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fruitless Splicing Specifies Male Courtship Behavior in *Drosophila*

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Summary

All animals exhibit innate behaviors that are specified during their development. *Drosophila melanogaster* males (but not females) perform an elaborate and innate courtship ritual directed toward females (but not males). Male courtship requires products of the *fruitless* (*fru*) gene, which is spliced differently in males and females. We have generated alleles of *fru* that are constitutively spliced in either the male or the female mode. We show that male splicing is essential for male courtship behavior and sexual orientation. More importantly, male splicing is also sufficient to generate male behavior in otherwise normal females. These females direct their courtship toward other females (or males engineered to produce female pheromones). The splicing of a single neuronal gene thus specifies essentially all aspects of a complex innate behavior.

Introduction

Animals are born not only with their characteristic body plan and morphology, but also a set of innate behaviors, or instincts, that are manifested as stereotyped responses to environmental stimuli (Tinbergen, 1951). Enormous progress has been made over the past several decades in elucidating the developmental processes that direct the formation of the body plan and its parts. In contrast, our understanding of how innate behaviors are specified is still rudimentary at best. It is not even clear whether the general principles gleaned from the study of morphological development also apply to the development of behavior. For example, body parts are often specified by "switch" or "selector" genes, the action of which is both necessary and sufficient to trigger the development of a complete anatomical structure (Garcia-Bellido, 1975). Might instincts be specified in a similar way? Are there behavioral switch genes that create the potential for a complex innate behavior (Baker et al., 2001)? Or, at the other extreme, do instincts emerge diffusely from the combined actions of the vast number of genes that contribute to nervous system development and function, so that no single gene can be said to specify any particular behavior (Greenspan, 1995)?

If behavioral switch genes exist, then one place in which they are likely to be found is in the specification of sexual behaviors (Baker et al., 2001). Males and females generally have dramatically distinct and innate sexual behaviors. These behaviors are essential for their reproductive success, and so strong selective pressure is likely to have favored the evolution of genes that "hardwire" them into the brain. The initial steps of sexual differentiation have been well characterized for several model organisms, and genetic perturbations in these sex-determination hierarchies can alter all aspects of the sexual phenotype—innate behaviors as well as gross anatomy. Several genes near the top of these sex-determination hierarchies thus qualify as developmental switch genes, but they cannot be considered specifically as behavioral switch genes. A switch gene for a sexual behavior should act to specify either male or female behavior, *irrespective* of the overall sexual phenotype of the animal. A candidate for such a gene is the *fruitless* (*fru*) gene of *Drosophila*, which is intimately linked to male sexual orientation and behavior (Baker et al., 2001).

Male courtship in *Drosophila* is an elaborate ritual that involves multiple sensory inputs and complex motor outputs (Hall, 1994; see Movie S1 in the Supplemental Data available with this article online). It is largely a fixed-action pattern, in which the male orients toward and follows the female, taps her with his forelegs, sings a species-specific courtship song by extending and vibrating one wing, licks her genitalia, and finally curls his abdomen for copulation. If the female is sufficiently aroused and has not recently mated, she accepts his advances by slowing down and opening her vaginal plates to allow copulation. An obvious but nonetheless remarkable aspect of this behavior is that mature males court only females, never other males, whereas females do not court at all.

Certain loss-of-function alleles of the *fru* gene disrupt both male courtship behavior and sexual orientation: performance of the courtship ritual is below par, and it is directed indiscriminately at either sex (Anand et al., 2001; Ito et al., 1996; Lee et al., 2001; Ryner et al., 1996; Villella et al., 1997). Strong *fru* alleles completely block courtship behavior, but weaker *fru* alleles variously disrupt individual steps, with each step affected in some allelic combination (Anand et al., 2001; Lee et al., 2001). This suggests that *fru* is required for every step of the courtship ritual, not just for a single critical step. For all of these *fru* alleles, female morphology and behavior appear normal.

Of the many genes known to be involved in male courtship behavior (Billeter et al., 2002), *fru* is unique in that it is sex-specifically spliced (Ito et al., 1996; Ryner et al., 1996). Alternative splicing at both the 5′ and 3′ ends of the *fru* locus generates a complex set of transcripts, all of which encode BTB domain-containing zinc finger proteins. Most of these transcripts are not sex specific, but those initiated from the most distal (P1) promoter are spliced differently in males and females. The alleles of *fru* that affect male courtship are all associated with chromosomal insertions, deletions, or rearrangements that specifically disrupt these sex-specific P1 transcripts (Anand et al., 2001; Goodwin et al., 2000). This has led to the hypothesis that the male-

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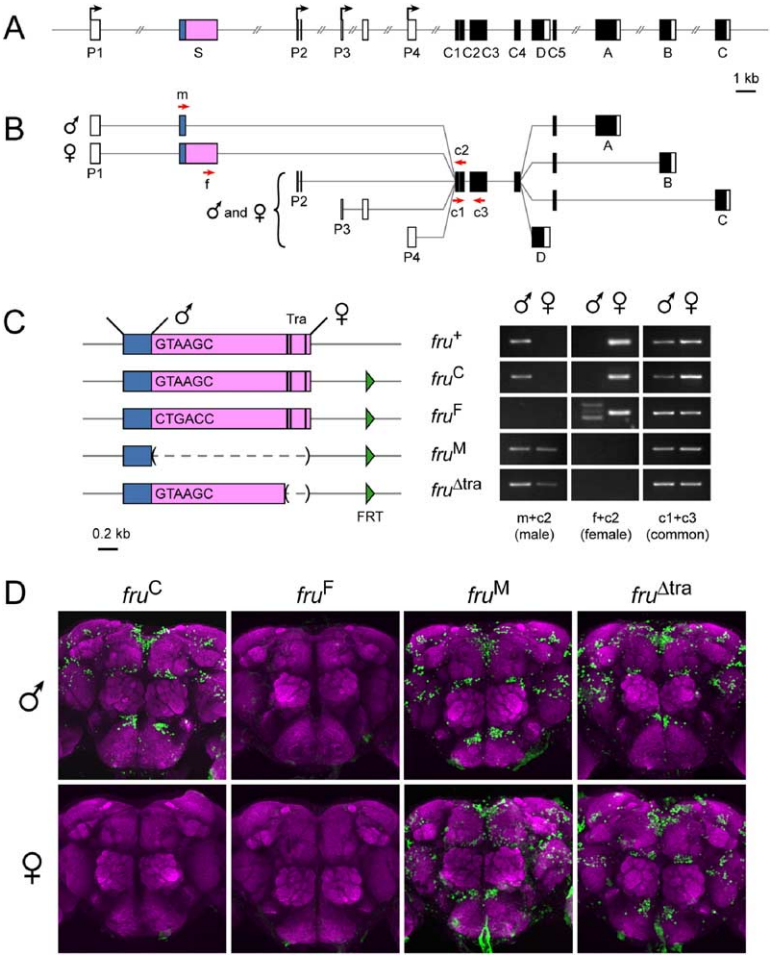

