

Functional Dissection of the Neural Substrates for Sexual Behaviors in *Drosophila melanogaster*

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ABSTRACT The male-specific Fruitless proteins (Fru^M) act to establish the potential for male courtship behavior in *Drosophila melanogaster* and are expressed in small groups of neurons throughout the nervous system. We screened ~1000 *GAL4* lines, using assays for general courtship, male–male interactions, and male fertility to determine the phenotypes resulting from the *GAL4*-driven inhibition of Fru^M expression in subsets of these neurons. A battery of secondary assays showed that the phenotypic classes of *GAL4* lines could be divided into subgroups on the basis of additional neurobiological and behavioral criteria. For example, in some lines, restoration of Fru^M expression in cholinergic neurons restores fertility or reduces male–male courtship. Persistent chains of males courting each other in some lines results from males courting both sexes indiscriminately, whereas in other lines this phenotype results from apparent habituation deficits. Inhibition of ectopic Fru^M expression in females, in populations of neurons where Fru^M is necessary for male fertility, can rescue female infertility. To identify the neurons responsible for some of the observed behavioral alterations, we determined the overlap between the identified *GAL4* lines and endogenous Fru^M expression in lines with fertility defects. The *GAL4* lines causing fertility defects generally had widespread overlap with Fru^M expression in many regions of the nervous system, suggesting likely redundant Fru^M-expressing neuronal pathways capable of conferring male fertility. From associations between the screened behaviors, we propose a functional model for courtship initiation.

GENETICS offers powerful approaches for (1) identifying the neural circuitry underlying complex behaviors, (2) elucidating how such neural circuits are organized during development or modified by experience, and (3) understanding how such circuits function in behaving animals. Indeed, distinct genetic elements may regulate the neural substrates of behaviors as diverse as courtship and mating,

aggression and avoidance, speech, language, and social behavior (Juntti *et al.* 2008; Robinson *et al.* 2008; Fisher and Scharff 2009; Siwicki and Kravitz 2009; Wu *et al.* 2009; Juntti *et al.* 2010; Robinett *et al.* 2010).

As reproductive behaviors are often innate, they provide excellent systems for genetic approaches (Manoli *et al.* 2006; Kimchi *et al.* 2007; Portman 2007; Juntti *et al.* 2008; Vilella and Hall 2008). Neuronal circuits mediating reproductive behaviors must act to discriminate relevant from nonrelevant stimuli, integrate information across multiple sensory modalities, and generate appropriate behavioral output. Understanding how sex-specific genetic functions organize this circuitry, as well as how that circuitry functions, will hopefully elucidate how complex behaviors are generated by the nervous system. Here, we use molecular tools derived from the *Drosophila melanogaster* sex determination gene *fruitless* to dissect the behavioral components of male courtship behavior.

The *D. melanogaster* male courtship ritual is an extensively studied, complex innate behavior that can be executed by males reared in isolation (Hall 1994; Greenspan and

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Ferveur 2000). Information perceived via multiple sensory modalities is integrated to direct both the initiation and progression of courtship. The initial identification of appropriate female targets is via visual and olfactory cues (Sturtevant 1915; Hall 1994; Greenspan and Ferveur 2000; Stockinger *et al.* 2005; Kurtovic *et al.* 2007; Krstic *et al.* 2009). Subsequent steps are mediated via contact-mediated chemosensory or mechanosensory cues perceived during tapping, licking, and attempted copulation (Acebes *et al.* 2003; Bray and Amrein 2003; Lacaille *et al.* 2007; Moon *et al.* 2009; Koganezawa *et al.* 2010). In addition, auditory cues (song) generated by males enhance male courtship drive and stimulate female receptivity (Kowalski *et al.* 2004; Ejima *et al.* 2005). Although most steps of courtship are innate, some are experience dependent (Griffith and Ejima 2009). Thus studies of male courtship behavior can contribute to understanding sensory processing and integration, coordination of motor programs, and motor output, as well as experience-dependent behavioral modifications.

In *Drosophila* a regulatory gene hierarchy governs all aspects of somatic sexual differentiation, including the potential for male courtship behavior. This cascade directs the synthesis of the sex-specific transcription factors encoded by the *fruitless* (*fru*) and *doublesex* (*dsx*) genes (Baker *et al.* 2001; Manoli *et al.* 2006; Dickson 2008; Villella and Hall 2008; Yamamoto 2008; Siwicki and Kravitz 2009). *fru* is the key effector through which the nervous system is sculpted for male behavior. Transcripts derived from the distal-most promoter (P1) of the *fru* locus (*fru^M*) are sex-specifically spliced to produce mRNAs that encode Fru proteins (Fru^M) in males and are untranslated in females (Ryner *et al.* 1996; Heinrichs *et al.* 1998; Lee *et al.* 2000; Usui-Aoki *et al.* 2000). Fru^M transcription factors are expressed in ~2% of neurons in the brain and ventral nerve cord (VNC), as well as in sensory components of the PNS (Ryner *et al.* 1996; Lee *et al.* 2000; Manoli *et al.* 2005; Stockinger *et al.* 2005; Cachero *et al.* 2010; Yu *et al.* 2010). Fru^M expression in the appropriate neurons is necessary and sufficient to generate the potential for nearly all aspects of male courtship behavior (Manoli *et al.* 2005; Stockinger *et al.* 2005; Manoli *et al.* 2006). Although the *fru^M* expression pattern is grossly similar between males and females (Manoli *et al.* 2005; Stockinger *et al.* 2005), visualization of subsets of *fru^M*-expressing neurons revealed several *fru^M*-dependent sexual dimorphisms (Kimura *et al.* 2005; Rideout *et al.* 2007; Datta *et al.* 2008; Kimura *et al.* 2008; Koganezawa *et al.* 2010; Mellert *et al.* 2010). More recent systematic anatomical characterizations of individual *fru^M* neurons in males and females have revealed extensive sexual dimorphism in the *fru* circuitry (Cachero *et al.* 2010; Yu *et al.* 2010).

The presence or absence of *fru^M* products also governs other sex-specific *Drosophila* behaviors, which include male- and female-specific aggression behaviors (Chen *et al.* 2002; Nilsen *et al.* 2004; Vrontou *et al.* 2006; Chan and Kravitz 2007), and at least some aspects of female reproductive behaviors (Kvitsiani and Dickson 2006; Yapici *et al.* 2008;

Häsemeyer *et al.* 2009; Yang *et al.* 2009). *fru^M*-expressing neurons appear to be dedicated to mediating these social interactions, since silencing these neurons via expression of the neural silencer *shi^{ts}* does not affect other general behaviors (Manoli *et al.* 2005; Stockinger *et al.* 2005), and activating them triggers courtship behavior (Kohatsu *et al.* 2011; Pan *et al.* 2011; von Philipsborn *et al.* 2011).

dsx plays an important role in the production of courtship song and the generation of sexually dimorphic numbers of neurons in parts of the central nervous system (CNS) and peripheral nervous system (PNS) (Demir and Dickson 2005; Manoli *et al.* 2005; Rideout *et al.* 2007; Kimura *et al.* 2008; Sanders and Arbeitman 2008; Mellert *et al.* 2010; Rideout *et al.* 2010). However, the overall effects of *dsx* null mutants on courtship behavior are subtle compared to those of *fru*.

The broad pattern of *fru^M* expression and the potent effects of impaired *fru^M* function on sexual behavior raises the question of which neurons regulate particular aspects of the behavior. Mosaic mapping experiments using XO//XX mosaics identified regions of the fly that need to be male for different steps of courtship to occur (Hotta and Benzer 1972; Hall 1977, 1978, 1979). Although these studies have inherent limitations that restrict their resolution (Kankel and Hall 1976), these results are consistent with the proposition that subsets of the CNS appear to function in specific steps of sexual behaviors, a key premise of the current study.

More recently, many studies focused on select groups of *fru^M* neurons have associated particular behavioral phenotypes with defined subsets of *fru^M*-expressing neurons (Ferveur *et al.* 1995; O'Dell *et al.* 1995; Ferveur and Greenspan 1998; Lee and Hall 2001; Lee *et al.* 2001; Manoli and Baker 2004; Couto *et al.* 2005; Fishilevich and Vosshall 2005; Chan and Kravitz 2007; Kurtovic *et al.* 2007; Clyne and Miesenböck 2008; Datta *et al.* 2008; Kimura *et al.* 2008; Koganezawa *et al.* 2010; Kohatsu *et al.* 2011; von Philipsborn *et al.* 2011). These studies provide entry points for the further understanding of the circuitry underlying male sexual behavior.

To more systematically assess the functional roles of subsets of Fru^M-expressing neurons, we have used >1000 random *GAL4* enhancer traps to target RNAi-mediated inhibition of Fru^M expression in reproducible, restricted subsets of Fru^M neurons (Figure 1). Our primary screen used assays for male fertility, elevated levels of male–male interactions, and perturbations in easily scored aspects of male–female courtship to dissect the functional components of *Drosophila* sexual behavior. From these assays, we identified lines producing altered latencies to courtship initiation, increased male chaining, male–male courtship or aggressive behaviors, and defects in male fertility. The *GAL4* lines identified in our initial screen were subjected to secondary screens that sought to differentiate between various neural processes that may have contributed to similar behavioral phenotypes, as well as to assess the contribution of certain types of neurons to the phenotypes observed. Finally, as an initial attempt to correlate these functionally defined phenotypic classes with the manipulation of particular subsets of *fru*-PI-expressing neurons, we determined which

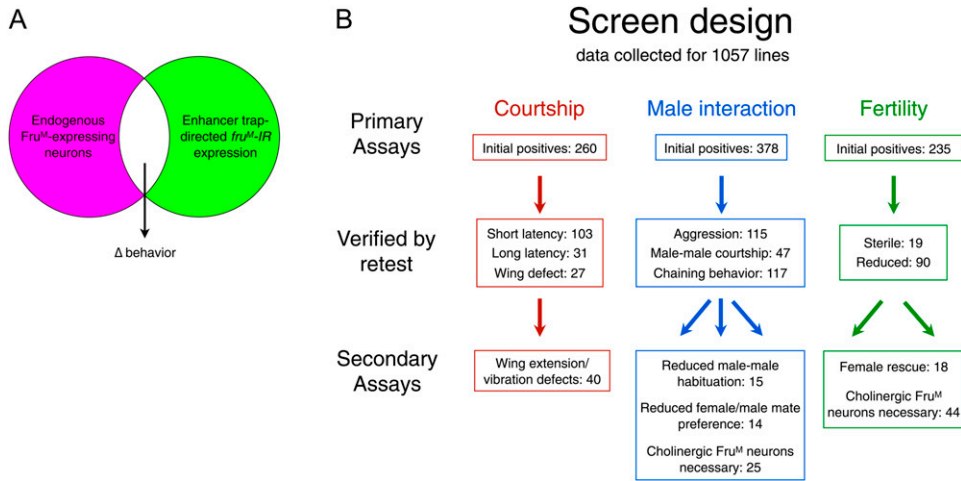


Figure 1 Regulation and targeting of Fru^M-specific elements of *Drosophila* sexual behavior. (A) Expression of UAS-*fru*^{MIR} produces effects only in those cells where enhancer-trap *GAL4* expression (green) intersects (white) with endogenous Fru^M expression (magenta). (B) Schematic illustrating behavioral screening for phenotypes arising from directed inhibition of Fru^M expression. *GAL4/UAS-fru*^{MIR} males were screened with three primary assays for (1) male fertility, (2) abnormal male–male interactions, and (3) courtship behavior. Lines that produced phenotypes in any of these screens were subjected to specific secondary screens in each primary phenotypic class.

neurons express the *GAL4* drivers that produced deficits in male fertility.

Materials and Methods

Drosophila stocks and cultures

Flies were raised on standard dextrose media. Crosses were performed at 29° to optimize *GAL4* and RNAi function. CO₂ was used to anesthetize flies. The UAS-*fru*^{MIR}, UAS-*GFP-IR*, and *fru-P1-LexA* stocks have been previously described (Manoli *et al.* 2005; Mellert *et al.* 2010). The *GAL4* enhancer trap collection was generated in the laboratory of Ulrike Heberlein (personal communication; University of California, San Francisco) and was produced via the mobilization of the pGAWB element into a *w Berlin* strain. The Canton-S strain (*CS*) used was the CS-A strain obtained from the laboratory of J. Hall (Brandeis University, Boston). The UAS-*fru*^{MIR}, *Cha-GAL80* stock was made by recombining the *Cha-GAL80* (Kitamoto 2001) transgene with the chromosome III UAS-*fru*^{MIR}. The *fru*^{Δ^{tra}} allele was obtained from the Dickson lab (Demir and Dickson 2005). We used a nuclear GFP reporter for *fru-P1-LexA*, *LexAop-Stinger-GFP*, made by replacing the UAS element of UAS-*Stinger-GFP* with four *LexAop* binding sites (Barolo *et al.* 2000; Lai and Lee 2006).

Primary assays

F₁ males containing each *GAL4* enhancer trap and two UAS-*fru*^{MIR} (experimental) or two UAS-*GFP-IR* (control) transgenes were collected within 12 hr of eclosion from 29° crosses and stored individually at 29° for the times indicated. To verify behavioral phenotypes, genotypes displaying altered behavior were produced from new crosses and subject to identical retests.

Fertility: Three to 5 days after collection, 5–10 F₁ males were crossed individually to a pair of ~1-week-old *CS* virgins. Crosses were carried out at 29° and the number of pupae counted once the first pharates appeared (7–8 days). *GAL4/UAS-GFP-IR* males were used as controls and pro-

duced an average of 45.7 pupae (standard deviation of 12.9). Genotypes with an average fecundity of <25 pupae were retested.

