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Tachykinin-Expressing Neurons Control Male-Specific Aggressive Arousal in *Drosophila*

Kenta Asahina,[1,2] Kiichi Watanabe,[1,2] Brian J. Duistermars,[1,2] Eric Hoopfer,[1,2,4] Carlos Roberto González,[3] Eyrún Arna Eyjólfsdóttir,[3] Pietro Perona,[3] and David J. Anderson[1,2,*]

[1]Howard Hughes Medical Institute, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125, USA
[2]Division of Biology, 156-29, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125, USA
[3]Division of Engineering and Applied Science, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125, USA
[4]Janelia Farm Research Campus, HHMI, 19700 Helix Drive, Ashburn, VA 20147, USA
*Correspondence: wuwei@caltech.edu
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SUMMARY

Males of most species are more aggressive than females, but the neural mechanisms underlying this dimorphism are not clear. Here, we identify a neuron and a gene that control the higher level of aggression characteristic of *Drosophila melanogaster* males. Males, but not females, contain a small cluster of FruM$^+$ neurons that express the neuropeptide tachykinin (*Tk*). Activation and silencing of these neurons increased and decreased, respectively, intermale aggression without affecting male-female courtship behavior. Mutations in both *Tk* and a candidate receptor, *Takr86C*, suppressed the effect of neuronal activation, whereas overexpression of *Tk* potentiated it. *Tk* neuron activation overcame reduced aggressiveness caused by eliminating a variety of sensory or contextual cues, suggesting that it promotes aggressive arousal or motivation. Tachykinin/Substance P has been implicated in aggression in mammals, including humans. Thus, the higher aggressiveness of *Drosophila* males reflects the sexually dimorphic expression of a neuropeptide that controls agonistic behaviors across phylogeny.

INTRODUCTION

Aggression is an innate, species-typical social behavior that is widespread in animal phylogeny. Expression of agonistic behavior is commonly observed between conspecific males in conflict over access to reproductively active females, food, territory, or other resources (Siegel et al., 1997). In many animal species, aggression is often quantitatively higher in males than in females (Lorenz, 1966). In humans, violent aggression constitutes a major public health problem (Filley et al., 2001) and its incidence is overwhelmingly higher among males than females (Craig and Halton, 2009). In addition, the behavioral expression of aggression is often qualitatively different between males and females, and may differ in the contexts in which it is exhibited (Lorenz, 1966).

Despite recent progress (reviewed in Manoli et al., 2013), the neurobiological mechanisms underlying the evolutionarily conserved sexual dimorphism in aggressiveness remain poorly understood. Pheromones are known to play an important role in intermale aggression (Chamero et al., 2007; Fernández et al., 2010; Liu et al., 2011; Wang and Anderson, 2010; Wang et al., 2011; reviewed in Stowers and Logan, 2010). However, in cases where the relevant receptors are known (Wang and Anderson, 2010), dimorphic expression of these molecules does not appear to explain sex differences in aggressiveness (Kurtovic et al., 2007; Ruta et al., 2010). Studies in numerous vertebrate species have identified sexual dimorphisms in the size of brain nuclei or their constituent neuronal subpopulations that are controlled by gonadal steroid hormones in a manner that parallels the influence of these hormones on aggressive behavior (reviewed in Wu and Shah, 2011). Recent studies have shown that genetic ablation of hypothalamic neurons expressing the progesterone receptor decreases both aggression and mounting in males, and mating behavior in females (Yang et al., 2013). These neurons display sexual dimorphisms in their projections, but whether this dimorphism is causally responsible for sex differences in levels of aggressiveness is not yet clear.

As in other species, *Drosophila* males flies are more aggressive than females and also exhibit qualitative differences in agonistic behavior (Nilsen et al., 2004; Vrontou et al., 2006). These sex differences in aggression are known to be under the control of *fruitless (fru)*, a master regulator of sexual differentiation of the brain (Lee and Hall, 2000; Siwicki and Kravitz, 2009; Vrontou et al., 2006). Although some efforts have been made to identify circuits through which *fru* exerts its influence on aggressive behavior (Certel et al., 2007, 2010; Chan and Kravitz, 2007; Mundiyanapurath et al., 2009), FruM$^+$ neurons that are necessary, sufficient, and specific for male-type aggression have not yet been identified.

Here, we have identified a small group of sexually dimorphic, FruM$^+$ neurons that promote aggressiveness in *Drosophila* males but have no influence on male-female courtship behavior. These neurons enhance aggression, at least in part, through the

release of a neuropeptide, *Drosophila* tachykinin (DTK) (Nässel and Winther, 2010; Winther et al., 2003). Tachykinin/Substance P has been implicated in certain forms of aggression in several mammalian species (Katsouni et al., 2009). Thus, the higher level of aggression that is characteristic of *Drosophila* males is promoted by sexually dimorphic neurons, which express a neuropeptide that regulates agonistic behavior across phylogeny.

RESULTS

Tachykinin-GAL4 Lines Label Aggression-Promoting Neurons

We reasoned that neural circuits that control aggression, like those that control other innate behaviors, are likely to be regulated by neuropeptides (Bargmann, 2012; Nässel and Winther, 2010; Taghert and Nitabach, 2012). To identify peptidergic neurons that control intermale aggression in *Drosophila*, we used the thermosensitive cation channel *Drosophila* TRPA1 (dTRPA1) (Hamada et al., 2008) to activate neurons labeled by a set of ~40 GAL4 driver lines created from putative promoter regions of ~20 different *Drosophila* neuropeptide genes (Hergarden et al., 2012; Tayler et al., 2012). We screened these lines for increases in aggressive behavior, using a high-throughput modification of hardware and software that permit automated detection of fly aggressive behaviors (Dankert et al., 2009; Figure 1A).

This screen identified two *Drosophila* Tachykinin (*Tk*)-*GAL4* lines that strongly increased aggression in combination with *UAS-dTRPA1* at 29°C (Figure 1A). Activation of a *Neuropeptide F-GAL4* line weakly enhanced aggression (Figure S1A available online), an effect opposite to that described previously using a different *NPF-GAL4* driver (Dierick and Greenspan, 2007). Secondary screens confirmed the genotype and temperature dependence of the enhanced aggression phenotype of the two *Tk-GAL4* lines (which we henceforth refer to as $Tk\text{-}GAL4^{1}$ and $Tk\text{-}GAL4^{2}$, respectively; Figures 1B and 1C). Aggression promoted by $Tk\text{-}GAL4^{1}$ neurons was particularly robust and intense (Movie S1). The enhanced aggression phenotype was not due to an increase in locomotor activity (Figures S1B and S1C). Two other *Tk-GAL4* lines, $Tk\text{-}GAL4^{3}$ and $Tk\text{-}GAL4^{GMR61H07}$, did not promote aggression (Figures S1I and S1J). Activation of $Tk\text{-}GAL4^{1}$ neurons also increased aggression toward a wild-type male (Figure S1D). Thus, the aggression-promoting effect is likely due to a fly-autonomous influence rather than to, e.g., an increased release of aggression-promoting pheromones (Fernández et al., 2010; Wang and Anderson, 2010; Wang et al., 2011).

Expression analysis of $Tk\text{-}GAL4^{1}$ and $Tk\text{-}GAL4^{2}$ using a *UAS-mCD8:GFP* reporter revealed a cluster of lateral protocerebral neurons that formed a ring-shaped arborization (Figures 1D and 1E), which resembled a previously characterized male-specific neuropil formed by *fruitless*-expressing neurons (Cachero et al., 2010; Yu et al., 2010). The ring-shaped arborizations were absent in the corresponding area of the female brain (D_3-G_3; Figures 1F and 1G). A sexually dimorphic labeling pattern was also evident in the ventral nerve chord (VNC) (Figures S1E–S1H).

Immunostaining against the male-specific isoform of *fruitless* (FruM) revealed that the lateral cluster of $Tk\text{-}GAL4^{1}$ neurons indeed expressed FruM (5.3 ± 0.5 cells/hemibrain, n = 6; Figure 2A). We did not find other FruM-expressing $Tk\text{-}GAL4^{1}$ neurons in the central brain, except for a few inconsistently labeled cells located ventrally to the lateral cluster. The $FruM^{+}$ lateral cluster neurons appeared to be labeled by $Tk\text{-}GAL4^{2}$ as well (Figure 2B; 7.6 ± 1.2 neurons/hemibrain, n = 6), although several other classes of $FruM^{+}$ neurons were also labeled in that GAL4 line. The other 2 *Tk-GAL4* lines that did not promote aggression did not contain the lateral cluster of $FruM^{+}$ neurons associated with the ring-shaped arborization (Figures S1K–S1N).

