A Novel Gene Controlling the Timing of Courtship Initiation in *Drosophila melanogaster*

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ABSTRACT Over the past 35 years, developmental geneticists have made impressive progress toward an understanding of how genes specify morphology and function, particularly as they relate to the specification of each physical component of an organism. In the last 20 years, male courtship behavior in *Drosophila melanogaster* has emerged as a robust model system for the study of genetic specification of behavior. Courtship behavior is both complex and innate, and a single gene, *fruitless* (*fru*), is both necessary and sufficient for all aspects of the courtship ritual. Typically, loss of male-specific Fruitless protein function results in male flies that perform the courtship ritual incorrectly, slowly, or not at all. Here we describe a novel requirement for *fru*: we have identified a group of cells in which male Fru proteins are required to reduce the speed of courtship initiation. In addition, we have identified a gene, *Trapped in endoderm 1* (*Tre1*), which is required in these cells for normal courtship and mating behavior. *Tre1* encodes a G-protein-coupled receptor required for establishment of cell polarity and cell migration and has previously not been shown to be involved in courtship behavior. We describe the results of feminization of the *Tre1*-expressing neurons, as well as the effects on courtship behavior of mutation of *Tre1*. In addition, we show that *Tre1* is expressed in a sexually dimorphic pattern in the central and peripheral nervous systems and investigate the role of the *Tre1* cells in mate identification.

KEYWORDS courtship; Fruitless; *Drosophila*; G-protein signaling; behavior

ONE of the goals of neuroscience is to understand how genetic programs direct neurodevelopment and how the resulting neural pathways interact with sensory inputs to result in behavior. *Drosophila melanogaster* courtship behavior provides an excellent model system to study this process. Male *Drosophila* carry out a complex courtship ritual, the correct performance of which affects their success in mating and reproduction (Baker *et al.* 2001). This ritual consists of a series of stereotyped behaviors. The male first orients toward and follows the female, then taps her with his forelegs, extends one wing toward the female and vibrates it to generate a species-specific courtship song, licks her genitalia, and, finally, curls his abdomen to attempt copulation (Hall 1994). If the female is receptive (*i.e.*, virgin or not recently mated) and

the ritual is performed correctly, copulation will follow. These behavioral outputs all require sensory input and integration (Dickson 2008), and all are dependent upon expression of the male-specific isoforms of the zinc-finger transcription factor Fru (Fru^M) (Demir and Dickson 2005).

The *fru* gene spans 130 kb of genomic DNA and generates at least 15 transcripts and six polypeptides (Demir and Dickson 2005). Most *fru* transcripts are common to males and females, but transcripts initiated from one of the four *fru* promoters (P1) are sex-specifically spliced under the control of the sex-determining splicing factors Tra and Tra-2 (Heinrichs *et al.* 1998). Male-specific splicing of *fru* P1 transcripts results in full-length Fru^M proteins, while female-specific splicing results in truncated Fru proteins. Genetic analysis of partial loss-of-function alleles of *fru* shows that Fru^M proteins are necessary for each step in the courtship ritual, rather than a single, early step (Anand *et al.* 2001). Finally, gain-of-function experiments show that male-specific splicing of *fru* is sufficient to confer male courtship behavior (Demir and Dickson 2005).

While disruption of male-specific splicing of *fru* (*fru*^M) expression typically leads to delays in, or absence of, normal

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courtship behaviors, we have defined a set of neurons (*Tre1*-*GAL4* neurons) in which downregulation of *fru^M* causes unusually rapid initiation of courtship. This phenotype is novel and suggests a previously uncharacterized function for *fru^M*-expressing neurons: delay of courtship.

The *Tre1* gene encodes an orphan rhodopsin family G-protein-coupled receptor (GPCR) required for transepithelial migration of germ cells (Kunwar *et al.* 2003) and asymmetrical neuroblast division (Yoshiura *et al.* 2012). *Tre1* was originally misidentified as a trehalose receptor (Ishimoto *et al.* 2000; Dahanukar *et al.* 2001; Ueno *et al.* 2001) due to its structural similarities to *Drosophila* gustatory receptors. However, *Tre1* appears to more closely align with receptors for hormones and neurotransmitters such as melatonin and histamine, as well as chemokine receptors (Kunwar *et al.* 2003). Thus, *Tre1* is a candidate chemoattractant receptor whose ligand remains unidentified.

We have found that *Tre1* is expressed in a subset of *Fru^M*-expressing neurons that both receive and process olfactory signals. Olfaction in *Drosophila* begins in olfactory sensory neurons (OSNs) housed in two organs: the third antennal segment (3AS), where ~90% of OSNs reside, and the maxillary palp (MP). OSNs are found in one of three types of olfactory hairs, or sensilla: basiconic (found on both the 3AS and the MP), trichoid, or coeloconic (both found exclusively on the 3AS) (Stocker 2001). Each OSN expresses a single specific olfactory receptor (OR) (Couto *et al.* 2005). OSNs project from the sensory organ to the antennal lobe (AL) in the brain, where all OSNs expressing the same OR target the same glomerulus (Couto *et al.* 2005). In the AL, OSNs synapse with olfactory projection neurons (PNs). These second-order neurons project to two major olfactory centers in the brain: the mushroom body (MB), the primary sites of olfactory-mediated learning and memory (Davis 2005), and the lateral horn (LH) of the protocerebrum, which is responsible for processing and integration of innate olfactory preferences, including pheromone signals (Marin *et al.* 2002; Wong *et al.* 2002; Jefferis *et al.* 2007; Ruta *et al.* 2010; Gupta and Stopfer 2012).