Figure 1. Generation of *fru* Sex-Specific Splicing Mutants

(A and B) Organization of the *fru* gene (A) and its transcripts (B). P1–P4 indicate alternative promoters, S the sex-specifically spliced exon found only in P1 transcripts, C1–C5 common exons, and A–D alternative 3′ exons.

(C) Targeted modifications of the S exon. Bars indicate Tra binding sites. Right panels show RT-PCR analysis of transcripts from the wild-type fru^{+} and targeted *fru* alleles. mRNA was extracted from heads of adults heterozygous for the indicated allele over $fru^{4\text{-}40}$. Primers are indicated by red arrows in (B).

(D) Whole-mount adult brains of males and females of the indicated genotypes, stained with anti-Fru^{M} (green) and mAb nc82 (magenta).

specific splicing of the *fru* P1 transcripts specifies male courtship behavior and sexual orientation (Baker et al., 2001). This is the hypothesis we test here.

We used gene targeting by homologous recombination to generate alleles of *fru* that are constitutively spliced in either the male or female mode. Forcing female splicing in the male results in a loss of male courtship behavior and orientation, confirming that male-specific splicing of *fru* is indeed essential for male behavior. More dramatically, females in which *fru* is spliced in the male mode behave as if they were males: they court other females. Thus, male-specific splicing of *fru* is both necessary and sufficient to specify male courtship behavior and sexual orientation. A complex innate behavior is thus specified by the action of a single gene, demonstrating that behavioral switch genes do indeed exist and identifying *fru* as one such gene.

Results

fruitless Splicing Mutants

The *fru* locus spans approximately 130 kb, and includes at least four promoters (P1–P4; Figure 1A; Ito et al., 1996; Ryner et al., 1996). Transcripts from the P2–P4 promoters are not sex-specifically spliced and encode a set of common Fru isoforms that have essential functions in the development of both sexes (Figure 1B; Anand et al., 2001; Ryner et al., 1996). Transcripts initiated from the distal P1 promoter include the S exon, which is sex-specifically spliced under the control of the sex-determination factors Tra and Tra-2 (Heinrichs et al., 1998; Ito et al., 1996; Ryner et al., 1996). In males, Tra is absent and the S exon is spliced at its default male-specific donor site. This results in an in-frame fusion to the exons common to all *fru* transcripts, adding a 101 amino acid N-terminal extension that is unique to these male-specific Fru^{M} isoforms. In females, Tra binds to *fru* P1 pre-mRNAs to promote splicing at a more 3′ donor site (Heinrichs et al., 1998) and to block translation of these transcripts (Usui-Aoki et al., 2000). Both mechanisms ensure that no full-length Fru^{M} proteins are produced in females.

We generated four alleles of *fru* by gene targeting: fru^{F}, an allele that should prevent male-specific splicing; fru^{M} and $fru^{\Delta tra}$, both of which should force male splicing; and fru^{C}, a control allele in which splicing should be unchanged (Figures 1C and S1). In fru^{F}, point mutations introduced at the male splice donor site of the S exon should abolish splicing at this site but not alter the coding potential of the unspliced transcripts. In fru^{M}, the entire 1601 bp female-specific part of the S exon is deleted, while $fru^{\Delta tra}$ contains a 261 bp deletion

that just eliminates the Tra binding sites. In fru^C, the only sequence modification is the insertion of an FRT site in the intron following the S exon. This FRT insertion is a footprint of the targeting procedure and is also present in the fru^F, fru^M, and $fru^{\Delta tra}$ alleles.

We established several independent lines for each of these four alleles and verified them by molecular, histological, and behavioral analyses. Independent derivatives of the same allele were indistinguishable in all of these assays. A single line for each allele was then backcrossed to the wild-type Canton S strain for four generations prior to more extensive behavioral tests. For all molecular, histological, and behavioral data presented here, unless otherwise stated, the fru^C, fru^F, fru^M, and $fru^{\Delta tra}$ alleles were examined in *trans* to $fru^{4\text{-}40}$. The $fru^{4\text{-}40}$ allele results from a deletion of at least 70 kb that removes all P1 and P2 transcripts and is genetically null for the *fru* behavioral phenotypes (Anand et al., 2001). Thus, any *fru* P1 product or activity detected in these assays can be assigned to the engineered fru^C, fru^F, fru^M, or $fru^{\Delta tra}$ allele.

PCR amplification and DNA sequencing of the entire 18 kb targeted region confirmed the predicted structure of the *fru* locus in each of the four alleles, and RT-PCR experiments confirmed that the predicted transcripts are indeed generated (Figure 1C; splicing at the female donor site is variable in fru^F males, presumably because no Tra is present to promote use of the normal female splice site.) Fru^M proteins could also be detected in the brains of fru^C males, and fru^M and $fru^{\Delta tra}$ flies of either sex, both in adults (Figure 1D) and 48 hr pupae. The distribution of Fru^M appears identical in each case and also matches the reported expression of Fru^M in wild-type males (Lee et al., 2000). We conclude that the modifications we have introduced into the *fru* locus eliminate the sex differences in Fru^M expression but do not alter its distribution.

fru Regulates Sexual Behavior but Not Gross Sexual Anatomy

The sex determination hierarchy in *Drosophila* bifurcates downstream of Tra. Like *fru*, the *doublesex* (*dsx*) gene is also differentially spliced under the control of Tra and produces either male (Dsx^M) or female (Dsx^F) isoforms of a DM-domain transcription factor (Burtis and Baker, 1989; Erdman and Burtis, 1993; Hoshijima et al., 1991). These Dsx proteins direct male or female morphological development, respectively, but have little influence on sexual behavior: males that lack Dsx^M still court, albeit at reduced levels (Villella and Hall, 1996), whereas females that produce Dsx^M resemble normal males but do not court (Taylor et al., 1994). This has led to the notion that *dsx* regulates gross sexual anatomy, while *fru* regulates sexual behavior (Taylor et al., 1994).

