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Control of sexual differentiation and behavior by the *doublesex* gene in *Drosophila melanogaster*

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Doublesex proteins, which are part of the structurally and functionally conserved *Dmrt* gene family, are important for sex determination throughout the animal kingdom. We inserted *Gal4* into the *doublesex* (*dsx*) locus of *Drosophila melanogaster*, allowing us to visualize and manipulate cells expressing *dsx* in various tissues. In the nervous system, we detected differences between the sexes in *dsx*-positive neuronal numbers, axonal projections and synaptic density. We found that *dsx* was required for the development of male-specific neurons that coexpressed *fruitless* (*fru*), a regulator of male sexual behavior. We propose that *dsx* and *fru* act together to form the neuronal framework necessary for male sexual behavior. We found that disrupting *dsx* neuronal function had profound effects on male sexual behavior. Furthermore, our results suggest that *dsx*-positive neurons are involved in pre- to post-copulatory female reproductive behaviors.

Substantial insights into the genetic and developmental logic supporting sex-specific behaviors have come from the genetically and behaviorally tractable model organism *Drosophila melanogaster*[1]. In *Drosophila*, genes of the sex-determination hierarchy orchestrate the development and differentiation of sex-specific tissues, establishing sex-specific physiology and neural circuitry[2]. Female-specific expression of *transformer* (*tra*), along with the non–sex-specific *transformer 2*, introduces a sex-specific splice in two pivotal downstream transcription factors, *dsx* and *fru*. In females, *dsx* transcripts are spliced to give rise to a female-specific isoform, dsx^F. In contrast, sex-specific splicing introduces a stop codon into female-specific *fru* mRNAs, which are not translated. In males, in the absence of Tra, *dsx* and *fru* transcripts undergo default splicing, generating the male-specific isoforms dsx^M and fru^M. Together, these transcriptional regulators establish most aspects of 'maleness' and 'femaleness'.

Dsx proteins are part of the Dmrt (*doublesex* and *mab-3*-related transcription factor) family, a structurally and functionally conserved group of zinc-finger transcription factors with important roles in sex determination throughout the animal kingdom[3]. In *Drosophila*, *dsx* directs most aspects of somatic sexual differentiation outside the CNS in both sexes. Many of these differences have been implicated in the performance or success of male sexual and reproductive behaviors[4–9].

Many recent studies have focused on the control of sexual behavior in males by *fru*[10–12]. Fru^M proteins are expressed from metamorphosis in the male CNS[13]. In the absence of Fru^M proteins, males perform little to no courtship toward females, fail to produce the pulse song component of courtship song, never attempt copulation and exhibit increased inter-male courtship[2]. Females expressing Fru^M display many male-specific courtship behaviors; however, they court less than wild-type males, generate aberrant song and never attempt copulation[11,14]. Thus, Fru^M expression is not sufficient to specify normal male courtship behavior, suggesting that other genes are required for a complete male courtship repertoire.

One obvious candidate is *dsx*. Males lacking *dsx* court at diminished levels fail to generate the sine song component of courtship song[15]. Dsx is found in the CNS[14,16,17] and Dsx^M and Fru^M proteins are coexpressed in several regions that are important for establishing male sexual behavior[14,18]. Recent studies have suggested that *dsx* and *fru* cooperate to dictate development of specific neural substrates underlying male sexual behavior[14,18–20]; thus, both genes are necessary for the specification of neural systems that generate male patterns of behavior[21]. However, little is known about the expression of *dsx* in the CNS and how this influences male and female sexual behavior.

To explore the anatomy and function of *dsx*-expressing neurons in both sexes, we targeted the insertion of the yeast transcription factor *Gal4* to the *dsx* locus. Clear differences in neuronal numbers and axonal projections were detected in the male and female CNS. These differences were behaviorally relevant, as disrupting *dsx* neuronal function led to highly aberrant behaviors in both sexes, suggesting these neurons instruct sex-specific neural programs. We propose that *dsx* coordinates the development of external sexual morphology and physiology with the development of sex-specific neurons that contribute to the circuitry required for male and female sexual behaviors.

RESULTS

Targeting *Gal4* to the *doublesex* locus

To allow detailed anatomical and functional analyses of *dsx*-expressing cells, we used ends-in homologous recombination to insert the *Gal4* coding sequence into the first, non–sex specific coding exon of *dsx*,

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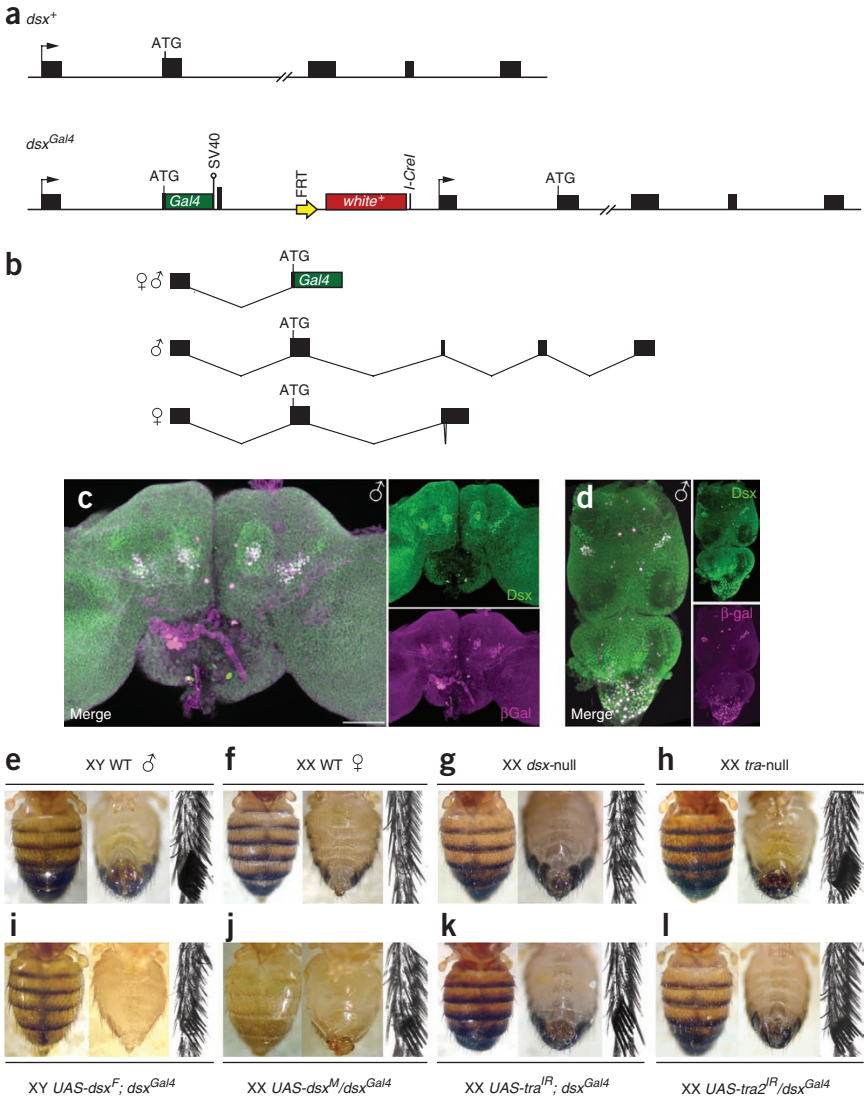

Figure 1 Expression of GAL4 from the dsx^{Gal4} locus recapitulates endogenous *dsx* expression. (**a**) Schematic of *dsx* (dsx^{+}) and *Gal4* knock-in allele (dsx^{Gal4}). Arrows indicate transcriptional start sites and black boxes indicate exons. (**b**) Male and female dsx^{Gal4} locus predicted transcripts. (**c**,**d**) dsx^{Gal4}, *UAS-nlacZ* 2-d-old male pupa brain (**c**) and VNC (**d**) stained with antibodies to Dsx (green) and β-galactosidase (magenta) (ventral views; anterior is up). The scale bar represents 50 μm. (**e**–**l**) dsx^{Gal4} transformation of secondary sexual characteristics, dorsal abdominal cuticular pigmentation, external genitalia and T1 leg basitarsal detail. Shown are XY (**e**) and XX (**f**) wild types, XX *dsx*-null intersexual (**g**), XX *tra*-null pseudo-male (**h**), XY $UAS\text{-}dsx^{F}$; dsx^{Gal4} pseudo-female (**i**), XX $dsx^{Gal4}/UAS\text{-}dsx^{M}$ pseudo-male (**j**), XX $UAS\text{-}tra^{IR};dsx^{Gal4}$ (**k**) and XX $dsx^{Gal4}/UAS\text{-}tra2^{IR}$ (**l**).

creating a tandem duplication at the locus (**Fig. 1a** and **Supplementary Fig. 1**). The resulting allele, dsx^{Gal4}, produced wild-type *dsx* transcripts and *Gal4*-containing transcripts in both sexes (**Fig. 1b** and **Supplementary Fig. 1**). When homozygous, or examined *in trans* to a deficiency of *dsx* ($Df(3R)dsx^{15}$), external sexual morphology (data not shown) and fertility of dsx^{Gal4} flies were unaffected ($P > 0.05$, $n > 45$). We concluded that the dsx^{Gal4} allele exhibits no overt disruption of gene function.