General courtship: F₁ males were entrained for 3 days in a 12-hr light:dark (12 l/d) cycle at 29°. Following entrainment, typically four males and four 2-day-old *CS* virgin females were anesthetized with CO₂ 2–4 hr after lights on [zeitgeber time (ZT) 2–4] and placed into individual wells of a 24-well tissue culture plate (Falcon). Animals were allowed to recover for 3–5 hr at 25° following anesthesia. For courtship assays, plates containing males were inverted onto those containing females, with a thin plastic barrier between the chambers at 25°. The barrier was moved allowing a row of males to be simultaneously introduced into the female chambers below and the barrier replaced, giving each well one male/female pair. Latency to courtship initiation (first wing extension) was measured, and any gross aberrations in courtship behavior were noted. Retests were performed on genotypes in which two or more males initiated courtship in <60 sec or failed to initiate courtship within 2 min.

GAL4 lines showing defects in wing extension and vibration were reexamined in detail on video recordings. Pairs were observed in a standard Plexiglas mating wheel with cylindrical chambers (diameter 10 mm, height 6 mm) (Vil-*lella et al.* 1997) at 25° and videotaped for 10–15 min or until copulation. Observations were conducted between ZT 2 and ZT 7. At least four males from each line were videotaped and for some assays a program called LifesongX programmed by J. Reiffel was used to count the number of abnormal behaviors, as well as to record time spent courting. Both *CS* and *GAL4/UAS-GFP-IR* males were used as controls.

Male–male interaction: Following 3–5 days at 29°, males were entrained at 29° for 3–4 days in a 12 l/d cycle. A total of seven to eight males per line were then anesthetized and placed together into a 24-well tissue culture plate (Falcon)

that contained ~1 mL of standard dextrose media at 29°. Males were observed for several minutes twice daily (ZT 2–3 and ZT 8–10) for 3 days and chaining behavior and male–male courtship and aggressive behaviors were scored qualitatively on a 0–3 scale, ranging from no aberrant behavior to very high levels of abnormal behavior. Chaining was defined as three or more males showing persistent courtship in a series for at least 5 sec, whereas male–male courtship was between pairs of males (supporting information, File S1). Aggressive behaviors were defined as previously described (File S2) (Chen *et al.* 2002; Nilsen *et al.* 2004).

Secondary Assays

Male–male habituation: Males from lines exhibiting chaining behavior or male–male courtship were isolated and entrained as in the male–male interaction assay. Typically 20 males were anesthetized using CO₂ in the circadian morning (ZT 2–4) and placed individually in wells of a 48-well tissue culture plate (Falcon). Animals were allowed to recover for 4–5 hr following anesthesia at 25°. Plates containing males were inverted onto those containing sibling males, with a thin plastic barrier between the chambers. For a given line (10 sibling pairs), the barrier was partially moved, the male from the upper chamber was introduced into the lower chamber, and the barrier replaced. Males were videotaped for 7 min immediately after pairing and for 5 min beginning 55 min after pairing, at 25°. Courtship indexes (time courting/observation period) were determined for minutes 2–7 (initial) and 55–60 (final).

Mate preference: Males from lines exhibiting chaining behavior or male–male courtship were tested for their sexual preference in a triad assay in which one test male was placed together with a target female and a target (decoy) male. Decoy males were genotypically *c155-GAL4/UAS-fru^{MIR}* and thus phenotypically male externally, but unable to court due to the expression of *fru* RNAi throughout their nervous system. For these assays, test males were isolated and entrained as in the male–male interaction assay. CS virgins were 4–6 days old at testing, not entrained, and kept at room temperature following collection. *c155-GAL4/UAS-fru^{MIR}* decoy males were 18 hr to 3 days old at the time of testing and were maintained at 29° until testing. Test males were anesthetized with CO₂ for loading and allowed to recover for at least 3.5 hr at 25°. All behavioral tests took place in the circadian afternoon (ZT 7–9). Chambers were 13 mm in diameter and 18 mm deep and split into three vertical sections by removable thin plastic dividers. Flies were separated until testing, then introduced together by removal of the dividers. Following divider removal and tapping down, trials were recorded for 5 min.

To calculate the courtship preference index (Cpi), test males were scored for 5 min or until copulation. The courtship index was calculated as follows: CI = (time spent courting)/(duration of trial); CI_f and CI_m represent the fraction of time courting the female and decoy male,

respectively; $Cpi = (CI_f - 0.5)/(CI_f + CI_m)$. Mean Cpi for each line was compared to mean wild-type Cpi and to zero by a one-sample *t* test.

Cha-GAL80 suppression: Enhancer-trap lines exhibiting chaining behavior or reduced fertility were tested as to whether the *fru* neurons through which these phenotypes were generated were cholinergic. F₁ *GAL4/UAS-fru^{MIR}* males with and without a *Cha-GAL80* transgene were collected and isolated within 12 hr of eclosion from parallel crosses raised at 29°. Fertility and male–male interactions were assayed as in *Primary Assays* above.

Female fertility rescue: Enhancer-trap lines that had reduced male fertility in the primary assay were subsequently examined for their ability to rescue female infertility induced by expression of the *fru*-masculinizing *fru^{Δtra}* allele (Demir and Dickson 2005). Males containing *UAS-fru^{MIR}* and the *fru^{Δtra}* allele were crossed to females from each *GAL4* line, and their *w*; *UAS-fru^{MIR}/GAL4*; *UAS-fru^{MIR}*, *fru^{Δtra}/+* male (as a control for the *fru^{Δtra}*) and female offspring were tested for fertility as previously. Males were crossed to two CS females, whereas females were crossed to two CS males.

Expression

To visualize *fru^M* expression independently of *GAL4*, we inserted the *LexA::VP16* transcriptional activator into the *fru* locus (Mellert *et al.* 2010). This transactivation system makes use of the *Escherichia coli* *LexA* gene fused with the *VP16* activation domain, which targets specific *LexAop* binding sites (Lai and Lee 2006).

We used a nuclear GFP reporter, *LexAop-Sti-GFP* with *fru-P1-LexA*. As has been reported (Lai and Lee 2006), *LexAop* reporters often suffer from leaky expression. We used an insertion (E) with the least leakage for examination of *fru^M* and *GAL4* overlap in the CNS and genitalia, and that insertion in combination with a second one (F, which does leak in the CNS and genitalia) for examination of expression in the antenna, maxillary palp, proboscis, and legs, where the stronger expression provided by two insertions is useful. We used *fru-P1-LexA*, *LexAop-Sti-GFP* in conjunction with an optimized nuclear DsRed, *UAS-Red-Sti* (Barolo *et al.* 2004) to simultaneously visualize *LexA* and *GAL4* expression.

For the visualization of Fru^M protein we used rat anti-Fru^M antibody at 1:300 dilution and Cy3 antirat secondary antibody from Jackson ImmunoResearch at a 1:800 dilution, as described (Lee *et al.* 2000). Samples were imaged at ×20 magnification on one of the following confocal microscopes: BioRad MRC 1024, Zeiss 510 Meta, or Zeiss 710, and processed with ImageJ software.

Statistical analysis

Statistical analyses were performed with Apple Numbers 2009, Microsoft Excel 2008, and SAS JMP 7. Multivariate regression analyses were performed relating expression and

infertility, including survival analysis and logistic regression. As quantitative fertility data were not collected for lines with high fertility, we used survival analysis to account for the right censored data, treating fertility as the time variable. In parallel, treating fertility as a binary result, we performed nominal logistic regression on the same dataset.

Results

Screen design

A total of 1057 independent *GAL4* enhancer-trap lines were crossed to a stock containing both second and third chromosome inserts of a *GAL4*-responsive RNAi transgene that targets male-specific Fru isoforms (*UAS-fru^MIR*) (Manoli and Baker 2004), and their male progeny were screened for alterations in sexual behaviors. Given the specificity of the RNAi for *fru^M* isoforms, only *fru^M*-expressing neurons in which *GAL4* is expressed should be affected by *UAS-fru^MIR* expression in these males (Figure 1A). To minimize background effects the *GAL4* lines were in a common Berlin genetic background and the *UAS-fru^MIR* transgenes were introgressed into the same genetic background.

Three high-throughput behavioral assays were used to screen multiple *GAL4; UAS-fru^MIR* male progeny from each *GAL4* line for courtship defects (Figure 1B). Males were tested for (1) reduced fertility, (2) the occurrence of chaining behavior (in which multiple males form a chain, each male courting another male), male–male courtship or aggression between pairs of males, and (3) overt courtship defects and alterations in the time to initiation of courtship (courtship latency). Following initial testing, all putative positive lines were retested in the same assays using progeny from independent crosses. To control for nonspecific effects of RNAi expression, a randomly selected subset of *GAL4* lines (see below) were crossed to an RNAi targeting *GFP* transcripts (*UAS-GFP-IR*). Behavioral defects seen with *UAS-fru^MIR* were not observed or were at a much lower rate with *UAS-GFP-IR*.

Male fertility

Previous studies have demonstrated that Fru^M is required for fertility, and that Fru^M-dependent reductions in male fertility can result from overall decreases in courtship, inability to initiate or successfully complete copulation, defective innervation of male internal genitalia, as well as defects in the transfer of sperm and seminal contents (Villella *et al.* 1997; Lee *et al.* 2001; Manoli and Baker 2004). Thus, we postulated that perturbations of courtship behavior at multiple stages might manifest as male infertility.

To measure fertility 5–10 *GAL4/UAS-fru^MIR* male progeny of each *GAL4* line were crossed individually with two CS virgin females and the number of offspring measured as described (see *Materials and Methods*). A total of 235 lines showing fertility defects were initially identified and through retesting, 19 lines were verified as sterile and 90 as having substantially reduced fertility.

Male–male interactions

Social behaviors require that an animal correctly identify a conspecific and its sex, as well as respond appropriately to both sexes in different ethological contexts. Intrasexual interactions occur both in the context of reproduction, as with courtship, as well as in aggressive interactions. As wild-type male reproductive and aggressive interactions are both dependent on *fru* (Hall 1978; Ito *et al.* 1996; Ryner *et al.* 1996; Villella *et al.* 1997; Goodwin *et al.* 2000; Anand *et al.* 2001; Vrontou *et al.* 2006), we assayed for abnormalities in interactions within groups of *GAL4/UAS-fru^MIR* males.

For male–male interaction assays, seven to eight *GAL4/UAS-fru^MIR* males were grouped and interactions scored as described (see *Materials and Methods*). Wild-type males displayed little in the way of male–male courtship interactions, likely due to inhibitory cues from males and habituation to positive cues (Miyamoto and Amrein 2008; Lacaille *et al.* 2009). Similarly, control males showed little aggression, likely due to our chambers having a large available food surface, as limited and localized resources appear to evoke territorial/aggressive behaviors in wild type (Chen *et al.* 2002; Nilsen *et al.* 2004).

From 378 lines initially selected as exhibiting elevated levels of male–male interactions, retesting using the same experimental regime identified 120 lines showing chaining behavior, 53 lines showing high levels of male–male courtship (between pairs of males), and 112 lines showing aggression (File S1, File S2, and see *Materials and Methods*). Lines showing both male–male courtship and chaining at different times were scored as chaining lines. Interestingly, for the aggression lines, only a single male per chamber showed aggressive behavior at each observation, often controlling the entire food surface. Because males were unmarked and only periodically observed rather than constantly monitored, it was not possible to determine whether a single male was aggressive across all observations (which might suggest the establishment of dominance hierarchies) (Nilsen *et al.* 2004; Vrontou *et al.* 2006). At different times of observation, 33 lines showed chaining and aggressive behaviors, whereas 20 other lines showed both male–male courtship and aggression, often contemporaneously. The high incidence of lines displaying male–male interactions is notable: of the 1057 *GAL4* lines tested, 288 (27%) had reproducibly elevated levels of one or more types of male–male interactions, with significant associations between these and other behavioral phenotypes (see *Discussion*).

Overt courtship defects

Fru^M function is required for all steps of courtship from its initiation through copulation and ejaculation (Villella *et al.* 1997; Lee *et al.* 2001; Manoli and Baker 2004). We thus assayed abnormalities in courtship latency (time to the first wing extension) and the more visually overt courtship behaviors (*i.e.*, wing extension, wing vibration, and copulation) of individual *GAL4/UAS-fru^MIR* males paired with a CS female.

Initial screening of four males per *GAL4* line yielded 260 lines that showed either reduced or extended courtship latencies. After retests, 86 lines with a short courtship latency (fast courtship initiation, median latency <30 sec), and 24 lines with a long latency (slow courtship initiation, median latency >4 min) were selected. Control flies expressing *UAS-GFP-IR* under control of randomly selected *GAL4* lines had a latency of 157 ± 97 sec. To determine whether the observed courtship latency phenotypes were due to changes in the overall activity of mutant males, we assayed locomotive activity of the fast and slow lines (using a Trikinetics DAM 5). The strongest fast and slow lines did not dramatically differ in activity from *GAL4/UAS-GFP-IR* and *CS* controls (data not shown).