These olfactory circuits are sexually dimorphic at multiple levels. *Fru^M* proteins are expressed in a subset of the trichoid OSNs, which project to three glomeruli (DA1, VA11m, and VL2a) in the AL. These glomeruli are present in both male and female fly brains, but they occupy a significantly greater volume in the male AL (Stockinger *et al.* 2005). DA1 is targeted by Or67d-expressing OSNs, which respond to the aversive pheromone 11-*cis*-vaccenyl-acetate (cVA) (Clyne *et al.* 1997; Ejima *et al.* 2007; Kurtovic *et al.* 2007; van der Goes van Naters and Carlson 2007; Billeter *et al.* 2009). Or67d-expressing OSNs are found in trichoid sensilla, which project to the region of the LH that is thought to be exclusively devoted to the processing of pheromone signals (Jefferis *et al.* 2007). DA1 PNs have been shown to have a sexually dimorphic arborization pattern in the LH (Datta *et al.* 2008). The DA1 PNs synapse with four clusters of cells in the LH, two of which are male-specific (DC1 and DC2), while a third is dimorphic in size and projection pattern (LC1) (Ruta *et al.* 2010).

Here we present data showing that feminizing the *Tre1*-expressing neurons in male flies leads to rapid courtship initiation, a phenotype that leads to increased reproductive success. We also show that the *Tre1* GPCR is itself required for normal courtship behavior. In addition, we show that *Tre1* is expressed in a sexually dimorphic pattern in the OSNs and the central nervous system, and that this pattern is consistent with a role in detection and processing of olfactory information. Finally, we show that the *Tre1* neurons are not required for distinguishing conspecific females, nor are they involved in distinguishing mated from unmated females.

Materials and Methods

Fly strains and genetics

Fly stocks were maintained at 25° on standard cornmeal/molasses medium. With the exception of RNAi strains from the Transgenic RNAi Project (TRiP), all mutant alleles and transgenes were introgressed for five generations into our standard lab background [*w¹¹¹⁸*; Wild Type Berlin (*w*; *WTB*)]. *GAL4⁹⁻²¹⁰* is a *P[GawB]* enhancer trap line kindly provided by Ulrike Heberlein. *Tre1^{EP496}*, *UAS-Tra^F*, and *UAS-mCD8-GFP* were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Indiana) (stock nos. 10089, 4590, and 5130). *D. simulans* and *D. mauritiana* strains were a gift from Theresa Logan-Garbisch.

GAL4⁹⁻²¹⁰ contains three independent *P[GawB]* insertions on the X chromosome. We isolated *Tre1*-*GAL4*, the insertion responsible for the rapid courtship phenotype, using meiotic recombination. Briefly, *GAL4⁹⁻²¹⁰* males were crossed to *w¹¹¹⁸*; *WTB* virgin females, and the resulting heterozygous virgins collected and crossed to *w¹¹¹⁸*; *WTB* males. From this second cross, 30 males with eye colors lighter than the *GAL4⁹⁻²¹⁰* parent strain (indicating fewer than three mini-*w⁺* insertions) were collected and crossed to *w¹¹¹⁸*; *WTB* virgin females and homozygous strains generated. For each strain, polymerase chain reaction (PCR) on genomic DNA was used to determine which *P[GawB]* insertions were present. Strains containing each isolated insertion were identified and crossed to *UAS-Tra^F* to determine which strain was responsible for the rapid courtship phenotype.

Inverse PCR

Inverse PCR to identify the *P*-element insertion sites was performed according to the Berkeley *Drosophila* Genome Project protocol. Briefly, genomic DNA was extracted with standard techniques, digested with *Sau3A* or *MspI* (New England Biolabs), ligated at low DNA concentration to favor circularization, then subjected to PCR with primers *GawB5'out* and *GawB5'in*. Resulting PCR products were cloned into the pCR4-TOPO-TA vector (Life Technologies) and sequenced with M13F or M13R primers.

Courtship assays

Courtship assays were performed according to published protocols (Villegla *et al.* 1997). Virgin males were kept in isolation for 2–3 days after eclosion. Each male was then

Figure 1 Feminization of *Tre1*-GAL4 neurons leads to a reduction in courtship latency. (A) *GAL4⁹⁻²¹⁰/Y; UAS-TraF* males initiate courtship in 18 sec, while control animals initiate courtship in an average of 28 or 36 sec ($N = 22$ –29 males, $**P < 0.01$, one-way ANOVA with Tukey HSD post hoc analysis). (B) Schematic diagram of the *Tre1* gene. *Tre1*-RA and *Tre1*-RB encode the same protein, but *Tre1*-RA is initiated ~3 kb further upstream. *P[GawB]Tre1*-GAL4 is inserted into the sixth exon of both transcripts. (C) *Tre1*-GAL4/*UAS-TraF* males initiate courtship in 8 sec, on average, compared with 24–33 sec in genotypic controls ($N = 8$ –10 males, $**P < 0.01$, one-way ANOVA with Tukey HSD post hoc analysis).

presented with a single 1- to 2-day-old *w¹¹¹⁸*; *WTB* virgin female. Single male and female pairs were placed into custom plexiglass chambers 10 mm in diameter and 6 mm in height, separated by plastic transparencies. Contact between pairs was initiated by removal of the transparencies. Courtship behavior was recorded in infrared light for 20 min.

Fertility assays

For the noncompetitive fertility assay, a single 2- to 3-day-old male fly was paired with a single 2- to 3-day-old virgin female fly. The flies were allowed to mate and lay eggs for 2 days, after which the adults were cleared from the vials. All flies eclosing from each vial were counted and averaged over all vials to calculate fertility of individual males.

For the competitive fertility assay, two experimental males and two *w¹¹¹⁸*; *WTB* males were placed in a vial with five *w¹¹¹⁸*; *WTB* virgin females. All flies were 2–4 days old. The adults were allowed to mate and lay eggs for 2 days, after which the adults were cleared from the vials. The resulting progeny were counted and scored for their eye color phenotype.