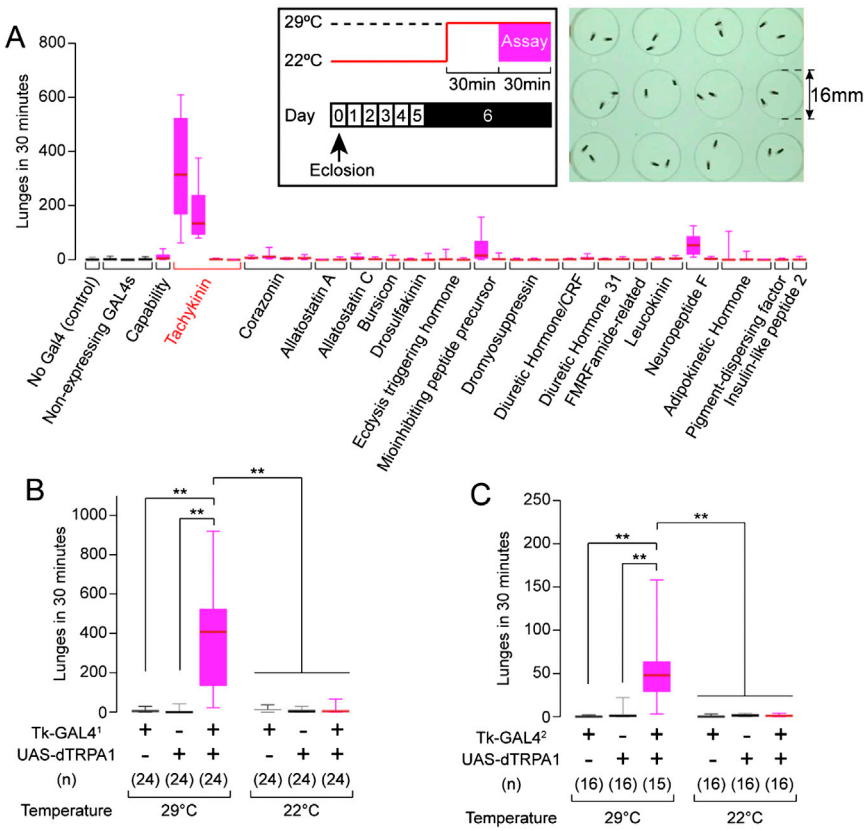
We confirmed the expression of FruM in the lateral cluster neurons using a genetic approach. Expression analysis of $Tk\text{-}GAL4^{1}$ and $fru^{P1.LexA}$ (Mellert et al., 2010) using dual reporters revealed that $fru^{P1.LexA}$ labels three to four of the approximately six $Tk\text{-}GAL4^{1}$ neurons in this cluster (Figure 2C; 3.1 ± 0.6 $tdTomato^{+}$ cells/5.5 ± 0.5 GFP+ cells/hemibrain, n = 10). Similar results were obtained using $fru^{P1.LexA}$ to drive *LexAop2-FLPL* in combination with FLP recombinase-dependent reporters (Figures 2D–2H), including a *UAS > stop > dTRPA1*^{mCherry} allele (Pan et al., 2012; Yu et al., 2010) used for functional manipulations (see below). Immunostaining with anti-FruM antibody confirmed that the $Tk\text{-}GAL4^{1}$ neurons identified by this intersectional strategy indeed express FruM protein (Figure 2H). The neurons revealed by $dTRPA1^{mCherry}$ expression (Figure 2G) appear similar to those in the aSP-f/g/5/6 $FruM^{+}$ cluster (Cachero et al., 2010; Yu et al., 2010), which was reported to contain four to 13 neurons in males. However, intersectional expression of $dTRPA1^{mCherry}$ labeled only 4.2 ± 0.8 neurons/hemibrain (n = 8). We hereafter refer to these neurons as $Tk\text{-}GAL4^{FruM}$ neurons.

TK-GAL4^{FruM} Neurons Control Male-Male Aggression

To determine whether $Tk\text{-}GAL4^{FruM}$ neurons are indeed responsible for the aggression phenotype, we carried out intersectional neuronal activation experiments (Pan et al., 2012; von Philipsborn et al., 2011; Figure 2D). This approach yielded $TRPA1^{mCherry}$ expression exclusively in the lateral cluster (Figure 2E, arrows) and in two pairs of VNC neurons (Figure 2F, arrows). No expression was detected when any one of the four genetic components used in the genetic intersection was omitted (Figures S2A–S2D).

Thermogenetic activation of these $Tk\text{-}GAL4^{FruM}$ neurons induced robust male-male aggression in a temperature-dependent manner (Figure 2I; Movie S2). To determine whether the two pairs of abdominal ganglion neurons play a role in aggression, we generated a transgene, *Otd-nls:FLPo*, that expresses FLP specifically in the central brain (see Experimental Procedures). $Tk\text{-}GAL4^{1}$; *Otd-nls:FLPo*, *UAS > stop > dTRPA1*^{mCherry} flies expressed $dTRPA1^{mCherry}$ selectively in the central brain, but not in the VNC (Figures S2E–S2J). These flies showed robust male-male aggression at high temperature (Figure S2K). Thus, activation of $Tk\text{-}GAL4^{FruM}$ neurons in the central brain alone is sufficient to promote intermale aggression.

To determine whether $Tk\text{-}GAL4^{FruM}$ neurons are necessary for aggression, we genetically silenced them in single-housed flies,



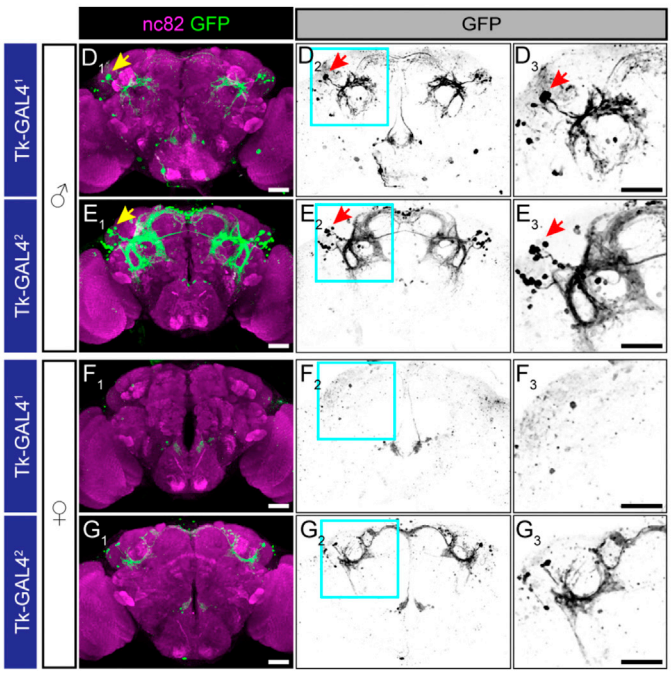


Figure 1. *Tk-GAL4* Lines Label Aggression-Promoting Neurons

(A) Number of lunges (boxplot) during dTRPA1-mediated thermogenetic activation of neurons labeled by 42 *neuropeptide-GAL4* drivers. A box indicates lower quartile, median, and higher quartile, from bottom to top. Whiskers represent the range of the remaining data points. The names of the genes from which the promoter fragments were generated are listed below the plots; n = 10–24. The inset illustrates the experimental design (left) and the 12-well aggression chamber used in this assay (right).

(B and C) Number of lunges (boxplot) during thermogenetic activation of *Tk-GAL4*^{1}(B) and *Tk-GAL4*^{2}(C) neurons. Genotypes, number of pairs, and temperature tested are indicated below the plot. **p < 0.01 (Kruskal-Wallis and post hoc Mann-Whitney U tests). Horizontal bar above 22°C data indicates pooling for statistical analysis (Kruskal-Wallis test, p > 0.05).

(D–G) Brains of *Tk-GAL4*^{1}; *UAS-mCD8:GFP* male (D), female (F), *Tk-GAL4*^{2}; *UAS-mCD8:GFP* male (E), and female (G) immunostained with anti-GFP antibody (green) and the neuropil marker nc82 (magenta) (D_1-G_1). D_2-G_2: GFP only. D_3-G_3: region within a cyan square in D_2-G_2 (magnified). Arrows: the lateral cluster neurons (see text).

See also Figure S1 and Movie S1. For the complete genotypes of animals used in all figures, please refer to Table S1.

which display higher levels of baseline aggression than group-housed flies (Wang et al., 2008). To do this, we expressed the inwardly rectifying potassium channel Kir2.1 selectively in the Tk-GAL4^{FruM} neurons by using *UAS* > *stop* > *Kir2.1*^{eGFP} in combination with *fru*$^{P1.LexA}$ and *LexAop2-FLPL*. Such flies showed significantly reduced aggression compared with genetic controls, although the level of baseline aggression between different controls varied (Figure 2J). Thus, the activity of Tk-GAL4^{FruM} neurons is necessary for aggression in single-housed flies. Together, these results identify a small subset of neurons that are necessary and sufficient for male-male aggression.

The Function of Tk-GAL4^{FruM} Neurons Is Specific to Aggression

FruM^{+} male neurons also control courtship behavior (Kimura et al., 2005; Manoli et al., 2005; Stockinger et al., 2005). Indirect evidence suggested that distinct populations of FruM^{+} neurons control intermale aggression versus male-female courtship (Chan and Kravitz, 2007); however, FruM^{+} neurons that control male aggression, but not male-female courtship, have not previously been identified. For this reason, it was of interest to explore the influence of Tk-GAL4^{FruM} neurons on male-female courtship.