For ~40 lines, *GAL4/UAS-fru^{MIR}* males exhibited defects in wing extension or vibration (on the basis of visual examination). In addition, one line exhibited defective copulatory behavior (File S3). These lines were examined in greater detail in smaller, standard courtship chambers and videotaped for 10 min or until copulation. Lines in which at least 75% of males tested demonstrated a given phenotype were selected for further analysis. Nine lines showed abnormal wing extension, in which the male rotates the wing upwards to ~30° above the horizontal plane, instead of the usual extension parallel to horizontal. While the wing is held at this upward angle, no vibration is observed. Seventeen lines showed simultaneous extension of both wings, varying between 30° and 80° in extent, instead of the usual unilateral wing extension (File S4). Seven lines showed scissoring behavior, in which both wings are rapidly extended somewhat outward and then retracted again (File S5). Five of the lines showing scissoring also showed double-wing extension, suggesting a relationship between the two behaviors. Eight lines showed permanent wing extension, in which one or both wings were permanently extended perpendicular to the body, and not visibly vibrated during courtship. This phenotype was exhibited even before the male was presented with the female, in contrast to the previous phenotypes. Control flies expressing *GFP-IR* under the control of a subset of these *GAL4* drivers did not show these defects.

From these three parallel behavioral screens of 1057 *GAL4* lines (2833 initial assays, as not every line was tested in all three screens, and ~6000 total assays with retests), we obtained 373 lines with robust behavioral changes, 83 of these with defects in more than one assay.

Secondary assays

We used a battery of secondary assays to examine whether each phenotypic class of *GAL4* lines could be divided into subgroups on the basis of additional neurobiological and behavioral criteria. That the groups of lines with similar behaviors in our primary assays were divisible into different subgroups on the basis of secondary assays suggests that the phenotypic classes identified in the primary screens were generated by multiple mechanisms.

Mechanisms underlying male–male courtship: We sought to determine whether chaining behavior resulted from a shared disruption or whether subsets of the chaining lines showed the same overall phenotype as the consequence of perturbations in different neural processes. We therefore examined whether chaining behavior in our lines resulted from: (1) a defect in habituation, by which naïve *D. melanogaster* males learn not to court other males or (2) from a change in mate preference, such that males are competitive with or preferred over females as courtship targets (Gailey *et al.* 1986; Griffith and Ejima 2009).

We examined habituation in the lines showing chaining or high levels of male–male courtship in a modified assay in which pairs of *GAL4/UAS-fru^{MIR}* males from each line were placed together and the amount of courtship measured both immediately after they were placed together and again after they had been together for 1 hr. Although control *GAL4/UAS-GFP-IR* and *CS* males, as well as the majority of *GAL4/UAS-fru^{MIR}* males showed robust decreases in courtship over 1 hr, we found that 15 of the 109 lines assayed initially had at least a moderate level of courtship (courtship index >0.05, Figure 2A), which was unchanged or increased after 1 hr. Thus, increasing attraction toward males or increasing indiscriminate levels of courtship drive may play a role in this behavior. As pairs of mutant males were used rather than one mutant and one wild type, it is also possible that the rejection behavior of the courted male has changed rather than that of the courting male. Thus, although we term the sustained courtship a failure to habituate, other mechanisms are not excluded.

To examine whether chaining behavior reflects a change in sexual orientation (Villella *et al.* 1997) we carried out a mate preference assay, in which a *GAL4/UAS-fru^{MIR}* test male was put together with a wild-type virgin female and a noncourting male, and the fraction of time the *GAL4/UAS-fru^{MIR}* test male spent courting each of the other two flies was measured. To create noncourting males that would not compete with the experimental male yet retained male somatic identity, we used the panneuronal *C155(elav)-GAL4* driver to drive *UAS-fru^{MIR}*, thus eliminating courtship by these males.

To quantify the data from these assays, a courtship preference index based on three parameters is calculated for each line from the analysis of video recordings (see *Materials and Methods*). The Cpi calculates the total relative courtship directed at each target and is defined as $Cpi = (Cif - 0.5)/(Cif + CIm)$, where Cif and CIm represent the fraction of time courting the female and decoy male, respectively (Villella *et al.* 1997). Many lines exhibited a reduced preference for females, with ~30% of the 46 tested lines showing similar courtship of both males and females (Figure 2B). That many chaining lines also show reduced female preference suggests that some *fru^M* neurons contribute to sexual preference by inhibiting inappropriate courtship of males. Most chaining males are willing to court males and females, but either retain a female preference or have no

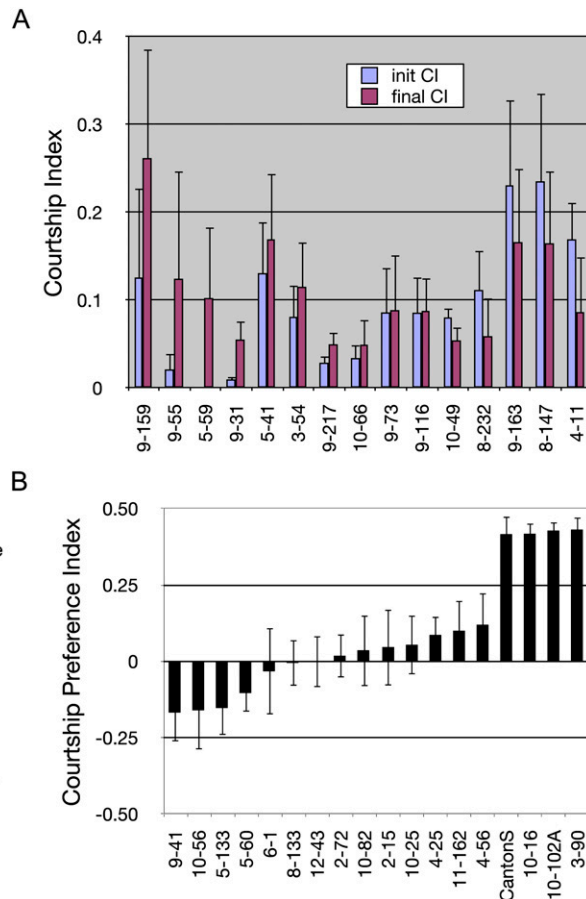
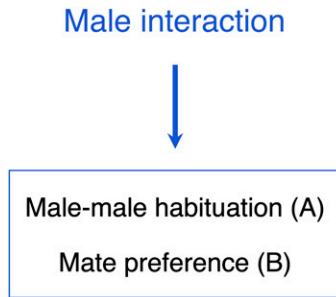


Figure 2 Male–male habituation and changes in mate preference in males that display chaining behavior. (A) Male–male habituation. *GAL4/UAS-fru^{MIR}* males from lines that produced male-chaining behavior were paired and observed over a 1-hr period. Shown are CI averages \pm SEM from minutes 2–7 (blue) and 55–60 (red) of the observation period. The lines shown are the subset with final CI > 0.05 and no significant decrease in CI. Figure S1A shows all tested lines. (B) Mate preference. *GAL4/UAS-fru^{MIR}* males from lines that produced chaining behavior were presented with a virgin Canton S female and a C155/*fru^{MIR}* male simultaneously and courtship preference index (Cpi) values are defined as $Cpi = (Cif - 0.5) / (Cif + Cim)$, where Cif and Cim represent the fraction of time courting the female and decoy male, respectively (Villella *et al.* 1997). Shown are Cpi values \pm SEM from $n = 10$ assays for the 14 lines with lowest Cpi and control lines. Figure S1B shows all tested lines.

preference for either sex, rather than a distinct bias for courtship toward males. Thus, it appears that inhibition of Fru^M expression is capable of reducing female preference while maintaining significant levels of courtship, supporting the separation of the mechanisms underlying arousal and courtship drive from those mediating the specificity of courtship targets.

Phenotypes involving cholinergic fru^M-expressing neurons:

To begin to elucidate the neural mechanisms underlying the behavioral phenotypes we observed, we examined the contribution of cholinergic fru^M neurons to fertility defects and chaining behaviors. Inhibition of neural activity in a subset of primarily cholinergic neurons has previously been associated with chaining behavior (Kitamoto 2001). A role in fertility was suggested by the finding that male-specific cholinergic neurons in the abdominal ganglion regulate the release of sperm and seminal fluids (Acebes *et al.* 2004). Thus, we examined the effects of rescuing Fru^M expression in cholinergic neurons on chaining behavior and fertility. We used the *GAL4* repressor GAL80 to selectively prevent the inhibition of Fru^M expression (by *GAL4* driven *UAS-fru^M-IR*) in the subset of neurons that express *choline acetyltransferase (Cha)* (Figure 3A), thus allowing us to determine the contribution of these neurons to the phenotypes we have identified. Thus we combined the *cha-GAL80* transgene (Kitamoto

2001) with *UAS-fru^M-IR* and each *GAL4* line to observe the extent of rescue.

Of 99 retested lines originally showing fertility defects, 44 lines more than doubled their fecundity when inhibition of Fru^M expression was prevented with *cha-GAL80* compared to simultaneous controls lacking the GAL80 (Figure 3B and data not shown). Thus perturbation of Fru^M expression in *cha-GAL80*-expressing neurons in these 44 lines substantially contributed to their decreased fertility. The failure to rescue the other infertile lines indicates that, consistent with previous studies, Fru^M function is also required in non-cholinergic neurons for full fertility (Lee *et al.* 2001).

Of the 123 retested lines showing high levels of chaining because of *GAL4*-directed fru^{MIR} expression, 8 lines showed a complete suppression of the aberrant behavior with *cha-GAL80* and 17 showed a substantial reduction (Figure 3C). Thus, similar to the fertility rescue results, it appears that both cholinergic and noncholinergic neurons contribute to the regulation of male–male interactions.

Rescue of female fertility: The general similarity of fru^M neuronal expression in males and females (Manoli *et al.* 2005; Stockinger *et al.* 2005) raised the question of whether the labeled neurons share homologous functions in both sexes. Masculinization of fru^M neurons in a female, via the use of a dominant male fru^{Δtra} allele, is sufficient to produce

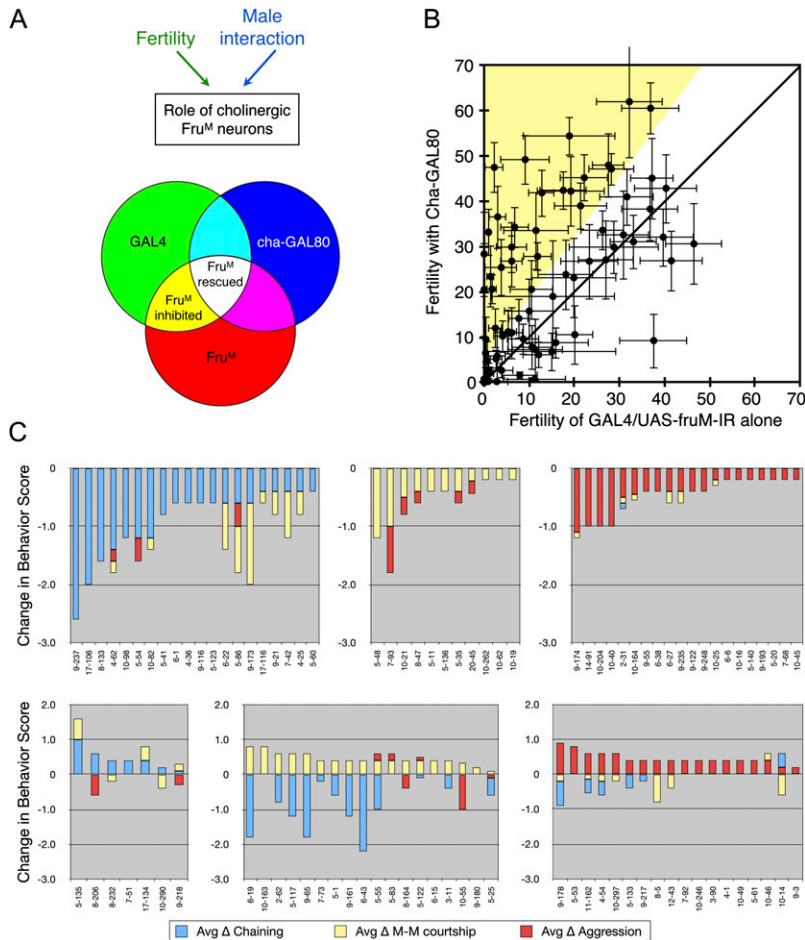


Figure 3 Subsets of fertility defects and male–male courtship phenotypes depend on cholinergic *fru^M* neurons. (A) Expression of *GAL80* in cholinergic neurons by *cha-GAL80* (blue) prevents *GAL4*-directed, *fru^M*IR-mediated inhibition (green) of *Fru^M* expression (red) in cholinergic *fru^M* neurons that are labeled by the enhancer trap (white). (B) Inhibition of *fru^M*IR-mediated disruption of *Fru^M* expression in cholinergic neurons suppresses male sterility or fertility defects in a subset of lines producing fertility defects. Shown are mean fertility counts \pm SEM for *GAL4/fru^M*IR (x axis) and *GAL4/fru^M*IR/*cha-GAL80* (y axis) males for individual lines. The yellow region indicates lines with substantially increased fertility with *cha-GAL80*. (C) Inhibition of *fru^M*IR-mediated disruption of *Fru^M* expression in cholinergic neurons prevents various aspects of male–male interaction phenotypes. Shown are relative changes in severity of behavioral phenotypes in *GAL4/fru^M*IR vs. *GAL4/fru^M*IR/*cha-GAL80* males for individual phenotypic classes. The top row of graphs includes lines with decreased severity of phenotypes, and the bottom row includes lines with combinations of increases and decreases.

sterility (Demir and Dickson 2005). Furthermore, *UAS-shi^{ts}*-based silencing of the *fru^M* neurons in females caused virgins to exhibit mated female behavior, including reduced courtship receptivity and increased egg laying, even though unmated (Kvitsiani and Dickson 2006). While these findings show that some *fru^M*-expressing neurons are necessary for female fertility, they do not address whether these are the same subset of *fru^M*-expressing neurons that are required for male fertility. As our screen identified a subset of *GAL4* lines that produced male infertility when driving *UAS-fru^M*IR, we asked whether the *fru^M*-expressing neurons in these lines are also important for female fertility.