Consistent with this view, our *fru* P1 splice mutations do not generally alter external or internal sexual anatomy: fru^F males have the normal male anatomy, whereas fru^M and $fru^{\Delta tra}$ females are anatomically normal females. The one exception is that fru^M and $fru^{\Delta tra}$ females have a male-specific muscle of Lawrence (MoL), and fru^F males lack this muscle (Figure S2). The MoL is a dorsal abdominal muscle that uniquely depends on *fru* rather than *dsx* for its sexual differentiation (Gailey et al., 1991; Lawrence and Johnston, 1984; Taylor, 1992), evidently requiring *fru* in the motor neurons that innervate it rather than the muscle itself (Currie and Bate, 1995). It is not involved in courtship behavior, but may facilitate the termination of copulation (Lee et al., 2001).

We also verified that correct splicing of *fru*, rather than *dsx*, is essential in the male nervous system for male courtship behavior. Ectopic expression of *tra* in all postmitotic neurons in males normally leads to a dramatic reduction in courtship vigor (*elav-GAL4/UAS-tra*; Kido and Ito, 2002). In these males, *fru*, *dsx*, and any other *tra* targets are presumably spliced in the female mode within the nervous system. However, restoring male splicing of *fru* alone, by introducing either a fru^M or $fru^{\Delta tra}$ allele, is sufficient to restore normal courtship levels (Figure S3).

Male Courtship Behavior and Sexual Orientation Require Male Splicing

Having generated alleles of *fru* that force either male or female splicing and confirmed that they do not affect general sexual anatomy, we were now in a position to test the hypothesis that *fru* splicing specifies sexual behavior (Ito et al., 1996; Ryner et al., 1996). First, we asked whether male behavior requires male splicing. If so, fru^F males should display little or no courtship, and, if they have any residual courtship activity at all, it should be directed at males as well as females. fru^M and $fru^{\Delta tra}$ males should behave normally. We used courtship, fertility, and chaining assays to test these predictions (Figure 2).

In male-female courtship assays, a test male is paired with a wild-type virgin female in a 10 mm observation chamber, and the percentage of time the male courts the female during the first 8 min or until copulation, is recorded as his courtship index (CI). In these assays, wild-type, fru^C, fru^M, and $fru^{\Delta tra}$ males are all avid courters (CI > 70%; Figure 2A and Movie S1). In contrast, fru^F males, like males carrying the classic P-induced *fru* alleles fru^3 and fru^4, barely court at all (CI < 5%). We also tested male courtship in competitive mating assays, in which a wild-type virgin female is placed in a chamber with two males—a test male and a wild-type (fru^+) competitor. The trio is then observed for up to 1 hr to record which of the two males succeeds in copulating with the female (Figure 2B). In these assays, fru^F males always lost out to the fru^+ control males, whereas fru^C, fru^M, and $fru^{\Delta tra}$ males were all at least as successful as their fru^+ competitors. Consistent with the loss of courtship behavior, fru^F males are also completely sterile (0% fertility; n = 196), whereas fru^+, fru^C, fru^M, and $fru^{\Delta tra}$ males are all fully fertile (>99%; n = 230, 140, 110, and 131, respectively). Together, these data establish that male-specific splicing of *fru* P1 transcripts is indeed essential for male courtship behavior.

To test for sexual orientation, we first performed courtship assays in which single *fru* test males were paired with wild-type males rather than females. Male-male courtship is low for all genotypes. However, fru^F males, like fru^3 and fru^4 males, court other males some-

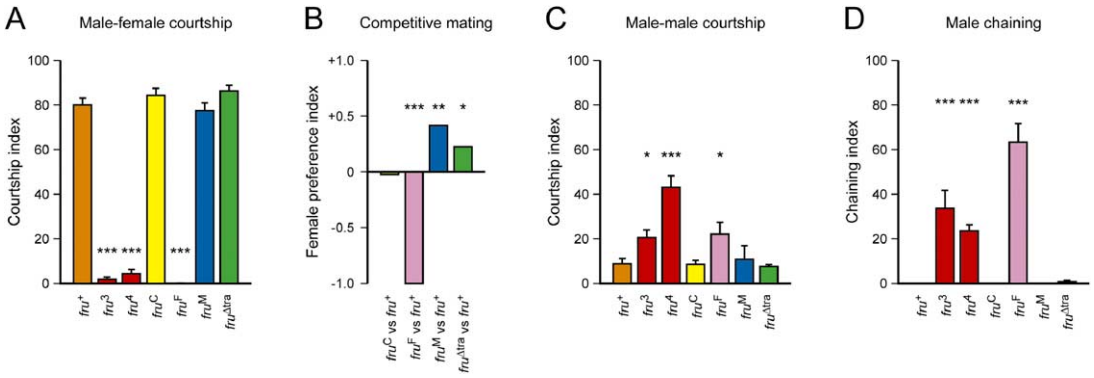

Figure 2. Male Splicing of *fru* Is Essential for Male Courtship Behavior and Sexual Orientation

(A) Courtship indices for males of the indicated genotypes paired with wild-type virgin females. Error bars indicate SEM; n = 43–57 for each genotype. ***p < 0.0001 compared to fru^{+} (Kruskal-Wallis ANOVA test).