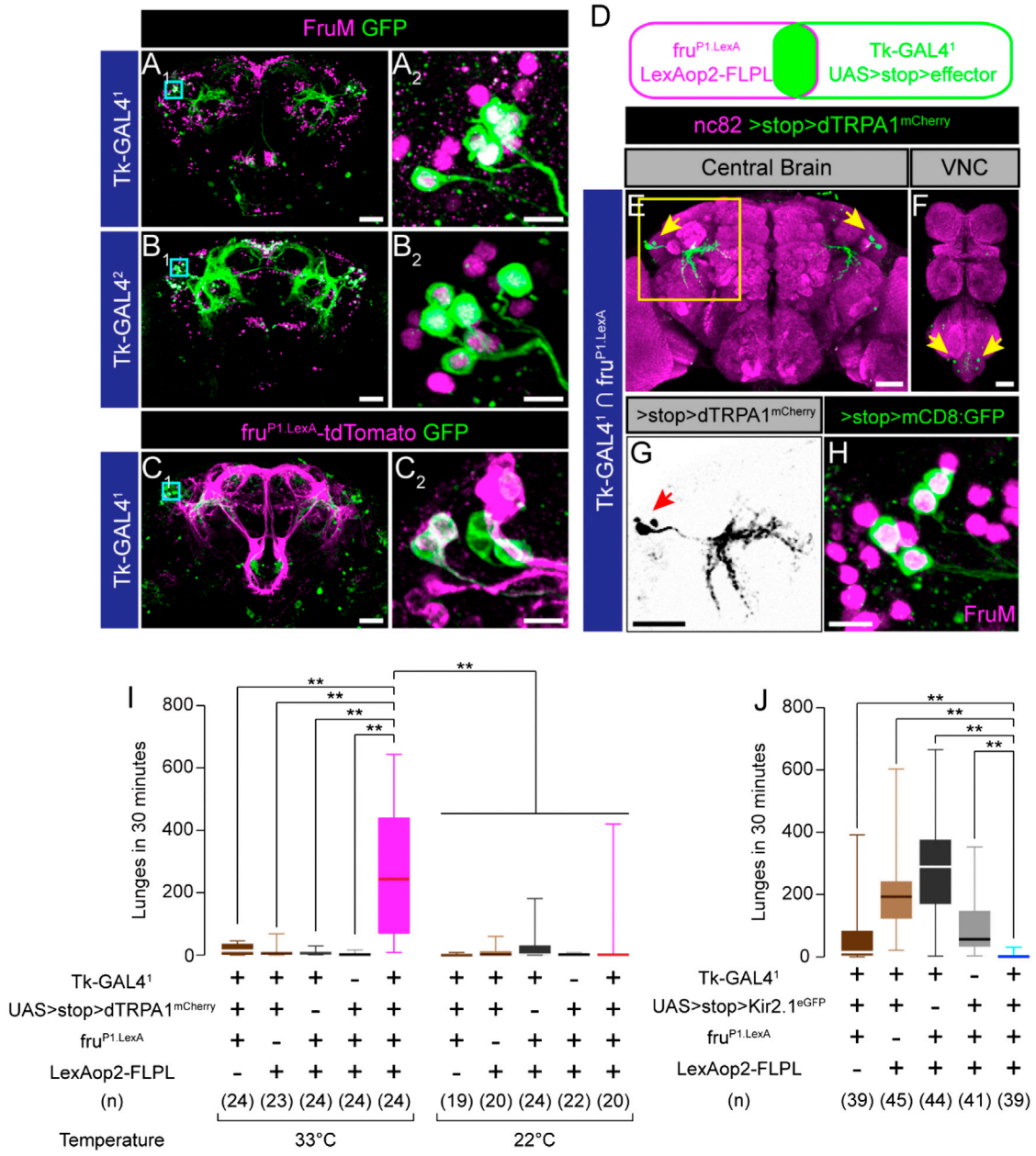


Figure 2. Tk-GAL4^{FruM} Neurons Control Male-Male Aggression

(A and B) *Tk-GAL4^{1}*; *UAS-mCD8:GFP* (A) and *Tk-GAL4^{2}*; *UAS-mCD8:GFP* (B) male brains immunostained with anti-GFP antibody (green) and anti-FruM antisera (magenta). A_2, B_2: region within a cyan square in A_1 and B_1 (magnified).

(C) *Tk-GAL4^{1}*; *fru$^{P1.LexA}$/ UAS-mCD8:GFP*, *LexAop2-tdTomato* male brain immunostained with anti-GFP antibody (green) and anti-DsRed antibody (magenta). C_2: region within a cyan square in C_1 (magnified).

(D) Schematic of the genetic intersectional strategy utilized in this study.

(E and F) Tk-GAL4^{FruM} neurons in the brain (E) and VNC (F) immunostained with anti-DsRed antibody (green) and nc82 (magenta). Arrows: cell bodies stained with anti-DsRed antibody.

(G) Region within a cyan square in (E) (magnified). Only the image of anti-DsRed is shown. Arrow: cell bodies stained with anti-DsRed antibody.

(H) Four Tk-GAL4^{FruM} neurons in a male brain, immunostained with anti-GFP antibody (green) and anti-FruM antisera (magenta). The magnification compares to images A_2, B_2, and C_2.

(I and J) Number of lunges during thermogenetic activation (I) and silencing (J) of Tk-GAL4^{FruM} neurons.

For (I) and (J), **p < 0.01 (Kruskal-Wallis and post hoc Mann-Whitney U tests).

See also Figure S2 and Movie S2.

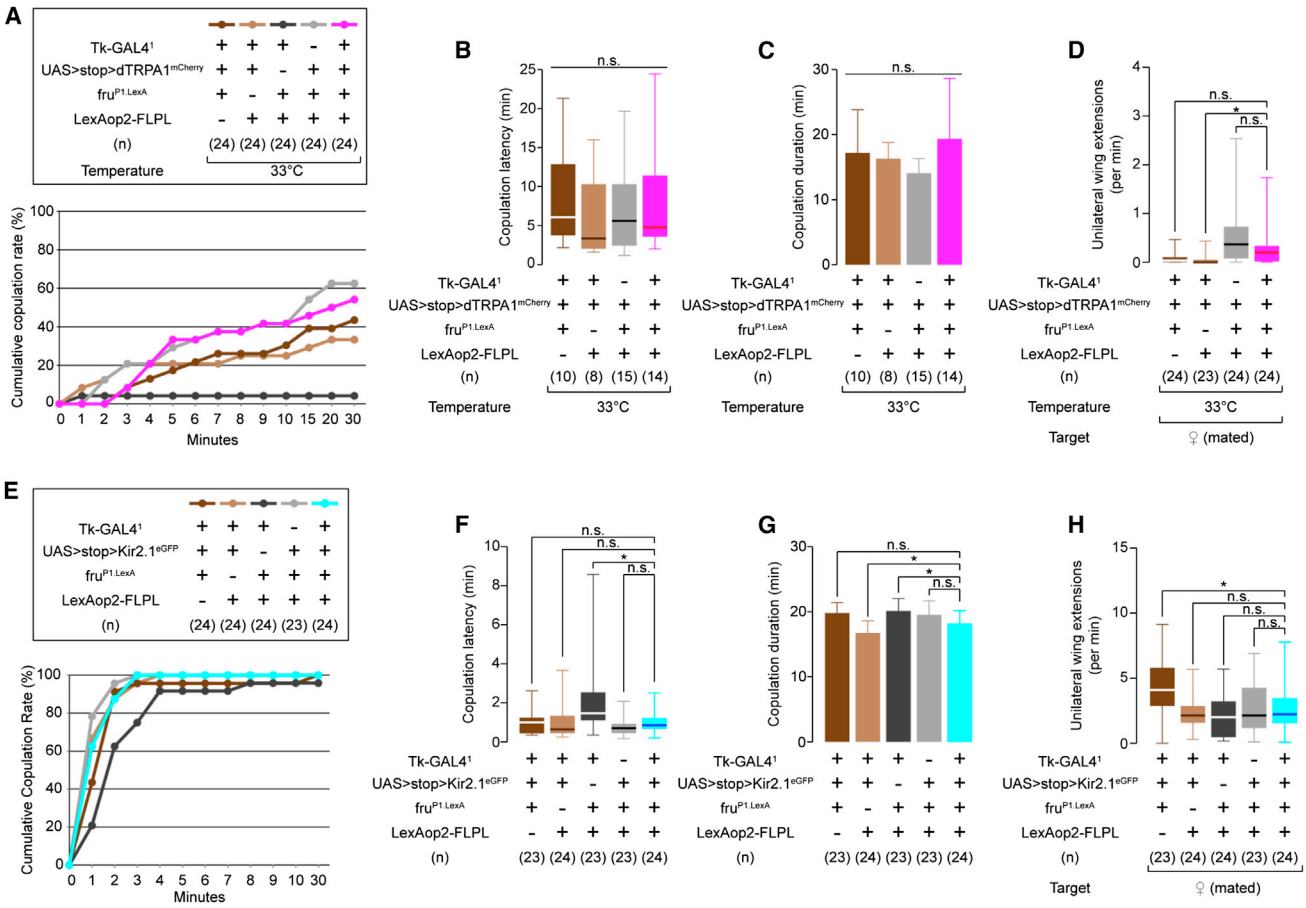


Figure 3. Tk-GAL4^{FruM} Neurons Do Not Modulate Courtship Behavior

(A) Cumulative copulation curve for males during thermogenetic activation of Tk-GAL4^{FruM} neurons. *Tk-GAL4^{1}*; *fru$^{P1.LexA}$*/ *LexAop2-FLPL* control males were eliminated from subsequent analyses.

(B–D) Copulation latency (B), duration (C, mean ± SD), and unilateral wing extension frequency (D) during thermogenetic activation of Tk-GAL4^{FruM} neurons.

(E) Cumulative copulation curve for males during silencing of Tk-GAL4^{FruM} neurons.