Immunofluorescence

The CNS and peripheral tissue were dissected and fixed according to Wu and Luo (2006) with the following modifications: tissues were fixed for 1 hr, incubated with NGS block for 24 hr, stained in primary antibody for 4 days, and secondary for

2 days. NC82 mouse anti-Brp was used at 1:50 (Developmental Studies Hybridoma Bank, AB 2314866). Rabbit anti-GFP was used at 1:750 (Life Technologies, A6455). Alexa Fluor 488 goat anti-rabbit was used at 1:1000 (Jackson ImmunoResearch, 111-545-144). Alexa Fluor 594 goat anti-mouse was used at 1:1000 (Jackson ImmunoResearch, 705-586-147).

Quantitative RT-PCR

For quantitative reverse transcriptase-mediated PCR (qRT-PCR), embryos were snap frozen on dry ice. Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, resuspended in RNase-free water, and stored at -80° until use. For qRT-PCR, 2 μ g of total RNA was reverse transcribed using the High-Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA), according to the manufacturer's instructions. cDNA was analyzed by quantitative, real-time PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). *rp49* transcript levels were used as an endogenous normalization control for RNA samples, and relative mRNA abundance was calculated using the comparative ΔC_t method (Schmittgen and Livak 2008). Each sample was analyzed in triplicate. As negative controls, we used both no-template and DNase-treated nonreverse-transcribed mRNA samples; no significant amplification was observed in these samples.

Primer sequences

The primer sequences are as follows—*Tre1*: left primer, 5'-TCGTTTCGTACTCGTGATC-3' and right primer, 5'-TGGAAGTTATCGTGGTTGCG-3'; *CG42343*: 5'-GTACTCCCTGTCCCACTCCA-3'; *GawB5'in*: 5'-CAATAATGGGTTCTTTGGC GACGG-3'; *GawB5'out*: 5'-GCCGCACGTAAGGGTTAATG-3'; *M13F*: 5'-GTAAAACGACGGCCAG-3'; *M13R*: 5'-CAGGAAACA GCTATGAC-3'; and *Rp49*: left primer, 5'-ACGTTGTGCAC CAGGAACCTT-3' and right primer, 5'-CCAGTCGGATCGAT ATGCTAA-3'.

Reagent and data availability

All strains and reagents are available upon request. The full dataset is available at http://figshare.com/articles/Luu_et_al_dataset/1507590.

Results

Feminization of *Tre1*-expressing neurons causes rapid courtship initiation

We have previously shown that driving a *fru^M*-RNAi construct (*UAS-fru^MIR*) with the GAL4 line *P[GawB]9-210* (*GAL4⁹⁻²¹⁰*) leads to a decrease in latency to courtship (Tran *et al.* 2014). We define courtship latency as the time from introduction of males to females to the first unilateral wing extension. Courtship latency in *GAL4⁹⁻²¹⁰; UAS-fru^MIR* males averages 18 sec, compared with 28–32 sec in heterozygous genetic background controls (Tran *et al.* 2014).

To confirm this result, we drove expression of the female-specific splice form of *transformer* (*UAS-Tra^F*) with *GAL4⁹⁻²¹⁰*. This splice form leads to the production of a full-length protein that directs female-specific splicing of *fru*, thus eliminating the expression of *Fru^M* proteins in all cells expressing GAL4. Courtship latency in *GAL4⁹⁻²¹⁰; UAS-Tra^F* males averaged 16 sec, compared with 27 and 24 sec in heterozygous background controls (Figure 1A).

We performed inverse PCR on the *GAL4⁹⁻²¹⁰* line to identify the site of the insertion. This analysis revealed three insertions of the *P[GawB]* transgene, all on the X chromosome. Two of these insertions were upstream of the genes *folded gastrulation* (*fog*) and *CG42343*, and the third was inserted into the sixth exon of *Trapped in endoderm 1* (*Tre1*) (Figure 1B). We next used meiotic recombination to isolate the independent insertions. Of the three insertions, only *Tre1-GAL4* results in rapid courtship initiation when used to drive *UAS-Tra^F*. The average latency to courtship of *Tre1-GAL4/Y; UAS-Tra^F* males is 8 sec, compared with 35 sec in heterozygous genetic controls (Figure 1C). These results demonstrate that elimination of *Fru^M* proteins in *Tre1-GAL4* neurons results in extraordinarily rapid courtship initiation.

Tre1-GAL4 is a weak loss-of-function allele of *Tre1*

Given the location of the transposon insertion (Figure 1B), it would be surprising if *Tre1-GAL4* did not affect gene expression. We performed quantitative reverse transcriptase-mediated PCR

Figure 2 *Tre1-GAL4* is a weak loss-of-function allele of *Tre1*. (A) Quantitative RT-PCR analysis indicates that *Tre1* expression is reduced in both *Tre1-GAL4* and *Tre1^{EP496}* embryos. (B) Half of *GAL4⁹⁻²¹⁰/Y* males show rapid courtship initiation. A total of 52% of *GAL4⁹⁻²¹⁰/Y* males initiate courtship in <20 sec, while only 18% of *UAS-TraF/+* males initiate courtship as quickly.

(qPCR) on *Tre1-GAL4* flies and found that *Tre1-GAL4* reduces *Tre1* mRNA expression to 63% of wild-type levels. We also performed qPCR on *Tre1^{EP496}*, a P{EP} insertion (Rørth 1996) ~2 kb upstream of the *Tre1*-RA transcriptional start site, in the predicted *Tre1* promoter (Ueno *et al.* 2001; Kunwar *et al.* 2003). *Tre1^{EP496}* reduces *Tre1* expression to 44% of normal levels (Figure 2A). Thus, both *Tre1-GAL4* and *Tre1^{EP496}* are hypomorphic alleles of *Tre1*.

Because *Tre1-GAL4* is a weak loss-of-function allele, this makes it an imperfect background control for the *Tre1-GAL4/Y; UAS-TraF/+* experiments. However, in all cases, the *UAS-TraF* control is consistently different from the *Tre1-GAL4/Y; UAS-TraF/+* experiment. In addition, *Tre1-GAL4/Y* males are often more similar to control animals than experimental animals. Finally, as shown below, the loss-of-function *Tre1^{EP496}* line shows the same courtship latency phenotype when compared with its control (see below), indicating that the rapid courtship phenotype is a consistent effect of loss of *Tre1* function.