We assayed whether the female infertility produced by *fru^{Δtra}* expression can be suppressed by *UAS-fru^M*IR expression driven by the *GAL4* lines that caused male fertility defects in our screen. We examined the female fertility of 75 *GAL4* lines carrying *UAS-fru^M*IR and *fru^{Δtra}* by crossing them individually to two CS males and counting their offspring (Figure 4). Eighteen of the lines showed significant rescue ($P < 0.05$, one-way ANOVA comparison to a negative control *GAL4* line). Male siblings of the test females generally showed fertility levels similar to what was observed in previous tests of the lines without the *fru^{Δtra}*, indicating that the *UAS-fru^M*IR is able to overcome any dominant effects of the *fru^{Δtra}* allele (data not shown). However, analysis

revealed no significant correlation between the level of reduction of male fertility and rescue of female fertility in this assay (Figure 4), suggesting that although some neurons may be important for fertility in both sexes, there are likely also neurons that are more important for fertility in just one sex. More detailed examinations of *fru^M* expression in males and females have also identified several sexually dimorphic patterns of cell survival and projections, which could also explain the lack of correlation (Kimura *et al.* 2005, 2008; Cachero *et al.* 2010; Yu *et al.* 2010).

Expression

As described, we used a large collection of *GAL4* enhancer-trap lines to inhibit *fru^M* expression in subsets of neurons in males, who were then screened for courtship defects. Beyond the immediate value of these behavioral genetic studies as discussed below, our larger goal has been to identify the particular neurons affected in each *GAL4* line and thus delimit the neurons that contribute to different aspects of sexual behaviors. Our general anatomical approach has been to fluorescently label *fru^M* and *GAL4* neurons using separate reporters and determine the neurons in which the reporters' expression overlapped. The neurons expressing both labels represent those affected by the RNAi against *fru^M* and thus the maximal set of neurons implicated in

Fertility
↓
Female rescue: 18

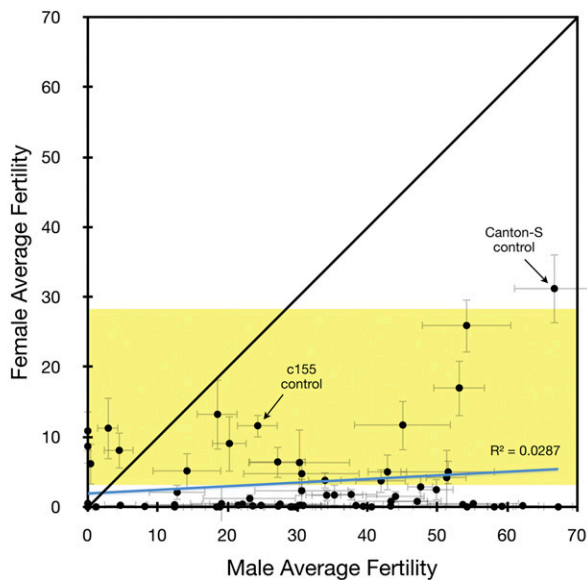


Figure 4 Rescue of *fru*^{Δtra}-induced female infertility by *GAL4* lines with reduced male fertility. Mean fertility ± SEM of *GAL4*; *UAS-fru*^{M/IR}, *fru*^{Δtra} males (x axis) is compared to females (y axis). The yellow region indicates the 18 lines (and positive controls) with substantially increased fertility in females compared with controls, highlighting that several lines did rescue female fertility. Comparison of male vs. female fertility with the same *GAL4* driver does not show a clear relationship, however ($R^2 = 0.03$).

the observed behavioral phenotypes, as the *UAS-fru*^{M-IR} has no known effect outside of *fru*^M-expressing neurons (Manoli and Baker 2004).

When we initiated studies to identify the overlaps in expression between *fru*^M and these *GAL4*'s we used anti-Fru^M antibodies and fluorescent-labeled secondary antibodies together with a UAS-driven nuclear GFP (*UAS-Stinger*) (Barolo *et al.* 2000) to detect *fru*^M and *GAL4* expression, respectively, in the brain and VNC, as *fru*^M expression was believed at that time to be restricted to the CNS (Ryner *et al.* 1996; Lee *et al.* 2000). We examined 183 lines for the concordance of nuclear GFP and Fru^M expression in 22 regions of the CNS using the Fru^M clusters described by Lee *et al.* (2000), as the basis for this CNS analysis. At the 0–24 hr posteclosion time examined, cluster 11 was not reliably labeled by our antibody and was excluded. We additionally subdivided VNC clusters 17–19 into dorsal (D) and ventral (V) subclusters due to their separation along the D–V axis.

The discovery of *fru*^M expression in extensive sets of peripheral sensory neurons (Manoli *et al.* 2005; Stockinger *et al.* 2005) significantly complicated initial attempts to associate the behavioral phenotypes identified in our screen with particular sets of neurons and substantially increased the scope of the project. Because of the tough cuticle surrounding peripheral sensory neurons, antibodies do not reliably penetrate to label these neurons, one of the reasons *fru*^M expression had not been identified there previously. Furthermore, although *fru-P1-GAL4* beautifully labels these PNS neurons, our screen of *GAL4* lines uses the same driver system, preventing us from unambiguously identifying the overlap between the two expression patterns.

This discovery of peripheral *fru*^M expression provided a potential explanation for our results up to this point, as our analyses had revealed only limited correlations of CNS expression and behavior (Figure S4 and see below). Primarily, lines with delayed courtship and/or chaining behavior

tended to have broad CNS overlap with Fru^M. But from that dataset, we were unable to tie a behavior to expression in any particular Fru^M cluster.

To label *fru*^M expression independently of *GAL4* in both the CNS and PNS, we used homologous recombination to insert the *LexA::VP16* transcriptional activator into the *fru-P1* locus (Lai and Lee 2006; Mellert *et al.* 2010), which allowed us to follow *fru*^M expression via expression of *LexAop-GFP*. To detect patterns of *GAL4* expression, we used an optimized nuclear DsRed, *UAS-Red-Stinger* (Barolo *et al.* 2004).

As a test case for this approach, we examined the concordance of nuclear GFP and RFP expression in lines that had fertility defects. The examined set included 96 lines with fertility defects and 48 control *GAL4* lines selected on the basis of normal behavior in our screen. Although we focused on lines with fertility defects, many lines had phenotypes in other assays as well, allowing a limited examination of other phenotypic classes. The results for these other assays may be biased by this selection, however.

We used the clusters described by Lee *et al.* (2000) as a basis for recording expression in the CNS, additionally noting Kenyon cell expression and subdividing the VNC clusters 16–19 into dorsal and ventral subsets. Cluster 15 could not be distinguished from cluster 14 with our reporter and the two were considered together as 14. We additionally scored expression in the following regions of the PNS: the second and third antennal segments, maxillary palp, and proboscis of the head; the five tarsal segments, tibia, and femoral chordotonal organ of the foreleg; and the anal plate, lateral plate, and claspers of the external genitalia. Together these observations identified the *fru*^M neurons in which each *GAL4* line is expressed.

Using this approach, we found that the vast majority of lines causing behavioral phenotypes had extensive, distributed overlap with different Fru^M clusters (Figure 5, Figure

S2, File S6, File S7). As a result, direct comparison of the *GAL4* and *fru^M* expression with observed behavioral defects has yet to indicate an individual cluster where expression is necessary or sufficient to cause fertility defects. Nonetheless, we did observe a general trend toward lines showing behavioral defects, and especially delayed courtship initiation or chaining, tending to have increased average expression compared to controls (Figure 5). Clustering by expression allowed for the visualization of the individual lines' expression and phenotypes but again did not indicate an obvious association beyond the general trend seen in the averaged data (Figure S3).

We used multivariate regression to look for an expression:phenotype relationship that eluded direct comparison. As fertility was not counted for vials with >50 offspring, thus right censoring the data, we first treated fertility as a time variable in multivariate parametric survival analysis, examining the contribution of each expression cluster to the likelihood of the observed fertility (Table S1). The analysis identified expression in foreleg tarsal segments 4 and 5 and anterior brain cluster 6 as significantly likely to result in lowered fertility. We separately treated fertility as a binary result and analyzed the same dataset with logistic regression. This approach also identified tarsal segment 4 and brain cluster 6 (but not tarsal segment 5) as significant drivers of infertility, supporting the result from the survival analysis. We are nonetheless hesitant to draw strong conclusions about these clusters without further experimental data to

directly test their behavioral effects, as basic assumptions of regression models—especially independence of the variables—are unlikely to be met in this complex biological system, but these neural populations remain potential targets for further study.

One explanation for the difficulty of relating expression and behavior from these results is that expression in some of the larger clusters was present in almost all of the lines, and such large clusters likely represent multiple functional classes of neurons, as suggested by their divergent projection patterns and lineage relationships (Cachero *et al.* 2010; Yu *et al.* 2010). For example 146 out of the 153 examined lines had overlap with *fru^M* expression in region 20, the abdominal ganglion, including 54 of 58 lines with normal fertility. Similarly, 123 lines (and 20 out of 58 with normal fertility) overlapped with *fru^M* in the third antennal segment, and 117 lines (and 24 out of 58 with normal fertility) overlapped with *fru^M* in region 14 on the posterior brain. This breadth of expression precluded drawing a direct relationship between expression and phenotype, as effects of expression in other clusters cannot be ruled out with the existing dataset.

Discussion

The studies presented here use an intersectional approach to probe the functional organization of the neural substrates underlying male sexual behaviors in *Drosophila*. The results

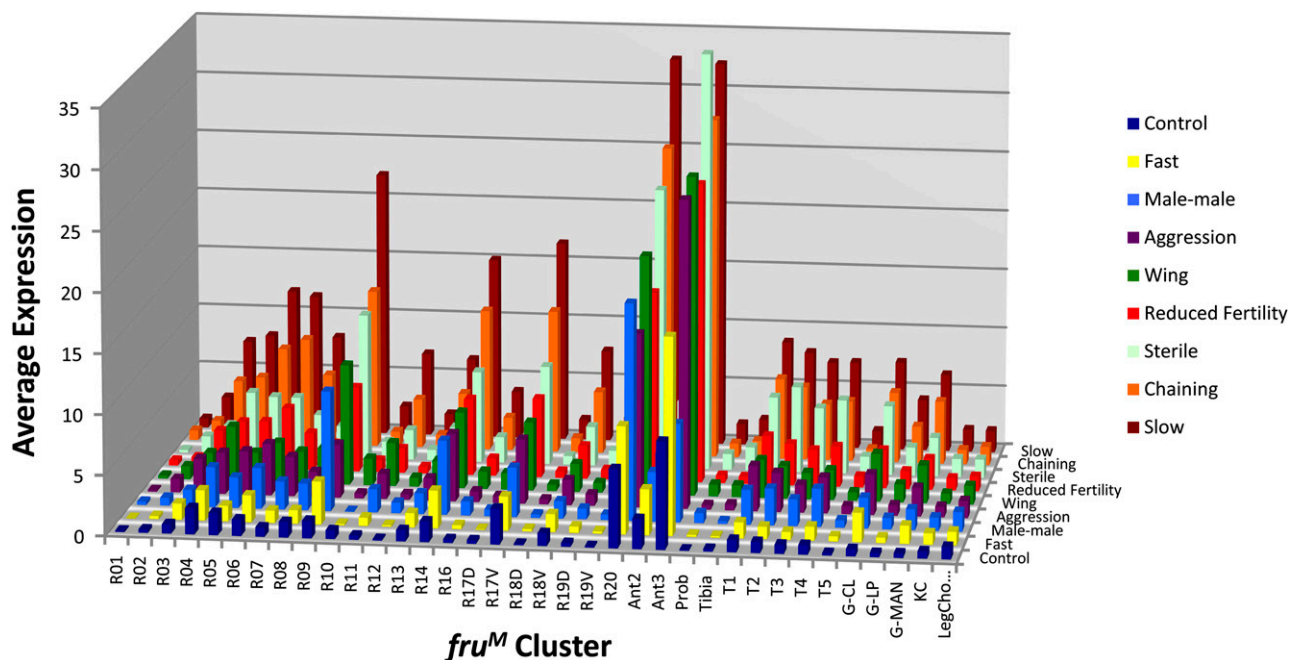


Figure 5 Average *GAL4* overlap with *fru^M* for each courtship phenotype. Phenotypes are sorted by total average expression, with control lines having lowest expression and lines with delayed courtship initiation having highest. Kenyon cell and leg chordotonal expression were scored on a qualitative 0–3 scale for strength of expression. R1–R20, regions 1–20 of the CNS as per Lee *et al.* (2000); Ant 2 and 3, antennal segments 2 and 3; Prob, proboscis; T1–T5, tarsal segments 1–5 of the forelimb; G-CL, genital claspers; G-LP, genital lateral plate; G-MAN, anal plate; KC, Kenyon cells; LegChord, foreleg chordotonal neurons of the femur. See Figure S2 for expression scaled by cluster size, Figure S4 for similar analyses of antibody data, and File S6 for an animation stepping through each phenotype.

of our screens—considered both with respect to the phenotypes we recovered and their underlying causes, as well as the relative frequencies with which certain phenotypic classes were recovered—provide insights into the organization of the *fru^M*-specified circuitry underlying courtship behaviors in *D. melanogaster*.