To determine whether dsx^{Gal4} reiterates endogenous *dsx* expression, we used dsx^{Gal4} to drive expression of a nuclear reporter and found that it colocalized with all previously described Dsx-expressing neuronal clusters[16] (**Fig. 1c**,**d**). Thus, dsx^{Gal4} is a sensitive, specific marker for Dsx-expressing cells.

dsx^{Gal4} cells dictate sexual differentiation

dsx is required to establish sexually dimorphic morphology and characteristics in male and female flies[22], but it is unclear where Dsx is expressed and whether *dsx* cells dictate all aspects of external sexual morphology. We determined the temporal and spatial distribution of dsx^{Gal4} cells (**Supplementary Fig. 2**). dsx^{Gal4} expression was observed in a number of tissues with sexually dimorphic features or physiology, such as the foreleg, cuticle, oenocytes, fat body and reproductive organs. dsx^{Gal4} expression, however, was not ubiquitous, suggesting that sex determination need not occur in every cell. Are these dsx^{Gal4} cells capable of controlling sexual differentiation?

dsx's ability to determine secondary sexual characteristics has been established through mutant analyses and ubiquitous overexpression of Dsx isoforms[22]. To determine whether dsx^{Gal4}-expressing cells are capable of directing a sex-specific program of development, we restrictively manipulated the sex of dsx^{Gal4}-expressing cells, assaying the consequences of expressing either Dsx^{M} or Dsx^{F} on external sexual morphology (**Fig. 1**). Dsx^{F} overexpression resulted in single-X males with female-like abdominal pigmentation, genitalia and no sex combs. (**Fig. 1i**); thus, Dsx^{F} was sufficient to direct a female-specific program of development even when competing with endogenous Dsx^{M} production. Dsx^{M} overexpression resulted in masculinized XX females that displayed male-like abdominal pigmentation and sex combs; however, the genitalia, although masculinized, were rotated and often malformed (**Fig. 1j**), possibly as a result of an inability to overcome the effects of endogenous Dsx^{F}.

XX flies homozygous for loss-of-function *tra* or *tra-2* mutations developed as pseudo-males; they were essentially indistinguishable from males in morphology and behavior but were sterile[23–25] (**Fig. 1h**). Using RNA interference, we specifically knocked down endogenous Tra and Tra-2 in dsx^{Gal4} cells. Notably, XX flies developed as close phenocopies (**Fig. 1k**,**l**) of *tra* and *tra-2* loss-of-function variants; they exhibited male-like abdominal pigmentation, genitalia and sex combs. dsx^{Gal4} cells are therefore capable of directing sex-specific morphological development.

dsx-specified sexually dimorphic neural circuitry

Although *dsx*'s specification of external sexual morphology influences sexual behavior[5,26], the anatomical foci central to determination of sex-specific behaviors lie in the CNS[2]. We used dsx^{Gal4} to determine the dimorphic *dsx* expression in the developing CNS (**Fig. 2**, **Supplementary Fig. 3** and **Supplementary Table 1**).

In addition to the previously described *dsx*-pC1, *dsx*-pC2 and *dsx*-aDN neuronal clusters in the adult posterior brain[16], we identified

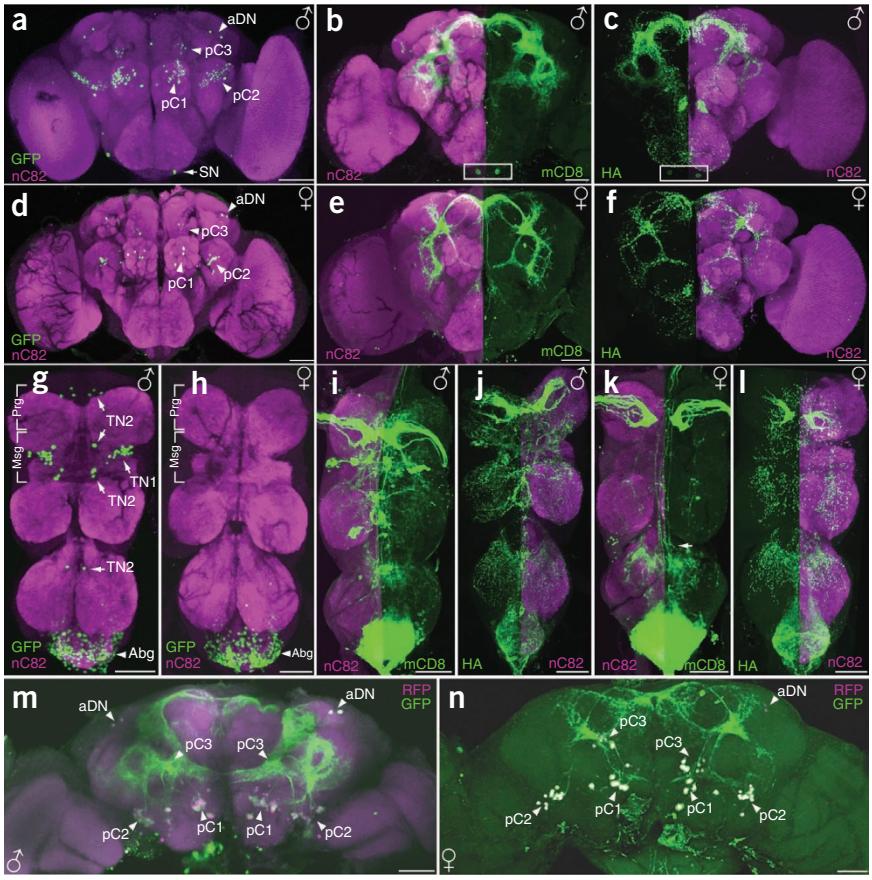

Figure 2 Sexually dimorphic expression of dsx^{Gal4}-neurons and associated projections in 5-d adult CNS. (**a**) Male brain, *dsx* neuronal clusters (arrowheads), male-specific SN neurons (only one cell in plane of focus, arrow). The cell bodies of pC1, pC2 and pC3 were located in the dorsal inferomedial, inferolateral and superomedial protocerebral areas, respectively. (**b**,**c**) Male brain (SN cells position is shown in the box). (**d**–**f**) Female brain; *dsx* neuronal clusters (arrowheads, **d**). (**g**) Male VNC, Abg cluster (arrowhead), male-specific TN1 and TN2 neurons (arrow). (**h**) Female VNC, Abg cluster (arrowhead). (**i**,**j**) Male VNC. (**k**) Female VNC, hindleg contralateral projection (arrow). (**l**) Female VNC. Neuronal cell bodies expressing *UAS-pStingerII* (nGFP) are shown in **a**, **d**, **g** and **h**. GFP staining is shown in green. Neuronal projections expressing *UAS-mCD8::GFP* (membrane-bound GFP) are shown in **b**, **e**, **i** and **k**. mCD8 staining is shown in green. Expression of *UAS-synaptotagmin* (pre-synaptic marker tagged with HA) is shown in **c**, **f**, **j** and **l**. HA staining is shown in green. Neuropil was counterstained with antibody to nC82 (magenta). Ventral views; anterior top. (**m**,**n**) *UAS-RedStinger*;dsx^{Gal4}, *UAS-mCD8::GFP* male (**m**) and female brain (**n**). Dsx neuronal clusters are indicated by arrowheads. GFP, green; RFP, magenta. Horizontal view, ventral top. Scale bars represent 50 μm.

another *dsx*-expressing cluster, designated *dsx*-pC3 (**Fig. 2a**,**d**,**m**,**n** and **Supplementary Table 1**). The pC1, pC2 and pC3 clusters have higher numbers of neurons in males (**Fig. 2a**,**d** and **Supplementary Table 1**). The pC1, pC2 and pC3 clusters lie in the dorsal inferomedial, inferolateral and superomedial protocerebrum, respectively, surrounding the mushroom body calyces; these sites have been implicated in sex-specific behaviors in both sexes[2] (**Fig. 2m**,**n** and **Supplementary Fig. 4**). In the suboesophageal ganglion (SOG), two male-specific neurons, *dsx*-SN, were present (**Fig. 2a**–**c**).