Tre1 is required for normal courtship behavior

Tre1-GAL4/Y males typically do not have an obvious courtship defect, as shown in Figure 1C. Additionally, in the experiments shown in Figure 1A, the *GAL4⁹⁻²¹⁰/Y* control and the *GAL4⁹⁻²¹⁰; UAS-TraF* experiment are not statistically different from one another. Upon closer observation, however, this is due to the fact that 15 of 29 (52%) of the *GAL4⁹⁻²¹⁰/Y* males

Figure 3 *Tre1* is required for normal courtship behavior. (A) *Tre1^{EP496}/Y* males display rapid courtship. *Tre1^{EP496}/Y* males initiate courtship in 36 sec, compared with 63 sec in control males. (* $P = 0.012$, Student's t -test assuming unequal sample variances). (B) *Tre1^{EP496}/Y* males have a copulation defect. A total of 27% of *Tre1^{EP496}/Y* males failed to initiate courtship during the 20-min observation window. (C) *Tre1^{EP496}/Y* males have one-third the mating success of control males during the 20-min assay window. Of the 16 males who initiated courtship, only 19% successfully copulated, while 54% of control males copulated.

initiated courtship in <20 sec, as quickly as the average *GAL4⁹⁻²¹⁰; UAS-Tra^F* male, while only 4 of 22 (18%) of *UAS-Tra^F/+* control males initiated courtship in <20 sec, leading to a large variance in the data (Figure 2B). As *GAL4⁹⁻²¹⁰* is a collection of X-linked insertions, one of which is *Tre1-GAL4* and responsible for the *GAL4⁹⁻²¹⁰/Y; UAS-Tra^F* phenotype (Figure 1C), it is possible that the *Tre1-GAL4* insertion itself causes a weak allele that sometimes causes rapid courtship initiation. Indeed, we find that the phenotype of

Figure 4 *Tre1-GAL4/Y; UAS-Tra^F* males have a competitive mating advantage over control males. (A) *Tre1-GAL4/Y; UAS-Tra^F/+* and *Tre1-GAL4/Y* males have normal fertility. *Tre1-GAL4/Y; UAS-Tra^F/+* males produced an average of 23 offspring/male, while *Tre1-GAL4/Y* males produced 25 offspring on average, compared with 21 offspring per *UAS-Tra^F/+* control male. (ns, not significant; one-way ANOVA.) (B) *Tre1^{EP496}/Y* males have normal fertility. *Tre1^{EP496}/Y* males produced an average of 15.5 offspring per male, while control males produced an average of 20 offspring ($P = 0.12$, Student's t -test). (C) The offspring of *Tre1-GAL4/Y; UAS-Tra^F* males are overrepresented in competitive mating assays. A total of 56.2% of offspring were nonwhite-eyed in a competitive cross where half the males were *Tre1-GAL4/Y; UAS-Tra^F* and half the males were *white* (two males of each genotype to five *white* virgin females per vial). This is an overrepresentation of 31% relative to the expected result for the null hypothesis ($P < 0.0001$, χ^2 analysis). All mating experiments were performed in the dark.

Figure 5 *Tre1-GAL4* is expressed in the CNS and in a male-specific pattern in the olfactory system. (A–D) Confocal reconstructions of tissue dissected from animals expressing *UAS-mCD8-GFP* under the control of *Tre1-GAL4*. (A) Antenna from a male of the genotype *Tre1-GAL4/Y; UAS-mCD8-GFP*. *Tre1-GAL4* is expressed in ~15 cells in the third antennal segment (3AS), as well as a small number of neurons in the second AS (2AS). (B) Brain from a male of the genotype *Tre1-GAL4/Y; UAS-mCD8-GFP*. *Tre1-GAL4* is expressed in cells in the antennal lobe (AL) lateral horn (LH), the antennal mechanosensory and motor center (AMMC), and the subesophageal ganglion (SOG). (C) Antenna from a female of the genotype *Tre1-GAL4/+; UAS-mCD8-GFP*. *Tre1-GAL4* expression is absent from the third AS, and significantly reduced in the second AS. (D) Brain from a female of the genotype *Tre1-GAL4/Y; UAS-mCD8-GFP*. *Tre1-GAL4* expression is still apparent in the SOG and AMMC, but there are far fewer projections in the LH and AL. Bars, 50 μ M in all panels.

Tre1-GAL4/Y control is sometimes intermediate between the *Tre1-GAL4/Y; UAS-Tra^F* experiment and the *UAS-Tra^F/+* control (see Figure 1A for an example).

To determine whether *Tre1* itself is involved in courtship, we tested the latency to court of *Tre1^{EP496}/Y* (Rørth 1996) males. *Tre1^{EP496}/Y* males had a courtship latency of 36 sec compared with 63 sec for control animals [wild-type males from the *w¹¹¹⁸*; Wild Type Berlin (WTB) strain, which is the background strain into which all *P*-element mutations and constructs have been introgressed] (Figure 3A). In addition, a subset of *Tre1^{EP496}* mutant flies had an additional (and opposing) phenotype. A total of 6 of 22 (27%) *Tre1^{EP496}* mutant males did not initiate courtship at all during the 20-min observation period, while all wild-type males initiated courtship within 6 min (Figure 3B). Finally, while 13 of 24 (54%) wild-type males successfully copulated within

Table 1 Results of competitive mating assays

Male genotype	Nonwhite offspring (%)	Expected nonwhite offspring (%)	χ^2 (<i>P</i> -value)
<i>Tre1-GAL4/Y; UAS-Tra^F</i>	304 (49.1)	232 (37.5%)	35.6 (<i>P</i> < 0.0001)
<i>Tre1^{EP496}/Y</i>	65 (9.9)	329 (50)	422.1 (<i>P</i> < 0.001)
<i>Tre1-GAL4/Y</i>	141 (43.8)	161 (50)	4.7 (<i>P</i> = 0.030)

w¹¹¹⁸ virgin females were presented with equal numbers of mutant/experimental males and *w¹¹¹⁸* males. While *Tre1-GAL4/Y; UAS-Tra^F* males have a competitive advantage over wild-type males, both *Tre1^{EP496}/Y* and *Tre1-GAL4/Y* were at a disadvantage when competing with wild-type males. All mating assays were performed in the dark.

the 20-min observation period, only 3 of 22 (14%) *Tre1^{EP496}* males copulated (Figure 3C). Thus, of the 16 males who initiated courtship, only 19% successfully proceeded to copulation. From these data, we conclude that *Tre1* is required for normal courtship behavior.