The frequency with which abnormalities in courtship behavior were detected in our three primary screens is at first glance surprisingly high. Of the *GAL4* lines successfully tested in all three primary assays, 44% (345/777) showed reproducible courtship phenotypes in at least one screen. When considered at the level of individual primary tests, 18% (482/2704) revealed reproducible behavioral defects. There are several factors that likely contribute to this high incidence of courtship phenotypes. First, the progenitor cells that give rise to the CNS are derived from most if not all segments of the body, and *fru^M*-expressing neurons comprise a widely dispersed set of ~2–3% of CNS neurons. Second, *fru^M* neurons make up significant proportions of the primary neurons of the olfactory, gustatory, auditory, and (to a lesser degree) mechanosensory systems, which are derived from imaginal discs. Thus collectively, *fru^M* neurons provide a broad set of targets across the fly for potential overlap with the expression of *GAL4* enhancer traps. In this context, it is reasonable that the screen we conducted identified a high frequency of lines in which *GAL4*-driven *UAS-fru^{MIR}* expression perturbed male courtship.

That the groups of lines with similar behaviors in our primary assays were divisible into subgroups on the basis of the secondary assays suggests that the phenotypic classes identified in the primary screens were generated via multiple mechanisms. Being able to make such subdivisions is important not only for specifying the functions perturbed in individual lines, but also for identifying subgroups of lines that are more likely to have a homogenous mechanism of action and thus a common set of affected *fru^M*-expressing neurons.

Comparisons of the individual classes of courtship behavior defects detected in our screens and secondary tests reveal that there are substantial differences in the relative frequencies with which different phenotypic classes were detected. As the same set of *GAL4* lines was used in all screens, the different frequencies with which particular courtship behavior defects are detected reflect properties of different parts of the courtship circuitry. Following discussion of the individual assays, we discuss associations between phenotypes and propose a simple functional model for courtship initiation that builds upon our observations.

Sterility

Reduced fertility was chosen as one of the diagnostic screen phenotypes because behavioral male sterility is a phenotype associated with many *fru* mutants and mutant combinations. Further, *fru* mutants defective at different steps of courtship were associated with male sterility (Hall 1978; Vilella *et al.* 1997; Manoli and Baker 2004). We thus anticipated that male sterility would be associated with the *GAL4/UAS-fru^{MIR}* silenc-

ing of *Fru^M* expression in multiple different sets of neurons and hence be found as a relatively common phenotype in our screens. Against this background, we were surprised that only 24 of the 911 lines tested produced sterile males. This relative refractoriness of those aspects of the *fru^M* circuitry necessary for fertility to RNAi perturbations suggests the possibility that there is functional redundancy in these aspects of the circuitry or that *fru^M* neurons essential for fertility are restricted with regard to gene expression and thus only rarely overlap with *GAL4*-expression patterns. From an evolutionary perspective, functional redundancy in the circuitry required for fertility makes sense. It is notable that this robustness of male fertility is not likely due to simple bilateral redundancy in the nervous system, since all the *GAL4*-expression patterns that we observed were bilaterally symmetrical. That many more lines showed perturbed behavior than sterility indicates that the observed insensitivity of fertility to *GAL4/UAS-fru^{MIR}* manipulations is also not simply due to a general ineffectiveness of the RNAi, although it is nonetheless possible that the neurons important for fertility are particularly refractive to the RNAi. In addition, the result that many lines altered aspects or specificity of sexual behaviors but only partially impaired these males' fecundity suggests many *fru^M* neurons may play a modulatory role in these behaviors rather than being strictly necessary for fertility.

Altered courtship latency

Our general courtship assay revealed *GAL4* lines with altered courtship initiation latencies toward females. More than three times as many lines were recovered with a shortened courtship latency (95 “rapid” lines), than lines with lengthened courtship latency (25 “delayed” lines). The isolation of rapid courtship lines *per se* suggests that the *fru^M*-specified courtship circuitry contains components that actively inhibit the initiation of courtship. Additionally, the relatively high incidence of rapid courtship lines may suggest that the mechanisms inhibiting courtship in inappropriate contexts are less robust to disruption compared to mechanisms driving courtship, perhaps due to there being more of a cost to delaying courtship than to triggering it inappropriately. Encouragingly, one group of *fru^M* neurons in the median bundle that appear to restrain courtship has been identified (Manoli and Baker 2004). That components of the *fru^M* circuitry function to slow progression through courtship is consistent with arguments that ancestral Dipteran courtship was rapid, similar to what is seen in solitary flies like *Musca* and *Calliphora*, where the strategy for courtship appears to be to identify an object of near optimal size and speed, grab it, then determine if it is an appropriate mating target (Spieth 1974; Collett and Land 1975). Under this view, during the course of evolution from rapid mating to expanded courtship, *D. melanogaster* would have acquired functions in its courtship circuitry that generate the relatively complex, ordered process seen today. Such a protracted courtship may have an evolutionary advantage in allowing the male to demonstrate his virility and both the female and male to receive sufficient

information to evaluate a potential mate. It remains to be determined whether lines that demonstrate rapid courtship onset also display specific deficits in discriminating appropriate courtship targets. It should be noted, however, that the previously characterized group of *fru^M*-expressing neurons that function to restrain courtship (Manoli and Baker 2004) do not have a *fru^M*-dependent role in the discrimination of females from males as appropriate courtship targets.

Male–male interactions

The most common phenotype detected in our screens was an elevated level of male–male interactions: 26% (232/908) of the *GAL4* lines tested had reproducibly elevated levels of one or more types of male–male interaction. There are several elements of note with respect to the high incidence of lines producing a male–male interaction phenotype.

First, male–male interactions encompasses at least two classes of male behavior that likely have different etiologies. The category includes 117 lines showing aggressive male–male interactions, 121 lines showing chaining behavior (involving three or more males), and 49 lines showing high levels of male–male courtship (between pairs of males). It is currently unclear whether the latter two categories are distinct phenotypic classes or just differ in the level of male–male courtship, although some lines showed very high levels of male–male courtship without chaining, perhaps due to differences in rejection behavior.

Second, even with this subdivision of male–male interacting lines the incidence of lines showing male–male courtship is quite high. Contributing to the high incidence of male–male courtship lines in our screen is the fact that this phenotype itself can be potentially further subdivided into classes of lines having different etiologies. *A priori* these include: (1) a change in sexual preference so that males are the preferred courtship targets, (2) an inability to distinguish females from males as appropriate courtship targets resulting in both being courted, (3) a failure of males to reduce initial courtship of other males via habituation (*i.e.*, learning not to court males; Gailey *et al.* 1982), or (4) an increase in sensitivity to general conspecific cues such that inhibitory cues that typically prevent male–male courtship are insufficient to do so. For example, there have been a number of reports of flies with altered gustatory perception showing male–male chaining behavior (Lacaille *et al.* 2007; Miyamoto and Amrein 2008; Moon *et al.* 2009). Our tests to investigate these possibilities revealed that in some lines increased male–male courtship may be attributed to a lack of male habituation or increased sensitivity to general cues. Many other lines showed reductions in their preference of females over males as courtship targets. However, none of the lines we examined showed a clear preference for males over females.

Cholinergic *fru^M*-expressing neurons

For the *GAL4* lines that produced chaining or fertility defects when driving *UAS-fru^M-IR* we extended our intersectional

strategy to examine the contribution of cholinergic *fru^M*-expressing neurons to these chaining and fertility defects. In particular, we used *Cha-GAL80* to selectively restore *Fru^M* expression in the subset of *GAL4/UAS-fru^M-IR*-expressing neurons that also likely express *choline acetyltransferase (Cha)* (Figure 3A). The expression of *Cha-GAL80* at least doubled the fecundity of ~40% (44/99) of lines with fertility defects, whereas it substantially or completely suppressed chaining in ~20% (25/123) of lines originally showing chaining. As cholinergic neurons are found in the CNS and also make up most if not all, olfactory, gustatory, auditory, and chordotonal sensory neurons (Salvaterra and Kitamoto 2001), the most informative aspect of these results are the findings of *GAL4* lines with *fru* phenotypes that are not suppressed by the restoration of *Fru^M* function in cholinergic neurons, as these likely identify cases where *fru^M* CNS expression is important for the observed phenotype. It is worth noting that the nine chaining lines in which restoration of *Fru^M* function produced the greatest suppression of chaining (and 20 of the top 25 such lines) showed normal behavior in the original courtship and fertility assays and retests. This suggests that *Fru^M* function in specific cholinergic neurons may be necessary to prevent chaining or the courtship of other males but that these neurons do not play a role in the proper performance of other courtship behaviors. Thus it appears that some elements underlying the discrimination of a courtship target are distinct from those subserving arousal or the drive to display specific sexual behaviors and appear to be mediated by separable subsets of *fru^M*-expressing neurons. That *Fru^M* expression in cholinergic neurons is important for selection of a courtship target is in part not surprising, as chemosensory cues contribute significantly to mate preference (Stockinger *et al.* 2005; Kurtovic *et al.* 2007; Datta *et al.* 2008; Krstic *et al.* 2009) and most if not all chemosensory neurons are cholinergic (Salvaterra and Kitamoto 2001). Similarly, that fertility in part was restored by rescue of expression in cholinergic neurons is consistent with the importance of cholinergic and noncholinergic neurons in copulation (Lee *et al.* 2001; Acebes *et al.* 2003; Acebes *et al.* 2004).

Behavioral associations

In one sense, our screens can be viewed as having isolated an allelic series of *fru^M* mutants, in each of which only some subset of *fru^M*-expressing neurons are mutant in phenotype. By examining the behavior of many lines with altered patterns of *Fru^M* expression, we can begin to gain insights into the functional units governing the interactions of male flies with other flies. For example, we observed that specific behavioral phenotypes tended to co-occur among sets of enhancer-trap lines (Figure 6), which could indicate that reduced *Fru^M* expression in neurons shared by the lines produces both behavioral outcomes. Alternatively, some enhancer traps may be expressed in several groups of neurons that independently give rise to subsets of the correlated phenotypes. Of the 343 lines showing robust behavioral changes, 84 lines showed changes for more than one type of behavior, providing a substantial group for comparison.

The two most striking correlations we found (1) that lines with longer courtship latencies tended to show chaining behavior, whereas (2) lines with shorter latencies tended to show aggression, are discussed below.

Delayed courtship associates with chaining: We initially hypothesized that Fru^M likely functions in some neurons to repress courtship, such that increased male–male courtship or chaining resulting from the suppression of Fru^M function might also be associated with rapid initiation of female courtship. We found in fact the opposite: a positive correlation between delayed courtship initiation toward females and chaining behavior. Whereas a minority of lines (120 out of 835, or 14.4%) showed chaining behavior, over half of the lines (14 of 24, or 58.3%) with slow courtship initiation also showed chaining ($P < 0.001$, Pearson’s chi square test; Figure 6). No lines showed delayed courtship and high male–male courtship without also exhibiting chaining behavior.

Four differences between the male–male interaction assay and the courtship assay may contribute to this association: (1) the target of courtship being male or female, thus changing the nature of the stimulus; (2) the number of flies in the chamber (two vs. eight), thus changing the overall level of general conspecific cues in the environment; (3) the time together being minutes vs. days, thus allowing for increased stimulation after longer, sustained interactions; and (4) the presence of food in the chamber, thus altering the environmental cues and perhaps arousal. Our examination of male habituation in the chaining lines also addresses whether lengthened exposure could stimulate courtship of males. Our results suggest that this may be the case in a subset of lines, as 15 of the 109 tested lines showed persistent or increasing male–male courtship over an hour. In general however, the lines showing both delayed courtship and chaining did not show defects in habituation. Similarly, the majority of lines that showed chaining behavior did not have a significant change in sexual orientation in the mate preference assay but, rather, appeared to have an overall decrease in the sex specificity of their courtship. Thus, while we cannot rule out a stimulatory role for the presence of food or overall increases in social behaviors or arousal due to cues from conspecifics, we hypothesize that these lines

have a decreased sensitivity to stimulation, which results in courtship only in the presence of multiple conspecifics, rather than when a single female or male was present.

Rapid courtship initiation associates with aggression: Lines showing rapid courtship initiation of females were more likely to show aggressive behavior toward males (20 out of 86 lines or 23.2%) than expected, on the basis of the fraction of total lines showing aggression (112 of 835 lines, 13.4%; $P < 0.01$, Pearson’s chi square test; Figure 6). A supportive, but borderline significant trend was seen with lines with delayed courtship, none of which showed aggression (0 of 24, $P = 0.0504$, Pearson’s chi square). Thus, it appears that the inhibition of Fru^M expression in regions sufficient to cause rapid courtship initiation toward females can be sufficient to increase other sexual behaviors in different contexts. This suggests that common mechanisms may control the stimulus thresholds necessary to elicit appropriate sexual behaviors in specific contexts, while others mediate the discrimination necessary to specify which behaviors occur in distinct ethological situations.

Mechanisms underlying the specificity and sensitivity of courtship initiation: On the basis of the assays described, the relative frequencies of lines producing distinct phenotypic classes, and the correlations described between different behaviors, we have developed a relatively simple functional model for the mechanisms underlying the initiation of sexual behaviors (Figure 7). Although other models can be arrived at from our data, this one is concise and has several attractive features that make it integrate well with our knowledge of *fru^M* circuitry as well as observations made by many groups regarding various aspects of *Drosophila* sexual behaviors. Notably, the model does not distinguish between different sensory modalities. We hope that it will prove useful in interpreting our data and understanding functional elements from expression studies (see File S8, Supplemental Model Discussion).

