EXTENDED EXPERIMENTAL PROCEDURES

Molecular Biology

Genomic DNA was extracted from whole fly body using phenol-chloroform method. Briefly, 30 flies were thoroughly crushed in 100mM Tris-HCl (pH = 9.0)/100mM EDTA/ 0.1% SDS with pestle and were incubated at 65°C for 30 min. Potassium acetate was added to the final concentration of 800mM, and the mix was chilled on ice for 30 min. Precipitation was pelleted by centrifugation at 13,000rpm for 15 min, and the supernatant was collected. Half the volume of isopropanol was added to the supernatant, and precipitation was pelleted by centrifugation at 13,000rpm for 5 min. The pellet was washed with 70% ethanol once, and the pellet was dried before resuspended in 100 μl of 100mM Tris-HC1/100mM EDTA. Then 5 μl of 10mg/ml RNase A was added and the mix was incubated at 37°C for 15 min. After this, 100 μl of phenol:chloroform:isoamyl alcohol, 25:24:1 (Sigma-Aldrich #P2069) was added. After vortexing, the mix was centrifuged for 5 min and the aqueous phase was collected. Then 70 μl of chloroform was added. After vortexing, the mix was centrifuged for 5 min and the aqueous phase was collected. Then 6 μl of 3M sodium acetate and 150 μl of ethanol was added, and the mix was chilled down at −20°C for 15 min. Precipitation was pelleted by centrifugation at 13,000rpm for 15 min. After washing the pellet with 70% ethanol once, the pellet of DNA was dried and resuspended in 100 μl of 100mM Tris-HC1/100mM EDTA.

cDNA of whole fly heads was synthesized by using SuperScript III (Invitrogen) with oligo-dT primer from while head RNA. Whole head RNA was extracted by first dissecting 30 fly heads and quickly transferring on dry ice. The frozen heads were then thoroughly crushed using a pestle in 700 μl of TRIzol (Ambion #15596026), followed by addition of 120 μl of chloroform. The mix was vortexed and centrifuged at 13,000rpm for 10 min. The aqueous phase was collected, and 400 μl of acid phenol:chloroform (Ambion #AM9720) was added. After vortexing, the mix was centrifuged at 13,000rpm for 10 min. The aqueous phase was collected, and 300 μl of chloroform:isoamyl alcohol (Sigma-Aldrich #C0549) was added. The sample was mixed well and then centrifuged at 13,000rpm for 5 min. The aqueous phase was collected, and 200 μl of isopropanol was added. After incubated at room temperature for 5 min, precipitation was pelleted by centrifugation at 13,000rpm for 10 min. After washing the pellet with 80% ethanol once, the pellet of RNA was dried and resuspended in 20 μl of DEPC-treated water.

Quantitative PCR was performed using 7300 Real Time PCR System (Applied Biosystems) and SYBR Green PCR Master Mix (Applied Biosystems #4309155), with the following primer sets:

Tk (CG14734): 5′-CATCCATGGCTCTACCTGTG-3′ and 5′-GCTCTAAGAGGAACTTCCATC-3′
mfas (CG3359): 5′-TTTGGTGCACTTCGATGACG-3′ and 5′-TTAGCCTTACGAACCAGCTC-3′
α-Tubulin at 84B (CG1913):5′-CCCTCGAGAAGGACTACGAG-3′ and 5′-AAGTGGCGTGACGCTTAGTA-3′

These primer sets were also used for RT-PCR in Figure 4C.

Quantification was done according to (Dorak, 2006) with "delta C_t" method with amplification efficiency correction. Briefly, the dilution series of 1/4, 1/16, 1/64, 1/256 were prepared for each sample and the amplification efficiency was calculated as the average amplification efficiency of all samples. The enrichment ratio of *Tk* or *mfas* transcripts to *α-Tubulin at 84B* transcripts, relative to Canton-S wild-type flies, was calculated as follows (Dorak, 2006):

$$\text{Ratio} = \left[(E_{\text{tubulin}})^{\text{Cp sample}} / (E_{\text{target}})^{\text{Cp sample}} \right] \Big/ \left[(E_{\text{tubulin}})^{\text{Cp Canton-S}} / (E_{\text{target}})^{\text{Cp Canton-S}} \right]$$

Where E_{tubulin} represents the amplification efficiency of *α-Tubulin at 84B*, E_{target} represents the amplification efficiency of *Tk* or *mfas*, $C_{p\ \text{sample}}$ represents the crossing point of the cDNA sample tested (+/*ΔTk*^{1}, *ΔTk*^{1}/*ΔTk*^{1}, +/*ΔTk*^{2} or *ΔTk*^{2}/*ΔTk*^{2}) and $C_{p\ \text{Canton-S}}$ represents the crossing point of the Canton-S cDNA sample. C_p was determined by the threshold generated by the 7300 SDS System Software (Applied Biosystems) with the default setting.

