The male-specific forms of Fru (FruM) act in a subset of the neurons within the male’s nervous system to establish the potential for social interactions such as courtship behavior and aggression (reviewed in ref. 3). In contrast, Dsx acts in subsets of both neural and nonneural tissues of males and females to regulate behavioral and nonbehavioral aspects of sexual development (reviewed in ref. 3).

Although the mechanisms regulating the production of the sex-specific isoforms of the Fru and Dsx proteins are well-established (4), how these proteins in turn function is only beginning to be elucidated. Several direct Dsx targets and a well-conserved 13-bp Dsx binding site have been identified (5–13). Many Dsx target genes encode well-known transcription factors and cell–cell signaling molecules that function sex-nonspecifically in most tissues in which they are expressed. However, in other tissues, Dsx directs the sex-specific expression of these genes to generate sex-specific aspects of development.

FruM regulatory function has thus far proven to be less tractable than that of Dsx. FruM appears to function in a complex with the transcription cofactor Bonus and either histone deacetylase 1 or heterochromatin protein 1a (14). Recent genome-wide screens of RNA expression levels or FruM binding activity have identified potential FruM targets, but have lacked independent confirmation of such regulation (15–17). Thus, our understanding of how FruM specifies the potential for sex-specific behavior remains limited.

Along with the study of the genetic targets of Dsx and FruM, studying the control of neuronal function by genomic enhancer elements has identified behavioral roles for Dsx- and FruM-expressing neurons (3, 18–20). In a screen for additional genomic enhancer elements that drive sexually dimorphic nervous system expression, we identified an enhancer derived from the Lgr3 gene. Lgr3 is a member of the leucine-rich-repeat G protein–coupled receptor (Lgr) family (21). The Drosophila genome contains four members of the Lgr family, including Lgr2, encoded by the rickets gene and necessary for tanning of the adult cuticle in response to the hormone bursicon (22). Lgr-related genes are also present in humans and include those encoding relaxin-family peptide receptors RXFP1 and RXFP2, which among other functions are necessary for normal reproduction in both sexes (reviewed in ref. 23).

We investigated the sex-specific regulation of Lgr3 expression and its functional importance in female sexual behavior. We identified roles for both FruM and Dsx in regulating Lgr3 expression in separate sets of neurons. One isoform of Fru inhibits Lgr3 expression in the male brain, whereas Dsx activates Lgr3 expression in some neurons in the female abdominal ganglion and represses it in others. To better understand the basis of this complex regulation of Lgr3 expression, potential enhancer fragments from the Lgr3 locus were used to narrow down the sites of Dsx and Dsx activity. We found that FruM interacts with a specific portion of an Lgr3 intron, suggesting its regulation may be direct. Finally, activation of a subset of Lgr3-expressing abdominal ganglion neurons reduced female receptivity to courtship, indicating the functional importance of sex-specific Lgr3 expression.

Results

We examined the expression of the Lgr3 gene using a bacterial artificial chromosome (BAC) reporter, Lgr3-GAL4:VP16, encompassing the Lgr3 locus, with the GAL4 DNA-binding domain and VP16 activation domain inserted in place of the first coding exon of Lgr3 (Fig. 1A). Lgr3-GAL4 drives expression of a UAS-GFP reporter construct in the abdominal ganglion, the site of functionally important Lgr3 expression. We used the UAS-GFP reporter to test the enhancer function of the Dsx protein. Lgr3-GAL4 downstream of FruM was used to test the enhancer function of FruM regulatory activity.

Significance

For individuals to develop sexually dimorphic body parts and behavior, their cells must know their sex. In the fruit fly Drosophila melanogaster, this process is carried out by a series of genes ending with fruitless (fru) and doublesex (dsx). We found that both Fru and Dsx regulate the expression of the leucine-rich repeat G protein–coupled receptor 3 (Lgr3) gene in separate sets of neurons, including neurons important for female sexual behavior. Thus, Lgr3 is important for sexual development and is a point of convergence after the fru/dsx split.

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Sexually dimorphic expression of function using \(12\text{.}8\) was significantly increased from control males (\(P < 0.001\), but still lower (\(PNAS < 0.01\)). Error bars indicate SEM.

To verify that the expression of the Lgr3-GAL4 reporter accurately reflects expression of the Lgr3 gene, we compared Lgr3-GAL4’s expression pattern to the pattern of in situ hybridization of Lgr3 probes to transcripts in sections of males and females (Fig. 1C and Fig. S1 B and C). Lgr3 antisense probe signal colocalized with Lgr3-GAL4 expression in both males and females, whereas the sense probe control lacked any observable coexpression (Fig. 1C). Expression of both GFP and the in situ probe appeared greater in females than males. Additional antisense probes targeting other regions of Lgr3 showed a similar pattern (Fig. S1D). Although otherwise coexpressed, hybridization signal in the abdominal ganglion was present in a few cells beyond the Lgr3-GAL4 pattern in both sexes.