Expression

General considerations: We have presented an unbiased approach to attempt to discern correlations between specific populations of *fru^M* neurons and distinct aspects of sexually dimorphic behaviors. However, it has proven challenging to

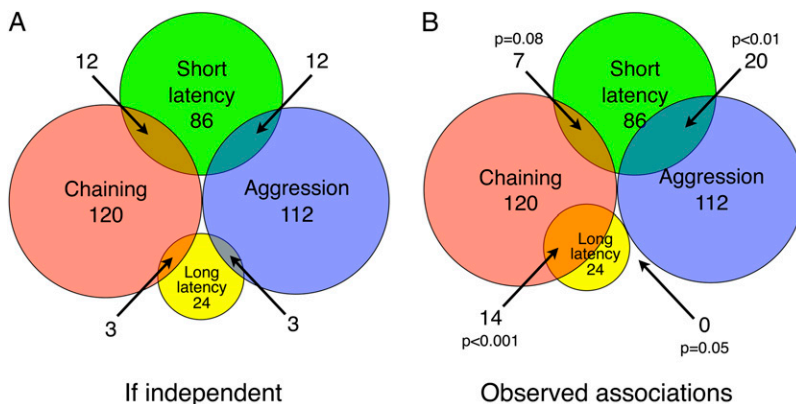


Figure 6 Associations between behavioral assays. (A) If chaining, aggression, and courtship behaviors were independent, the indicated number of GAL4 lines would show each phenotype or combination thereof. (B) The observed associations significantly (P values indicate Pearson’s chi square significance) deviate from the expected values. In particular we observed an increase in lines with both long courtship latency and chaining behavior, as well as lines with both short courtship latency and aggressive behavior.

correlate common sets of *fru^M* neurons with specific aspects of sexual behavior. Factors likely contributing to these difficulties include: the nature of the *GAL4* enhancer-trap lines, functional diversity between neighboring populations of *fru^M* neurons, and redundancy in neural circuitry underlying sexual behaviors.

Functional considerations: Several factors have directed our thinking as to how best to use this data to identify the *fru^M*-expressing neurons responsible for the various courtship phenotypes we observed. Our initial observations with the Fru^M antibody, that *GAL4* expression commonly overlapped many Fru^M clusters per line, agreed with our expectation that some overlapping neurons may not be involved in the specific behavioral phenotype(s) we observed. There are several ways in which we can envisage expressing *UAS-fru^{MIR}* in a subset of *fru^M* neurons might not elicit a behavioral phenotype in our assays. First, a set of *fru^M* neurons might be functionally redundant with some other subset of *fru^M* neurons. Second, subtle phenotypes in sexual behaviors may not have been detected in our assays. Third, some *fru^M* neurons may be involved in conveying information from the circuitry mediating sexual behaviors to parts of the nervous system involved in other behavioral processes. Taken together these thoughts suggest two approaches that might be used to associate particular behavioral phenotypes with individual groups of *fru^M* neurons.

First, the neurons most likely to contribute to a given behavioral phenotype are those that are common to multiple drivers that produce that behavioral deficit. Thus, to narrow

down the set of neurons to those most likely responsible for an observed behavior, we took advantage of the many lines that showed similar phenotypes. We assume that the neurons not responsible for the phenotype will be labeled in some but not other *GAL4* lines producing the same phenotype, whereas the essential neurons will be more consistently labeled in multiple *GAL4* lines producing the same phenotype. By examining the neurons affected in each such line and comparing them between the lines, one expects to find the shared subset responsible for the observed phenotype.

However, our studies revealed some important challenges to this initial theoretical framework. One is that, as we observed from subdividing the initial behavioral classes by our secondary assays, similar phenotypes can have multiple underlying causes. Thus, multiple independent sets of neurons may be able to cause these phenotypes, preventing isolation of a single group of neurons responsible for a given phenotype. We hope to have reduced this concern by increasing our behavioral resolution with the secondary assays, but likely have not eliminated it. Thus, our analysis must allow for multiple foci for a phenotype rather than seeking a single neural population. Second, as described above, while covering several ethological contexts, our assays by no means exhaust the myriad of conditions in which sexual dimorphisms in behaviors may occur. Thus, neuron populations that overlap with regard to phenotypic class in our assays may do so secondary to deficits in pathways that function in contexts outside of those tested, thus preventing their separation.

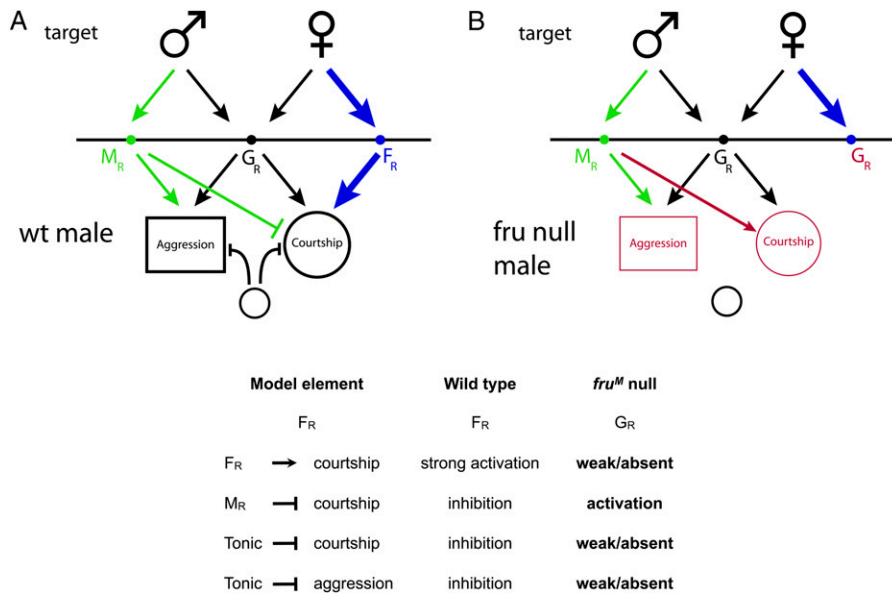


Figure 7 Functional model of mechanisms mediating sex specificity and stimulation during courtship initiation. (A) In a wild-type male, arousal cues common to both sexes act via Fru^M-independent mechanisms to increase the likelihood of either aggressive or courtship behaviors, but are insufficient to elicit behavioral programs themselves (black). Female-specific cues act and are enhanced via Fru^M-dependent mechanisms to drive the initiation of courtship and also likely maintain courtship drive until successful mating (blue). Furthermore, distinct mechanisms modulated by Fru^M regulate the threshold of stimulation necessary to elicit courtship. Thus, we propose that Fru^M functions to allow the detection of female-specific cues to increase the strength of signaling downstream of this perception to drive courtship and to modulate the activation threshold for initiation of courtship. The detection of male-specific cues via Fru^M-dependent mechanisms additionally provides stimulus toward both the initiation of aggression and the inhibition

of courtship initiation (green). Fru^M-dependent mechanisms distinct from the detection of and response to male-specific cues act to modulate the threshold of stimulation necessary to elicit aggressive or courtship behaviors when such cues are perceived. Thus, in addition to regulating the processing of sex-specific cues, distinct Fru^M-dependent mechanisms modulate the activation thresholds for specific behavioral programs, enabling the alteration of thresholds for sexual behaviors without affecting the specific contexts within which these behaviors occur. (B) In males lacking Fru^M function, Fru^M-dependent mechanisms are either eliminated or exist in a default state (red). In the absence of Fru^M function, these pathways may represent female-like mechanisms of stimulus detection or processing.

An alternative to the approach just outlined picks out the *GAL4* line(s) with a given phenotype that have the most restricted overlap with *fru^M* expression. Fewer potential neurons responsible for a phenotype are naturally more straightforward to interpret. Both of these approaches have proved useful in delimiting groups of neurons important for particular behaviors (O'Dell *et al.* 1995; Broughton *et al.* 2004; Manoli and Baker 2004; Stockinger *et al.* 2005; Luan *et al.* 2006; Chan and Kravitz 2007; Kimura *et al.* 2008; Luo *et al.* 2008; Häsemeyer *et al.* 2009; Yang *et al.* 2009).

Neuroanatomical considerations: Perhaps unsurprisingly, screening for behavioral defects without regard to expression patterns of their drivers has yielded many *GAL4* lines with interesting and informative behaviors but with complex expression patterns. The challenge then is to design constraints upon the manipulators either into the initial screen or efficient secondary tests. The primary constraint upon which we relied when we began the screen in 2003 was the restriction of *GAL4/UAS-fru^{MIR}* effects to *fru^M*-expressing neurons. Discovery of *fru^M* expression in the peripheral nervous system substantially expanded the scope of this set of neurons and required new tools to efficiently label *fru^M* expression (Manoli *et al.* 2005; Stockinger *et al.* 2005; Mellert *et al.* 2010). Additionally, we did not anticipate the breadth of typical *GAL4*-driver expression across the nervous system, resulting in simultaneously altering *Fru^M* levels in diverse sets of neurons. Finally, while the initial neuroanatomical analysis of *fru^M* expression revealed likely lineage-related populations based on the proximity of nuclei, recent analyses of the morphology of *fru^M* neurons suggests that even developmentally related populations appear functionally diverse on the basis of their projection patterns and presumed connectivity (Datta *et al.* 2008; Kimura *et al.* 2008; Cachero *et al.* 2010; Yu *et al.* 2010; and data not shown). These observations suggest more refined neuroanatomical parameters are likely also necessary to functionally subdivide populations of *fru^M* neurons and establish behavioral correlations.

Conclusion

We anticipate that the set of lines we have isolated and the richness of behavioral data based on phenotypic classes that we have generated presents a resource that will advance our understanding not only of the neural substrates of sexual behavior in the fly, but perhaps also the fundamental principles that underlie the specification and function of circuitry that subserves complex behaviors in general. Once identified, the characterization and manipulation of these distinct sets of neurons will allow us to begin to understand the representation of information relevant to specific behavioral states within the nervous system and how such information is processed to generate and execute innate behavioral programs.

Finally, it is also worth noting that the formal logic of our approach—carrying out a large-scale screen for genetic var-

iants that have phenotypes perturbing a process of interest, validating those phenotypes by retests, and then sorting them into classes and subclasses on the basis of subsequent secondary assays—is the basic paradigm that has been used across all model organisms to dissect developmental and physiological processes of interest. Our experiments establish that this approach can be extended to the analysis of an innate behavior and provide guides for designing similar studies of other behaviors and their underlying genetic and neural substrates.

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GENETICS

Supporting Information

<http://www.genetics.org/content/suppl/2011/06/24/genetics.111.129940.DC1>

Functional Dissection of the Neural Substrates for Sexual Behaviors in *Drosophila melanogaster*

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David J. Mellert, David H. Tran, and Bruce S. Baker

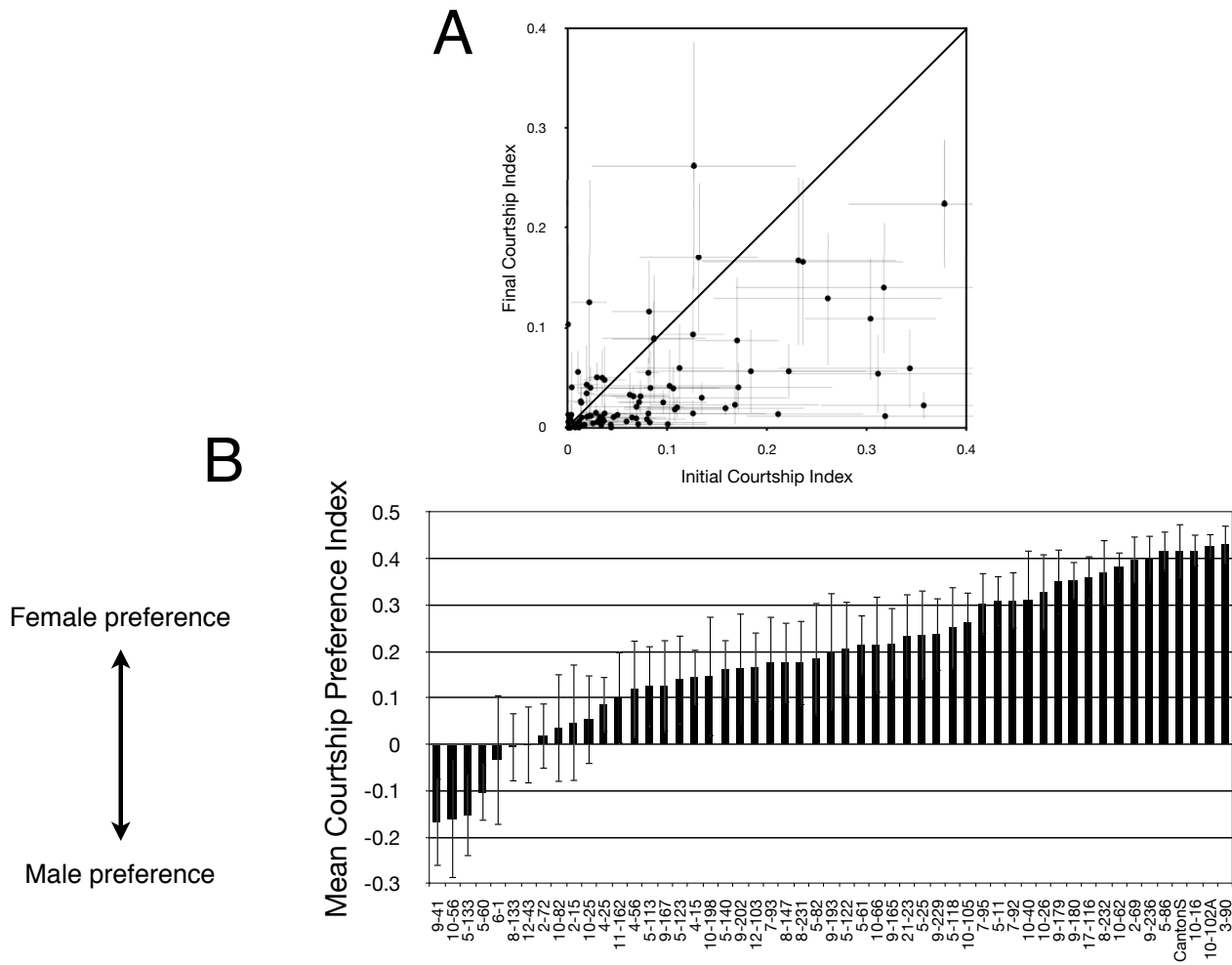


Figure S1 Male-male habituation and changes in mate preference in males that display chaining behavior; complete data. A) Courtship index decreases over time in most *GAL4* lines, when initial (x axis) courtship is compared to that after one hour (y axis). A subset of lines, highlighted in Figure 2A, have substantial courtship and no decrease over the course of the assay. B) Mate preference data for complete set of lines tested, with a subset highlighted in Figure 2B.