(F–H) Copulation latency (F), duration (G; mean ± SD), and unilateral wing extension frequency (H) during silencing of Tk-GAL4^{FruM} neurons.

For (B), (D), (F), and (H), *p < 0.05, n.s.: p > 0.05 (Kruskal-Wallis and post hoc Mann-Whitney U tests. For (C) and (G), *p < 0.05, n.s.: p > 0.05 (one-way ANOVA or one-way ANOVA and post hoc Student's t tests).

See also Figure S3.

We initially performed gain-of-function studies using intersectional expression of dTRPA1 in Tk-GAL4^{FruM} neurons. Thermogenetic activation did not induce any aggressive behaviors toward females (zero lunges toward females by any of 24 males). Rather, the males copulated at a rate comparable to that observed in the control strains (Figure 3A). (An exception was a control*Tk-GAL4^{1}*; *fru$^{P1.LexA}$*/ *LexAop2-FLPL* strain, which failed to copulate at 33°C, perhaps reflecting transcriptional "squelching" by the GAL4 activation domain in the absence of an upstream activating sequence (UAS)-binding site [Gill and Ptashne, 1988].) We also measured copulation latency and the average duration of copulation, and found no significant difference between the experimental strains and the other three control strains (Figures 3B and 3C).

To quantify the intensity of courtship behavior, we measured the frequency of unilateral wing extension or "singing" (Hall, 1994) using a new automated, unilateral wing extension classifier (see Experimental Procedures; Figures S3A and S3B). Thermogenetic activation of Tk-GAL4^{FruM} neurons did not produce any consistent change, compared with controls, in the frequency of unilateral wing extension toward mated females (Figure 3D). Selective silencing of Tk-GAL4^{FruM} neurons with Kir2.1 did not reduce male copulation efficiency (Figure 3E), copulation latency (Figure 3F), copulation duration (Figure 3G), or the frequency of unilateral wing extension (Figure 3H) compared with the controls. These results suggest that Tk-GAL4^{FruM} neurons do not influence male courtship behavior toward females.

We next investigated whether genetic manipulations of Tk-GAL4^{FruM} neuronal activity influence male-male courtship, which normally occurs at low frequency. Silencing of Tk-GAL4^{FruM} neurons did not consistently influence the frequency of unilateral wing extensions in comparison with control strains (Figure S3C). Similarly, thermogenetic activation of

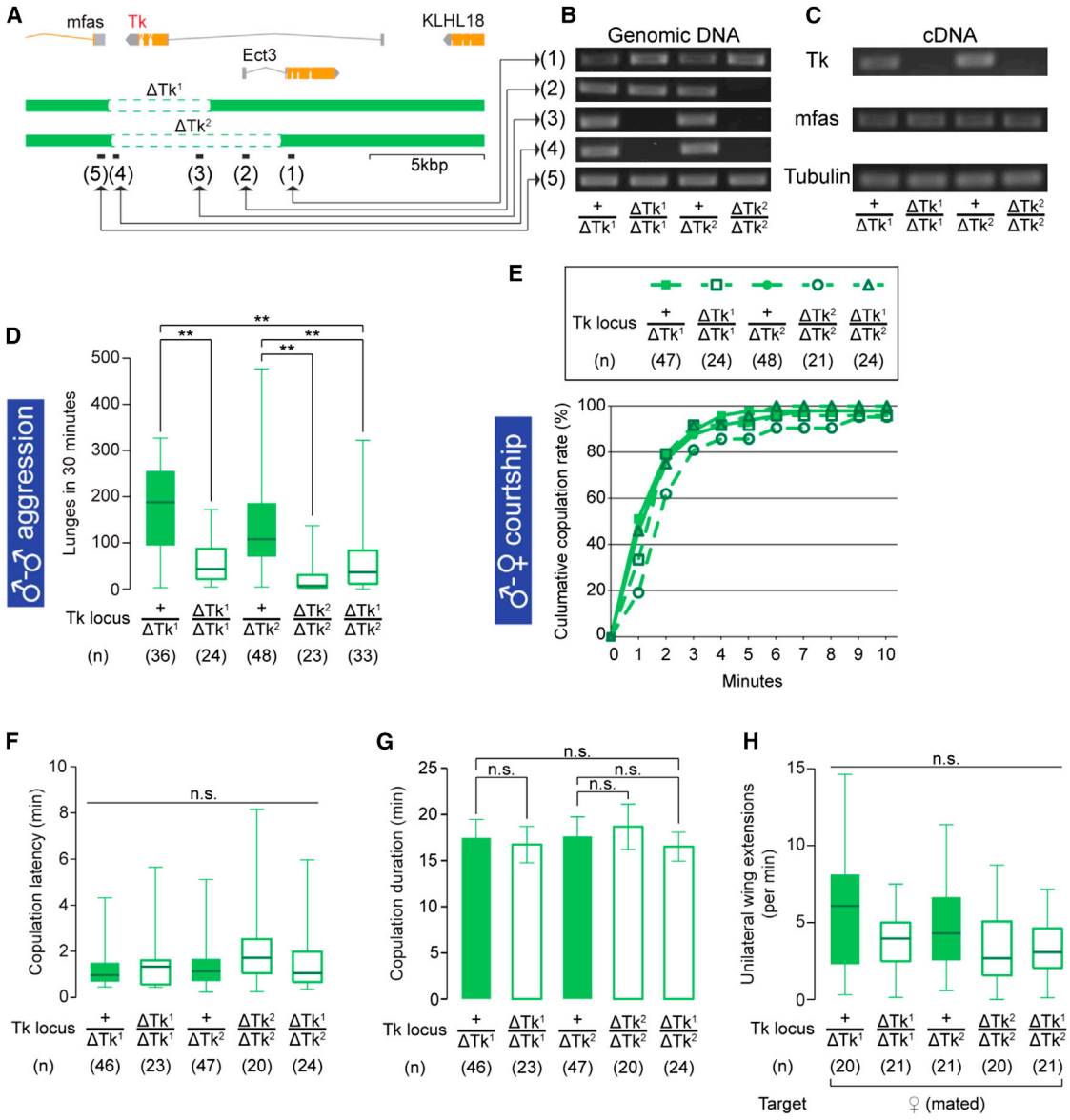


Figure 4. Null Mutations on *Tk* Specifically Affect Male-Male Aggression

(A) Schematic view of the *Tk* gene locus and deletions by *ΔTk*^{1} and *ΔTk*^{2}. Black bars (1)–(5) represent the regions targeted in the PCR analysis in (B).

(B) PCR analysis against regions (1)–(5) in (A) from genomic DNA samples of *Tk* deletion mutants.

(C) RT-PCR targeted to the *Tk*, *mfas*, and *Tubulin* (*α-Tubulin at 84B*) (positive control) gene transcripts from cDNA samples of *Tk* deletion mutants.

(D–H) Number of lunges (D), cumulative copulation curve (E), copulation latency (F), copulation duration (G; mean ± SD), and unilateral wing extension frequency (H) performed by *Tk* deletion mutants.

For (D), (F), and (H), **$p < 0.01$, n.s.: $p > 0.05$ (Kruskal-Wallis or Kruskal-Wallis and post hoc Mann-Whitney U tests). For (G), n.s.: $p > 0.05$ (one-way ANOVA and post hoc Student's t test).

See also Figure S4.

Tk-GAL4^{FruM} neurons using the *UAS > stop > dTRPA1*^{myc} allele (von Philipsborn et al., 2011; Figure S3D) did not increase male-male courtship (Figure S3F), although it did increase aggression (Figure S3E; also see Figure S6B). Thus, thermogenetic activation of Tk-GAL4^{FruM} neurons under the conditions of our assays produced consistent and significant effects on male-male aggressive behavior, but not on male-female or male-male courtship behavior.

The *Drosophila Tachykinin* Gene Is Required for Normal Levels of Intermale Aggression

The foregoing observations raised the question of whether *Tk* itself plays a role in aggression, and if so, whether it acts through Tk-GAL4 neurons. To address this question, we created two deletion alleles of *Tk* using FLP-mediated chromosome translocation (Parks et al., 2004), which we term *ΔTk*^{1} and *ΔTk*^{2}, respectively (Figure 4A). We confirmed the deletion of *Tk* by PCR

analysis at the *Tk* locus on genomic DNA samples (Figure 4B) and by RT-PCR (Figure 4C). The expression level of a neighboring gene, *mfas*, was not affected by the *Tk* deletion (Figures 4C and S4D). These deletion mutants were fully viable and fertile, and exhibited no obvious anatomical abnormalities or differences from controls in locomotor activity (Figure S4B). Homozygotes of both ΔTk^1 and ΔTk^2, as well as $\Delta Tk^2/\Delta Tk^1$ transheterozygotes, showed a significant decrease in lunging compared with heterozygous controls (Figure 4D). This phenotype is fully recessive, as heterozygous males did not differ from wild-type males of the same genetic background in lunging frequency (Figure S4A) or *Tk* mRNA expression (Figure S4C).

We next examined the effect of the *Tk* deletions on male reproductive behaviors. Both homozygous and transheterozygous males copulated with virgin females at efficiencies comparable to those observed for heterozygous control males (Figure 4E). Copulation latency (Figure 4F) and duration (Figure 4G) were also unaffected, as was the frequency of unilateral wing extension toward mated females (Figure 4H). The frequency of unilateral wing extension toward other males was also not consistently different from controls (data not shown). Thus, a null mutation in the *Tk* gene reduced male-male aggression without affecting male-female or male-male courtship behavior.