Despite the marked dimorphism in neuronal numbers in the brain, the topology of *dsx*-neuronal projections was notably similar between male and female brains. The most overt difference was the marked increase in density of synapses and projections in males compared with females, as determined by visualization of pre-synaptic and membrane markers in dsx^{Gal4} neurons (**Fig. 2b**,**c**,**e**,**f**,**m**,**n** and **Supplementary Video 1**).

In both sexes, these bilateral dorsal clusters are extensively interconnected (ipsilaterally, with projections extending throughout the medial and lateral protocerebrum, and contralaterally, via an extensive commissural bridge in the superior protocerebrum, to clusters in the opposing hemisegment)[20]. These connections also extend to the SOG, the point of termination for projections originating directly and indirectly from tarsal gustatory neurons, which has been implicated in mate choice via processing of nonvolatile pheromonal cues[27].

In the ventral nerve cord (VNC), adult males exhibit dsx^{Gal4} expression inthe male-specific *dsx*-TN1 and *dsx*-TN2 neuronal clusters (**Fig. 2g**)[16]. The TN1 neuronal clusters appeared to communicate directly with each other, with locally associated TN2 cells and with regions in the brain responsible for higher-order processing of sensory cues via projections through the cervical connective (**Fig. 2i**,**j** and **Supplementary Video 2**). It is noteworthy that the ventral mesothoracic ganglion (Msg), a focus for male-specific unilateral wing extension and courtship song, is innervated by male-specific TN1 and TN2 neurons[14,28,29]. Thus, dsx^{Gal4} revealed that there was substantial dimorphism between males and females in neuronal number, axonal projections and synaptic density in behaviorally relevant regions of the brain.

Extensive innervation in the ventral prothoracic ganglia (Prg) occurs in both sexes (**Fig. 3**). In males, these project ipsilaterally, connecting with TN2 cells, and contralaterally, forming a distinct commissural bridge (**Figs. 2i**,**j** and **3f**). Females have only ipsilateral projections (**Figs. 2k**,**l** and **3h**). Previous studies have shown that gustatory receptors in the foreleg exhibit a similar sexual dimorphism[30]. There were significantly more dsx^{Gal4} cells ($P < 0.05$) in the male foreleg, in both metatarsus and tarsi 2–5, than in the female foreleg (**Fig. 3a**,**d**). Some of these cells were neuronal, as fewer cells were observed when GAL80, a *Gal4* repressor, was expressed in postmitotic neurons using the *elav* promoter (**Fig. 3e**). More cells were repressed in males than females, indicating there are more neurons in the male foreleg.

As axons degenerate when severed[31], we amputated the forelegs of dsx^{Gal4} flies expressing membrane-bound green fluorescent protein (GFP). Amputating male forelegs below the sex comb, or an equivalent point in females, did not cause an overt degeneration in projections (data not shown). When the amputations were performed above the sex comb, or an equivalent point in females, no foreleg projections were observed (**Fig. 3g**,**i**). As only gustatory neurons cross the midline in male VNCs[30], at least some of the male dsx^{Gal4} projections must have been from gustatory neurons involved in nonvolatile taste sensation.

The abdominal ganglion (Abg), the anatomical foci for copulatory behaviors in males and females[1], is the sole region of the VNC

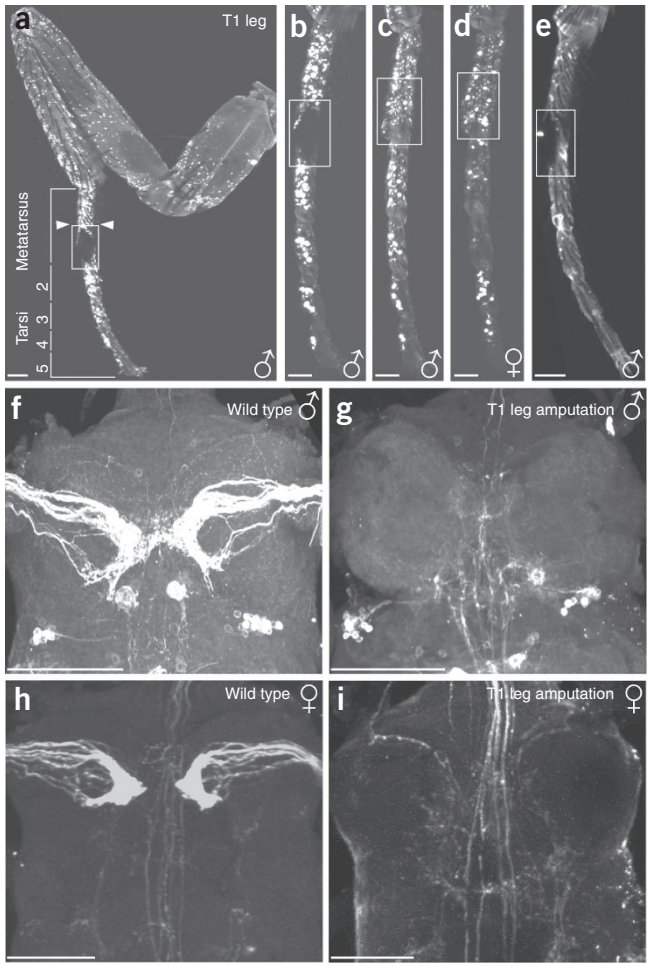

Figure 3 Sex-specific dsx^{Gal4} expression in the foreleg and effect of basitarsal amputations on axonal projections. (**a**–**d**) Sexually dimorphic expression in T1 foreleg. (**a**) Male T1 leg (medial aspect), sex comb (boxed). We found 96 ± 14.4 (n = 7) dsx^{Gal4}-expressing cells in the metatarsus and 72 ± 10.0 (n = 7) in tarsi 2–5. (**b**,**c**) Medial (**b**) and lateral (**c**) aspect male T1 tarsi and metatarsus, sex comb (boxed). (**d**) Female T1 tarsi and metatarsus (lateral aspect), area consistent with sex comb (boxed). We found 77 ± 12.4 (n = 8) dsx^{Gal4}-expressing cells in the metatarsus and 58 ± 9.7 (n = 7) in tarsi 2–5. (**e**) Male T1 tarsi and metatarsus (medial aspect), *elav-Gal80* repression in subset of dsx^{Gal4} cells, metatarsal sex comb (boxed). (**f**,**h**) Wild-type male (**f**) and wild-type female (**h**) prothoracic axonal projections (close-up from **Fig. 2k**). (**g**,**i**) Atrophied male (**g**) and atrophied female (**i**) prothoracic axonal projections, post-amputation. The point of amputation is indicated by arrowheads in **a**. Scale bars represent 50 µm.

in which dsx^{Gal4} was expressed in both sexes, with a larger number of cells expressing dsx^{Gal4} in females than in males (**Fig. 2g**,**h** and **Supplementary Table 1**). Neurons from this region send projections through the abdominal nerve trunk to ramify the internal genitalia in both sexes (data not shown). In males, four bilateral pairs of axonal fascicles ran through the cervical connection, connecting the brain and VNC (**Fig. 2i**). In females, there were five bilateral pairs of axonal fascicles, perhaps reflecting the increased neuronal expression apparent in the Abg (**Figs. 2k** and **3h**).