(B) Competitive mating assays in which various *fru* mutant males were pitted against wild-type (fru^{+}) males for copulation with a wild-type virgin female. The female preference index is the relative advantage of the *fru* mutant male over the fru^{+} male (i.e., the excess copulations with the *fru* mutant male divided by the total number of copulations). n = 43, 17, 41, and 62 for fru^{C}, fru^{F}, fru^{M}, and $fru^{\Delta tra}$, respectively. ***p < 0.0001; **p < 0.001; *p < 0.05 (χ^2 test).

(C) Courtship indices for males of the indicated genotypes paired with wild-type males. n = 11–42 for each genotype. ***p < 0.0001; *p < 0.05 compared to fru^{+} (Kruskal-Wallis ANOVA test).

(D) Chaining indices for groups of males of the indicated genotypes. n = 4–7 groups. ***p < 0.0001 compared to fru^{+} (Kruskal-Wallis ANOVA test).

what more actively than do any of the control males (fru^{+}, fru^{C}, fru^{M} or $fru^{\Delta tra}$; Figure 2C). Comparing courtship levels in these single-pair assays is more difficult for male-male assays than for male-female assays, as courtship levels are generally much lower. A more reliable way to test for male-male courtship is to monitor chaining behavior in groups of males. If groups of *fru* mutant males are left on food plates for several hours or days, they begin to form courtship chains in which each male courts the one ahead of him (Hall, 1978). It is not clear how this chaining behavior relates to normal courtship, and it probably involves environmental and social stimuli that are absent in the single-pair assays. Nevertheless, it is a robust male-male courtship behavior displayed by classical *fru* mutants but not wild-type males and can be readily quantified by a chaining index (ChI, the percentage of time three or more males form a chain during a 10 min observation period). Using this assay, we observed dramatically elevated levels of male-male courtship amongst fru^{F} males (ChI = 63%, p < 0.0001; Figure 2D and Movie S2) compared to fru^{+}, fru^{C}, fru^{M}, or $fru^{\Delta tra}$ males (ChI < 1%). We conclude that male-specific *fru* splicing not only promotes male-female courtship, it also inhibits male-male courtship.

Intriguingly, in the competition assays, fru^{M} and $fru^{\Delta tra}$ males had a slight but significant edge over their fru^{+} competitors, winning 71% (n = 41, p = 0.0002) and 61% (n = 62, p = 0.01) of assays, respectively (Figure 2B). This prompted us to compare individual courtship steps performed by fru^{C}, fru^{M}, and $fru^{\Delta tra}$ males in single-pair assays with wild-type virgin females (Table 1). Qualitatively, courtship performed by fru^{M} and $fru^{\Delta tra}$ males is indistinguishable from courtship by fru^{C} males. However, fru^{M} and $fru^{\Delta tra}$ males initiate courtship more rapidly than fru^{C} males (p < 0.01 for both genotypes). Once courtship is initiated, fru^{M} and $fru^{\Delta tra}$ males spend as much time as fru^{C} males performing each of the other steps (tapping, wing extension, licking, and attempted copulation; p > 0.05 for each step). Although we have not been able to detect any differences by molecular or histological means (Figure 1), it is possible that forced male splicing results in slightly elevated levels of Fru^{M} proteins in at least some cells in fru^{M} and $fru^{\Delta tra}$ males. A tentative inference from this result is therefore that Fru^{M} is not only essential for male courtship behavior but may also contribute quantitatively to its initiation.

Fru^{M} Inhibits Female Reproductive Behaviors

We next examined the sexual behaviors of females for each of the *fru* splicing mutants, focusing first on female reproductive behaviors (Figure 3). fru^{C} and fru^{F} females are as fertile as fru^{+} controls (>99%), but less than 25% of fru^{M} and $fru^{\Delta tra}$ females are fertile (Figure 3A). We could not detect any gross morphological abnormalities in the genitalia or reproductive organs of these females, including their innervation, suggesting that the reduced fertility might be due to behavioral rather than anatomical defects (L. Tirián and B.J.D., unpublished data). We therefore examined two female behaviors critical for reproduction: mating receptivity and egg laying.

In mating assays in which a single virgin test female was paired with a wild-type male, fru^{C} and fru^{F} females almost always copulated within 60 min (>94%), but less than 16% of fru^{M} and $fru^{\Delta tra}$ females copulated (Figure 3B). Similarly, in competition assays in which a wild-type male was offered a choice of two virgin females, one *fru* mutant and one wild-type, the fru^{C} and fru^{F} females competed equally with the wild-type females but fru^{M} and $fru^{\Delta tra}$ females were never chosen (Figure 3C).

We took the females that did mate in the single-pair

Table 1. Comparison of Courtship by fru^C Males and fru^M and $fru^{\Delta tra}$ Males and Females

	fru^C Male	fru^M Male	$fru^{\Delta tra}$ Male	fru^M Female	$fru^{\Delta tra}$ Female
n	13	10	11	21	15
All courtship steps (CI)	72.94 ± 3.41	89.75 ± 2.87	81.86 ± 4.23	42.40 ± 3.58**	45.76 ± 3.55*
Courtship latency (s)	50.00 ± 15.23	6.90 ± 2.64*	10.27 ± 3.82*	48.86 ± 10.57	41.73 ± 7.72
Tapping (s)	51.23 ± 10.28	73.00 ± 8.95	56.09 ± 6.79	86.67 ± 9.49*	147.20 ± 18.02***
Wing extension (s)	128.77 ± 16.66	181.30 ± 19.63	153.36 ± 22.08	105.57 ± 16.14	86.20 ± 12.87
Licking	3.77 ± 0.76	5.60 ± 0.62	9.09 ± 3.03	0.62 ± 0.22*	0.27 ± 0.12*
Attempted copulation	1.54 ± 0.29	2.40 ± 0.65	3.36 ± 1.06	0 ± 0**	0 ± 0**

Courtship assays for fru^C males and fru^M and $fru^{\Delta tra}$ males and females paired with wild-type virgin females were recorded at higher magnification to monitor individual courtship steps. Values are mean ± SEM. For tapping and wing extension, the total time engaged in these steps was recorded; for licking and attempted copulation, it was the total number of events. *p < 0.01; **p < 0.001; ***p < 0.0001 compared to fru^C males (Kruskal-Wallis ANOVA test). p > 0.05 for all other comparisons.