**Fru\({\text{M}}\)** Inhibits Expression of Lgr3 in the Male Median Bundle. To understand the basis of sexually dimorphic Lgr3 expression in the median bundle, we examined the dependence of Lgr3-GAL4 expression on Fru\({\text{M}}\) and Dsx. Dsx expression has not been detected in the median bundle (24, 25), and dsex loss-of-function mutants had no effect on Lgr3-GAL4 expression in the median bundle (Fig. S2). In contrast, Fru\({\text{M}}\) is expressed in the median bundle (26), and we observed coexpression of a LexA reporter for fru\({\text{M}}\) (fru\({\text{M}}\text{-}\text{LexA}\) (27) and Lgr3-GAL4 when examined with fluorescent nuclear reporters in the median bundle (Fig. 1D). Similar coexpression was seen when LexAop2-FlipL and UAS > stop > myrGFP were used to perform Flippase-mediated genetic intersection, in which fru-LexA and Lgr3-GAL4 are both needed to cause Flip to remove the stop codon in UAS > stop > myrGFP and the production of GFP (Fig. 1E). In a fru\({\text{M}}\) loss-of-function mutant (fru\({\text{M}}^-\text{fru}\text{-}\text{LexA}\), Lgr3-GAL4, UAS-GFP expression in males was restored to nearly female levels, indicating that wild-type Fru\({\text{M}}\) likely inhibits Lgr3-GAL4 expression in males (Fig. 1 F and G).

Differences in the numbers of cells expressing Lgr3-GAL4 could be a result of Lgr3-GAL4 expression being altered in existing cells, or to the death of some of those cells. Prior studies of Fru\({\text{M}}\) expression reported cell number dimorphism in other subsets of Fru\({\text{M}}\) neurons, but not in the median bundle (28, 29).

**Fig. 1.** Sexually dimorphic expression of Lgr3. (A) To create Lgr3-GAL4::VP16, GAL4::VP16 was inserted into a BAC containing the Lgr3 locus, along with \(\sim 1\text{-kb upstream and 6.6-kb downstream sequence, the latter including several neighboring genes. Sequence encoding the GAL4 DNA-binding domain and VP16 activation domain replaced the first coding exon of Lgr3. (B) Lgr3-GAL4 (attP40), UAS-myrGFP (attP2) shows higher expression in the female median bundle compared with the male median bundle. The brain and VNC were imaged in separate 20x tiles and composited. (Inset) Lgr3-GAL4 expression in the abdominal ganglion at a higher gain and approximately 2.4x higher magnification. (Scale bar, 100 \(\mu\text{m}\).) (C) DIG-labeled in situ hybridization of Lgr3 antisense probe (in red) shows correspondence with Lgr3-GAL4 (attP40), UAS-myrGFP (su(Hw)attP5) (in green) in horizontal sections through the male and female brain and abdominal ganglion. The sense probe did not show appreciable overlap with GFP. The membrane-bound GFP generally surrounds the mRNA signal in neurons with both labels. DAPI nuclear label (in blue) is included for reference. (Scale bar, 50 \(\mu\text{m}\).) (D) Lgr3-GAL4, UAS-Stinger-GFP (green) overlaps with fru\({\text{M}}^-\text{LexA}, \text{LexAop2-FlipL} > \text{tdTomato} (\text{magenta}) in females, but less so in males. Overlap is in white. (Scale bar, 50 \(\mu\text{m}\).) (E) Intersection of Lgr3-GAL4 (attP40) with fru\({\text{M}}^-\text{LexA}, \text{LexAop2-FlipL} (\text{attP40}) and UAS > stop > myrGFP (su(Hw)attP5) leads to GFP expression primarily in females (\(8.6 \pm 0.9\) cells in females vs. \(0.6 \pm 0.2\) in males, \(P = 0.001\) by Student’s \(t\) test, \(n = 12–14\) hemispheres). GFP expression occurs only in cells expressing both Lgr3 and fru\({\text{M}}^-. \) Scale bar, 50 \(\mu\text{m}\).) (F) Lgr3-GAL4 (attP40), UAS-myrGFP (su(Hw)attP5) shows dependence on fru\({\text{M}}^-\text{stop}\text{fru}\text{-}\text{LexA}\) elimination of fru\({\text{M}}^-\text{stop}\text{fru}\text{-}\text{LexA}\) brings male expression close to female levels. (Scale bar, 50 \(\mu\text{m}\).) (G) Cell counts for F. Expression in control females was significantly broader than in control males (\(20.6 \pm 0.7\) cells vs. \(6.1 \pm 0.7\), \(P < 0.001\) by ANOVA and Tukey post hoc test, \(n = 8–10\) brain hemispheres). fru\({\text{M}}^-\text{fru}\text{-}\text{LexA}\) male expression (17.9 \(\pm 0.8\)) was significantly increased from control males (\(P < 0.001\)), but still lower than in the female (20.9 \(\pm 0.7\)), by a small amount (\(P < 0.01\)). Error bars indicate SEM.
Cachero et al. (28) in particular counted the cells produced by neuroblast clones in both sexes and did not detect a difference. We verified that the number of Fru<sup>4</sup>-expressing median bundle neurons did not significantly differ between females and males (66.6 ± 2.3 cells vs. 68.1 ± 1.8, P = 0.6 by Student’s t test, n = 22–26 hemispheres). Thus, differences in Lgr3-GAL4 median bundle expression do not appear to result from cell death.