The pBPGUw-Otd-nls:FLPo construct was generated by first adding theSV40 large T-antigen nuclear localization signal (nls, ATGGCCCCCAAGAAGAAGCGCAAGGTG) at the 5′ of FLPo coding sequence (Raymond and Soriano, 2007). The resulting nls:FLPo was then placed in pBPGUw (Addgene #17575) by replacing it with the coding region of GAL4. The promoter of the *Drosophila Otd* was cloned from the genomic DNA as the EcoRV-BglII fragment (1.8kbp) of the previously characterized *Drosophila Otd* promoter (Gao and Finkelstein, 1998), and then subcloned to the EcoRV-BglII site of the pBPGUw-nls:FLPo.

The pJFRC2-UAS-Tk ("UAS-Tk") construct was generated by first amplifying the entire coding region of Tk transcript from the cDNA of the Bloomington #2057 strain using PrimeSTAR HS DNA polymerase (Takara Bioscience). XhoI site and the CAAA Kozak sequence was added right upstream of the ATG, and XbaI site was added right downstream of the stop codon. The amplified DNA fragment was subcloned into pCR4Blunt (Invitrogen) and sequenced. Next, the plasmid was digested with XhoI and XbaI, and was subcloned to the backbone of pJFRC2-10XUAS-IVS-mCD8::GFP (Pfeiffer et al., 2010; Addgene #26214), by replacing the mCD8:GFP coding region at XhoI-XbaI site.

All DNA constructs were verified by sequencing.

Fly Strains

The following strains were obtained from Bloomington Stock Center (Indiana University); *Akh-GAL4* (#25683, #25684), *NPF-GAL4* (#25681, #25682), *PDF-GAL4* (#6900), *dilp2-GAL4* (#37516), *Takr86C*^{KG07724} (#14552), *Takr99D*^{MB09356} (#26471), *Δ2-3* (#3629), *y1*; *Gr22b*^{1} *Gr22d*^{1} *cn*^{1} *CG33964*$^{R4.2}$ *bw*^{1} *sp*^{1}; *LysC*^{1} *MstProx*^{1} *GstD5*^{1} *Rh6*^{1} (#2057). The following strains were obtained from the Exelixis Collection at Harvard Medical School: *Tk*^{f03824}, *Tk*^{d08303}, *Tk*^{f06233} and *Tk*^{d02777}.

UAS-dTRPA1 (Hamada et al., 2008) was a gift from Dr. Paul Garrity (Brandeis University). *Tk-GAL4*^{GMR61H07} (Bloomington #39282; (Jenett et al., 2012; Pfeiffer et al., 2008)), *pBDPGAL4U* in attP2 (Pfeiffer et al., 2008, 2010), *pJFRC2-10XUAS-IVS-mCD8:GFP* in attP2, denoted as "UAS-mCD8:GFP in main text and figures (Bloomington #32185; (Pfeiffer et al., 2010)), *pJFRC48-13XLexAop2-myr:tdTomato* in VK00005, denoted as "LexAop2-tdTomato" in main text and figures (Pfeiffer et al., 2010), *pJFRC79-8XLexAop2-FLPL* in attP2, denoted as "LexAop2-FLPL" in main text and figures, (Pan et al., 2012) were gifts from Barret D. Pfeiffer and Dr. Gerald Rubin (Janelia Farm Research Campus, Virginia). *UAS > stop > mCD8:GFP* (Yu et al., 2010), *UAS > stop > dTRPA1*^{mChery}, *UAS > stop > dTRPA1*^{myc} (von Philipsborn et al., 2011) and *UAS > stop > Kir2.1*^{eGFP} was a gift from Dr. Barry Dickson (IMP, Vienna). *fru*$^{P1.LexA}$ (Mellert et al., 2010) is a gift from Dr. Bruce Baker (Janelia Farm Research Campus, Virginia). *Cha-GAL80* (Kitamoto, 2002) is a gift from Dr. Gero Miesenbock (CNCB, University of Oxford).