assays and counted the number of eggs they laid over each of the next 3 days. Mated fru^C and fru^F females laid on average over 65 eggs during this period, whereas the fru^M or $fru^{\Delta tra}$ females laid on average less than two eggs (Figure 3D). By mating fru^M and $fru^{\Delta tra}$ females to males whose sperm are labeled by GFP (*dj-GFP*; Santel et al., 1997), we confirmed that sperm are transferred and stored in the spermathecae of ~30% of these females (L. Tirián and B.J.D., unpublished data). At least some of these sperm are used, as we often observed fully developed embryos in the uterus of a mated fru^M female and occasionally even witnessed a "live birth" as a larva attempted to crawl out through the vagina. Thus, male specific *fru* products inhibit at least two female reproductive behaviors: copulation and egg laying.

Females Behaving Like Males

If *fru* is a behavioral switch gene, then fru^M and $fru^{\Delta tra}$ females should not only lose female reproductive behaviors, they should also gain male behaviors. Specifically, they should court other females. We tested this prediction in single-pair courtship assays and in chaining assays (Figure 4).

Remarkably, fru^M and $fru^{\Delta tra}$ females court wild-type females, with courtship indices over 40% in single-pair assays (Figures 4A and 4B and Movie S3). Placed together on food plates, groups of fru^M and $fru^{\Delta tra}$ females also form courtship chains similar to those formed by fru^F males (Figure 4C and Movie S4). In these assays, fru^M and $fru^{\Delta tra}$ females had chaining indices of over 40%. Neither fru^C nor fru^F females show any female-female courtship, either in single-pair assays (CI < 0.1%) or in chaining assays (ChI < 0.1%).

Qualitatively, courtship of wild-type virgin females by fru^M and $fru^{\Delta tra}$ females resembles normal male courtship, as shown for example by fru^C males (Table 1). fru^M and $fru^{\Delta tra}$ females perform all steps of the courtship ritual, with the obvious exception of copulation, which is anatomically impossible, as well as abdominal bending in attempt to copulate, which may be inhibited by the much larger abdomen of the female. There are also some quantitative differences in the individual courtship steps performed by fru^M or $fru^{\Delta tra}$ females and fru^C

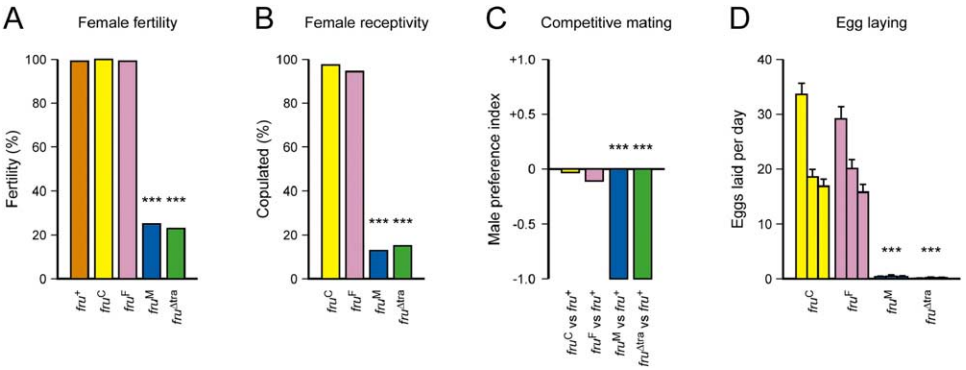

Figure 3. Male Splicing of *fru* Suppresses Female Reproductive Behaviors

(A) Fertility of females of the indicated genotypes. n = 100–128. ***p < 0.0001 (χ^2 test).

(B) Receptivity of females of the indicated genotypes. n = 39, 54, 93, and 66, respectively. ***p < 0.0001 (χ^2 test).

(C) Competitive mating assays in which various *fru* mutant females were pitted against wild-type (fru^+) females for copulation with a wild-type male. The male preference index is the relative advantage of the *fru* mutant female over the fru^+ female (i.e., the excess copulations with the *fru* mutant female divided by the total number of copulations). n = 23–31. ***p < 0.0001 (Kruskal-Wallis ANOVA test).

(D) Eggs laid by single mated females of the indicated genotype for each of the first three days after copulation with a wild-type male. The three bars for each genotype indicate the average number of eggs laid on the first, second, and third days, respectively. Error bars indicate SEM. n = 89, 69, 22, and 30 for fru^C, fru^F, fru^M, and $fru^{\Delta tra}$, respectively. ***p < 0.0001 (Kruskal-Wallis ANOVA test).

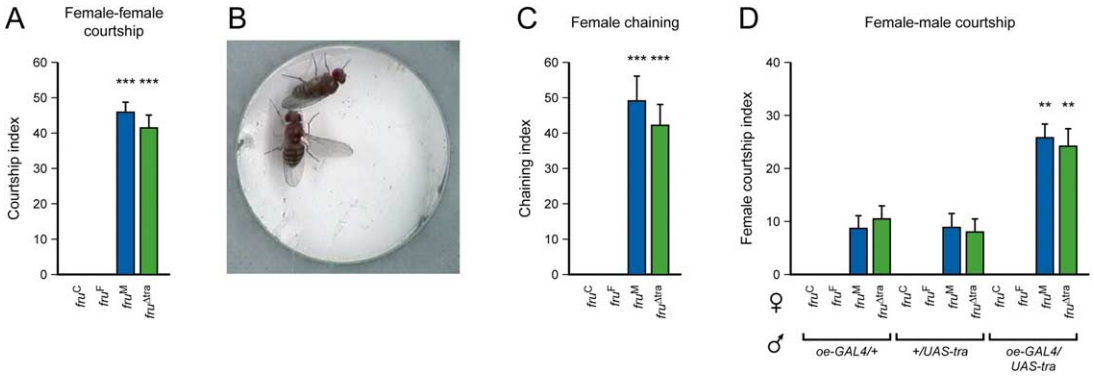

Figure 4. Male Splicing of *fru* Is Sufficient for Male Courtship Behavior and Sexual Orientation

(A) Courtship indices for females of the indicated genotypes paired with wild-type virgin females. Error bars indicate SEM; n = 38–49 for each genotype. ***p < 0.0001 (Kurskall-Wallis ANOVA test).