***Tre1-GAL4/Y; UAS-Tra^F* males have increased mating success**

Because feminizing the *Tre1-GAL4* neurons leads to rapid courtship initiation, and over the course of a 20-min observation *Tre1^{EP496}* mutant males successfully copulate at a rate one-third that of wild-type males, we tested the reproductive and mating success of *Tre1-GAL4/Y; UAS-Tra^F* and *Tre1* mutant males in two ways. First, we tested their fertility in a noncompetitive assay with *w*; WTB virgin females. *Tre1-GAL4/Y; UAS-Tra^F* males produce an average of 23 offspring per male, compared with 21 offspring for the *UAS-Tra^F/+* heterozygous background control, indicating that their fertility is unaffected (Figure 4A). Similarly, *Tre1-GAL4/Y* males were just as fertile as control flies, producing an average of 25 offspring per male (Figure 4A). *Tre1^{EP496}* males have a trend toward reduced fertility when compared with *w¹¹¹⁸*; WTB control males, but this trend did not achieve significance (Figure 4B).

We next tested *Tre1-GAL4/Y; UAS-Tra^F* and *Tre1* mutant males in competitive mating assays. In this assay, two experimental males and two *w¹¹¹⁸*; WTB males were allowed to attempt mating with five *w¹¹¹⁸*; WTB virgin females. Relative success in mating was determined by counting the number of offspring with white eyes and comparing it to the number of offspring with nonwhite eyes. In competitive crosses between *Tre1^{EP496}* or *Tre1-GAL4* males and *w¹¹¹⁸*; WTB males, 50% of the offspring are predicted to be white if mating success is equal. For *Tre1-GAL4/Y; UAS-Tra^F/+* males, 75% of the offspring will be nonwhite-eyed; thus, if mating success between the two genotypes is equal, 37.5% of all offspring will be nonwhite.

We found that in 9 of 12 vials, *Tre1-GAL4/Y; UAS-Tra^F/+* males were at a competitive advantage compared with control males. Overall, 49% (304 of 619) of offspring were nonwhite, an overrepresentation of 31% relative to the expectation for equal success in mating (Figure 4C and Table 1). Thus, under laboratory conditions, the rapid courtship initiation seen in *Tre1-GAL4/Y; UAS-Tra^F* males provides a competitive advantage over wild-type males.

Table 2 Courtship latency of *Tre1-GAL4/Y; UAS-Tra^F/+* males with virgin females of sibling *Drosophila* species

Male genotype	<i>D. melanogaster</i> virgins	<i>D. simulans</i> virgins	<i>D. mauritiana</i> virgins
<i>UAS-Tra^F/+</i>	29.8 ± 2.7 s	38.7 ± 6.3 s (NS)	93.3 ± 15.1 s (<i>P</i> < 0.01)
<i>Tre1-GAL4/Y</i>	36.5 ± 5.0 s	50.2 ± 7.8 s (NS)	76.4 ± 16.7 s (<i>P</i> < 0.05)
<i>Tre1-GAL4/Y; UAS-Tra^F/+</i>	13.9 ± 6.2 s	57.7 ± 12.5 s (<i>P</i> < 0.01)	87.2 ± 23.3 s (<i>P</i> < 0.01)

Tre1-GAL4/Y; UAS-Tra^F/+ males display a significant increase in courtship latency with both *D. simulans* and *D. mauritiana* virgin females. *N* = 18–24 males per assay. Statistics were performed using Student's *t*-test assuming unequal sample variances.

Intriguingly (though perhaps unsurprisingly given the proportion of males that fail to copulate within 20 min), unlike *Tre1-GAL4/Y; UAS-Tra^F/+* males, *Tre1^{EP496}* males are at a substantial disadvantage when competing with wild-type males (Table 1). Similarly, *Tre1-GAL4/Y* males showed a slight and weakly significant disadvantage when in competition with wild-type males (Table 1), consistent with *Tre1-GAL4* being a weak allele of *Tre1*. Thus, while *Tre1* mutant flies share a similar courtship initiation phenotype with *Tre1-GAL4/Y; UAS-Tra^F/+* flies, *Tre1* mutant males display additional and opposing phenotypes that are absent from males in which the *Tre1-GAL4* neurons are feminized.

***Tre1-GAL4* is expressed in a sexually dimorphic pattern in both the CNS and PNS**

We have used *Tre1-GAL4* to drive expression of *UAS-mCD8-GFP* (a mouse transmembrane glycoprotein fused to green fluorescent protein, under the control of the yeast UAS promoter) and examined the neuronal expression pattern in the adult CNS and PNS. We found that *Tre1-GAL4* drives GFP expression in ~15 neurons in the third antennal segment (Figure 5A), and this expression pattern is male specific (Figure 5C). In addition, *Tre1-GAL4* is expressed in several small clusters of neurons in the CNS. The expression pattern in the CNS includes two to three antennal lobe glomeruli that are only seen in brains from male flies (Figure 5, B and D). These presumably represent projections from the OSNs. In addition, we observe projections in the antennal mechanosensory and motor center (AMMC), which is consistent with expression of *Tre1-GAL4* seen in the second antennal segment (Figure 5A). The second antennal segment houses the auditory neurons, which project to the AMMC (Kamikouchi *et al.* 2006). Additional projections are present in (but not limited to) the subesophageal ganglion (SOG) and the lateral horn (LH). The projections in the area of the LH are particularly interesting because, while the SOG and AMMC expression is very similar between male and female brains, we observe fewer projections in the region of the LH in female brains (Figure 5D). Understanding the significance of this pattern will depend on further characterization of the neurons expressing *Tre1-GAL4*. However, the identity of *Tre1* as a gene encoding a GPCR that is predicted to respond to chemoattractant signals that is required for germ cell migration in *Drosophila* (Kunwar *et al.* 2003), combined with the role of the LH in the processing of innate olfactory preferences, including pheromone signals, suggests that the *Tre1-GAL4* cells may be involved in the reception and/or processing of a chemosensory signal involved in mate selection.