**FrU<sup>4</sup> Acts Through a Small Region of an Lgr3 Intron.** To determine the region of the Lgr3 locus through which Fru<sup>4</sup> regulates Lgr3 median bundle expression, we asked whether various fragments of the Lgr3 gene contained enhancer elements sufficient to confer Fru<sup>4</sup>-dependent enhancer-GAL4 expression (Fig. 2A) (30). Of the five Lgr3 fragments tested in this manner, only R19B09-GAL4, a fragment from the largest Lgr3 intron, conferred sexually dimorphic median bundle expression similar to that exhibited by Lgr3-GAL4 (Fig. 3). R19B09 thus likely contains the enhancer sequences necessary for the median bundle expression observed with Lgr3. To further localize these enhancer sequences, we subdivided the 3,683-bp R19B09-GAL4 reporter into smaller fragments, each of which was assayed for sexually dimorphic GAL4 expression (Fig. 2B and Fig. S3). Two rounds of such subdivisions yielded a 484-bp reporter termed R19B09.3A-GAL4 that maintained dimorphic median bundle expression (Fig. 2 B–D). As with Lgr3-GAL4, the expression of R19B09.3A-GAL4 both colocalizes with fru-LexA in females and is inhibited by Fru<sup>4</sup> in males (Fig. 2 E–H).

The fru gene encodes multiple transcripts through the use of alternative promoters in combination with sex-specific and sex-nonspecific alternative pre-mRNA splicing. The fru mRNAs thus generated are translated into proteins with different C-terminal zinc-finger DNA-binding domains. Of these, isoforms A, B, and C have detectable nervous system expression and are candidates for regulators of Lgr3 expression in the male nervous system (31). We examined the expression of R19B09.3A in flies individually mutant for the A, B, or C isoforms (13, 28), and found that R19B09.3A expression was only affected by the absence of Fru<sup>B</sup> (Fig. 3 A and B). Fru<sup>A</sup> and Fru<sup>B</sup> mutants had no effect on R19B09.3A expression, whereas in Fru<sup>C</sup> mutant males R19B09.3A expression is increased to female levels, suggesting that Fru<sup>B</sup> negatively affects R19B09.3A-dependent expression. Using pan-neuronally expressed microRNAs targeting transcripts of the A–C fru isoforms, we similarly observed that knockdown only of Fru<sup>B</sup> brought male expression to female levels.

![Fig. 2. R19B09.3A-GAL4 recapitulates dimorphic Lgr3 median bundle expression.](image-url)

(A) Rubin GAL4 enhancer fragment lines contain sequences from the indicated regions of the Lgr3 locus. (B) R19B09-GAL4 was subdivided in two rounds to identify a smaller region driving dimorphic median bundle expression. Fragments in red showed dimorphic median bundle expression, whereas those in black had other patterns of expression (Fig. S3). (C) Expression of R19B09.3A-GAL4, UAS-myRFP (attP40) is sexually dimorphic in the female and male median bundle. UAS-GFP in (attP40) has basal expression in the labial nerve (arrowheads) (44). The brain and VNC were imaged in separate 20× tiles and composited. (Scale bar, 100 μm.) (D) R19B09-LexA, LexAop2-IVS-nlsTomato (VK22) colocalizes with Lgr3-GAL4, UAS-Stinger-GFP in females, but both have low male expression. (Scale bar, 50 μm.) (E) R19B09.3A-GAL4 (attP2), UAS-myRFP (attP40) shows dependence on Fru<sup>4</sup>. Elimination of Fru<sup>4</sup> function using fru<sup>4</sup>fru<sup>4-40</sup> brings male expression to female levels. (Scale bar, 50 μm.) (F) Cell counts for E. Expression in control females was significantly broader than in males (14.2 ± 0.8 cells vs. 3.4 ± 0.5, P < 0.001 by ANOVA and Tukey post hoc test, n = 4–10 brain hemispheres). fru<sup>4</sup>fru<sup>4-40</sup> male expression was indistinguishable from females (13.6 ± 0.5 vs. 13.9 ± 0.5, P > 0.5). Error bars indicate SEM. (G) R19B09.3A-GAL4, UAS-RedStinger colocalizes with fru<sup>4</sup>-LexA, LexAop-Stinger-GFP in females but not males. (Scale bar, 50 μm.) (H) Intersection of R19B09.3A-GAL4 (attP2) with fru<sup>4</sup>-LexA using LexAop2-Fpl (attP40) and UAS > stop > myRFP (su(Hw)attP1) shows expression in females but not in males. (Scale bar, 50 μm.)
levels (Fig. S4). Thus, FruB appears likely to inhibit Lgr3 median bundle expression via this fragment of an Lgr3 intron.