(B) fru^{M} female courting a wild-type virgin female (from Movie S3). The fru^{M} female is below the wild-type female, and has one wing extended to "sing."

(C) Chaining indices for groups of females of the indicated genotypes. Error bars indicate SEM. n = 3, 5, 11, and 15 groups, respectively. ***p < 0.0001 compared to fru^{+} (Kruskal-Wallis ANOVA test).

(D) Courtship indices for virgin females toward males in single-pair courtship assays. Female and male genotypes are indicated. Females were aged in isolation, but males were aged in groups. Error bars indicate SEM; n = 17–58 for each genotype (n = 58 and 49 for fru^{M} and $fru^{\Delta tra}$ females, respectively, courting *oe-GAL4/UAS-tra* males). **p < 0.001 compared to females of the same genotype courting *oe-GAL4/+* males (Kruskal-Wallis ANOVA test). fru^{M} and $fru^{\Delta tra}$ females display a low level of courtship directed toward control *oe-GAL4/+* and *+/UAS-tra* males, consistent with the low level of male-male courtship performed by wild-type and fru^{C} males (Figure 2C). All males showed negligible courtship (CI < 1%). This can be explained by their conditioning (Siegel and Hall, 1979). When $fru^{\Delta tra}$ females were instead paired with naive *oe-GAL4/UAS-tra* males (i.e., aged in isolation rather than in groups), these males vigorously court the females (CI = 88.4 ± 3.3%), while the $fru^{\Delta tra}$ females reject the males but are unable to sustain any courtship activity of their own.

males (Table 1). The fraction of the total courtship time devoted to tapping is slightly but significantly higher in fru^{M} or $fru^{\Delta tra}$ females, whereas licking is much less frequent. fru^{M} and $fru^{\Delta tra}$ females also tend to spend less time than fru^{C} males extending and vibrating their wings, although this difference did not reach statistical significance in our assays. Although fru^{M} and $fru^{\Delta tra}$ females spend less time courting than fru^{C} males, they initiate courtship just as rapidly.

Nevertheless, despite these subtle differences in the pattern of courtship, it is clear from these experiments that fru^{M} and $fru^{\Delta tra}$ females have male sexual instincts: they perform the male courtship ritual, and, like normal males, direct their courtship toward females. Male splicing of *fru* is therefore not only necessary for male sexual orientation and behavior, it is also sufficient.

Reversing the Sex Roles

Males normally court females, not the other way around. This courtship is driven at least in part by female pheromones produced in subcuticular cells called oenocytes. Thus, if the oenocytes of a male are feminized by the ectopic expression of *tra* (*oe-GAL4/UAS-tra*), then these males acquire a female pheromone profile and become attractive to other males (Ferveur et al., 1997). Since we had now generated females with male sexual instincts, we anticipated that pairing such fru^{M} or $fru^{\Delta tra}$ females with *oe-GAL4/UAS-tra* males would result in a reversal of the courtship roles—the females should now court the males. Indeed, this is exactly what happens (Figure 4D). Thus, by engineering females to produce male-specific Fru^{M} proteins and males to produce female pheromones, we have been able to reverse the sex roles during *Drosophila* courtship.

Discussion

Development endows an animal with the morphology and instinctive behaviors characteristic for its species, preparing it for survival and reproduction in the environment into which it is likely to be born. An animal's instinctive behaviors are just as stereotyped and just as characteristic for its species as its morphology, and so one might expect to find a similar logic underlying the genetic programs that specify morphology and behavior. Yet, whereas morphological development has now largely succumbed to the attack of classical forward genetics in a few model organisms, the same approach has made only modest inroads into the developmental origins of complex innate behaviors. Does this reflect a fundamental difference in the ways behavior and morphology are specified during development or just a lack of attention to the problem of behavioral development?

One of the lessons from the genetic analysis of morphological development is that anatomical features are often specified by switch genes, the action of which is both necessary and sufficient to direct the formation of a particular feature. A striking example of such a morphological switch gene is the *eyeless* gene of *Drosophila*, which is both necessary and sufficient for eye development (Halder et al., 1995). If analogous genetic principles guide the emergence of both morphology and behavior, then we should also expect that at least some innate behaviors are specified by switch genes.

The action of such a behavioral switch gene would be both necessary and sufficient to hardwire the potential for the behavior into the nervous system. Until now, such behavioral switch genes have been elusive. Here, we have demonstrated that the *fruitless* (*fru*) gene of *Drosophila* is a switch gene for a complex innate behavior: the elaborate ritual of male courtship.

fru as a Switch Gene for Male Courtship Behavior

fru has long been known to be required for male courtship behavior (Gill, 1963). In this regard, however, *fru* is not particularly unusual. Many other genes have also been implicated in male courtship behavior, and in one way or another, a substantial fraction of the genome is likely to be required for a male to be capable of and inclined to court a female. *fru* only assumed its more prominent position when it was molecularly characterized, revealing that some of its transcripts are spliced differently in males and females (Ito et al., 1996; Ryner et al., 1996). This led to the hypothesis that splicing of *fru* specifies male courtship behavior (Ito et al., 1996; Ryner et al., 1996). Although widely discussed (e.g., Baker et al., 2001), this hypothesis has remained untested for almost a decade. We have now confirmed the key predictions of this hypothesis by showing that male splicing is indeed necessary for male courtship behavior (Figure 2) and is also sufficient to generate male behavior by an otherwise normal female (Figure 4).