Tk Exerts Its Aggression-Promoting Effect through Tk-GAL4^{FruM} Neurons

To investigate the relationship between the *Tk* gene and Tk-GAL4^{FruM} neuronal function, we first asked whether *Tk* gene products (DTK peptides) are present in Tk-GAL4^{FruM} neurons, using immunostaining. We used two antisera against DTK peptides: one raised in rabbits (Winther and Nässel, 2001) and one raised in guinea pigs (see Experimental Procedures). In double-labeling experiments, only the staining of cells labeled by both antibodies was eliminated in $\Delta Tk^2/\Delta Tk^1$ transheterozygous male brains, suggesting that single-labeled cells reflected nonspecific staining (Figures S5A–S5C). *Tk*-expressing neurons defined by this criterion were distributed throughout the brain, in a pattern similar to that previously reported (Winther et al., 2003).

More detailed analysis indicated that a subset of Tk-GAL4^{FruM} neurons expressed *Tk* (Figures 5A, 5B, and 5E). This DTK immunoreactivity was undetectable in the $\Delta Tk^2/\Delta Tk^1$ transheterozygotes (Figure 5C), but was recovered when the mutation was rescued using the *Tk-GAL4*^{1} driver and a *UAS-Tk* transgene (Figure 5D). The distribution of cell bodies and gross morphology of *Tk-GAL4*^{1} neurons remained unchanged in the $\Delta Tk^2/\Delta Tk^1$ transheterozygotes (Figures S5B and S5C) and in the rescue genotype (Figure S5D). These genetic and immunohistochemical data suggest that at least a subset of Tk-GAL4^{FruM} neurons expresses one or more DTK peptides.

To determine whether the *Tk* gene exerts its influence on aggression in Tk-GAL4 neurons, we performed genetic rescue experiments. Expression of *UAS-Tk* under the control of *Tk-GAL4*^{1} restored levels of aggression to those observed in $+/\Delta Tk^1$ heterozygous controls (Figure 5G, left). Similar results were obtained using *Tk-GAL4*^{2} (Figures S5E and S5G), which also labels aggression-promoting *Tk-GAL4*^{FruM} neurons. In contrast, *Tk-GAL4*^{3} and *Tk-GAL4*^{GMR61H07}, which label different subsets of *Tk*-expressing neurons (Figures 5F and S5F), but not Tk-GAL4^{FruM} neurons, failed to rescue the reduced aggression of the transheterozygotes (Figures 5G, right, and S5H). This result suggests that the rescue obtained using the *Tk-GAL4*^{1} and *Tk-GAL4*^{2} drivers is not due simply to extracellular diffusion of the DTK peptides. These data suggest that *Tk* is required specifically in Tk-GAL4^{FruM} neurons to maintain normal levels of male-male aggression.

Tk Expression Levels Are Limiting for the Aggression-Promoting Influence of *Tk-GAL4*^{1} Neuron Activation

To determine whether release of DTK peptides from Tk-GAL4 neurons plays a role in aggression, we analyzed the effect of *Tk* gene dosage on the aggression-promoting phenotype of *Tk-GAL4*^{1} neuron thermogenetic activation. In the transheterozygous *Tk* deletion mutant background, the aggression-promoting effect of *Tk-GAL4*^{1} neuron activation was significantly suppressed (by ~60%) in comparison with heterozygous controls (Figure 6A, 29°C). This incomplete suppression could reflect the presence of classical transmitters in *Tk-GAL4*^{1} neurons that also contribute to aggression. Immunostaining experiments indicated that Tk-GAL4^{FruM} neurons did not contain GABA (Figure S5K), serotonin (5-HT; Figure S5L), or dopamine (Figure S5M), but a *Cha-GAL80* transgene strongly suppressed GFP expression in these cells (Figures S5I and S5J), suggesting that they are likely cholinergic.

We next examined the effect of increasing *Tk* gene dosage on aggressive behavior. Inclusion of the *UAS-Tk* transgene significantly potentiated the aggression-promoting effect of activating *Tk-GAL4*^{1} neurons (Figure 6B). The supplemental *Tk* also increased the amount of tussling, a high-intensity agonistic behavior that is observed only infrequently in wild-type flies (Chen et al., 2002; Figure S6A). Importantly, *Tk-GAL4*^{1}; *UAS-Tk* flies lacking *UAS-dTRPA1* did not show increased aggression at either 22°C or 29°C (Figure 6B), suggesting that increased DTK expression requires increased neuronal activity to enhance aggression. When *Tk-GAL4*^{1}*; UAS-dTRPA1; UAS-Tk* flies were tested at different temperatures, the inclusion of *UAS-Tk* had the effect of shifting the "dose-response" curve for behavior versus temperature to the left (Figure S6C). In contrast, *Tk* deletion reduced the maximal lunge number in activated flies (Figure S6C). These results demonstrate a strong interaction between thermogenetic activation of *Tk-GAL4*^{1} neurons and levels of *Tk* expression in *Tk-GAL4*^{1} neurons, supporting the idea that release of this peptide plays a role in the effect of these neurons to promote aggression.

The Aggression-Promoting Effect of *Tk-GAL4*^{1} Neuron Activation Is Suppressed by a Mutation in *Takr86C*

To obtain further evidence that the effects of Tk-GAL4 neuron activation are mediated by DTK peptide release, we investigated whether these effects could be suppressed by putative loss-of-function mutations in DTK receptor genes. Two such genes have been identified in the fly genome: *Takr86C* (Poels et al., 2009) and *Takr99D* (Birse et al., 2006). We created a mutation in *Takr86C* by imprecise excision of a P-element insertion; we call this allele *Takr86C*$^{\Delta F28}$. This deletion removes most of the first exon of *Takr86C*, including the start codon and the first 60 amino acids,

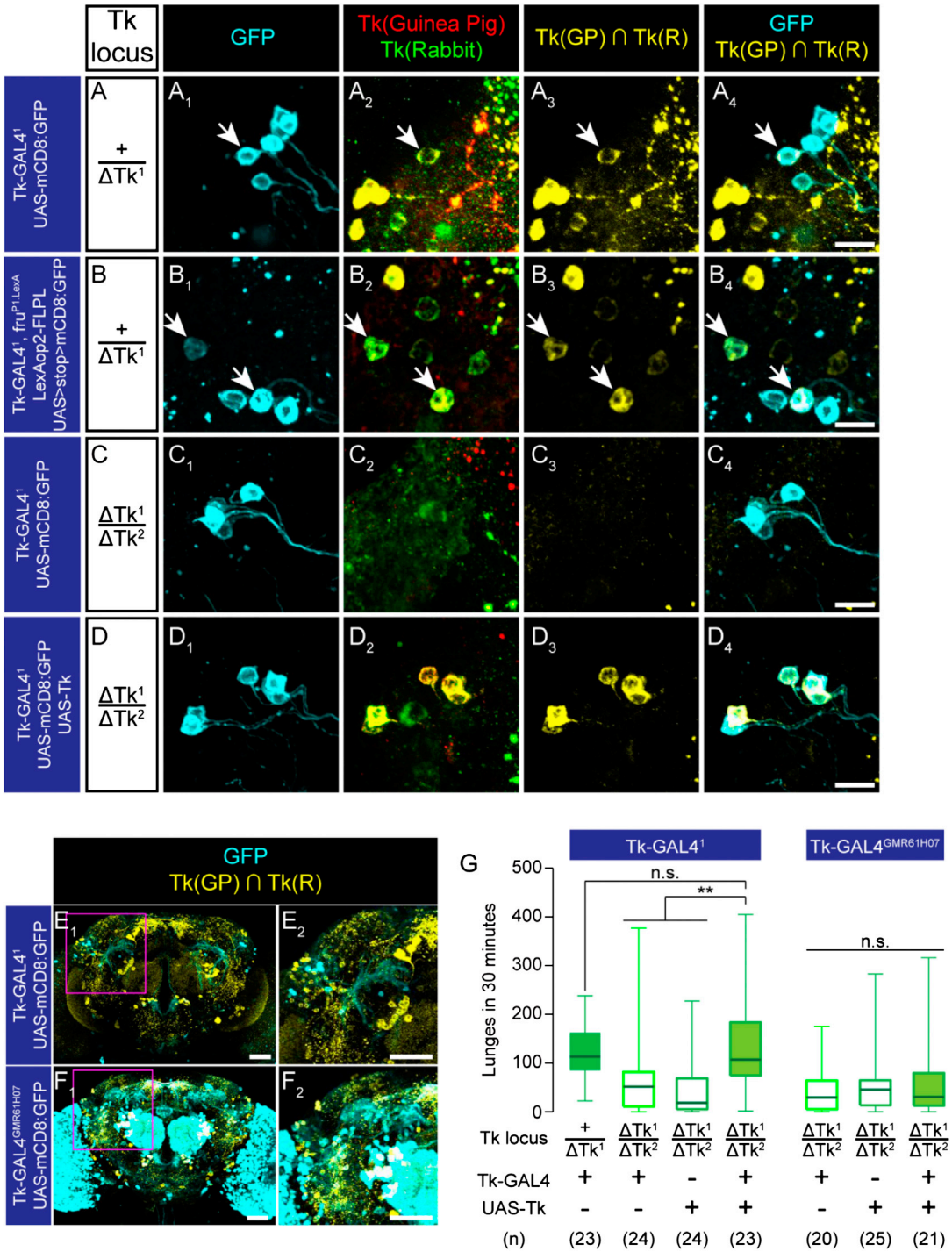


Figure 5. Tk Gene Products in the Tk-GAL4^{FruM} Neurons Are Sufficient to Maintain Normal Levels of Aggression

(A–D) *Tk-GAL4^{1}* lateral cluster neurons in *+/ΔTk^{1}* (A), *ΔTk^{2}/ΔTk^{1}* (C), *ΔTk^{2}/ΔTk^{1}* plus *UAS-Tk* (D) backgrounds, and Tk-GAL4^{FruM} neurons in the *+/ΔTk^{1}* (B) background, immunostained with anti-GFP antibody (cyan; A_1-D_1, A_4-D_4), anti-DTK guinea pig antiserum (red, A_2-D_2) and anti-DTK rabbit antiserum (green, A_2-D_2). The overlap of the two antisera is shown in yellow in A_3-D_3 and A_4-D_4. Arrows: GFP^{+}, DTK^{+} neurons.