Neuropeptide-GAL4 lines (Hergarden et al., 2012; Tayler et al., 2012) were generated by first cloning putative promoter regions of *Drosophila* neuropeptide genes into pCaSpeR3-GAL4 vector, and injecting the resultant plasmids to fly embryos to obtain transformants. In particular, the 1,634bp putative promoter of *Drosophila* Tachykinin was amplified from fly genome DNA region of 7,829,512-7,831,146 on the 3R chromosome by using the primers 5′- ATC ACA AAT TGC TTG TCT ATC GAT GGG CAA T-3′ and 5′-GAG AAG TAT GCA ACA ACT GAG CGA CAG ATC G-3′. All *Neuropeptide-GAL4* lines used in this study are available from Bloomington Stock Center (*Tk-GAL4*^{1} (5Fa): Bloomington # 51975, *Tk-GAL4*^{2} (3Ma): Bloomington # 51974, *Tk-GAL4*^{3} (2Ma): Blooomington # 51973).

The *UAS-Tk* line was generated by targeting the pJFRC2-UAS-Tk construct with φ31C-mediated transgenesis to the attP2 site on the third chromosome. The *Otd-nls:FLPo* line was generated by targeting the pBPGUw-Otd-nls:FLPo construct with φ31C-mediated transgenesis to the attP40 site on the second chromosome. The Takr86C BAC transformant was generated by targeting the p[acman] CH322-17N40 (BACPAC Resources, Children's Hospital Oakland Research Institute, Oakland, CA) with φ31C-mediated transgenesis to the attP2 site on the third chromosome.

ΔTk^{1} and *ΔTk*^{2} were generated following the procedure described previously (Parks et al., 2004). *Tk*^{f03824} and *Tk*^{d08303} were used for *ΔTk*^{1}, and *Tk*^{f06233} and *Tk*^{d02777} were used for *ΔTk*^{2}. Candidates of *ΔTk*^{1} were first screened for the loss of mini white marker, and subsequently for the presence of the recombinant DNA signature by performing PCR on the genomic DNA using the XP5′ plus and WH3′ minus primers (Parks et al., 2004). Candidates of *ΔTk*^{2} were first screened for brighter red eyes resulting from two copies of mini whites, and subsequently characterized by the loss of DNA band in the deleted areas by PCR on the genomic DNA, as shown in Figure 4B. Primer sequences used for regions (1)-(5) in Figure 4B are as follows:

Region (1): 5′-GCAATGACCACTCCACATAG-3′ and 5′-TGAAGTTCAGTGTGGTGGTC-3′
Region (2): 5′-AAGCCATCCAAACGATCTCC-3′ and 5′-TTGCTCAACAATGTTGCCGC-3′
Region (3): 5′-ACTTTTCTGCTCGATGCTGC-3′ and 5′-TAAGTGCAACATCCCATGCC-3′
Region (4): 5′-ACCAGACACCACAGTGATTG-3′ and 5′-ACCCAAACTAAAACGGTGTAG-3′
Region (5): 5′- ACATCGTCGGATCAAGCAAC-3′ and 5′- GCGATGGATGGAAAAACAGC-3′

Takr86C^{ΔF28} was generated by imprecise excision of the P-element insertion *Takr86C*^{KG07724} on the 5′-UTR of *Takr86C* using *Δ2-3*. The candidates of imprecise P-element excision mutants were screened for the loss of mini white marker, and PCR on genomic DNA were carried out to determine the area of deletion. Among 62 candidates, 2 were found to have a large deletion near the insertion site. Of these two, one was characterized by sequencing of the genomic DNA to have 1,020bp deletion spanning 6,576,803-6,575,782 of the 3R chromosome. In addition, this allele contains 678bp leftover of the KG element at the chromosome break point. We named this mutant as *Takr86C* ^{ΔF28}.

"oe+" and "oe-" flies were created following the protocol previously described (Billeter et al., 2009; Wang et al., 2011). In summary, the crosses of +; *UAS-StingerII, UAS-hid/CyO*; + (for oe-) or +; *UAS-StingerII*; + (for oe+) females and +; *PromE(800)-GAL4*, *tub-GAL80*^{ts}; + males were reared at 18°C, and male progeny was collected upon eclosion. The males were kept at 25°C for one day, and then 31°C during night (9PM-9AM) and 25°C during the day (9AM-9PM) for another 3 days. The flies were then maintained at 25°C for 1-2 days before use.