***Tre1-GAL4* neurons are not required for identification of conspecific females**

Since the rapid initiation of courtship in *Tre1-GAL4/Y; UAS-Tra^F* males (in the absence of other courtship deficiencies seen in *Tre1* mutant males) does not result in reduced mating success, at least under lab conditions, we conclude that it does not reflect reduced courtship quality. Alternatively, the *Tre1-GAL4* cells may participate in mate identification—they may receive a signal that helps to distinguish an appropriate mate from an inappropriate mate. In this case, rapid courtship initiation would reflect the “skipping” of a mate-identification step.

One important aspect of mate choice is the identification of a member of one's own species. To test whether the *Tre1-GAL4* neurons are required to distinguish conspecific females, we tested their latency to court virgins of the sibling species *D. mauritiana* and *D. simulans*. Wild-type *D. simulans* males show increased courtship latency toward *D. melanogaster* females compared with conspecific females (Billeter *et al.* 2009); however, the reverse may not be true (von Schilcher and Manning 1975; Carracedo *et al.* 2000). We found, however, that *D. melanogaster* males display a profound increase in courtship latency when paired with *D. mauritiana* virgin females, and that *Tre1-GAL4/Y; UAS-Tra^F* males display a similar increase (Table 2). Latency to courtship initiation in *UAS-Tra^F/+* males was 93.3 sec, an increase of >1 min over the courtship latency for this genotype with *D. melanogaster*. Similarly, the courtship latency of *Tre1-GAL4/Y; UAS-Tra^F* males increased from an average of 14 sec to 87 sec.

In addition to *D. mauritiana*, we also tested the behavior of *Tre1-GAL4/Y; UAS-Tra^F* males with *D. simulans* virgins. We found that courtship latency of control *UAS-Tra^F/+* males with *D. simulans* virgins was only modestly and insignificantly increased, while *Tre1-GAL4/Y; UAS-Tra^F* males showed an even greater increase in courtship latency (Table 2). It is clear from these data that *Tre1-GAL4/Y; UAS-Tra^F* males are able to identify females of sibling species as inappropriate mate choices.

***Tre1-GAL4* neurons are not required to distinguish mated from unmated females**

We next tested the latency to court of *Tre1-GAL4/Y; UAS-Tra^F* males toward nonvirgin females. Normally, males will avoid courting mated females, leading to a significant courtship delay. If the *Tre1-GAL4* cells are involved in distinguishing virgin from nonvirgin females, we would predict courtship latency of *Tre1-GAL4/Y; UAS-Tra^F* males with nonvirgins to be similar to that with virgin females, and significantly faster than control males when presented with nonvirgin females.

Table 3 Courtship latency of *Tre1-GAL4/Y; UAS-Tra^F/+* males with mated females

Male genotype	Virgin female	Mated female
<i>UAS-Tra^F/+</i>	34.5 ± 3.2 s	49.8 ± 8.5 s (<i>P</i> = 0.019)
<i>Tre1-GAL4/Y</i>	32.5 ± 3.4 s	46.4 ± 12.6 s (NS)
<i>Tre1-GAL4/Y; UAS-Tra^F/+</i>	19.2 ± 3.7 s	34.2 ± 4.0 s (<i>P</i> = 0.0022)

Tre1-GAL4/Y; UAS-Tra^F/+ males display an increase in courtship latency when presented with mated females. Statistics were performed with Student's *t*-test assuming unequal sample variances. *N* = 18–23 males per assay. Statistics were performed using Student's *t*-test assuming unequal sample variances.

The results of this experiment demonstrate that the *Tre1-GAL4* neurons are not required for recognition of mated females. *UAS-Tra^F/+* males showed a modestly significant increase in courtship latency with nonvirgin females (34.5 sec with virgin females, 50 sec with mated females; Table 3). *Tre1-GAL4/Y; UAS-Tra^F* males similarly demonstrated delayed courtship with nonvirgin females relative to their performance with virgin females (19 sec vs. 34 sec, Table 3). These results indicate that the ability of *Tre1-GAL4/Y; UAS-Tra^F* males to distinguish virgin from mated females remains intact.

***Tre1-GAL4* neurons are not required to distinguish male flies from female flies**

Finally, we tested the function of the *Tre1-GAL4* neurons in distinguishing male from female flies by testing male–male courtship in *Tre1-GAL4/Y; UAS-Tra^F/+* males. Wild-type males typically do not court other males at an appreciable rate, and when they do, they display a profound courtship delay. As expected, both the *UAS-Tra^F/+* and *Tre1-GAL4/Y* males show a dramatic increase in courtship latency when presented with male virgin courtship targets (Table 4). *Tre1-GAL4/Y; UAS-Tra^F/+* males show a similar delay; in fact, *Tre1-GAL4/Y; UAS-Tra^F/+* males show a greater delay in courting virgin males, though this effect is not statistically significant.

Because *Tre1^{EP496}* males display courtship defects in addition to rapid courtship initiation, and because one of the primary phenotypes associated with loss of *fru^M* expression is an increase in male–male courtship, we also tested male–male courtship behavior in *Tre1^{EP496}* mutant males. *Tre1^{EP496}* mutant males display a significant increase in courtship initiation when presented with virgin males, nearly identical to that seen in *Tre1-GAL4/Y* males (Table 4).