(E and F) *Tk-GAL4^{1}* (E) and *Tk-GAL4^{GMR61H07}* (F) neurons in male brains immunostained with anti-GFP antibody (cyan), anti-Tk guinea pig antiserum, and anti-Tk rabbit antiserum (shown as the overlap in yellow). (E_2, F_2): region within a magenta square in E_1 and F_1 (magnified).

(G) Number of lunges in *ΔTk^{1}/ ΔTk^{2}* rescued by *Tk-GAL4^{1}* or *Tk-GAL4^{GMR61H07}* driving *UAS-Tk*. Left: **p < 0.01, n.s. p > 0.05 (Kruskal-Wallis and post hoc Mann-Whitney U tests). Right: n.s.: Kruskal-Wallis test, p > 0.05.

See also Figure S5.

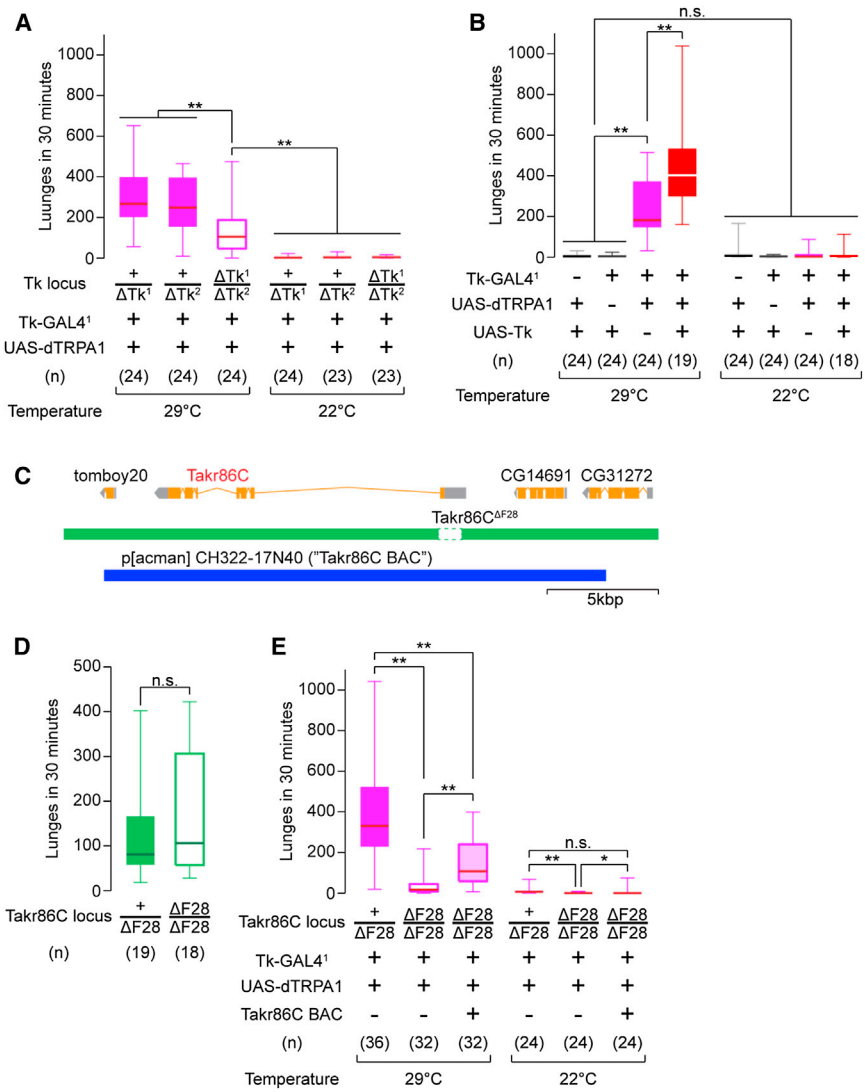


Figure 6. The *Tk* System Gates the Aggression-Promoting Effect of *Tk-GAL4¹* Neurons

(A and B) Number of lunges during thermogenetic activation of *Tk-GAL4¹* neurons in the *Tk* null mutant background (A) or with *Tk* overexpression (B).

(C) Schematic of the *Takr86C* gene locus, its deletion in *Takr86C^ΔF28*, and the region covered by p[acman] CH322-17N40.

(D) Number of lunges performed by *Takr86C^ΔF28* mutants.

(E) Number of lunges during thermogenetic activation of *Tk-GAL4¹* neurons in the *Takr86C^ΔF28* mutant background.

For (A), (B), (D), and (E), **p < 0.01, *p < 0.05, n.s.: p > 0.05 (Kruskal-Wallis and/or post hoc Mann-Whitney U tests).

See also Figure S6.

which contain the signal peptide and most of the N-terminal extracellular domain (Figure 6C).

In *Takr86C^ΔF28* homozygotes, the aggression-promoting effect of activating *Tk-GAL4¹* neurons was significantly suppressed (Figure 6E). A similar suppression was observed for *Tk-GAL4²* neuron activation (Figure S6D). In contrast, a putative loss-of-function insertional mutation in *Takr99D* (*Takr99D^MB09356* [Metaxakis et al., 2005]) had no effect in either *Tk-GAL4* line (Figure S6E and data not shown). The suppressing effect of the *Takr86C^ΔF28* mutation could be partially rescued by a bacterial artificial chromosome (BAC) (Venken et al., 2006, 2009) containing the complete *Takr86C* transcription unit (Figures 6C and 6E). These data provide further evidence that the effect of *Tk-GAL4* neuron activation to promote aggression involves the release of DTK peptides. Surprisingly, the *Takr86C^ΔF28* homozygous deletion on its own did not diminish baseline aggression in single-housed flies (Figure 6D). This likely reflects compensation by *Takr99D*, since double mutants showed a strong reduction in baseline as well as induced aggression (Figures S6F and S6G). The reason why *Takr99D^MB09356* does not suppress *TK-GAL4¹* neuron activation-induced aggression is not clear, but could reflect the circuitry where this receptor acts, or its relative affinity or specificity for different DTK peptides. Further studies are required to understand the respective roles of these two receptors in the control of aggression.

Activation of Tk-GAL4 Neurons Promotes a State of Aggressive Arousal

High levels of arousal or motivation have been proposed to diminish the requirement for an optimal specificity or salience of releasing signals that promote innate behaviors (Tinbergen, 1951). We therefore investigated whether activation of *Tk-GAL4¹* neurons could overcome the requirement for conspecific sensory cues or environmental factors in *Drosophila* aggression.

The presence of a resource such as food is essential for aggression in *Drosophila* (Chen et al., 2002; Svetec and Ferveur, 2005). We therefore tested whether activation of *Tk-GAL4¹* neurons could overcome the effect of eliminating food from the aggression arena. On a pure agarose substrate, *UAS-dTRPA1*; *UAS-Tk* control flies showed almost no aggression compared with the same genotype on an apple juice-agarose substrate (Figure 7A). Strikingly, activation of *Tk-GAL4¹* neurons partially restored aggression on pure agarose (Figure 7A, blue-shaded area, magenta box). Supplementation of DTK using *UAS-Tk* further increased the level of thermogenetically induced aggression on agarose to levels that were not significantly different from those exhibited by control flies on apple juice-agarose (Figure 7A, gray box on left versus red box in shaded area).