In summary, we found that dsx^{Gal4} was expressed in ~900 neurons (nine groups) in males and ~700 neurons (five groups) in females in the adult CNS (**Supplementary Table 1**). In the development of these neurons, we found that 48 h pupae is a critical point of divergence between the sexes. At this stage, SN and TN2 cells arose in both sexes (later disappearing in females), TN1 neuronal clusters appeared in males (but never females) and, until this stage, the number of dsx^{Gal4}-Abg neurons was consistently higher in males (**Supplementary Fig. 3** and **Supplementary Table 1**). These results indicate that there are substantial differences in cell numbers, projections and synaptic density in regions of the CNS associated with sex-specific behavior. We speculate that assembly of these neural networks and their sex-specific differences are responsible for the differences in male and female behavioral programs.

dsx and *fru* specify sexual dimorphisms in the CNS

Dsx^M and Fru^M are coexpressed in a restricted number of regions in the male CNS[14,18,20]. We found that dsx^{Gal4}-expressing neurons were likewise colocalized with Fru^M (**Fig. 4**). Both Dsx^M and Fru^M expression are required for the complete development of a number of specific neuronal populations in the male CNS[14,18,20]. Therefore, to examine the contributions of *fru* and *dsx* to the specification of dsx^{Gal4}-expressing neurons, we counted the number of dsx^{Gal4} neurons in *fru* and *dsx* mutant backgrounds (**Fig. 5** and **Table 1**).

In Fru^M-null males (expressing Dsx^M but lacking Fru^M), *dsx*-pC1, *dsx*-pC2 and *dsx*-pC3 neuronal numbers were significantly reduced compared with wild type ($P < 0.0001$, $P < 0.005$ and $P < 0.0001$, respectively; **Fig. 5c**,**d** and **Table 1**). A substantial reduction in the density of axons was observed in the cervical connection and in the contralateral connections in the Prg (**Fig. 5f**). There was no reduction in *dsx*-SN, *dsx*-TN1 or *dsx*-TN2 clusters (**Fig. 5e**); however, we previously found a Fru^M-dependent reduction in *dsx* cells in the Abg[18]. Fru^M is therefore required

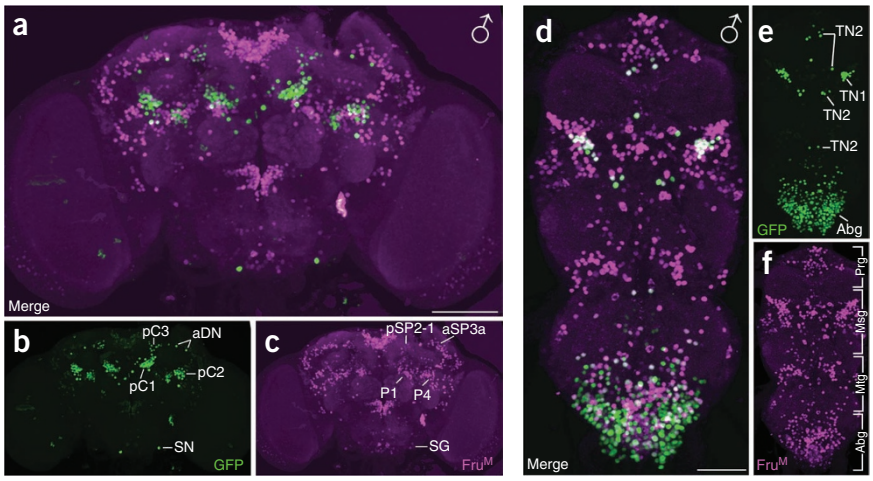

Figure 4 Colocalization of Fru^M neuronal cells and dsx^{Gal4}-expressing cells (expressing UAS-nGFP) in 3-d-old adult male flies. (**a**–**c**) Brain (dorsal view, anterior up, *dsx* and Fru^M neuronal cells and clusters are designated). (**d**–**f**) VNC (ventral view, anterior up, *dsx* and Fru^M neuronal cells and clusters that colocalized are designated). Fru^M clusters as previously described[20]. Scale bars represent 50 µm. Fru^M, magenta; nGFP, green.