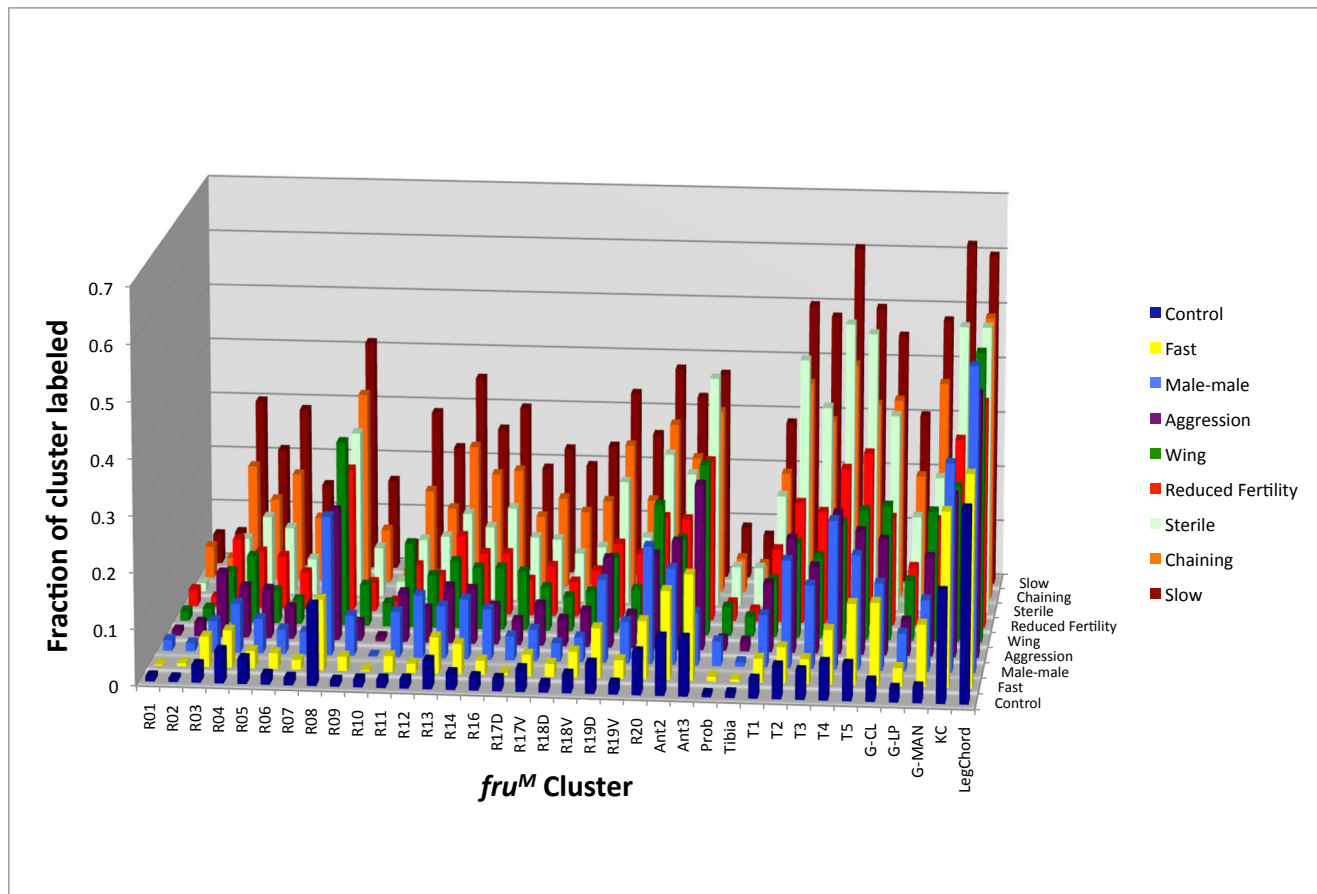
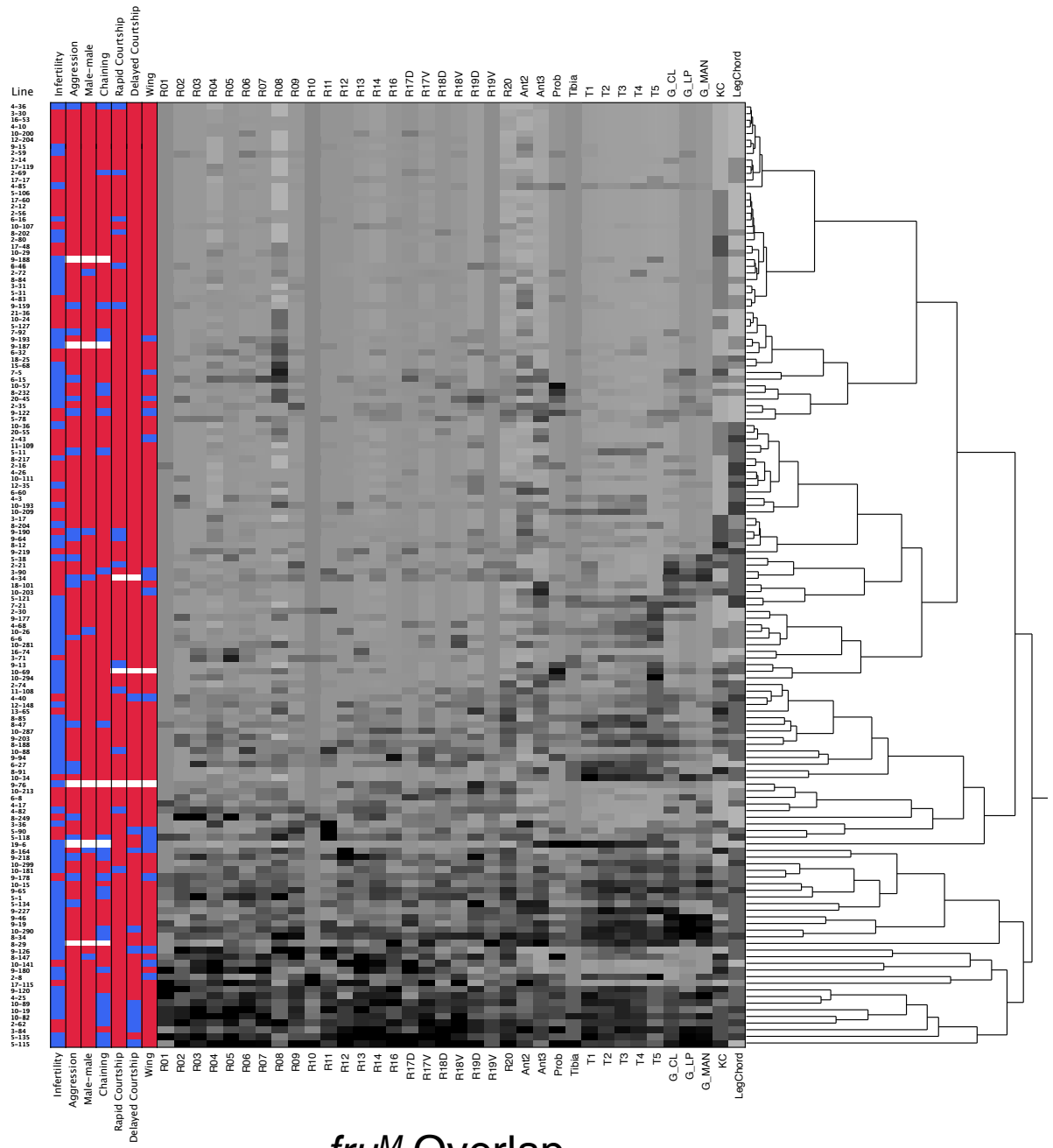


Figure S2 Average *GAL4* overlap with *fru*^M for each courtship phenotype, rescaled relative to cluster size. To compensate for size differences in the *fru* clusters presented in Figure 5, expression was scaled independently relative to each cluster. See File S7 for an animation of each phenotype in sequence.