UAS > dTRPA1^{mCherry} was generated by removing the "stop cassette" of *UAS > stop > dTRPA1*^{mCherry} as follows. First, *w*^{1118}, *hs-FLP*; *Adv*^{1}*/CyO*; + females (Bloomington #6) and *w*^{11118}; *UAS > stop > dTRPA1*^{mCherry}; + males were crossed, and the F1 embryos were given heat shock of 37°C for 1 hr at 1, 2 and 3 days old. The F1 adult males were collected and individually crossed to *w*^{1118}; *BI/CyO*; *TM2/TM6B* females. The F2 adult males from this cross were collected and again individually crossed to *w*^{1118}; *BI/CyO*; *TM2/TM6B* females. After 4 days, males were collected, and crude DNA solution from individual F2 males was prepared. PCR using primers 5′-GAG GCG CTT CGT CTA CGG AGC GAC-3′ and 5′-GGC GAA ATC TTC CCT CTT AG-3′ was performed for these crude DNA solutions. This primer set was expected to generate ~3kbp band from intact *UAS > stop > dTRPA1*^{mCherry} transgene, and ~400bp band from the transgene in which the stop cassette was removed (*UAS > dTRPA1*^{mCherry}). Offspring from the male which had the 400bp band was kept for the experiment.

Injection of plasmids to the embryos was performed by Genetic Systems, Inc. (Cambridge, MA) and BestGenes (Pomona, CA). All insertions were backcrossed to Canton-S background for at least 6 generations. In this study, flies that did not include the *Tk-GAL4*^{1}

transgene were wild-type for the *w* gene locus (*w+*). *Tk-GAL4*^{1} transgene is located on the X chromosome and was resistant to recombination with wild-type *w* gene locus.

Behavior Assays

All aggression assays (except experiment in Figure S1D) were done in the "12-well" chamber, which was briefly described previously (Dankert et al., 2009). This chamber, made of acrylic plates, contains 12 cylindrical arenas with the dimension of 16mm diameter x 10mm height. The floor food substrate was made of 2.25% w/v agarose in commercial apple juince (Minutes Maid) and 2.5% (w/v) sucrose, and was sandwiched by the arena and the floor acrylic plate. The ceiling acrylic plate has 1.5mm diameter holes, which can be used as a port to introduce flies into arenas by sliding the lid. The inner surface of arenas was coated with Insect-a-Slip (BioQuip Products, Inc., CA, #2871), and the ceiling was coated with SurfaSil Siliconizing Fluid (Thermo Scientific, IL, #42800) to prevent flies from climbing the wall and ceiling. Flies were introduced through a port by gentle aspiration, and were allowed to acclimate to the chamber for 5 min before the recording began. The assay for Figure S1D was done in the 40mm x 50mm x 100mm aggression chamber described in (Hoyer et al., 2008) and (Dankert et al., 2009).

The mating assay (Figures 3, 4E–4H, and S3B) was done in a chamber similar to the 12-well aggression chamber. This chamber has arenas with identical dimensions, plus a thin slit in the middle of the acrylic plate. This slit accommodates an aluminum plate to keep females in the lower half of arena separated from males in the upper half of arena separated until the plate is removed. This way, males in all arenas were simultaneously exposed to the female. Canton-S virgin females were 3-4 days old. Mated females were generated by introducing 4 Canton-S males to 6 4-day-old virgin Canton-S females and rearing them for 2 additional days. All females were reared at 25°C, 10AM:10PM Light:Dark cycle. As courtship started immediately after the first encounter of a male and a female, recording began right after the separations were removed.

The travel distance by single flies (Figures S1B, S1C, and S4B) was measured by placing a single male fly in each well of the 12-well aggression chamber and tracking the total distance each fly traveled in 30 min using the newly developed fly tracker program (see below).