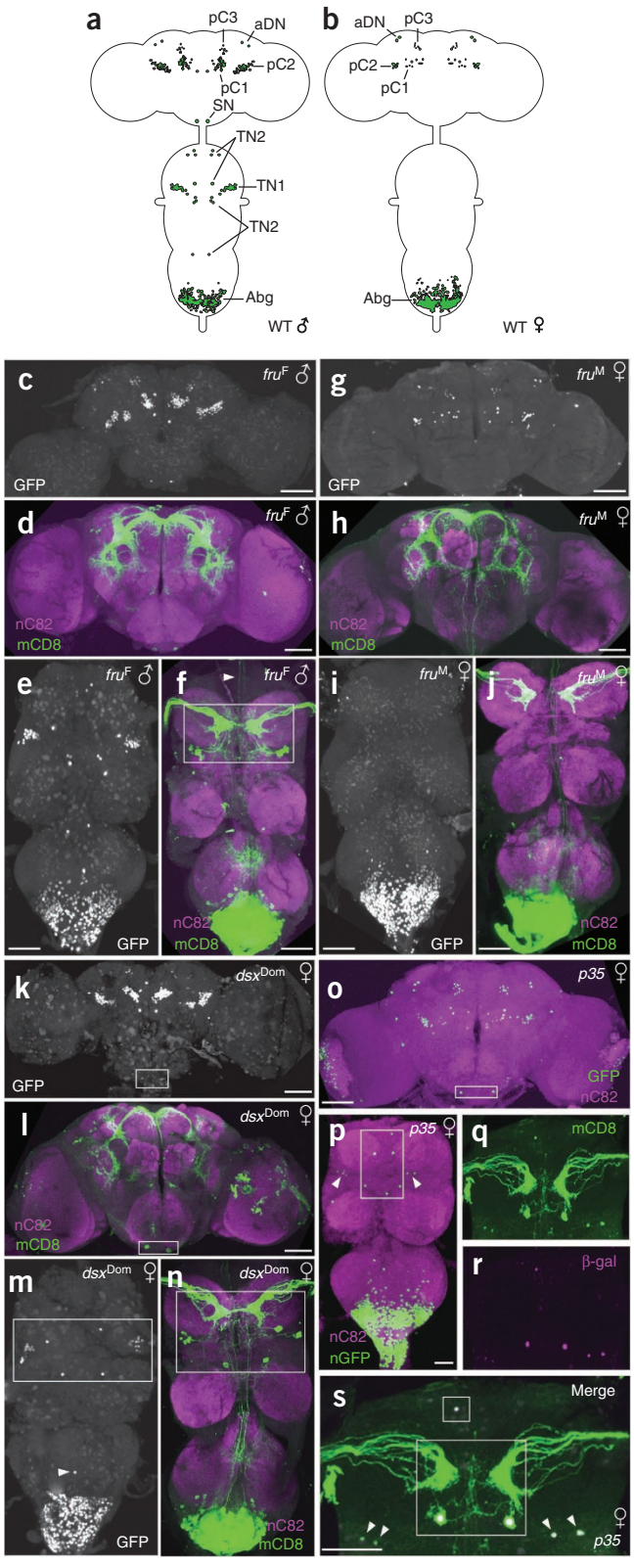

Figure 5 dsx^{Gal4} expression in CNS of Fru^M-null males and females expressing Fru^M, Dsx^M or the anti-apoptotic transgene *p35*. (**a**,**b**) Schematic of dsx^{Gal4}-*nGFP* expression in wild-type adult male (**a**) and female (**b**) CNS. Individual neuronal clusters are designated. (**c**,**d**) nGFP (**c**) and membrane-bound GFP (**d**) in Fru^M-null adult male brain. (**e**,**f**) nGFP (**e**) and membrane-bound GFP (**f**) in Fru^M-null adult male VNC, reduction in prothoracic contralateral (boxed area) and cervical connection projections (arrowhead). (**g**,**h**) nGFP (**g**) and membrane-bound GFP (**h**) in adult female brain expressing Fru^M. (**i**,**j**) nGFP (**i**) and membrane-bound GFP (**j**) in adult female VNC expressing Fru^M. (**k**,**l**) nGFP (**k**) and membrane-bound GFP (**l**) in adult female brain expressing both Dsx^F and Dsx^M. Supernumerary SN cells are shown in the boxed area. (**m**) nGFP adult female VNC expressing both Dsx^F and Dsx^M. Supernumerary male-specific TN1 and TN2 cells are shown in the boxed area, indicated by the arrow. (**n**) Membrane-bound GFP in adult female brain expressing both Dsx^F and Dsx^M. Prothoracic contralateral projections (boxed area). (**o**) *UAS-p35;* dsx^{Gal4} adult female brain, supernumerary SN cells (boxed area). (**p**) *UAS-p35;*dsx^{Gal4} adult female VNC, supernumerary TN1 (arrowheads) and TN2 (boxed area) neurons. (**q**–**s**) *UAS-nLacZ,-mCD8::GFP,-p35;*dsx^{Gal4} adult female Prg. Staining with membrane-bound GFP (**q**), nuclear β-galactosidase (**r**) and a merged image are shown (**s**). Supernumerary TN1 (arrowheads) and TN2 cells (boxed area) and ectopic contralateral projections (ventral views, anterior up). Scale bars represent 50 μm.

for the full complement of dsx^{Gal4}-expressing neurons. To test whether Fru^M is sufficient to specify dsx^{Gal4}-expressing neurons, we counted dsx^{Gal4} neurons in females expressing Fru^M and found that the number of dsx^{Gal4}-expressing neurons was not significantly different from that of wild-type females ($P > 0.05$; **Fig. 5g–j** and **Table 1**). This indicates that Fru^M expression is not sufficient to specify complete development of male-specific circuitry, as previous studies have suggested[11].

To determine whether *dsx* is sufficient to specify this neural circuitry, we counted dsx^{Gal4}-expressing neurons in females expressing both Dsx^F and Dsx^M. Neuronal numbers were significantly increased in *dsx*-pC1, *dsx*-pC2 and *dsx*-SN (but not *dsx*-pC3) in the brain and in all of the VNC clusters compared with wild-type females with a concomitant increase in projections and formation of contralateral connections ($P < 0.0001$; **Fig. 5k–n** and **Table 1**). However, the number of *dsx*-pC1 and *dsx*-pC2 neurons was still significantly lower than in wild-type males ($P < 0.0001$; **Table 1**). Because Dsx^F precipitates programmed cell death (PCD) in ~20 cells of the *fru*-P1 neuronal cluster[20] and this cluster is a subpopulation of *dsx*-pC1, a reduction in overall cell number in Dsx^M-expressing females is expected. Similar cell-death processes may occur in the pC2 and pC3 clusters.

Sex-specific PCD is one mechanism for creating sexual dimorphism in the brain[12,20]. Thus, we investigated its role in sculpting the *dsx* circuitry by expressing the cell-death inhibitor *p35* in dsx^{Gal4}-expressing cells[32] and counting the number of neurons in adult females. *dsx*-SN and *dsx*-TN2 cells, present in both sexes in 48 h pupa but specific to males in the adult (**Supplementary Table 1**), were protected from PCD, as their numbers were not significantly different from wild-type males (**Fig. 5o,p** and **Table 1**). Additional neurons were observed in *dsx*-pC2 and *dsx*-pC3 clusters as well as cells of the TN1 cluster (3 ± 0.4, $n = 14$; **Fig. 5o–s** and **Table 1**), which are never observed in females. Notably, when TN1 and TN2 neurons are present in females, they have contralateral projections that are ordinarily seen only in wild-type males (**Fig. 5q**).

Taken together, these results demonstrate that Dsx^M and Dsx^F are the primary regulators of dimorphisms in dsx^{Gal4} neurons, although Fru^M function is required to obtain a full complement of male-specific dsx^{Gal4}-expressing neurons, and that sex-specific PCD is one mechanism used to assemble this dimorphic circuitry. Notably, we found that small changes in neuronal populations can substantially alter the organization and connectivity of the neural network that presumably forms the anatomical basis for sex-specific behaviors.

dsx^{Gal4} neurons are required for male sexual behavior

dsx mutant males display aberrant courtship, but the neural etiology for this abnormality is unknown[2]. Having shown that *dsx* is required

Table 1 dsx^{Gal4}-driven nGFP expression in CNS of Fru^M-null males and females expressing Fru^M, Dsx^M or p35

	5-d-old adult male		5-d-old adult female			
	Wild type (*CS*)	fru^F/dsx^{Gal4}, $Df(3R)fru^{A-40}$	Wild type (*CS*)	fru^M/dsx^{Gal4}, $Df(3R)fru^{A-40}$	dsx^{Gal4}/dsx^{Dom}	*UAS-p35; dsx*^{Gal4}
Fru^M	+	–	–	+	–	–
Dsx^M	+	+	–	–	+	–
Dsx^F	–	–	+	+	+	+
Neuronal clusters						
Brain						
dsx-PC1	56.9 ± 5.0	48.2 ± 8.6**	8.7 ± 2.0	9.6 ± 1.1	45.8 ± 6.4 **	11.1 ± 2.8
dsx-PC2	78.6 ± 3.1	67.4 ± 8.8*	11.2 ± 1.9	10.7 ± 1.5	56.3 ± 4.7 **	16.1 ± 1.8*
dsx-PC3	13.6 ± 1.0	10.7 ± 1.2**	6.4 ± 1.4	6.3 ± 1.9	7.0 ± 1.3	11.7 ± 2.1**
dsx-SN	1 ± 0	1 ± 0	0 ± 0	0 ± 0	1 ± 0 **	1.0 ± 0 **
dsx-aDN	2 ± 0	2 ± 0	2 ± 0	2 ± 0	2 ± 0	2.2 ± 0.4
VNC						
dsx-TN1	22.4 ± 1.7	23.0 ± 2.2	0 ± 0	0 ± 0	15.5 ± 2.2**	3.0 ± 0.6**
dsx-TN2	6.9 ± 3.0	6.6 ± 0.7	0 ± 0	0 ± 0	6.0 ± 2.5**	7.0 ± 2.0**

The presence or absence of Fru^M, Dsx^M and Dsx^F expression is noted below genotypes. Counts represent one cluster per hemisegment. Mean ± s.d. $n = 10$ for all genotypes. $^*P < 0.005$, $^{**}P < 0.0001$.

in the construction of sex-specific neural substrates, we asked whether *dsx*-expressing neurons have an active role in male courtship behavior (**Fig. 6**). We quantified fertility and courtship behavior in males expressing *UAS–tetanus neurotoxin light chain* (*TNT*), which targets neuronal synaptobrevin[33], in dsx^{Gal4}-expressing neurons. After 1 week in the presence of several virgin females, these males were completely infertile ($n = 60$; **Fig. 6a**). We next determined whether the infertility was a consequence of defects in courtship and/or mating. The time taken for *UAS-*TNT_G*;* dsx^{Gal4} males to initiate courtship was significantly increased ($P < 0.05$; **Fig. 6b**) and, once courtship was initiated, the amount of courtship performed was severely reduced and consisted entirely of intermittent following and orientation with no attempted copulation (**Fig. 6c**). These males did not extend their wings and there was a complete absence of sine- and pulse-courtship song (**Fig. 6e**).

During an extended 4-h observation period, ~95% of control flies successfully copulated, but no *UAS-*TNT_G*;* dsx^{Gal4} males copulated (**Fig. 6d**). Courtship defects in *UAS-*TNT_G*;* dsx^{Gal4} males cannot be explained by more general defects in morphology or sensorimotor function, as *UAS-*TNT_G*;* dsx^{Gal4} males did not exhibit gross anatomical abnormalities in their genitalia or reproductive systems, including neuronal innervation (data not shown), and performed at least as well as wild-type and control males in locomotion, flight, olfaction and taste assays (**Supplementary Fig. 5**). Thus, inhibition of dsx^{Gal4} neuronal function in males disrupts the early steps of courtship (orientation and following) and causes a complete absence of the later steps (wing extension, courtship song and attempted copulation), suggesting that *dsx*-expressing neurons directly and specifically contribute to male courtship behaviors.