Male courtship behavior performed by fru^{M} and $fru^{\Delta tra}$ females is a remarkable mimic of courtship by wild-type or control fru^{C} males. Some courtship steps, such as initiation, orientation, following, and wing extension, are indistinguishable in fru^{M} (and $fru^{\Delta tra}$) females and fru^{C} males. Other steps are clearly abnormal. fru^{M} females do not, for obvious reasons, copulate. But licking, which should be anatomically possible, is also significantly reduced. Qualitatively, this pattern of courtship resembles that of *dsx* males (Villella and Hall, 1996). This is perhaps not surprising, as fru^{M} females resemble *dsx* males in that they lack male-specific Dsx isoforms (Dsx^{M}) and hence are anatomically female, yet they express the male-specific Fru isoforms (Fru^{M}).

The distinct roles of *fru* and *dsx* in sexual development are clearly illustrated by the differences between animals that produce either only Fru^{M} or only Dsx^{M}. Animals that express Dsx^{M} but not Fru^{M} (either fru^{F} males or dsx^{M} females) resemble normal males but do not court (Figure 2A; Taylor et al., 1994). Conversely, animals that express Fru^{M} but not Dsx^{M} (either fru^{M} females or *dsx* males) do court, even though they resemble normal females (Figure 4A; Villella and Hall, 1996). Thus, Fru^{M} is both necessary and sufficient for male courtship, whereas Dsx^{M} is neither necessary nor sufficient. The role of Dsx^{M} in courtship may simply be to provide the gross male anatomy needed for its optimal execution. This anatomical contribution of Dsx^{M} includes the formation of male reproductive organs and external genitalia (Burtis and Baker, 1989), the generation of the neurons that innervate these organs (Taylor and Truman, 1992), and the formation of male-specific taste sensilla on the forelegs that may house pheromone-detecting neurons (Bray and Amrein, 2003).

An open question is whether *fru* specifies male-like behavioral patterns more generally or is exclusively involved in male courtship behavior. We have focused our study on courtship behavior because this is the most dramatic, most robust, and best understood of the sexually dimorphic behaviors in *Drosophila*. But other behavioral patterns, such as aggression (Chen et al., 2002; Nilsen et al., 2004), are also sexually dimorphic, and it will be interesting to determine to what extent these behaviors depend on *fru*.

How Does *fru* Specify Male Courtship Behavior?

A behavioral switch gene such as *fru* must act through the relevant neural circuits. In the accompanying paper (Stockinger et al., 2005 [this issue of *Cell*]), we begin the anatomical and functional characterization of the neurons in which Fru^{M} is expressed and present evidence that they form a neural circuit that is largely dedicated to male courtship behavior. As the same circuit seems to be present in the female, we reason that Fru^{M} most likely exerts its effect by modulating the function rather than the assembly of this circuit. Nevertheless, the critical period for Fru^{M} to do so is evidently during development, as adult males begin courting soon after eclosure, without any prior exposure to another fly. Moreover, experiments involving conditional expression of *tra* have suggested that male behavior is irreversibly programmed during the early- to midpupal stages (Arthur et al., 1998), coincident with the onset of Fru^{M} expression in increasing numbers of neurons in the male nervous system (Lee et al., 2000).

By analogy to other members of the BTB-zinc finger family, Fru^{M} proteins are thought to be transcription factors and as such would specify sexual behavior by regulating the expression of one or more target genes. In the simplest scenario, *fru* may regulate one and the same target gene in all of the neurons in which it is expressed—acting merely as a switch that sets another switch. Alternatively, *fru* might directly regulate a large number of target genes, with different targets in different neurons. Several observations favor this latter scenario. The set of Fru^{M} proteins includes isoforms with at least four different DNA binding domains, which are likely to homo- and heterodimerize through their common BTB domain. Fru^{M} may also interact with other BTB-domain-containing zinc finger transcription factors such as Lola, which itself has at least 20 different DNA binding domains (Goeke et al., 2003; Horiuchi et al., 2003). Thus, Fru^{M} has the potential to form a large set of distinct regulatory complexes, as might be expected if it is to regulate different genes in different neurons. That at least some of this potential is utilized is suggested by the fact that we could not rescue a *fru* mutant with cDNAs encoding just a single isoform (even when using fru^{GAL4} [Stockinger et al., 2005] to drive expression in the correct neurons; D. Kvitsiani and B.J.D., unpublished data) and that we have already isolated mutations in two different DNA binding domains in an ongoing screen for revertants of the gain-of-function $fru^{\Delta tra}$ phenotype (L. Tirián and B.J.D., unpublished data).

The *fru* target genes themselves are unknown, as are, for the most part, their effects. The few cellular func-

tions so far ascribed to *fru* are the regulation of the number or size of synaptic terminals in specific glomeruli of the antennal lobe (Stockinger et al., 2005) and at the MoL (Billeter and Goodwin, 2004), as well as the production of serotonin in certain male-specific neurons of the abdominal ganglion (Lee and Hall, 2001). A fascinating question for the future is whether profound differences in sexual behavior arise as the sum of many subtle differences such as these, or are instead primarily due to a still unknown action of Fru^M in a few key "decision" neurons.

Single Genes and Complex Behaviors

Complex behaviors require the actions of vast numbers of genes, and so it is quite easy to isolate mutations in single genes that disrupt a particular behavior. Almost all of these mutations are relatively uninformative as to the genetic basis of that behavior or indeed the relationship between genes and behavior more generally. More useful are genes for which different allelic states result in different manifestations of the behavior. Several fascinating examples of such genes have been found, and all have provided important insights into the behaviors they influence. This includes, for example, the *npr-1* neuropeptide receptor gene in the control of social feeding in *C. elegans* (de Bono and Bargmann, 1998), the *foraging* cGMP-dependent protein kinase gene in *Drosophila* social feeding behavior (Osborne et al., 1997), the vasopressin 1a receptor gene in affiliative behavior in voles (Lim et al., 2004; Young et al., 1999), and, more controversially, the serotonin transporter gene in human depression (Ogilvie et al., 1996). Importantly, *fru* differs from "behavior genes" such as these in one critical aspect: it does not influence a behavior as it happens, but rather acts during development to create the potential for a behavior (Baker et al., 2001).