Preparation of Flies

Male flies for dTRPA1-mediated thermogenetic activation were collected upon eclosion and reared in a vial containing standard fly food medium as a group of 15 (except flies used in Figures 7A–7I, which were singly reared) at 22°C with 10AM:10PM Light:Dark cycle. For thermogenetic activation with the UAS-dTRPA1 transgene (Figures 1B, 1C, 6A, 6B, 6E, 7A–7C, 7E, 7H, S1A–S1D, S1I, S1J, S6A–S6E, and S6G), flies were pre-warmed in 29°C water bath for 20 min prior to being introduced to the arena. For thermogenetic activation with mCherry- or myc- tagged dTRPA1 transgene (Figures 2I, 3A–3D, S2K, S3E, and S3F), flies were prewarmed in 33°C water bath for 20 min. Consistent with the previous report (von Philipsborn et al., 2011), the higher temperature was necessary to observe robust phenotype with the tagged dTRPA1. However, it should be noted that *Tk-GAL4*^{1}; *UAS-dTRPA1* males showed mild locomotor defects in temperatures above 30°C. This phenotype was accompanied by bent abdomen, suggesting that the VNC neurons labeled by *Tk-GAL4*^{1} might cause the phenotype. Experiments for Figures 2J, 3E–3H, 4D–4H, 5G, 6D, 7A–7C, 7E, 7H, S3C, S4A, S4B, S5G, S5H, and S6F were carried out using single-housed male flies, which were isolated upon eclosion into individual vial and reared at 25°C (22°C for flies used in Figures 7A–7C, 7E, and 7H) with 10AM:10PM Light:Dark cycle. Except experiments in Figures 7A–7C, 7E, and 7H, behavior assays using single-housed male flies were also carried out at 25°C. All flies were used 5-7 days after eclosion except in experiments for Figures 2I, 3A–3D, and S3E–S3F, in which flies were aged to 15 days old following a condition used in a previous report (von Philipsborn et al., 2011).

For "mixed genotype pair" aggression assays (Figure 7C, 7E, and S1D), one of the flies were marked with acrylic paint dot on the thorax for genotype identification. During this marking and antennae removal surgery (Figure 7A), flies were briefly (less than 2 min) anesthetized with CO_2 on the 3rd day after the eclosion. Marking was performed on both the target and tester flies to balance the influence of this operation, although marking did not result in detectable behavioral changes (data not shown). Consistent with previous observations (Wang et al., 2011), the group-housed "target" males (Figures 7C and S1D) seldom lunged toward tester males. This lack of aggression from the tester males, and consequently the lack of fight escalation, are likely the reasons why fewer lunges were observed in these experiments than in other aggression assays.

To obtain "tester" male flies with smaller body (Figures 7D–7F), a group of parental flies (70 females and 30 males) were introduced to a food bottle containing 5ml of standard fly food medium. Flies were allowed to deposit embryos for 24 hr, and then were transferred to the next bottle. The bottles were subsequently kept at 22°C until eclosion. Canton-S "target" flies with regular size, as well as all other male flies used in this study, were obtained by rearing no more than 20 females and 10 males in a bottle containing 50ml of standard fly food medium. Flies were allowed to deposit embryos for 2 days, and then were transferred to the next bottle. Body size were measured as the area (pixel^{2}) projected by a fly body to the floor, which was captured by the video camera (see below) and was calculated by the tracking program (see below).

Acquisition of Digital Behavioral Data

All behavioral assays were carried out between 5PM-10PM. Digital movies were recorded by HandyCam DCR-HC52 (Sony) as .wmv format at 30 frames per second. Number of lunges was counted by CADABRA software (Dankert et al., 2009) except "mixed genotype pair" aggression assays (Figures 7C, 7E, and S1D), lunges of which were manually counted to keep track of genotype identities. We confirmed that CADABRA counted about 70% of manually counted lunges in the 12-well chambers (K.A., E.H. and D.J.A., data not shown).

In order to count the number of wing extensions, we first used software that detects, segments, and tracks the flies in the video, and then applied a wing extension classifier that takes as input data from the tracker and outputs frames where wing extensions occurred. Both the tracking software and the wing extension classifier were written in MATLAB (MathWorks).

Detection and Segmentation

In order to detect flies in a video a background image was computed from a subset of frames in the video. The background image was subtracted from each frame and pixels with absolute difference above a noise threshold were considered as foreground pixels. The foreground was segmented into body and wing using constraints, morphological operations, and Gaussian fitting on its pixel values. A user can adjust parameters to optimize the accuracy of body and wing segmentation, depending on movie resolution, lighting and contrast conditions, prior to the tracking process. Wing segmentation necessitated that flies were on the floor, with body and wing fully visible from the top. Thus, flies on the wall or flies that were attempting to climb the wall would not be correctly segmented. Such incidents were relatively rare due to the coating of the wall.