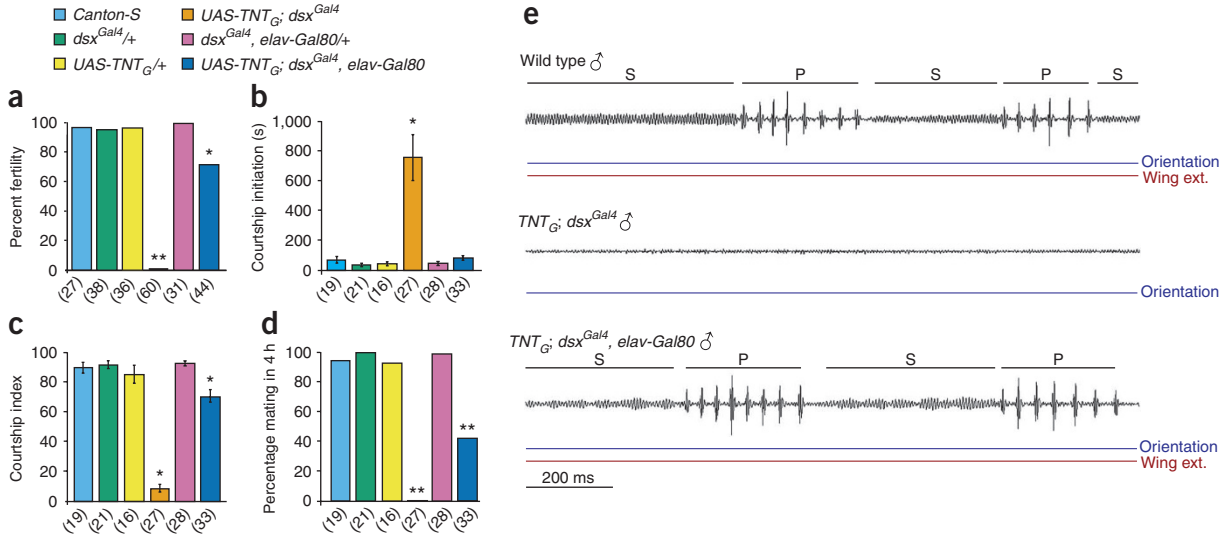

Figure 6 dsx^{Gal4} neurons control male sexual behavior. (**a**) Male fertility ($^*P < 0.05$, $^{**}P < 0.0001$, Fisher exact test). (**b**) Courtship initiation (mean ± s.e.m., $^*P < 0.05$, Tukey-Kramer HSD statistical test). (**c**) Courtship index (mean ± s.e.m., $^*P < 0.05$, Tukey-Kramer HSD test). (**d**) Percentage males mating in 4 h ($^{**}P < 0.0001$, Fisher exact test). Data represent mean ± s.e.m. *n* values are shown in parentheses. (**e**) Song recording, 5–7-d-old males. Pulse (P) and sine (S) song components are indicated above traces and courtship is shown below. Each trace represents a fraction of a 10-min recording. Scale bar represents 200 ms. Genotypes indicate males. Target females were wild type. For 15 wild-type males, there were 18.6 ± 2.2 sine bouts per min, 19.9 ± 1.4 pulse trains per min, 8.1 ± 0.3 mean pulses per train and a 31.7 ± 3.0–ms interpulse interval. For 10 *UAS-*TNT_G*;* dsx^{Gal4} males, there was no recordable data. For 10 *UAS-*TNT_G*;* dsx^{Gal4}, *elav-GAL80* males, there were 18.4 ± 1.5 sine bouts per min, 26.6 ± 3.8 pulse trains per min, 10.0 ± 0.4 mean pulses per train and a 34.0 ± 0.4–ms interpulse interval.

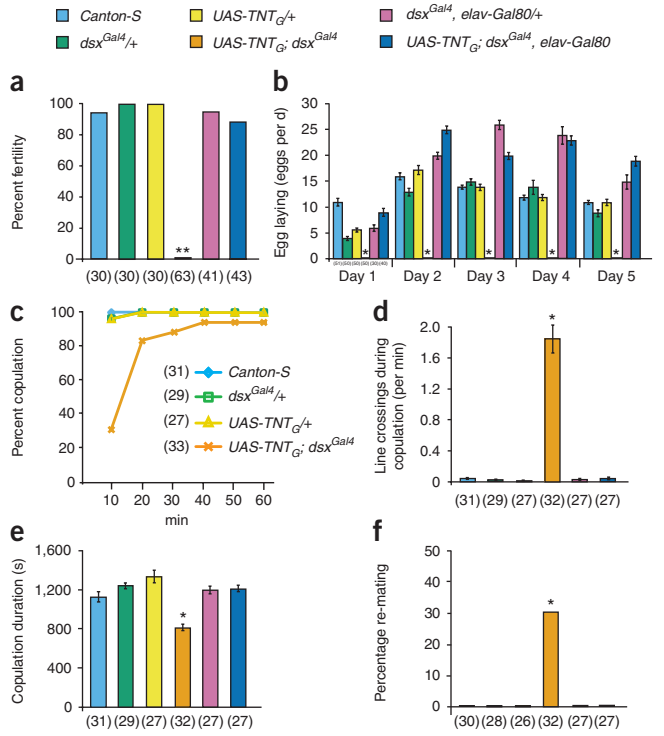

Figure 7 *dsx*^{Gal4} neurons control female sexual behavior. (**a**) Female fertility (**$P < 0.0001$, Fisher exact test). (**b**) Egg-laying (mean ± s.e.m., *$P < 0.0001$, Dunnett's test). (**c**) Percent copulation over time (10-min intervals for 1 h). (**d**) Line crossings during copulation (mean ± s.e.m., *$P < 0.05$, Tukey-Kramer HSD test). (**e**) Copulation duration (mean ± s.e.m. *$P < 0.05$, Tukey-Kramer HSD test). (**f**) Percentage females re-mating with the same male in 4 h (*$P < 0.05$, Tukey-Kramer HSD test). Genotypes indicate females. Target males were wild type. *n* values are shown in parentheses.

Because *dsx*^{Gal4} is expressed in a variety of tissues outside of the nervous system (**Supplementary Fig. 2**), we asked whether aberrant courtship behavior in *UAS-TNT*$_G$*; dsx*^{Gal4} males results from disruption of neural function. We used *elav-Gal80* to inhibit GAL4-driven expression of TNT specifically in neurons. *elav-Gal80* specifically and comprehensively inhibited neural expression of GFP in *dsx*^{Gal4}*; UAS-nGFP* flies, validating the efficacy of this tool (**Supplementary Fig. 3**). Removing TNT expression specifically from neurons reversed the observed courtship defects. Males expressing *UAS-TNT*$_G$*; elav-GAL80; dsx*^{Gal4} showed greatly improved levels of fertility (**Fig. 6a**) and courtship initiation (**Fig. 6b**), and there was a significant recovery in their courtship indexes (with consequent restoration of courtship modalities such as licking, tapping and wing extension) when compared with *UAS-TNT*$_G$*; dsx*^{Gal4} males ($P < 0.05$; **Fig. 6c**). The percentage of males copulating in a 4-h observation period was also significantly improved ($P < 0.0001$; **Fig. 6d**), as was their ability to produce both sine- and pulse-song bouts (**Fig. 6e**). These results suggest that the aberrant courtship phenotypes of *UAS-TNT*$_G$*; dsx*^{Gal4} males were largely a consequence of disrupting *dsx*^{Gal4} neuronal function. The fact that not all behaviors were restored to control levels likely reflects the inability of GAL80 to fully repress GAL4 function. Collectively, these results indicate that *dsx*^{Gal4}-expressing neurons are required for male courtship behavior.

dsx^{Gal4} neurons are critical for female sexual behavior

The neurobiological basis of female sexual behavior is poorly defined, although it has been suggested that *dsx*'s influence on the sex of the CNS might be critical for female behavior[8]. We investigated the effects of disrupting *dsx*^{Gal4} neuronal function on female behavior using TNT expression (**Fig. 7**). To confirm the neuronal contribution of *dsx*^{Gal4} cells to female sexual behavior, we also used *elav-GAL80* to restrict expression of TNT to non-neuronal tissues. *UAS-TNT*$_G$*; dsx*^{Gal4} females were completely infertile (**Fig. 7a**) and laid no eggs over five consecutive days post-mating (**Fig. 7b**). Over time, the abdomens of these females became distended, with extruded ovipositors and mature eggs atrophying in their oviducts (**Supplementary Fig. 6**).