Having delimited a relatively short Lgr3 fragment that confers regulation by FruM, we asked whether this regulation is direct. To do so, we performed EMSAs examining the binding of the Fru A, B, and C DNA-binding domains (DBDs) to probes tiled across the R19B09.3A fragment (Fig. 3 C–E and Fig. S5). We observed several regions that are bound by Fru-DBD-A (Fig. S5A) or Fru-DBD-B domains (Fig. 3C), suggesting Fru DBDs can directly bind sequences from the Lgr3 gene in vitro.

We subdivided each bound probe roughly into thirds and used additional EMSAs to further refine the regions important for Fru binding. In addition, we designed competitor oligos with mutations tiled across the smaller probes (Fig. 3D and Fig. S5B). Competitors with mutations at residues unimportant for binding outcompete labeled probes, reducing or eliminating the labeled probe/protein complex. Competitors with mutations at residues important for binding leave the complex unaffected, allowing identification of necessary bases (e.g., competitor m4 in Fig. 3D). We made single base mutations in the competitor to provide

Fig. 3. FruB regulates R19B09.3A-GAL4 expression. (A) Expression of R19B09.3A-GAL4, UAS-myrGFP (attP40) in females and males carrying heteroallelic combinations of fruLexA with either a wild-type, fruΔA, fruΔB, or fruΔC fru isoform mutant as indicated. (Scale bar, 50 μm.) (B) Cell counts for A. Expression in males was significantly lower than in females for flies carrying heteroallelic combinations of fruLexA with either wild-type, fruΔA, or fruΔC (P < 0.001 by Mann–Whitney u test with Bonferroni correction on each male/female pair, n = 18–30 hemispheres). However, in fruLexA/fruΔB individuals the expression of R19B09.3A-GAL4, UAS-myrGFP (attP40) did not differ between males and females (P > 0.1). Error bars indicate SEM. (C) EMSA showing binding of purified Fru-DBD-B protein to probe #3 from R19B09.3A (Fig. S5 C and D). A decreasing gradient in the amount of protein was used to show the binding kinetics. The bands for free probe and protein-DNA complex are indicated. (D) Tiled substitutions in a shorter probe derived from probe #3 were used as competitors in the EMSA to identify the specific position of Fru-DBD-B binding. The sequence of the labeled probe and the mutant sequences used as competitors are listed below the gel with the substituted nucleotides indicated in red lowercase. (E) Single base substitutions in the probe were used as competitors in the EMSA to examine the contribution of each position to the Fru-DBD-B binding site.
maximal resolution of binding specificity (Fig. 3E). Potential Fru binding sites have been explored previously (15, 16). The sites we identified generally agree with the consensus sites previously reported. Notably, although Dalton et al. (15) identified Fru binding motifs in Lgr3, it did not rise above their thresholds for responding to Fru expression, perhaps because of the small number of neurons affected.

Anticipating that binding at multiple sites may be necessary for Fru function, we mutated our identified sites individually and in multiple combinations in the R19B09.3A reporter (Fig. S5 E and F). Examining these combinations in females and males identified several sites necessary for expression in both sexes, but no tested single mutation or combination thereof increases expression in males to female-like levels (Fig. S5 G and H). As the fourth Fru-binding site has particularly strong effects on expression, we explored further single base changes across it, but observed minimal effects on expression (Fig. S5 G and H). We hypothesize that a transcriptional activator may bind the same sites as Fru, such that mutations of the shared binding site reduce or eliminate expression in both sexes. Alternatively, the full-length Fru protein in vivo could require a longer binding site or additional cofactors for binding.