The male–male courtship comparisons do not achieve statistical significance in *Tre1-GAL4/Y; UAS-Tra^F/+* due to the very small number of males that initiate courtship with other males during the assay window. As shown in Figure 6, only 17–21% of *Tre1-GAL4/Y; UAS-Tra^F/+* and *UAS-Tra^F/+* males court other males during this window. However, 58% of *Tre1-GAL4/Y* males displayed wing song during the courtship assay, further suggesting that *Tre1-GAL4* is a weak allele of *Tre1*. More strikingly, 83% of *Tre1^{EP496}/Y* males courted other males, compared to 56% of their *w¹¹¹⁸*; WTB (wild type) control males. These observations confirm that the phenotypes associated with feminizing the *Tre1-GAL4* neurons via

Table 4 Male–male courtship in *Tre1-GAL4/Y; UAS-Tra^F/+* and *Tre1* mutant males

Male genotype	Virgin female	Virgin male
<i>UAS-Tra^F/+</i>	34.5 ± 3.2 s	386 ± 128 s (<i>P</i> = 0.026)
<i>Tre1-GAL4/Y</i>	32.5 ± 3.4 s	205 ± 65 s (<i>P</i> = 0.0099)
<i>Tre1-GAL4/Y; UAS-Tra^F/+</i>	19.2 ± 3.7 s	494 ± 291 s (NS)
Wild type	63.9 ± 8.6 s	415 ± 97.6 s (<i>P</i> = 0.0018)
<i>Tre1^{EP496}</i>	35.8 ± 8.4 s	210 ± 69.9 s (<i>P</i> = 0.014)

Tre1-GAL4/Y; UAS-Tra^F/+ and *Tre1* mutant males display an increase in male–male courtship latency. *N* = 3–24 males per assay (see text). Statistics were performed with Student's *t*-test assuming unequal sample variances.

Tre1-GAL4/Y; UAS-Tra^F/+ are distinct but overlapping with those associated with loss of function in the *Tre1* gene.

Discussion

Courtship in *D. melanogaster* is a complex and stereotyped repertoire of behaviors established by sex-specific genetic pathways, mediated by sensory inputs via the peripheral nervous system, and integrated in the central nervous system (Hall 1994; Baker *et al.* 2001; Demir and Dickson 2005; Dickson 2008; Pavlou and Goodwin 2013). The resulting behavioral outputs are dependent upon expression of the male-specific isoforms of the zinc-finger transcription factor Fru (*Fru^M*) (Demir and Dickson 2005). Here we have identified a unique role for *Fru^M* proteins. When cells expressing *Tre1-GAL4* are feminized via expression of the female splice form of *tra* (*tra^F*) (leading to female-specific splicing of *fru* transcripts), male flies initiate courtship much more quickly than wild-type flies. Thus, in the *Tre1-GAL4* cells, *Fru^M* proteins are uniquely required to reduce the speed of courtship initiation.

We show that mutation of the *Tre1* GPCR itself can lead to rapid courtship, but can also cause courtship delays, reduced reproductive success, and increased male–male courtship, phenotypes that are typical of flies mutant for *fru^M*, and that are not recapitulated by feminization of the *Tre1-GAL4* neurons, indicating a complex requirement for *Tre1* in courtship behavior. In addition, we find that *Tre1-GAL4* is expressed in a sexually dimorphic pattern in the adult OSNs and central nervous system, and that the *Tre1-GAL4* neurons are not required for distinguishing conspecific females, unmated females, or males from females.

***Drosophila* mating behavior and the role of the *Tre1-GAL4* neurons**

We have identified a *GAL4* transgene that is inserted into the coding sequence of the *Tre1* GPCR gene (*Tre1-GAL4*). When this *GAL4* line is used to drive expression of either an RNAi targeting *fru^M* or the feminizing transgene *UAS-Tra^F*, it results in male flies that initiate courtship and achieve copulation much more quickly than control animals. This phenotype is unique: the typical result of loss of *Fru^M* is slowed, abnormal, or absent courtship (Demir and Dickson 2005).

Figure 6 *Tre1-GAL4/Y; UAS-TraF/+* males do not court other males. Only 17% of *Tre1-GAL4/Y; UAS-TraF/+* males initiated courtship with other males during the 20-min observation window, comparable to *UAS-TraF/+* control males. Intriguingly, 58% of *Tre1-GAL4/Y* males and 83% of *Tre1^{EP496}/Y* males initiated courtship during the assay, indicating that global mutation of *Tre1* does result in male–male courtship behavior. *N* = 18–24 trials per genotype.

While the *Fru^M*-expressing neuronal projection patterns have been well characterized (Yu *et al.* 2010), the functions of all cell clusters expressing *Fru^M* have not been completely elucidated. The *Tre1-GAL4* expressing cells represent a category of *Fru^M* cells that have not previously been identified: cells whose function appears to reduce the speed of courtship initiation. At least three different hypotheses may explain these results. First, the cells may participate in “quality control,” ensuring the correct performance of the courtship ritual. This hypothesis predicts that courtship quality would be reduced in animals with rapid courtship initiation, leading to reduced mating and reproductive success, which we do not observe. On the contrary, rapid courtship initiation, in the absence of other phenotypes, appears to provide a reproductive advantage.

Second, the *Tre1-GAL4* cells may also function in correct mate identification, allowing males to distinguish an appropriate mate from a less appropriate mate. This would predict that rapid courtship reflects the skipping of such a step. If that was the case, we would expect *Tre1-GAL4/Y; UAS-TraF^F* males to have trouble distinguishing the “correct” mate from an inappropriate mate. Our experiments indicate that the *Tre1-GAL4* cells are not involved in the ability of *D. melanogaster* males to identify conspecific females, nor do they appear to be required to distinguish virgin from nonvirgin females or males from females. It is also important to note that the *Tre1* mutations used in these analyses are not null mutations, so it is possible that some of the complexity of phenotype we are seeing is due to varying levels of residual *Tre1* function. This could be resolved through the generation and analysis of null mutations through excision of the *Tre1^{EP496}* or *Tre1-GAL4* *P*-elements.