We next observed the courtship behavior of *UAS-TNT*$_G$*; dsx*^{Gal4} virgin females paired with individual wild-type males. Initially, the percentage of females copulating in a 1-h period was investigated (**Fig. 7c**). Virgin *TNT*$_G$*; dsx*^{Gal4} females were less receptive than control females in the first 10 min (<30% versus >96%, respectively), but by 40 min, the percentage of *TNT*$_G$*; dsx*^{Gal4} females that had copulated was near control levels (>93% and 100%, respectively).

We then investigated *UAS-TNT*$_G$*; dsx*^{Gal4} females' behavior during and several hours after copulation. During copulation, these females actively rebuffed males, exhibited sustained wing flicks, kicking and failed to remain stationary (**Supplementary Video 3**). Although males often manage to grasp hold of a female, they never manage to spread the female's wings during copulation. The result of this aberrant copulation with *UAS-TNT*$_G$*;dsx*^{Gal4} females was a marked increase in locomotion of copulating pairs (**Fig. 7d** and **Supplementary Video 3**) compared with controls. We found a significant decrease in the length of copulation with *UAS-TNT*$_G$*; dsx*^{Gal4} females as compared with controls ($P < 0.05$), presumably as a consequence of the vigorous rejection displayed by these females (**Fig. 7e**). During a 4-h observation period, approximately 30% of *UAS-TNT*$_G$*; dsx*^{Gal4} females were observed to re-mate with the same male, some as many as four times (**Fig. 7f**), although they continued to exhibit vigorous rejection behaviors throughout. Control females never re-mated during the observation period.

The observed infertility of *UAS-TNT*$_G$*;dsx*^{Gal4} females was not a result of a lack of sperm transfer during the shorter copulation time, as the reproductive tracts of females who copulated with males with the sperm-enriched mitochondrial marker Don Juan–GFP were positive for GFP (**Supplementary Fig. 6**)[34]. We also confirmed transfer of seminal fluids, required for post-mating rejection responses, using a transgene encoding the accessory gland protein Sex-peptide fused to GFP (**Supplementary Fig. 6**)[35]. That no 'live births' were observed suggests this infertility is a result of an inability to deposit eggs into the uterus, preventing sperm, stored in the seminal receptacle and spermathecae, from fertilizing the mature oocyte. *UAS-TNT*$_G$*; dsx*^{Gal4} females exhibited no gross anatomical abnormalities in the genitalia or reproductive system, including their neuronal innervation (data not shown), and they performed at least as well as wild-type and control females in locomotion, flight, olfaction and taste assays (**Supplementary Fig. 5**).

To interrogate the unusual re-mating phenotype, we compared post-mating responses in mated *UAS-TNT*$_G$*; dsx*^{Gal4} females with those of mated control females and retested for receptivity 24 h later with a second naive male. During a 1-h observation period, 69% of mated *UAS-TNT*$_G$*; dsx*^{Gal4} females re-mated ($n = 13$), whereas no mated control females re-mated ($n = 14$). In addition, male courtship behaviors were not suppressed by mated *UAS-TNT*$_G$*; dsx*^{Gal4} females, as wild-type *Drosophila* females ordinarily do post-mating[2]; instead, they continued to elicit vigorous courtship (courtship index = 82.4% ± 4.4) compared with mated control females (courtship index = 28.4% ± 6).

To confirm that the infertility and courtship defects that we observed were a consequence of disrupting *dsx*^{Gal4} neurons, we used

elav-Gal80 to inhibit GAL4-driven neuronal expression of TNT. Fertility and egg laying in *UAS-TNT*$_G$*; elav-GAL80; dsx*^{Gal4} females was not appreciably different from controls (**Fig. 7a**,**b**). Similarly, locomotion during copulation, copulation duration and re-mating after copulation were restored to wild-type levels (**Fig. 7d–f**). This suggests that the abnormal behaviors exhibited by *UAS-TNT*$_G$*; dsx*^{Gal4} females were a result of disrupting *dsx*^{Gal4}-expressing neurons. These results indicate that *dsx*^{Gal4}-expressing neurons are critical for female courtship and reproductive behaviors. In particular, *dsx*^{Gal4}-expressing neurons appear to be important for receptivity to copulation and mating-induced behavioral changes.

DISCUSSION

Using the *dsx*^{Gal4} allele, we characterized *dsx* expression in neuronal and non-neuronal tissues throughout development. By manipulating the sex of *dsx*^{Gal4}-expressing cells, we found that they were able to direct a sex-specific program of morphological development. We also identified marked dimorphisms in neural circuitry in males and females: from differences in cell numbers in homologous clusters to sex-specific neuronal development to density, organization and connectivity of axonal projections. Specific inhibition of the function of these neurons in the males and females resulted in disruption of distinct sex-specific behavioral outputs, suggesting that differences in neuroanatomy instruct sex-specific behaviors.

In males, substantial overlap between *fru*- and *dsx*-expressing neurons occurs, allowing functional roles for certain *dsx* neurons to be inferred from their intersection with *fru* neurons with defined roles in male sexual behavior[14,20,36]. In males, *dsx*-pC1 colocalized with the medial *fru*-P cluster (**Fig. 4a–c**), a focus for licking and copulatory behaviors[37–39]. *dsx*-pC1 intersects with a subset of *fru*-P1 neurons, in which have been shown to be involved in courtship initiation and whose sexual differentiation and axonal morphology is dependent on both *fru* and *dsx*[20]. *dsx*-pC2 colocalized with the lateral *fru*-P cluster[20] (specifically P2–4; **Fig. 4a–c**) and *dsx*-pC3 colocalized with the *fru*-pSP2 cluster (**Fig. 4a–c**), both regions of the brain associated with initiation, following, tapping and wing extension[37–39]. Projections to the ventral SOG had intense synapses positionally associated with the *fru*-mCAL, a region that has been implicated in control of sequential courtship steps[13,40] (**Fig. 2c**). This coexpression of Fru^M and Dsx^M implies cooperation in shaping a shared male neural circuitry, an observation that is supported by the reduction of *dsx*^{Gal4}-expressing neurons in pC1, pC2 and pC3 clusters in Fru^M-null males, analogous to the cooperation seen in the Abg or Msg[14,18]. Early studies identified the ventral Msg as a neural focus for wing extension and song production[28]. More recently, successful song production has been correlated with the presence of a male-specific neuronal population in the Msg and the existence of a localized song pattern generator in the Msg[14,29]. Again, substantial overlap between *fru*- and *dsx*-expressing neurons was apparent in the VNC (**Fig. 4d–f**). *dsx* increases neural complexity in this region by specifying the appearance of male-specific neurons and dimorphic projection patterns. Further complexity arises from the *dsx*-dependent dimorphism in axonal projection paths in the Prg (ispilateral in females versus contralateral in males), which appear to arise as a consequence of inputs from male-specific gustatory receptor neurons in the foreleg[30]. These receptors have been shown to be involved in nonvolatile pheromonal gustation associated with male-specific tapping behaviors in courtship[27]. That these *dsx*^{Gal4}-expressing neurons direct male-specific behaviors is supported by our finding that male courtship is impaired when their neural function is specifically disrupted. Therefore, although *fru* is necessary in specifying male sexual behaviors, it is not sufficient; instead, *dsx* is also required for complete specification of male courtship behaviors.