Gal4 Line

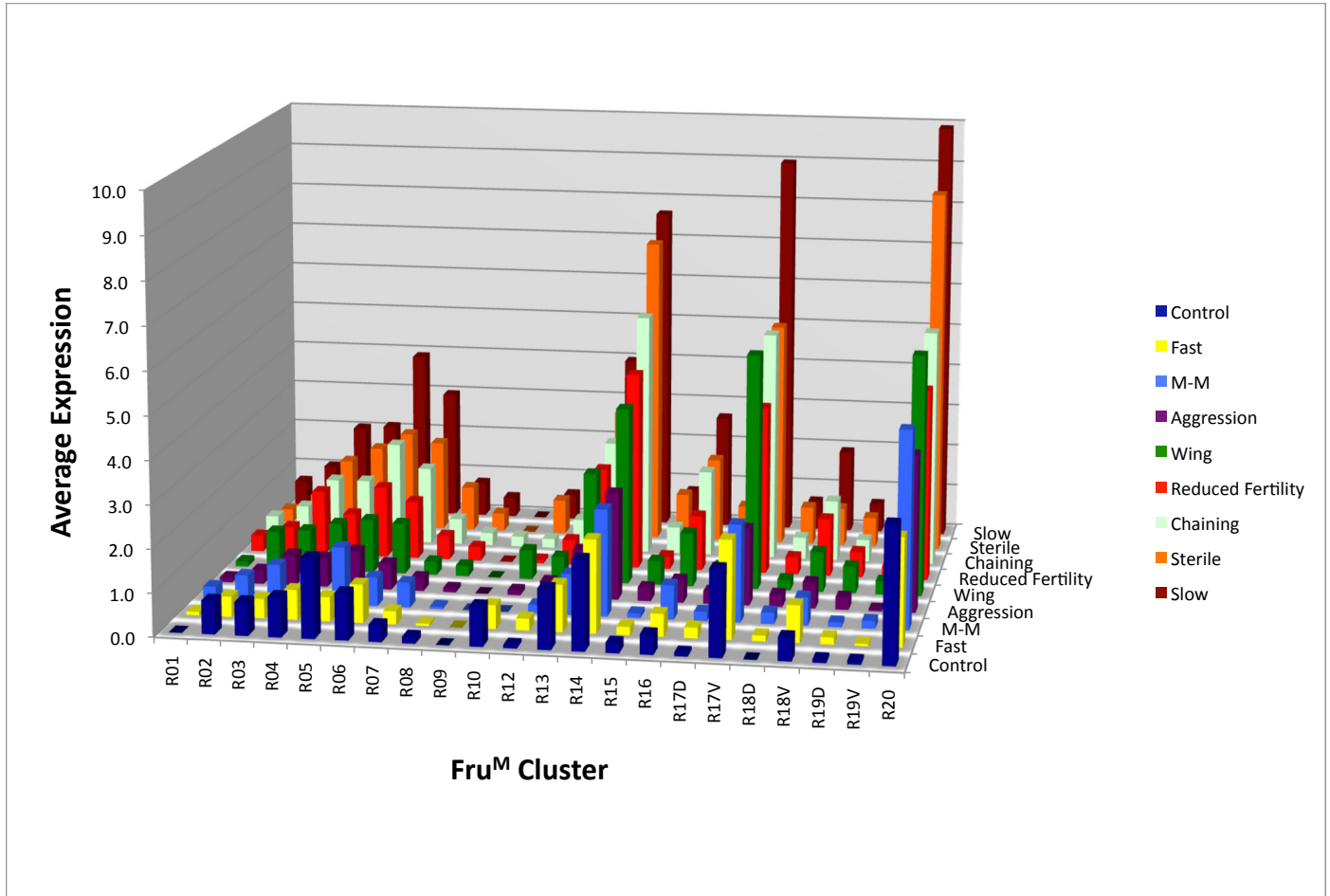


Phenotype

fru^M Overlap

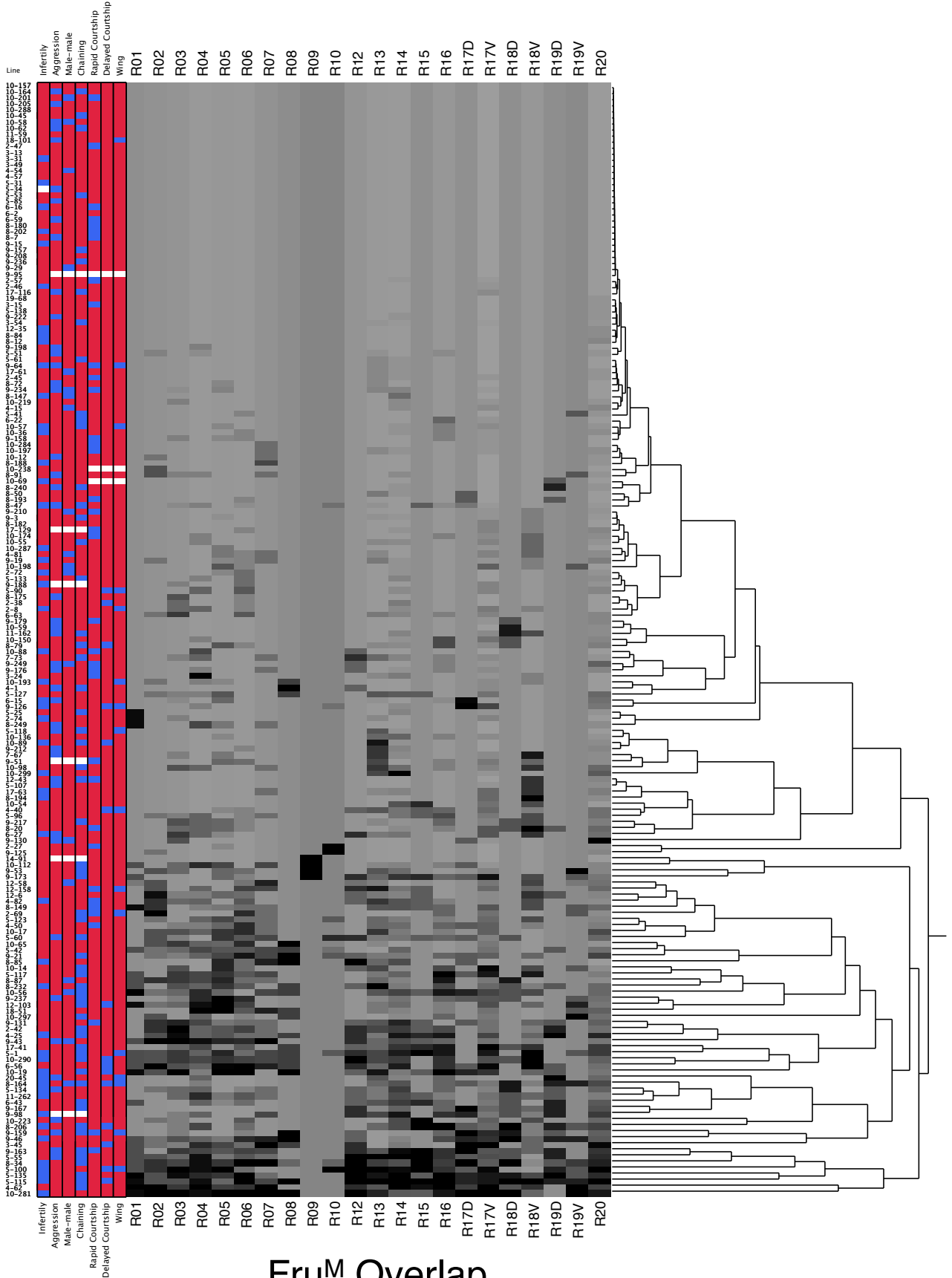
Figure S3 Phenotypic and overlap data for each line, clustered by overlap. *GAL4* lines were clustered by patterns of overlap with *fru^M* to determine if phenotypes would associate accordingly. Each *fru^M* cluster is independently normalized, with darker grey expression indicating stronger overlap. For phenotypic data, blue indicates an altered phenotype, red indicates wild-type, and white indicates no data. Order of *fru^M* clusters and abbreviations follow Figure 5.

A



Gal4 Line

B



Phenotype

Fru^M Overlap

Figure S4 *GAL4* overlap with Fru^M antibody for each courtship phenotype, related to Figure 5 and S3. Analyses were performed as in A) Figure 5 and B) Figure S3, but limited to CNS clusters.

Files S1-S7

Supporting Movies

Files S1-S7 are available for download as .mov files at

<http://www.genetics.org/content/suppl/2011/06/24/genetics.111.129940.DC1>.

FILE S1. Line 7-73 expressing *UAS-fru^MIR* demonstrates high levels of chaining behavior, in which multiple males court each other in an extended chain.

FILE S2. Line 10-164 expressing *UAS-fru^MIR* demonstrates high levels of aggressive behavior, with one male chasing others on the food surface, pulling them and lunging at them.

FILE S3. Line 19-6 expressing *UAS-fru^MIR* appears to temporarily become stuck in a bent position after attempted copulation.

FILE S4. A male from line 9-178 expressing *UAS-fru^MIR* frequently extends both wings simultaneously during courtship of the female.

FILE S5. A male from line 5-118 expressing *UAS-fru^MIR* frequently displays scissoring behavior when courting the female. His wings are rapidly extended and retracted from their rest position to a roughly 15 degree angle.

FILE S6. Animated version of Figure 5.

FILE S7. Animated version of Figure S2.

Supplemental Model Discussion

We propose that the elicitation of sexual behaviors occurs via the perception, integration, and translation of sex-specific (Figure 7A, green and blue) as well as non-sex-specific (Figure 7A, black) cues to activate appropriate behavioral responses. Thus, in a wild-type male, arousal cues common to both sexes, perhaps signifying conspecifics, act via Fru^M-independent mechanisms to increase the likelihood of either aggressive or courtship behaviors, but are insufficient to elicit behavioral programs themselves (black). In addition female-specific cues, acting via multiple sensory modalities, act and are enhanced via Fru^M-dependent mechanisms to drive the initiation of courtship, and also maintain courtship drive until successful mating (blue). Finally, distinct mechanisms modulated by Fru^M regulate the threshold of stimulation necessary to elicit courtship (black circle). Thus, we propose that Fru^M functions to allow the detection of female-specific cues, to amplify signaling downstream of this perception to drive courtship, and to modulate the activation threshold for initiation of courtship.

We propose that in a wild-type male, in addition to Fru^M-independent mechanisms that process general arousal cues as described above, the detection of male-specific cues via Fru^M-dependent mechanisms provides stimulus towards both the initiation of aggression, and the inhibition of courtship initiation (Figure 7A, green). As in the response to female cues, Fru^M-dependent mechanisms distinct from the detection of and response to male-specific cues act to modulate the threshold of stimulation necessary to elicit aggressive or courtship behaviors (black circle). Thus, for the responses of a wild-type male to males, we propose that Fru^M functions to inhibit courtship initiation by processing signals downstream of the detection of male cues to inhibit courtship initiation and promote aggressive displays. Thus, when Fru^M expression is perturbed, cues that normally inhibit male-male courtship become activating and cause chaining behavior with males rather than aggression, while female-specific cues are either equally or less attractive, resulting in increased courtship latency, and either non-specific or reversed sexual orientation.

As discussed for the response to males and females, Fru^M-dependent mechanisms appear to modulate the activation thresholds for specific behavioral programs, enabling the alteration of stimulus thresholds for sexual behaviors without affecting the contexts within which these behaviors occur (Figure 7A, black circle). For example, we observe that lines that show a rapid onset of courtship towards females are more likely to show increased aggression towards males. We propose that males with such phenotypes have decreased Fru^M-dependent inhibition of the initiation of sexual behaviors, but are likely to have no

disruption in the pathways that mediate the processing of cues dictating whether courtship or aggressive programs are appropriate. The frequency with which we observe rapid courtship initiation and aggression, either together or separately, suggests that the mechanisms regulating the threshold(s) for behavior initiation are less robust to perturbation than other Fru^M-dependent processing, perhaps indicating recent evolutionary changes from other species.

In contrast to an alteration of stimulus thresholds for the display of specific behaviors, we propose that multiple mechanisms involved in detection or processing of sex-specific cues may induce chaining behavior in grouped males. Although disruptions in both processes may result in chaining behavior, each mechanism can be distinguished by its distinct courtship latency phenotype (Figure 7B). For example, in males that display chaining with no changes in courtship latency, we propose a partial loss of the Fru^M-dependent processing of inhibitory signals from the perception of males to the stimulation of courtship, thus yielding increased male-male courtship in grouped males, with no changes affecting female courtship. In males that display chaining behavior along with increases in courtship latency, we propose a partial loss in the perception of female-specific cues in exchange for sensitivity to more general arousal cues, thus allowing for stimulation by males of courtship behaviors, but a decreased response to females.

Based on these correlations between phenotypic classes and the frequencies at which individual classes arise, we propose a model for Fru^M-dependent regulation of the initiation of sexual behaviors in specific contexts. We suggest that Fru^M function affects the perception or processing of sensory cues that stimulate the drive towards distinct programs for sexual behaviors. The processing affected by Fru^M function thus includes 1) the detection of sex-specific and general cues, 2) the strength of signaling downstream of such detection, 3) the stimulatory or inhibitory function of such signaling, and 4) the thresholds for the activation of distinct behavioral programs. From data in this and other studies, we conclude that the mechanisms that determine the contexts in which distinct behavioral programs are initiated in response to sensory cues, and the strength of these cues to elicit behavior, are largely separable from the mechanisms that set the thresholds for activation of these behavioral programs.

A number of general principles found within this model make it both concise and consistent with current knowledge of Fru^M function. First, we propose that Fru^M functions as a binary switch at specific points in the circuit mediating initiation, a feature consistent with the exclusively binary mechanisms underlying most sex-determination mechanisms as well as *fruitless* regulation itself. Second, in accordance with the existence of isolated examples of Fru^M-dependent changes in gross neuronal

morphology or specification of male-specific neural structures, we propose that Fru^M primarily functions to alter either fine synaptic structure or neuronal physiology (KIMURA et al. 2005; MANOLI et al. 2005; STOCKINGER et al. 2005; DATTA et al. 2008; KIMURA et al. 2008; CACHERO et al. 2010; YU et al. 2010). For example, changes in the strength of signaling downstream of sex-specific cues may be mediated by increased synaptic density at the termini of *fru*^M neurons relaying such information, consistent with Fru^M dependent changes in the volume of antennal lobe glomeruli innervated by *fru*^M-expressing neurons (KONDOH et al. 2003; STOCKINGER et al. 2005). As another example, Fru^M dependent changes in neural physiology may occur via changes in neurotransmitter expression, consistent with previously described Fru^M-dependent sexual dimorphisms in neural function (LEE and HALL, 2001). Via a different mechanism, but also consistent with Fru^M-dependent function as a binary switch, subsets of *fru*^M neurons survive in a male-specific manner, thus potentially providing male-specific detection or processing of information (KIMURA et al. 2005). Third, based on the phenotypic categories defined here, we have been able to show that only one change is required for all observed categories of behavior. Thus, we find, for example, that relatively few changes in a Fru^M-dependent pathway inhibiting stimulation of courtship by males can result in higher levels of male-male courtship while still preserving a female bias in mate preference.

Table S1 Multivariate statistical tests relating overlap to fertility data. Survival analysis was used for the first analysis, as vials with fecundity over 50 were not counted. As a complementary approach, a nominal logistic regression between expression and the presence or absence of a fertility defect was performed. **A)** Both analyses applied to *fru^M GAL4* overlap found a significant relationship for clusters T4, R06, and LegChord, with expression in the former two being associated with reduced fecundity and in the latter with increased fertility. **B)** Parallel analysis of *GAL4* overlap with FruM antibody found a relationship for R08 only in the survival analysis.

A

Parametric Survival					Logistic Regression				
Source	Chi Square	P > ChiSq	Estimate	Std Error	Source	Chi Square	P > ChiSq	Estimate	Std Error
T4	17.15	<.0001*	-0.614	0.135	LegChord	11.72	0.0006*	1.024	0.339
LegChord	8.54	0.0035*	0.497	0.180	R06	9.47	0.0021*	-0.361	0.136
T5	8.30	0.0040*	-0.610	0.205	T4	8.28	0.0040*	-0.961	0.382
R02	7.88	0.0050*	0.707	0.263	R18D	3.21	0.0731	1.302	0.717
R06	6.32	0.0119*	-0.126	0.050	R01	2.83	0.0925	-1.008	0.620
R07	3.80	0.0512	-0.153	0.071	R17V	2.78	0.0954	0.258	0.165
R11	3.74	0.0532	0.178	0.093	KC	1.54	0.2144	-0.402	0.328
R04	3.47	0.0625	0.172	0.094	T5	1.52	0.2176	-0.647	0.559
R01	2.79	0.0946	-0.636	0.374	Tibia	1.49	0.2220	0.446	0.331
R18D	2.70	0.1005	0.751	0.454	R12	1.38	0.2405	-0.854	0.741
Weibull Distribution					N				
N					144				
Degrees of Freedom					36				
Chi Square					67.0				
Prob > Chi Square					0.0013*				

B

Parametric Survival					Logistic Regression				
Source	Chi Square	P > ChiSq	Estimate	Std Error	Source	Chi Square	P > ChiSq	Estimate	Std Error
R08	5.35	0.0207*	-1.489	0.624	R02	2.65	0.1033	0.477	0.320
R18V	3.80	0.0511	-0.395	0.201	R03	2.46	0.1168	-0.272	0.178
R19D	3.49	0.0617	-0.827	0.409	R09	2.37	0.1239	1.903	10.703
R04	2.28	0.1308	0.549	0.393	R19D	2.13	0.1449	-0.447	0.314
R03	2.16	0.1417	-0.356	0.248	R08	1.54	0.2142	-0.561	0.454
R17V	2.15	0.1424	0.225	0.170	R17V	1.44	0.2297	0.104	0.090
R09	1.91	0.1671	6.517	1362.305	R13	1.44	0.2304	-0.127	0.106
R19V	1.53	0.2155	-0.518	0.367	R18V	1.16	0.2824	-0.151	0.138
R02	1.36	0.2428	0.516	0.469	R19V	0.97	0.3247	-0.311	0.295
R13	1.21	0.2711	-0.149	0.132	R12	0.84	0.3582	-0.353	0.386
Weibull Distribution					N				
N					182				
Degrees of Freedom					22				
Chi Square					30.0				
Prob > Chi Square					0.1182				