Dsx<sup>+</sup> Activates and Inhibits Lgr3 Expression in Different Abdominal Ganglion Neurons. Although the sexually dimorphic pattern of Lgr3 expression in the median bundle is relatively straightforward, with females having broader expression than males, we observed a more complex Lgr3-GAL4 pattern in the abdominal ganglion, where both females and males express Lgr3-GAL4, but in different patterns (Fig. 4A). Mutants in fru<sup>−</sup> did not appear to change these Lgr3-GAL4 abdominal ganglion expression patterns (Fig. 3E), but mutations in dsx did alter these patterns (Fig. 4A). As with Lgr3 expression in the brain median bundle, we examined the expression of Lgr3 enhancer fragment lines (Fig. 2A) to determine if any confer sex-specific expression in the abdominal ganglion. Of the five lines examined, R17G11-GAL4 and R17H01-GAL4 replicate subsets of the Lgr3 pattern in the abdominal ganglion. As before, we examined how these two enhancer fragments relate to Lgr3, fru, and dsx to determine the basis of their sexually dimorphic expression and how it relates to the sexual dimorphisms seen in Lgr3.

The first of the two fragments mentioned above, R17G11-GAL4, is expressed in several abdominal ganglion cells in males, but not in females (Fig. 4B). R17G11-GAL4 also directs non-dimorphic expression in the brain. To compare the abdominal ganglion expression driven by the R17G11 enhancer fragment to that driven by Lgr3-GAL4 and dsx<sup>−</sup>LexA<sup>−</sup>, we replaced the GAL4 sequence in R17G11-GAL4 with LexA to make R17G11-LexA. R17G11-LexA showed coexpression with Lgr3-GAL4 in the male abdominal ganglion, but not in the female (Fig. 4C).

We examined the intersection between R17G11-LexA and dsx<sup>−</sup>LexA<sup>−</sup> as well as between R17G11-GAL4 and fru-LexA, and
in both cases we observed expression in the male abdominal ganglion, but little or none in females (Fig. 4 D and E). Examination of R17G11-GAL4 expression in fru and dsx loss-of-function mutants indicated that Dsx (Fig. 4F), but not Fru (Fig. S6B), regulates R17G11. Unlike Fru, Dsx has functional forms in both sexes. As loss-of-function mutations of dsx led to expression of R17G11-GAL4 in females, it appears that the female form of Dsx, Dsx⁻, either inhibits R17G11 expression or the survival of these neurons in females.

The second of the two fragments mentioned above, R17H01-GAL4, showed a pattern of regulation in the abdominal ganglion opposite to that of R17G11-GAL4. R17H01-GAL4 has expression in several abdominal ganglion cells in females, but fewer cells in males (Fig. 5A). As with R17G11-GAL4, we replaced the GAL4 in R17H01-GAL4 with LexA to make R17H01-LexA to compare it with Lgr3-GAL4 and dsx-GAL4. Coexpression of R17H01-LexA with Lgr3-GAL4 was seen in females, but not in males (Fig. 5B). Comparisons of the expression patterns of R17H01-LexA with dsx and R17H01-GAL4 with fru-LexA indicated minimal coexpression with fru, but coexpression with dsx especially in females (Fig. 5 C and D). Examination of R17H01-GAL4 expression in fru and dsx loss-of-function mutants indicated that Dsx (Fig. 5E), but not Fru (Fig. S6C), regulates R17H01-GAL4. Loss-of-function mutations of dsx largely eliminate R17H01-GAL4 expression in the female abdominal ganglion but have no clear effect in males. Thus, it appears that Dsx⁻ again plays the controlling role, but in this case it activates R17H01 expression in females, as opposed to the inhibition of R17G11. The effects of Dsx⁻ on Lgr3-GAL4 expression in the abdominal ganglion may be the simple additive sum of its effects on R17H01 and R17G11, with each enhancer driving expression in the appropriate subsets of neurons.

The above results indicate that Dsx regulates Lgr3 expression in the abdominal ganglion, but do not reveal whether this regulation is

![Image](https://example.com/image1.png)