Tracking

Fly identities were kept throughout the video by computing the cost of assigning each detected fly in frame i to each detected fly in frame $i + 1$, and minimizing the total assignment cost at each time step. The cost function consists of distance between centroid of detections, and body overlap, such that when flies are close to each other body overlap is the dominating cost and when they are at least one body length away from each other centroid distance becomes the dominating factor.The software comes with an interface that allows users to manually tune constraints used to detect and segment body components such that they are optimized for their specific filming conditions (resolution, lighting and contrast).

Output

The software outputs for each frame contain 36 features in a form of vectors, calculated for every single frame. Some features are concerned with motions of individual flies, such as the centroid, orientation, and axes of fly's body ellipse; orientation, length and angle of their wings; and derived features such as velocity and acceleration of the body, angular velocity of its axis, etc. Other features are concerned with the relation between the pair of flies, such as distance between them, facing angle, etc., and the corresponding derived features.

The more detailed description of the tracking software will be found elsewhere (E. A. E. and P. P., manuscript in preparation), and the complete code of the software is available upon request.

Wing Extension Classifier

We employed a machine learning algorithm assisted by human-defined filters to accurately classify unilateral wing extensions. The basic idea of the classifier is to use the vector output of the tracking software to separate frames that contain unilateral wing extension from frame that do not, in the 35-dimensional space. To train the classifier, we first manually annotated a 20 min long movie of a pair of wild-type Canton-S males, filmed at a 200 fps rate, which contained around 4000 frames of wing extensions (J. Schor, B. Duistermars and D.J.A., unpublished data). From this sample, a linear discriminating function was used to separate wing extension frames from others. That is, if we located in this 35-dimensional space each frame of a fly, some points would be labeled as wing extensions or positives and some as negatives. The simplest classifier would be a hyper-plane that separates the two classes in this space. However, since the two classes might not be completely separable in such a space, the 35-dimensional vectors were projected into an even higher dimensional space (2 degree polynomial kernel transformation) to make the data separable by a simple hyper-plane. The hyper-plane was found by using a weighted regression, so that more weight was given to positive samples when minimizing the classification error. Although this hyper-plane was effective in detecting unilateral wing extensions with high recall rate (i.e., few "false negatives") when initially applied to tester movies, it was found to inadvertently include many false positives (thus low precision rate). These false positives included wing threats, grooming, events in which a fly tried to climb up the wall, and so on. To selectively eliminate such commonly observed false positive events, several hard-written filters for specific output features were introduced. These include (a) the minimum angle of the wing used for unilateral wing extension relative to the body axis, (b) the maximum angle of the wing not used for unilateral wing extension relative to the body axis, (c) minimum relative body axis ratio, (d) the maximum facing angle of the performing fly to the target fly, (e) the minimum length of the wing used for unilateral wing extension, and (f) the minimum duration of the event. These values were modifiable. In this study, the values used were (a) 62 degree, (b) 30 degree, (c) 0.8. (d) 35 degree, (e) 1.07, and (f) 4 frames ($\sim$132 msec). The complete MATLAB code of the wing extension classifier is available upon request.

Immunohistochemistry

The following antibodies were used: mouse nc82 (1:10 Developmental Studies Hybridoma Bank), chicken anti-GFP (1:1,000, abcam #13970), rabbit anti-FruM (1:10,000 (Stockinger et al., 2005) a gift from Dr. Barry Dickson), rabbit anti-DsRed (1:1,000, Clontech #632496), rabbit anti-LemTRP-1 (1:2,000, code K9836/8, Winther and Nässel, 2001; a gift from Dr. Dick Nässel), anti c-*myc* (1:10, Developmental Studies Hybridoma Bank 9E10), DN-EX #8 (1:10, Developmental Studies Hybridoma Bank), rabbit anti-GABA (1:2,000, Sigma-Aldrich #A2052), mouse anti-Tyrosine Hydroxylase (TH) (1:200, ImmunoStar #22941), rabbit anti-serotonin (1:5,000, Sigma-Aldrich #S5545), goat anti-chicken Alexa 488 (1:100, Molecular Probe #A11039), goat anti-rabbit Alexa 568 (1:100, Molecular Probe #A11011), goat anti-mouse Alexa 568 (1:100, Molecular Probe #A11004), goat anti-mouse Alexa 633 (1:100, Molecular Probe #A21050), goat anti-guinea pig Alexa 633 (1:100, Molecular Probe #A21105). The nc82 antibody developed by E. Buchner, anti c-myc antibody developed by J.M. Bishop, and DN-EX #8 developed by T. Uemura were obtained from the

Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Guinea Pig anti-Tk antibody was generated by using the peptide N'-MAALSDSYDLRGKQQRFADFNSKFVAVRGK-C', which corresponds to the predicted DTK-6 Tachykinin peptide from the Tk prepropeptide (Poels et al., 2009), as antigen. The antiserum was both positively and negatively purified, and used at 1:2000 dilution. The antigen peptide synthesis and antiserum production were performed by Covance (Denver, PA).

Whole fly brain immunostaining was performed essentially as previously described (Hummel and Zipursky, 2004; Van Vactor et al., 1991). Briefly, fly brains were dissected out in 1x PBS and fixed in 2% paraformaldehyde/32.25mM Na_2HPO_4/ 9.41mM NaH_2PO_4/ 75mM L-Lysine-HCl (pH = 7.4) at room temperature for 90 min. Brains were then extensively washed in 1xPBS/0.3% Triton X-100, and were blocked in 10% normal goat serum/1x PBS/ 0.3% Triton X-100 for 30 min. Primary antibodies were diluted in 10% normal goat serum/0.3% Triton X-100, and brains were incubated in the primary antibody dilution at 4°C for 2-3 days. Brains were then extensively washed in 1xPBS/0.3% Triton X-100, and were blocked in 10% normal goat serum/1x PBS/ 0.3% Triton X-100 for 30 min. Secondary antibodies were diluted in 10% normal goat serum/0.3% Triton X-100, and brains were incubated in the secondary antibody dilution at 4°C overnight. Brains were then extensively washed in1xPBS/0.3% Triton X-100, and incubated in 1xPBS/ 50% glycerol for 2 hr at 4°C. Lastly, brains were mounted on slide glass in Vectashield (Vector Laboratories). Images were taken with Olympus FV-1000 confocal microscopy, and processed in ImageJ (NIH) software. Despecling and the maximum intensity stack method were applied when creating a z-stack image.

Statistical Analysis

Sigmoidal curve fit on Figure S6C was performed using Prism7 with Y = Min+ (Max-Min)/(1+$10^{(LogEC_{50}-X)}$) (where X = [Temperature], Y = [Lunges in 30 min]) as a model equation and least-squares fit as a calculation method. 95% confidence intervals of "EC50" temperature and "top value" of lunges were calculated by Prism7 as well. Each data point contains 18 – 24 pairs.