Might there be other behavioral switch genes like *fru*, and if so, how will we find them? The lack of obvious candidates is no reason to doubt that other behavioral switch genes exist. Indeed, in many ways it is almost fortuitous that this function of *fru* has been discovered at all. Mutations that eliminate all *fru* function are lethal and hence uninformative as regards to *fru*'s role in male courtship. This role only came to light through the isolation of relatively rare alleles that disrupt specific transcripts (Gill, 1963). Even then, it was not until its molecular cloning that *fru* acquired any particular significance (Ito et al., 1996; Ryner et al., 1996) and only now, through precise gene manipulations, that its role as a switch gene has been established. Classical forward genetics might not be the most effective way to search for behavioral switch genes, particularly if, like *fru*, the genes also have essential but unrelated functions during development.

Perhaps even more challenging will be recognizing a behavioral switch gene when we find one. Formally, this requires a sufficiency experiment, which involves asking if ectopic expression can specify a novel behavioral pattern in an otherwise normal animal. It is difficult to envision how such an experiment might be performed for anything other than a sexually dimorphic behavior. Hence, if we are to identify switch genes for behaviors that are not sex specific, then we must relax this strict criterion. What other features of *fru* could serve as a guide in assessing other candidate switch genes? Four aspects of *fru* stand out. First, as already noted, it acts during development to create the potential for the behavior, rather than directly influencing the behavior itself. Second, it appears to be involved in most or all aspects of the behavior, not just a single component. Third, loss-of-function mutations do not result in a general impairment of neural function, but a specific behavioral deficit. Fourth, it is required in a diverse set of neurons with little in common except their role in this behavior, to which they may also be dedicated. Candidate vertebrate genes that fulfill at least some of these criteria have been linked to behaviors at opposite extremes of complexity: the ETS transcription factor genes *Er81* and *Pea3* in the spinal stretch reflex (Lin et al., 1998) and, more speculatively, the forkhead-domain transcription factor gene *FoxP2* in human language ability (Vargha-Khadem et al., 2005).

Finally, the concept that a switch gene can specify an entire innate behavior in no way denies the critical role of complex gene networks, just as the concept of a morphogenetic switch does not deny the existence of complex regulatory networks among the genes it regulates. These networks add both detail and robustness to the behavioral or morphological pattern initially laid down by the switch gene at the top of the hierarchy. The notion of a behavioral switch gene does, however, imply that at least some instinctive behaviors develop according to the familiar genetic logic of morphological development. Given the appropriate genetic tools, behavioral instincts should ultimately succumb to the same kind of molecular genetic analysis that has so successfully revealed the principles of morphological development.

Experimental Procedures

Generation of *fru* Splicing Mutants

Gene targeting by homologous recombination was performed essentially as described by Rong and Golic (2000) and illustrated in Figure S1. Four "5′ half" donor elements were used to derive targeted lines containing one of each of the desired modifications in the 5′ part of the *fru* locus, followed by the FRT insertion. Similarly, a single "3′ half" donor construct was used to derive targeted lines consisting of an FRT insertion and the 3′ part of the *fru* locus. Targeted lines were selected by mobilizing and linearizing the original donor using *hsFLP* and *hsI-SceI* and crossing these virgin females to *eyFLP* (Newsome et al., 2000) males so that reintegration can be detected in the progeny by the stable expression of the $white^+$ reporter. Between two and ten independent lines were obtained from each of the original donor elements. We selected two independent 3′ lines and recombined each with one of two different 5′ lines (for each of the four alleles), using *hsFLP* to induce recombination at the FRT site. This generated two completely independent lines for each allele. For genomic sequencing, PCR was used to amplify nine overlapping fragments of 1.1–3.1 kb, which were directly sequenced. For RT-PCR, flies were frozen and passed through a sieve to isolate heads, legs, and wings, which were then homogenized and used to prepare mRNA using the Quick Prep Micro mRNA purification kit (Amersham Biosciences). Random hexamers were used for first strand synthesis, and gene-specific primers were used for second strand synthesis.

Behavioral Assays

Flies were raised on semidefined medium (Backhaus et al., 1984) at 25°C in a 12hr:12hr light:dark cycle and aged for 5–7 days after

eclosure. Test flies were collected shortly after eclosure and aged individually in small food vials. Flies used as courtship objects were aged in pools of 30–50 in large food vials, with the exception of the naive males used in the *oe-GAL4/UAS-tra* experiments, which were aged individually. The *oe-GAL4* line is line C described in Ferveur et al. (1997).

Single-Pair Courtship

Single-pair courtship assays (Villella et al., 1997) were performed using a round chamber of 10 mm diameter and 4 mm height containing a white nitrocellulose filter. For same-sex courtship assays, genotypes were distinguished by applying a terra cotta color marker to the thorax of one fly at the time of initial collection. In control experiments, the marker did not influence the courtship vigor of either the marked or unmarked flies.

Chaining

Chaining assays (Villella et al., 1997) were performed using groups of eight flies placed in a 35 mm × 10 mm petri dish containing food. Flies were left undisturbed for one day and observed for 10 min on the second day.

Competitive Mating

In the competitive mating assays (Bray and Amrein, 2003), a terra cotta marker was used to distinguish flies of the same sex. For each pair of genotypes, the marker was applied to each genotype in approximately half the assays. No difference in outcome was observed between the two sets of assays for a given pair of genotypes, and the data were therefore pooled.

Fertility

Single virgin males or females were placed in food vials with five wild-type virgin females or eight males, respectively, and scored as sterile if the flies were still alive but there was no progeny after 20 days.

Receptivity

In receptivity tests (Aigaki et al., 1991), females were scored as receptive if they mated within 60 min.

Egg Laying

Single virgin females were paired with three wild-type males in a food vial. After copulation, the female was removed and transferred every 24 hr to a new food vial. The number of eggs in each vial was counted.

Histochemistry

Staining of pupal and adult brains was performed as described in Stockinger et al. (2005). Dorsal abdominal muscles were stained with rhodamine-labeled phalloidin as described in Taylor (1992).

Supplemental Data

Supplemental Data include three figures and four movies and can be found with this article online at http://www.cell.com/cgi/content/full/121/5/785/DC1/.

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