**Fig. 5.** R17H01-GAL4 expression depends on Dsx⁻. (A) R17H01-GAL4 (attP2), UAS-myrGFP (attP40) expression in females and males. Sexually dimorphic abdominal ganglion expression is circled. The brain and VNC were imaged in separate 20x tiles and composited. (Scale bar, 100 μm.) (B) Expression of R17H01-LexA::p65 (attP2) and Lgr3-GAL4 (VK33) driving UAS-IvS-mCD8::RFP (attP18) and LexAop2-mCD8::GFP (su(Hw)attP8) in females and males. Coexpression is seen in females but not males. (C) R17H01-LexA::p65 (attP2), LexAop2-IvS-nlsTomato (VK22) has some colocalization with dsx-GAL4. UAS-Stinger-GFP in females, but less so in males. (D) Intersection of R17H01-GAL4 (attP2) with fru-LexA using LexAop2-Fpl (attP40) and UAS > stop > myrGFP (su(Hw)attP7) (Scale bar, 50 μm.) (E) R17H01-GAL4 (attP2), UAS-myrGFP (attP40) expression in the female and male abdominal ganglion with dsx-GAL4 and either the TM6B balancer or dsx-MdR15. (Scale bar, 50 μm.) (F) R17H01dm-GAL4 (attP2), UAS-myrGFP (attP40) expression in the female and male abdominal ganglion. (Scale bar, 50 μm.) (G) Line scans of R17H01-GAL4 and R17H01dm-GAL4 expression in the abdominal ganglion and metathoracic neuromere. R17H01dm-GAL4, UAS-myrGFP (attP2) females and males were compared with a mixture of R17H01-GAL4, UAS-myrGFP (attP40) heterozygous controls (dsx⁻¹⁶⁴⁹⁻¹⁰⁹⁸⁻¹⁺, dsx⁻¹⁶⁴³⁻¹⁰⁸⁵⁻¹⁺, and fru⁻¹⁶⁴⁳⁻¹ and R17H01-GAL4, UAS-myrGFP (attP40) dsx mutants (dsx⁻¹⁶⁴³⁻¹⁰⁹⁸⁻¹⁺, dsx⁻¹⁶⁴⁹⁻¹⁰⁹⁸⁻¹⁺, dsx⁻¹⁶⁴⁹⁻¹⁰⁹⁸⁻¹⁺, and dsx⁻¹⁶⁴³⁻¹⁰⁹⁸⁻¹⁺).
direct, or a result of indirect effects such as Dsx-dependent cell death. We attempted to address this question, as described below. Previous work identified regions of likely Dsx DNA-binding activity and a consensus 13-bp Dsx binding motif (GCAACAATGTGGTTCG) (5, 13, 32). The Lgr3 locus contains three potential Dsx binding sites: a 9/13-bp partial binding site match in R17G11 (GagACAATGTGaC, with mismatches in lowercase), and two 10- and 11-bp partial matching sites in R17H01 (GCAACAATGTGaGT and GtaACAATGTGGTC). We focused on R17H01 because of its closer matches to the 13-bp motif identified previously (5). We mutated these putative binding sites to disrupt Dsx binding, and hence suggest whether Dsx directly regulates R17H01 expression. Mutation of the R17H01-GAL4 Dsx binding sites (changed to GCAtgcagGT and GtTCAcacGC, respectively, with mutated bases in lowercase) created R17H01dm-GAL4, where “dm” denotes the presence of mutated DSX binding sites.

R17H01dm-GAL4 driving UAS-GFP yielded a moderate decrease in female abdominal ganglion expression compared with R17H01-GAL4 and no change in males (Fig. 5 F–H). Because the change in expression in Fig. 5F is subtle, several ventral nerve cords (VNCs) were registered together, and expression of R17H01dm-GAL4 in the abdominal ganglion was compared with R17H01-GAL4 in wild-type and dsx loss-of-function mutants (Fig. 5G). To quantitate the changes in expression, a line scan analysis was performed along the two lines shown in Fig. 5G, the upper control line and the lower line across the abdominal ganglion (Fig. 5H). R17H01dm-GAL4 shows a reduction in expression intensity across the female abdominal ganglion, but not across the mesothoracic neuropere. This reduction was intermediate between control and dsx loss-of-function mutant R17H01-GAL4 females.

Thus, it appears that DxsF may directly regulate a fraction of R17H01-GAL4, and hence Lgr3, expression. However, other aspects of Lgr3 abdominal ganglion expression may be regulated indirectly or by enhancers in R17H01 outside the Dsx binding sites. The latter case was recently observed with Dsx regulation of Fmo-2 expression, in which both a Dsx binding site and nearby enhancers were necessary for the full pattern of sexually dimorphic expression (12).