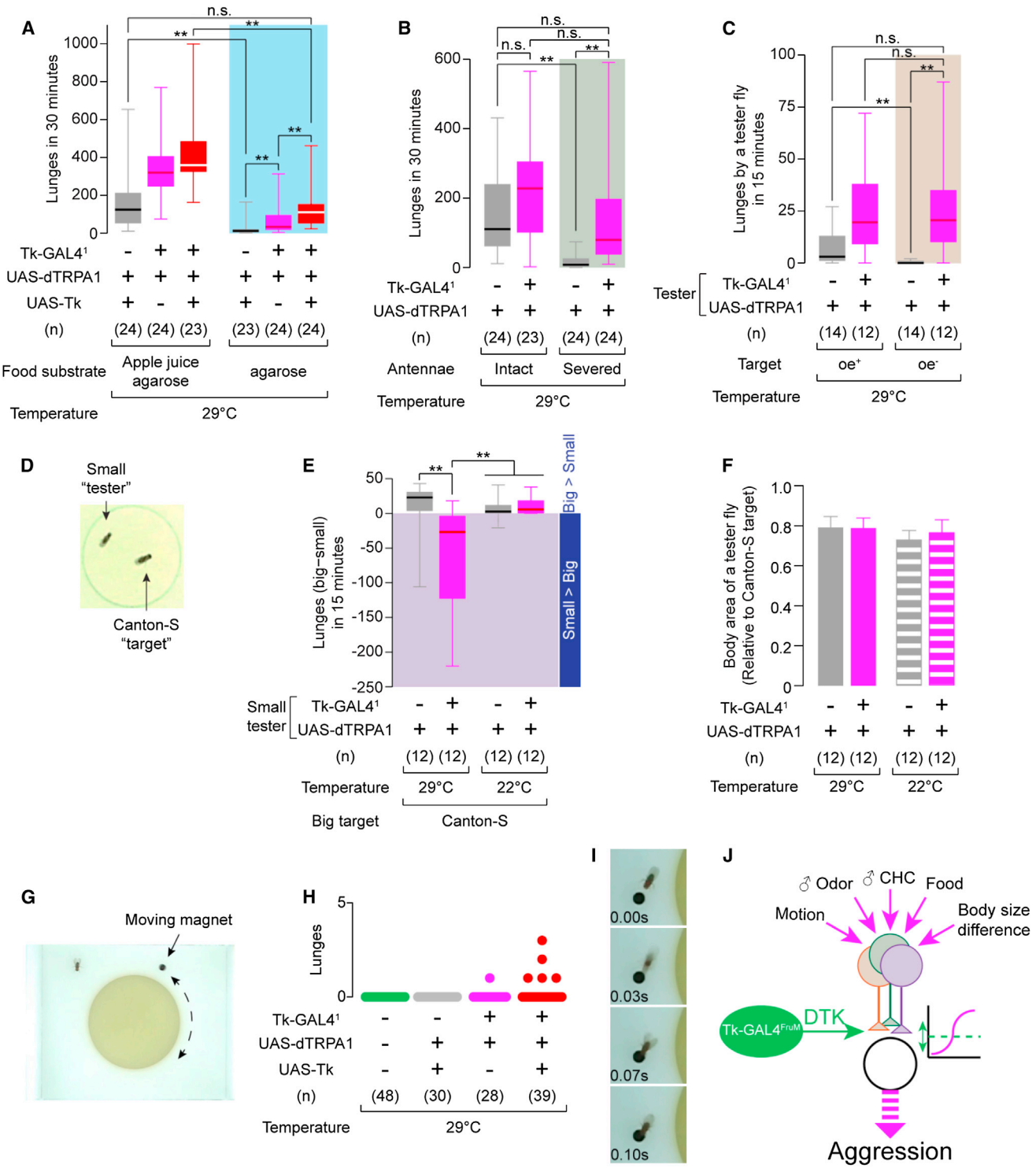


Figure 7. Activation of *Tk-GAL4¹* Neurons Overrides the Absence of Aggression-Promoting Cues

(A–C) Number of lunges during thermogenetic activation of *Tk-GAL4¹* neurons (A) on an apple juice agarose (left) or pure agarose (right) substrate; (B) with intact (left) or surgically removed (right) antennae; and (C) toward oe+ (left) or oe− (right) "target" males.

(D) Example image of a normal-sized "target" Canton-S male and a small "tester" male fly (70% the size of the target fly).

(E) Lunge number difference between smaller tester and larger target flies. Values in the shaded area indicate that the smaller testers performed more lunges than the larger targets.

(F) Relative body sizes (mean ± SD) of "tester" versus target flies.

(legend continued on next page)

We next investigated whether activation of *Tk-GAL4*^{1} neurons could overcome the requirement for olfactory cues in aggression, one of which is the male-specific pheromone 11-*cis*-vaccenyl acetate (Wang and Anderson, 2010). To do this, we surgically removed the third antennal segment in *Tk-GAL4*^{1}; *UAS-dTRPA1* and control males. Although antennae-less +; *UAS-dTRPA1* control flies showed a profound reduction in lunging (Figure 7B, gray boxes), activation of *Tk-GAL4*^{1} neurons restored lunging to a level that was not significantly different from that of control flies with intact antennae (Figure 7B, gray box on left versus magenta box in shaded area). Thus, the activation of *Tk-GAL4*^{1} neurons can also compensate for loss of sensitivity to aggression-promoting olfactory cues.

Male-specific cuticular hydrocarbon (CH) pheromones, which are detected by the gustatory system (Lu et al., 2012; Thistle et al., 2012; Toda et al., 2012), are a second class of chemosensory cues required for male-male aggression in *Drosophila* (Fernández et al., 2010; Wang et al., 2011). To test whether activation of *Tk-GAL4*^{1} neurons could compensate for the absence of these cues, we paired thermogenetically activated tester flies with target "oe− flies," which are depleted of most CHs via genetic ablation of oenocytes (Billeter et al., 2009). The oe− target flies evoked significantly less aggression from +; *UAS-dTRPA1* flies than did control oe+ flies (Figure 7C, gray boxes; Fernández et al., 2010; Wang et al., 2011). Remarkably, thermogenetic activation of *Tk-GAL4*^{1} neurons in tester flies restored aggression toward oe− target flies to a level indistinguishable from that exhibited toward oe+ targets (Figure 7C, magenta boxes).

To explore further the idea that *Tk-GAL4*^{1} neurons promote aggressive arousal, we investigated whether activation of these neurons could overcome the well-known size disadvantage in intermale aggression (Briffa and Sneddon, 2007). In *Drosophila,* as in many other species, the smaller male of a pair is far less likely to attack its larger opponent (Hoffmann, 1987; Hoyer et al., 2008). We generated smaller males (21%–27% body area size reduction compared with the wild-type "target" males; Figures 7D and 7F) by larval caloric restriction (see Experimental Procedures), and paired them with normal-sized wild-type males. At 22°C, the majority of lunges were performed by the normal-sized males toward the smaller fly, regardless of its genotype (Figure 7E). Thermogenetic activation of *Tk-GAL4*^{1} neurons reversed this trend (Figure 7E, two left columns; Movie S3). Thus, *Tk-GAL4*^{1} neuron activation can overcome the decreased likelihood of attack toward goal objects that lack appropriate cues or exhibit unfavorable properties, such as larger size.

Classic experiments by Tinbergen (1951) using dummy fish suggested that increased levels of arousal in a male can promote aggression toward a goal object exhibiting suboptimal "releasing stimuli." We therefore investigated whether activation of *Tk-GAL4*^{1} neurons could promote attack toward a fly-sized inanimate object. To do this, we engineered a system similar to that described by Zabala et al. (2012), in which a small, computer-controlled magnet circled a food patch in the presence of a single tester fly (Figure 7G). In close to 80 experiments, we did not observe a single lunge toward the magnet by either wild-type (CS) or genetic control males (Figure 7H, green and gray). Remarkably, however, thermogenetic activation of *Tk-GAL4*^{1} neurons in the presence of supplemental *Tk* (*UAS-Tk*) elicited lunges toward the magnet from 5/39 tester males (Figures 7H, red, and 7I); a single lunge was detected among 28 males activated without supplemental *Tk* (Figure 7H, magenta). Although the frequency of such attacks toward a "dummy fly" was low, its occurrence was striking given that we never observed such behavior from control or wild-type flies.

Taken together, these data suggest that the activation of *Tk-GAL4*^{1} neurons, particularly when supplemented with higher levels of *Tk*, can override the requirement for several categories of cues or conditions that are necessary for normal levels of male fly aggression, including social isolation, food, volatile and CH pheromones, and fly-specific visual cues (Figure 7J).

DISCUSSION

Here we have identified a sexually dimorphic neuron and a gene that play a critical and specific role in the expression of intermale aggression in *Drosophila*. The gene encodes a neuropeptide homologous to mammalian Substance P, and its release from the identified neurons is important for aggression. Substance P has been implicated in aggression in several mammalian systems (Halasz et al., 2009; Katsouni et al., 2009; Siegel et al., 1997). Together, our data suggest that the higher level of aggressiveness in *Drosophila* males may be controlled by the expression in sexually dimorphic neurons of a neuropeptide that regulates forms of agonistic behavior across phylogeny.

Sexually Dimorphic Neurons that Control Higher Levels of Aggression in Males

Previous studies have investigated the role of FruM^{+} neurons in aggression versus courtship. Selective masculinization of certain groups of neurons in females masculinized courtship behavior, but not aggression, suggesting that distinct subsets of FruM neurons may control these behaviors (Chan and Kravitz, 2007); however, a selective masculinization of aggression, but not courtship, was not observed. Feminization of most or all octopaminergic (OA) or cholinergic neurons, via expression of *UAS-Tra*, altered the balance between male-male courtship and aggression (Certel et al., 2007), or enhanced aggression (Mundiyanapurath et al., 2009), respectively. Feminization of

(G) Image of the "moving magnet" setup.

(H) Number of lunges (scatterplot) toward the magnet during thermogenetic activation of *Tk-GAL4*^{1} neurons. The total recording time per session was 4 min (see Experimental Procedures).

(I) Example of a lunge performed by a male *Tk-GAL4*^{1}, *UAS-dTRPA1*; *UAS-Tk* fly toward a magnet.

(J) Schematic illustrating the possible influence of Tk-GAL4^{FruM} neurons in the male fly brain in relation to the processing of sensory cues regulating aggression.