Male courtship behaviors, being robust and quantifiable, have long been the focus of behavioral genetics; however, males are not the only participants in copulatory bouts. Females appear superficially passive, but exhibit subtle behaviors, consisting mostly of rejection behaviors such as wing flicking or kicking[2]. Little is known about the effects these rejection responses have on courting males and what stimuli trigger these behaviors. From the female's perspective, however, she must be able to assess a courting male to make an assured judgment of species type before she will sanction mating. Increased receptivity is indicated when a female slows down and ceases rejection behaviors. It is worth noting that some species of drosophilids actually exhibit an acceptance posture. Although no clear acceptance posture has been demonstrated in *D. melanogaster*, our findings suggest female cooperation does occur to facilitate copulation. A neural focus for receptivity has been identified in the dorsal female brain[2]; it seems likely that *dsx*^{Gal4}-expressing neurons in this region contribute to female mating decisions, just as homologous neurons in males are involved in male decisions[20,41]. This is supported by our finding that disrupting synaptic activity of *dsx*^{Gal4}-expressing neurons in females impaired distinct female courtship behaviors. These females were seemingly incapable of sampling the male's display and were thus incapable of providing any acceptance response. When copulation occurred, lack of female cooperation was evidenced by continuous movement and rejection behaviors. Although sperm and seminal fluids were successfully transferred to *UAS-TNT*$_G$*; dsx*^{Gal4} females, they laid no eggs, re-mated and remained incapable of actively rejecting or suppressing further courtship. This indicates that disrupting *dsx* neuronal function in females also suppresses post-mating behaviors and implies that some of these neurons relay information from *fru*-positive sensory neurons in the female reproductive tract in response to Sex-peptide[42,43].

We found that *dsx*^{Gal4}-expressing cells can not only reprise the functional roles of endogenous *dsx* in establishing external sexual morphology, but also establish a dimorphic neuroanatomy capable of directing distinct sex-specific behavioral outputs. In addition, *dsx*^{Gal4} expression occurs in sexually physiologically relevant non-neuronal adult tissues, such as the fat body and oenocytes, whose correct sexual identity is critical for normal sex-specific behaviors[8,9,44]. Our findings indicate that adult sex-specific behaviors may arise as a consequence of changes to distinct cell groups during development, creating dimorphic neural circuitry in equivalent regions of the male and female brain. Our results suggest a fundamental requirement for *dsx* in the sexual development of both neuronal (sex-specific circuitry) and non-neuronal tissues (sex-specific physiology). Future studies need to be aimed at identifying how *dsx* effects these dimorphic changes, refining our understanding of how individual *dsx* neurons instruct sex-specific neural programs and at identifying the relative contributions of both mind (fly brain) and body to these behaviors.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS
E.J.R., A.J.D., M.C.N. and S.F.G. designed experiments and wrote the paper. E.J.R., A.J.D. and M.C.N. all contributed equally to performing the experiments. S.E. provided technical assistance.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Targeted insertion of *Gal4* into the *dsx* locus. GAL4 was targeted to the *dsx* locus by ends-in homologous recombination as previously described[45] (and as illustrated in **Supplementary Fig. 1**). Three fragments with homology to the *dsx* genomic region were cloned sequentially into the targeting vector pED22 (generously provided by B. Dickson, IMPA) using the following primers (5′ to 3′): Fragment I gga tcc cac taa acg aga atc aaa aac and tct aga gag aaa gac cct gct ggc tat cag, Fragment II cct agg ctt aaa gtg gct ctt cgg agg and gga tcc gat tcc agc ttc tga tat cct a, Fragment III gcg gcc gca caa aac ttt cac cca act caa ta and gcg gcc gct tgt ctg cca ggg gtt ttt cag. The resulting construct was injected into a w^{1118} strain (Genetic Services). One of the ensuing second chromosome transformant lines was crossed (2,000 virgin females) to males of the genotype *y,w/Y,hs-hid; Sco,hs-I-SceI,hs-FLP/CyO*; first instar larvae were heat shocked for 1.5 h at 38 °C on the third day following the cross and again on the following day for 1 h. The following fly stocks were used to identify and balance all third chromosomal $white^{+}$ recombinants: *y,w; ey-FLP, y,w,ey-FLP; Pin/CyO, y,w,ey-FLP;;Ly/TM3,Sb* (provided by B. Dickson). Approximately 150,000 flies were screened and 11 independent targeted recombination events were recovered. PCR, sequencing and Southern blot analysis were used to confirm the predicted recombination event in multiple lines (data not shown). Two lines were selected for further analysis using multiple *UAS* reporter transgenes and both exhibited equitable patterns of expression (data not shown); a single line was then chosen for all further studies. Using a *hs-CreI* expressing line (w^{1118}; *P{hs-ICreI.R}1A,Sb*^{1}*/TM6*), we attempted to reduce the duplication at the *dsx* locus; however, after screening >50,000 flies, we were unable to recover a resolved line. This is likely a result of a polymorphism subsequently discovered in the *I-CreI* site in the targeting vector pED22 (J. Walker, unpublished data).

***D. melanogaster* strains and crosses.** Fly strains were raised as previously described[46], unless stated otherwise. Wild-type flies were obtained from *Canton-S* strain. The dsx^{Gal4} allele strain was generated as described in **Supplementary Figure 1**. The dsx^{Gal4} lines and all transgenes were in a w^{+} background for behavioral studies. dsx^{Dom} allele was dsx^{Swe}. fru^{M} and fru^{F} alleles were examined *in trans* to *Df(3R)fru*$^{4-40}$. Transformation experiments employed a *Dp(1:Y)B*^{S}; dsx^{Gal4}*/TM3, Sb* stock; haplo-X versus diplo-X progeny were identified by *Bar*-marked *Y* chromosome. RNAi crosses were performed at 29 °C and used $UAS\text{-}tra^{IR\ No.2560}$ and $UAS\text{-}tra\text{-}2^{IR\ No.8868}$ (purchased from the Vienna *Drosophila* RNAi Center). We also used *UAS-pStingerII*, *UAS-mCD8::gfp*, *UAS-RedStinger/CyO, UAS-LacZ.NZ* J312 (Bloomington *Drosophila* Stock Center), *UAS-synaptotagmin–HA*, $UAS\text{-}dsx^{M}$, $UAS\text{-}dsx^{F}$, *elav-GAL80* (provided by S. Sweeney, University of York), *y,w FRT19a; UAS-nlacZ,UAS-mCD8::gfp, UAS-p35; MKRS/+* (provided by F. Hirth, King's College London), *w*; P{dj-gfp}*, *w*; P{SP-gfp}* and $UAS\text{-}TNT_{G}$ transgenic fly lines.

Immunohistochemistry. All samples were dissected and treated as previously described[13,46] (see **Supplementary Table 2** for antibody details). Images and cell counts acquired as previously described[13,46]. Epifluorescence microscopy was performed on a Zeiss SteREO Lumar V.12 Stereomicroscope and captured via a Zeiss Axiocam. Axonal degeneration experiments involved severing newly eclosed flies' legs and dissecting the CNS 7 d later.

Behavioral assays. For $UAS\text{-}TNT_{G}$ experiments, flies were raised at 21 °C in a 12 h:12 h light:dark cycle. Individual virgin adults were collected and aged for 5–7 d post-eclosion at 25 °C and assays carried out at 25 °C. Locomotion, male courtship index and song were recorded in round courtship chambers (1-cm diameter × 4-mm height), courtship latency measured in larger round chambers (2-cm diameter × 4-mm height). Fertility, latency, courtship index and duration were measured as previously described[18]. Sperm/Sex-peptide transfer, song analyses and locomotion during copulation were measured as previously described[8,14,35].

Statistics. Behavioral means were compared using Tukey-Kramer HSD statistical test where indicated. For Fisher's exact test, two-tail *P* values were compared with wild type. Egg laying data was subjected to Dunnett's test with wild type as the set control. Cell counts were subjected to Student's *t* test. Statistical tests were performed with JMP v6.0 software (SAS Institute).

45. Rong, Y.S. & Golic, K.G. Gene targeting by homologous recombination in *Drosophila*. *Science* **288**, 2013–2018 (2000).
46. Billeter, J.C. & Goodwin, S.F. Characterization of *Drosophila* fruitless-gal4 transgenes reveals expression in male-specific fruitless neurons and innervation of male reproductive structures. *J. Comp. Neurol.* **475**, 270–287 (2004).