**Female Lgr3-Expressing Neurons Regulate Sexual Behavior.** To begin to understand the functional significance of sex-specific Lgr3 regulation, we stimulated Lgr3 neurons using the thermogenetic activator UAS-dTrpA1 and assayed changes in courtship behavior. We placed virgin male and female pairs in courtship chambers and (i) measured latency to courtship initiation by males (indicated by their first wing extension directed at females), (ii) measured latency to successful copulation, and (iii) looked for other obvious changes in behavior. Activation in females of R17H01-GAL4 neurons inhibits female receptivity to female courtship, as indicated by delayed or no copulation during the observation period (Fig. 6). Control females consisted of pBDP-GAL4u (which lacks an enhancer insertion) with UAS-dTrpA1 and R17H01-GAL4 without UAS-dTrpA1. Performance of the two controls was not significantly different (P = 0.2 for courtship latency and P = 0.4 for copulation latency, by log-rank test, n = 130 and 47, respectively), and the genotypes were pooled for further comparisons. Although males initiated courtship of R17H01 > dTrpA1 and control females at indistinguishable speeds (P = 0.15, log-rank test) (Fig. 6A), R17H01 > dTrpA1 female receptivity to courtship was significantly reduced at the Trp-activating temperature of 29 °C (P < 0.001, log-rank test).
test, n = 116–177) (Fig. 6B). No difference was seen between the experimental and control groups in courtship or copulation latency at 22 °C, where Trp is inactive (courtship latency P = 0.3, copulation latency P = 0.6, log-rank test, n = 78–115) (Fig. 6C and D).

After observing their courtship, as described above, we briefly anesthetized the male/female pairs and placed them into food vials to measure their fecundity. Pairs were maintained at 29 °C or 22 °C until offspring were counted at the pharate adult stage. R17H01 > dTrpA1 females displayed significantly lower fecundity than pbDPGAL4u > dTrpA1 or R17H01/+ females at 29 °C (P < 0.001 for both, Kruskal–Wallace test then Dunn post hoc with Bonferroni correction, n = 82–90) (Fig. 6E). This difference was not simply because of a failure to copulate: rather than being a mix of completely infertile and unaffected females, most R17H01 > dTrpA1 females produced fewer offspring, (Fig. 6E).

At 22 °C, fecundity was somewhat variable between control genotypes, but all were significantly above R17H01 > dTrpA1 at 29 °C (P < 0.001 for each, Kruskal–Wallace test then Dunn post hoc with Bonferroni correction) (Fig. 6F).

Although the effects of Dsx-binding-site mutations on R17H01dm-GAL4 expression were subtle, we asked whether they were sufficient to alter the phenotype observed in tests of R17H01 > dTrpA1 females (Fig. 6). At 29 °C, R17H01dm > dTrpA1 females were counted as prompt courtship inducers (courtship latency P = 0.2, log-rank test, n = 48–116) (Fig. 6D) but showed a strong increase in receptivity (P < 0.001, log-rank test) (Fig. 6B). R17H01dm > dTrpA1 female receptivity was statistically indistinguishable from controls (P = 0.3, log-rank test, n = 46–178) (Fig. 6B). At 22 °C there was a slight, but significant, delay in courtship toward R17H01dm > dTrpA1 females (P = 0.043, log-rank test, n = 36–78) (Fig. 6C), but no difference in receptivity (P = 0.3, log-rank test) (Fig. 6D). Similarly, R17H01dm > dTrpA1 females showed a significant recovery in fecundity at 29 °C compared with R17H01 > dTrpA1 (P < 0.001, Kruskal–Wallace test then Dunn post hoc with Bonferroni correction, n = 47–90) (Fig. 6E), but no difference at 22 °C (P = 1, Kruskal–Wallace test then Dunn post hoc with Bonferroni correction, n = 36–69) (Fig. 6E and F).

Thus, although GFP expression was only partially reduced in R17H01dm flies, it appears that the Dsx binding site-dependent effects on R17H01 expression account for most or all of its effects on female receptivity and fecundity.

Other recent studies have also examined the abdominal ganglia for Dsx-expressing neurons competitive for female reproductive behavior in Drosophila (18, 19, 33–35). One question is whether the neurons we report here are the same or different from those previously reported. Gou et al. (33) focused on ascending neurons from the reproductive tract, and both Fang et al. (34) and Bussell et al. (35) reported stimulation of neurons favoring receptivity. In contrast, we observed that stimulation of R17H01-GAL4 neurons inhibits receptivity, suggesting the neurons examined here differ from the ones reported above.

Rezával et al. also identified two populations of Dsx-expressing neurons in the female abdominal ganglion that when activated cause females to become less receptive (18, 19). The relationship between the two populations, labeled by either the ET1L.P250 enhancer trap flippase or octopaminergic/tyraminergic Tdc2-GAL4 driver, is not entirely clear, but their overall morphology does not strongly resemble that of Lgr3-GAL4 or R17H01-GAL4. We compared the intersection of ET1L.P250 and dsl.GAL4[A2] with that of ETFL.P250 and Lgr3-GAL4 or R17H01-GAL4 (Fig. S7). ET1L.P250 intersected with R17H01-GAL4 does show a pattern similar to a subset of ETFL.P250 intersected with dsl.GAL4[A2], but ETFL.P250 intersected with Lgr3-GAL4 does not. Thus, it appears that ETFL.P250 coexpresses with separate populations of neurons in the two drivers, despite their otherwise strong correspondence (Fig. 5B). It is also notable that Rezával et al. (18) observed a strong effect of ETFL.P250, dsl.GAL4, UAS > stop > TrpA1 in females inhibiting the initiation of male courtship, whereas we observed prompt courtship initiation toward R17H01-GAL4, UAS-dTrpA1 females. As a result, although we cannot exclude the possibility that our observed reproductive phenotypes result from effects on the same neurons, it appears unlikely.