For (A–C) and (E), $^{**}p < 0.01$, $^{*}p < 0.05$ (Kruskal-Wallis and post hoc Mann-Whitney U tests). For (F), n.s.: $p > 0.05$ (one-way ANOVA and post hoc Student's t test). See also Movie S3.

a small subset of OA neurons increased male-male courtship, but not aggression (Certel et al., 2010). Specific OA and dopaminergic neurons that influence aggression have been identified (Alekseyenko et al., 2013; Zhou et al., 2008), but these neurons are not sexually dimorphic. The present results identify sexually dimorphic Tk-GAL4^{FruM} neurons that are necessary, sufficient, and specific for the quantitatively higher level of aggressiveness that is characteristic of *Drosophila* males. The neurons responsible for the qualitative sex-specific differences in the behavioral expression of aggression remain to be identified.

Studies in mice have localized aggression-promoting neurons to the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) (Lin et al., 2011). Genetic ablation of anatomically dimorphic neurons within VMHvl that express the progesterone receptor (PR) was shown to partially reduce aggressive behavior (Yang et al., 2013). However, this effect of this ablation was not specific to aggression, since male mating behavior and female mating behavior were attenuated as well. In contrast, the Tk-GAL4^{FruM} neurons identified here control aggression, but not mating behavior. Unlike PR^{+} neurons, moreover, these cells are not detectable in females.

The fact that the Tk-GAL4^{FruM} neurons were not observed in females suggests that either the developmental generation of these neurons and/or their expression of the neuropeptide is male specific. Whatever the case, the absence of these neural elements from the female brain is likely to contribute to their lower level of aggressive behavior. Our data suggest that sex-typical features of some innate behaviors in *Drosophila* may be achieved, at least in part, by the sexually dimorphic expression in specific neurons of neuropeptides that coordinate male-specific behavioral subprograms (see also Tayler et al., 2012). Dimorphic populations of FruM-expressing neurons also regulate sexually dimorphic behaviors through the release of classical fast neurotransmitters that act on sexually dimorphic chemical synapses (Ruta et al., 2010).

Tk-GAL4^{FruM} Neurons as Regulators of Aggressive Arousal

Several lines of evidence presented here argue that Tk-GAL4^{FruM} neurons influence aggressive arousal or motivation, rather than simply acting as ''command neurons'' for aggressive actions. First, activation of these neurons did not trigger a single aggressive action, as would be expected for a command neuron (Bentley and Konishi, 1978), but rather increased the frequency of multiple agonistic behaviors, including wing-threat, lunging, and tussling. Second, thermogenetic activation of these neurons supervened the requirement for several aggression-permissive conditions and cues, some of which (such as male-specific pheromones) could be construed as ''releasing signals'' (Tinbergen, 1951). The activation of *Tk-GAL4^{1}* neurons was even able to promote lunging toward a moving dummy fly (albeit in a minority of trials). To the extent that increased arousal decreases the requirement for specific releasing signals to evoke innate behaviors (Tinbergen, 1951), activation of Tk-GAL4^{FruM} neurons may generate an arousal-like state that is specific for aggression. Alternatively, Tk-GAL4^{FruM} neurons may enhance behavioral sensitivity to multiple releasing signals that characterize an attackable object, either at the level of parallel sensory processing pathways or at a locus downstream of the integration of these multisensory cues (Figure 7J), analogous to the neuropeptide regulation of feeding behavior in *C. elegans* (Macosko et al., 2009).

Tachykinins Modulate Agonistic Behavior across Phylogeny

Several lines of evidence presented here suggest that the release of DTK peptides indeed contributes to the aggression-promoting function of Tk-GAL4^{FruM} neurons. Nevertheless, the release of a classical neurotransmitter, probably acetylcholine (Figures S5I–S5M), likely contributes to the behavioral influence of Tk-GAL4^{FruM} neurons as well. Furthermore, while our data implicate *Takr86C* as a receptor for *Tk* in the control of aggression, they do not exclude a role for *Takr99D*.

Among three species of vertebrate Tachykinin neuropeptides (Severini et al., 2002), Substance P has been implicated, directly or indirectly, in various forms of aggression, including defensive rage and predatory attack in cats (reviewed in Katsouni et al., 2009; Siegel et al., 1997), and intermale aggression in rats (Halasz et al., 2008, 2009). Although not all functions of Substance P are necessarily conserved (such as nociception in mammals [Woolf et al., 1998] and olfactory modulation in the fly [Ignell et al., 2009; Winther et al., 2006]), these data suggest that this neuropeptide is broadly involved in the control of agonistic behavior in both vertebrates and invertebrates. They therefore add to the growing list of neuropeptide systems that show a remarkable evolutionary conservation of functions in the regulation of innate ''survival behaviors'' such as feeding and mating (reviewed in Bargmann, 2012; Taghert and Nitabach, 2012). Biogenic amines also control aggression across phylogeny (Alekseyenko et al., 2010, 2013; Certel et al., 2007; Baier et al., 2002; Dierick and Greenspan, 2007; Hoyer et al., 2008; Zhou et al., 2008). However, in the case of serotonin, the directionality of its influence is opposite in flies and humans (reviewed in Zwarts et al., 2012).

Our findings indicate that studies of agonistic behavior in *Drosophila* can identify aggression-regulating genes with direct relevance to vertebrates. Interestingly, in humans, the concentration of Substance P-like immunoreactivity in cerebrospinal fluid has been positively correlated with aggressive tendencies in patients with personality disorders (Coccaro et al., 2012). Substance P antagonists have been tested in humans as anxiolytic and antidepressant agents, although they failed to show efficacy (Keller et al., 2006; Steckler, 2009). The present findings, taken together with mammalian animal studies, suggest that it may be worthwhile to investigate the potential of these antagonists for reducing violent aggression in humans.

EXPERIMENTAL PROCEDURES

Fly Strains

In the main text and figures, short names are used to describe genotypes for clarity. The complete genotypes of animals used in this study are available in Table S1. The origins of these animals are described in Extended Experimental Procedures.

Behavior Assays

In most experiments, behavioral assays (aggression, courtship, and locomotion) were done in a "12-well" chamber (Figure 1A; Dankert et al., 2009), which contains 12 cylindrical arenas with the dimension of 16 mm diameter × 10 mm height. The floor food substrate was made of 2.25% w/v agarose in commercial apple juice and 2.5% (w/v) sucrose. Male flies were reared either singly or as a group of 15 individuals for 5–7 days under a 10AM:10PM light/dark cycle. For experiments using dTRPA1 transgenes, flies were reared at 22°C. In other experiments, flies were reared at 25°C. Most behavioral assays lasted for 30 min, and all were digitally recorded for further analysis.

The details of the "moving magnet" setup (Figures 7G–7I) will be described elsewhere (B.J.D. and D.J.A., unpublished). Briefly, 1 min after the start of the movie recording, a rare-earth magnet (1.6 mm diameter, 0.8 mm thick) was moved 1.25 revolutions (450°) at 18 mm/s 12 times, at 7 s intervals. Recording continued 30 s after the final revolution (total movie length 4 min), and the total number of lunges that a fly performed toward the magnet was counted.

The number of lunges performed by a pair of males was counted using CADABRA (Dankert et al., 2009) unless otherwise specified. Copulation latency and duration were measured by observation. In order to count wing extensions, we developed a new tracking and annotation software in MATLAB. Details regarding these programs and the behavioral assays used are available in Extended Experimental Procedures.

Statistical Analysis

Statistical analysis was performed using Prism7 (GraphPad Software) or MATLAB (MathWorks) software. All data, except for copulation-duration data (Figures 3C, 3G, and 4G), were analyzed with nonparametric tests. For comparison of more than three genotypes, we first performed a Kruskal-Wallis test. If the null hypothesis that medians of all genotypes were the same was rejected ($p < 0.05$), we performed a post hoc Mann-Whitney U test between a pair of interest to test whether the medians of these two genotypes were significantly different. If data in a given group (such as 22°C in Figure 1B) were not significantly different from each other, by either Kruskal-Wallis test (for more than two groups) or Mann-Whitney U test (for only two groups), we pooled these data for statistical comparison with other data points. Such pooled data points are indicated by a horizontal bar above the plot.

Copulation-duration data (Figures 3C, 3G, and 4G) were considered normally distributed because these data passed the D'Agostino and Pearson omnibus normality test ($p > 0.05$), and therefore were analyzed first by one-way ANOVA to test the null hypothesis that the means of all genotypes were the same. If the null hypothesis was rejected ($p < 0.05$), we performed a post hoc Student's t test between a pair of interest to test whether means of these two genotypes were significantly different.

In both cases, Bonferroni correction was applied when more than one pairwise tests were performed on a single set of data.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, one table, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.11.045.

AUTHOR CONTRIBUTIONS

K.A. generated most new transgenic animals and mutants, performed all experiments, and cowrote the manuscript. K.W. generated the *Otd-nls:FLPo* transgenic strain. B.J.D. set up the "moving magnet" arena and performed experiments for Figure 7H with K.A. E.H. established the 12-well aggression chamber assay system, including modification of CADABRA software to run on computer clusters. E.A.E. created a new fly tracking program. C.R.G., with feedback from K.A., wrote a code for the unilateral wing extension classifier. P.P. supervised E.A.E. and C.R.G. and provided useful suggestions to K.A., E.H., and D.J.A. D.J.A. supervised the project and cowrote the manuscript.

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