Third, feminization of the *Tre1-GAL4* cells may be causing gain-of-function effects, leading to rapid courtship initiation. This hypothesis may be tested by determining whether the *Tre1-GAL4/Y; UAS-TraF^F* courtship phenotype is due to loss, or gain, of neuronal activity. We are currently undertaking a

series of experiments to drive expression of a temperature-sensitive allele of *shibiri* (*shi^{ts}*), the gene encoding the fly homolog of dynamin (Gonzalez-Bellido *et al.* 2009), as well as the heat-sensitive TrpA1 (Rosenzweig *et al.* 2005) channel in the *Tre1-GAL4* neurons. If rapid courtship results from loss of function of the *Tre1-GAL4* cells, expression of *shi^{ts}* should recapitulate the phenotype. On the other hand, if rapid courtship results from inappropriate neuronal activity, activation of TrpA1 in the *Tre1-GAL4* cells should phenocopy *Tre1-GAL4/Y; UAS-TraF^F*.

The *Tre1* receptor

Tre1-GAL4 is an insertion into the coding sequence of the GPCR-encoding gene *Tre1*. *Tre1* is required for initiation of transepithelial migration in *Drosophila* germ cells (Kunwar *et al.* 2003, 2008), neuroblast polarity during asymmetric stem cell division in the developing central nervous system (Yoshiura *et al.* 2012). The ligand for *Tre1* is not known, but based on sequence similarity, belongs to a family of proteins related to the GPCR CXCR4, which, together with its ligand SDF1, is required for germ cell migration in vertebrates (Kunwar *et al.* 2003). *Tre1* is most closely related to the vertebrate histamine and melatonin receptors, and more distantly related to chemokine receptors (Kunwar *et al.* 2003).

Both the cell migration and cell polarity roles for *Tre1* reflect activation of heterotrimeric G-protein signaling that ultimately leads to polarization of the cytoskeleton (and, in the case of germ cell migration, polarized distribution of E-cadherin) (Kunwar *et al.* 2008; Yoshiura *et al.* 2012). In migrating germ cells, the role of *Tre1* appears to be to change the polarity of the cells such that they are oriented toward their ultimate target, and to facilitate the loss of cell–cell adhesion between germ cells, thereby priming the cells to be receptive to further guidance signals (Kunwar *et al.* 2008).

Based on these data, *Tre1* is likely a neuropeptide or protein hormone receptor that, upon activation, leads to a polarized reorganization of the cytoplasm. This proposed function for *Tre1* is consistent with a role in axon pathfinding or synapse formation. We find that *Tre1* is expressed in a sexually dimorphic pattern in the adult nervous system. One attractive mechanism for the requirement for *Tre1* in courtship behavior is that *Tre1* may allow sex-specific responses to a guidance signal that is present in all developing fly brains.

Tre1 and courtship behavior

Feminization of the *Tre1-GAL4* neurons causes rapid courtship initiation, and this phenotype leads to increased reproductive success in competition with normal males. This advantage indicates that *Tre1-GAL4/Y; UAS-TraF^F* males perform the courtship ritual correctly and completely, and our data indicate that *Tre1-GAL4/Y; UAS-TraF^F* males successfully copulate at a rate identical to wild-type males (not shown). However, males mutant for *Tre1* display reduced competitive reproductive success and successfully copulate at a rate far below that seen in wild-type males under noncompetitive conditions Table 1 and Figure 3C). In addition, males mutant

for *Tre1* display the “classic” *fru^M* mutant phenotype: increased male–male courtship behavior (Figure 6). These data suggest two possibilities: first, that *Tre1-GAL4* recapitulates a subset of the full *Tre1* expression pattern, and second, that the *Tre1-GAL4*-expressing neurons are involved only in courtship initiation. Based on our analyses of *Tre1* hypomorphic mutations compared with the phenotype of *Tre1-GAL4/Y; UAS-Tra^F* males, it seems very likely that *Tre1-GAL4* identifies a set of neurons with a novel function in courtship initiation relative to what has previously been shown for *fru^M*-expressing neurons. These questions could be addressed by generating an antibody against Tre1 and performing immunofluorescence experiments to determine the full Tre1 expression pattern in both the larval and adult nervous systems.

Alternatively, the difference may reflect a difference in temporal requirement for Tre1 function. In *Tre1-GAL4/Y; UAS-Tra^F* males, the *Tre1*-expressing neurons are feminized during development due to female-specific splicing of *fru* transcripts. This could result in changes in neurite outgrowth and synapse formation that results in aberrant courtship initiation. However, in *Tre1* mutant males, the *Tre1* neurons are presumably correctly masculinized. The developmental absence of Tre1 would still be expected to result in rapid courtship initiation; however, the reduced copulation success and competitive disadvantage seen in *Tre1* mutant males may result from later requirements for *Tre1*. These hypotheses could be tested by characterizing the critical developmental period for each of the *Tre1* courtship and copulation phenotypes.

Conclusions

We have identified a novel effect of loss of *Fru^M* expression: rapid courtship initiation. Males lacking *Fru^M* expression in the *Tre1-GAL4*-expressing neurons initiate courtship much more quickly than wild-type males, and this rapid courtship initiation results in a competitive reproductive advantage. In addition, we have identified a gene (*Tre1*) that was previously unknown to be involved in courtship behavior. Mutation of this gene leads not only to rapid courtship initiation, but, unlike males in which the *Tre1* cells are feminized, *Tre1* mutant males also frequently fail to copulate and are at a reproductive disadvantage relative to wild-type males. Future studies will focus on characterization of the downstream components of the Tre1 signal transduction pathway, which likely include the trimeric G-protein subunits *Gγ1* and *Gβ13f* (Kunwar *et al.* 2008) as well as identification of the Tre1 ligand.

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