**Discussion**

We have shown that DsxF and FruM regulate the expression of Lgr3 in separate populations of Drosophila neurons. FruM inhibits Lgr3 expression in the median bundle in males, whereas DsxF inhibits Lgr3 expression in one population of abdominal ganglion neurons and activates expression in another population. This combined regulation of Lgr3 marks a point of convergence in sex determination after the Dsx/Fru split. Furthermore, we found that activation of Lgr3-expressing neurons inhibits female receptivity toward male courtship and lowers female fecundity, indicating the functional importance of these neurons.

In vitro binding of Fru-DBD-B to R19B09.3A suggests FruM could directly regulate Lgr3 expression in vivo in the median bundle. However, the functional consequences of this regulation remain unclear. FruM-expressing median bundle neurons have previously been shown to play an important role in coordinating steps of male courtship (36). Preventing Lgr3 expression in these neurons may play a role in specifying their male-specific function. Alternatively, Lgr3 median bundle expression could help specify female behavior, with FruM inhibition in males preventing a male-specific side effect.

Expression analysis of Drosophila Lgr3 and Lgr4 at the level of whole tissue was recently reported (37). Although significant differences in expression were reported between males and females, these were outside the central nervous system. The largest reported adult dimorphisms were higher male expression in salivary glands and higher female expression in the fat body. A lack of nervous system changes reported by Van Hiel et al. (37) may not conflict with our results, because only a small number of neurons were affected in our observations, and the changes in the abdominal ganglion were bidirectional.

The distant homology of Lgr3 to mammalian relaxin receptors RXFP1 and RXFP2 presents the possibility of related function, as well as related structure. These receptors and their ligands, relaxin and insulin-like peptide 3, respectively, are necessary for normal reproductive physiology in the male testis and female ovary, along with other functions (reviewed in refs. 23 and 38). This homology has led to the proposal that insulin-like peptides, especially Dilp8, function as ligands for Lgr3 in Drosophila (39). Dilp8 has been implicated in the regulation of developmental timing and growth in the larva (40, 41), and Lgr3 has recently been found to be necessary for this Dilp8 function (42–44). Lgr3 mutants appear to develop similarly to dilp8 mutants and prevent effects of Dilp8 overexpression, and some evidence has been found for direct interaction between them (44; see ref. 43).

It is unclear whether Dilp8 signaling is also important for Lgr3's reproductive function, but it is notable that Dilp8 is highly expressed by the adult female ovary (45). Thus, we hypothesize that the ovaries may signal their state via Dilp8 to Lgr3-expressing neurons in the abdominal ganglion, which then regulate aspects of female reproductive behavior. Regulation of Lgr3 expression by the sex-determination hierarchy may thus pattern the neural components of a sex-specific signaling pathway.

**Materials and Methods**

Transgenic Drosophila. Lgr3-GAL4::VP16 was generated by recombination-mediated replacement of the coding portion of the first Lgr3 coding exon with GAL4::VP16 in a bacterial artificial chromosome, which was then inserted into the Drosophila genome at defined attP sites. Other GAL4 reporter stocks were generated by standard methods (46). See SI Materials and Methods for detailed transgenic methods.

Immunohistochemistry, in Situ Hybridization, and Microscopy. For immunohistochemistry, adult fly central nervous systems were dissected in Schneider's insect medium and fixed in 4% (v/v) paraformaldehyde. Primary and secondary antibodies were applied to labeled neuropil and neuronal membranes.
Protein Expression and EMSA. Fzr A, B, and C DNA-binding domains were obtained by RT-PCR from wild-type flies and expressed in bacteria. EMSA was performed as described previously (5). See SI Materials and Methods for further description.

Courtship and Fecundity Assays. Four- to 7-d-old flies were aspirated into standard courtship chambers and allowed to recover. The divider between males and females was removed, and the latency until courtship initiation and successful copulation were measured. After filming, flies were CO2 narcotized, sexed, and moved to food vials. Numbers of pupae offspring were counted one week later. See SI Materials and Methods for a full description of behavioral methods.

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