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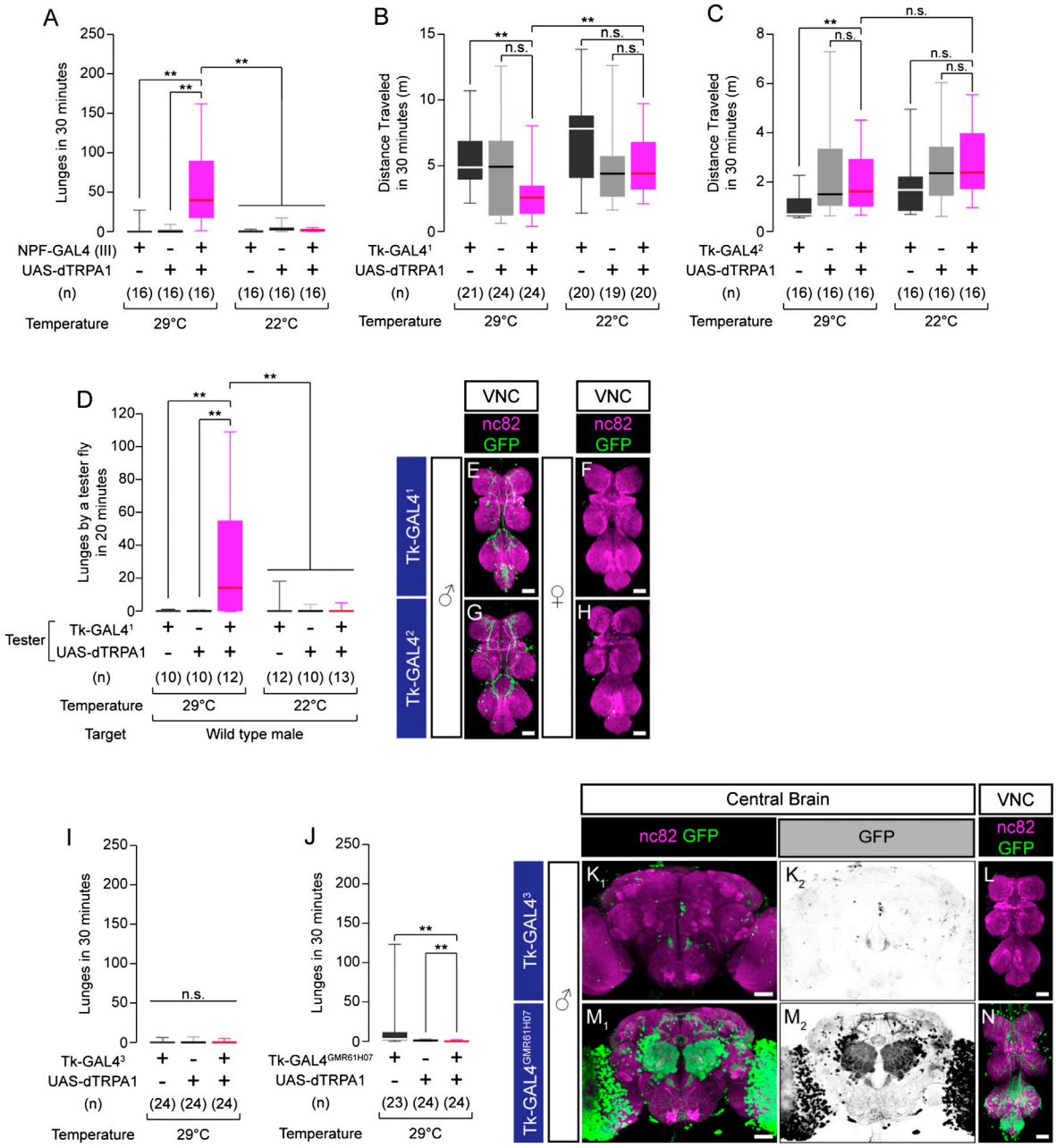

Figure S1. Behavioral and Immunohistochemical Analysis of Tk-GAL4 Lines Used in This Study, Related to Figure 1

(A) Number of lunges during thermogenetic activation of *NPF-GAL4* (III) neurons.

(B and C) Total distance traveled (boxplot) during thermogenetic activation of *Tk-GAL4*^{1} (B) and *Tk-GAL4*^{2} (C) neurons. Flies were singly placed in a well of 12-well aggression chamber and distance traveled by each individual was tracked (see Experimental Procedures).

(D) Number of lunges during thermogenetic activation of *Tk-GAL4*^{1} neurons toward "target" Canton-S male.

(E and F) Male (E) and female (F) *Tk-GAL4*^{1}; *UAS-mCD8:GFP* VNCs immunostained with anti-GFP antibody (green) and neuropil marker nc82 (magenta).

(G and H) Male (G) and female (H) *Tk-GAL4*^{2}; *UAS-mCD8:GFP* VNCs immunostained with anti-GFP antibody (green) and neuropil marker nc82 (magenta).

(I and J) Number of lunges during thermogenetic activation of *Tk-GAL4*^{3} (I) and *Tk-GAL4*^{GMR61H07} (J) neurons.

(K and L) *Tk-GAL4*^{3}; *UAS-mCD8:GFP* male brain (K) and VNC (L) immunostained with anti-GFP antibody (green) and neuropil marker nc82 (magenta) (K_1). K_2: GFP only.

(M and N) *Tk-GAL4*^{GMR61H07}; *UAS-mCD8:GFP* male brain (M) and VNC (N) immunostained with anti-GFP antibody (green) and neuropil marker nc82 (magenta) (M_1). M_2: GFP only.

For (A–D), (I), and (J), **$p < 0.01$, n.s.: $p > 0.05$ (Kruskal-Wallis or Kruskal-Wallis and *post-hoc* Mann-Whitney U-test).

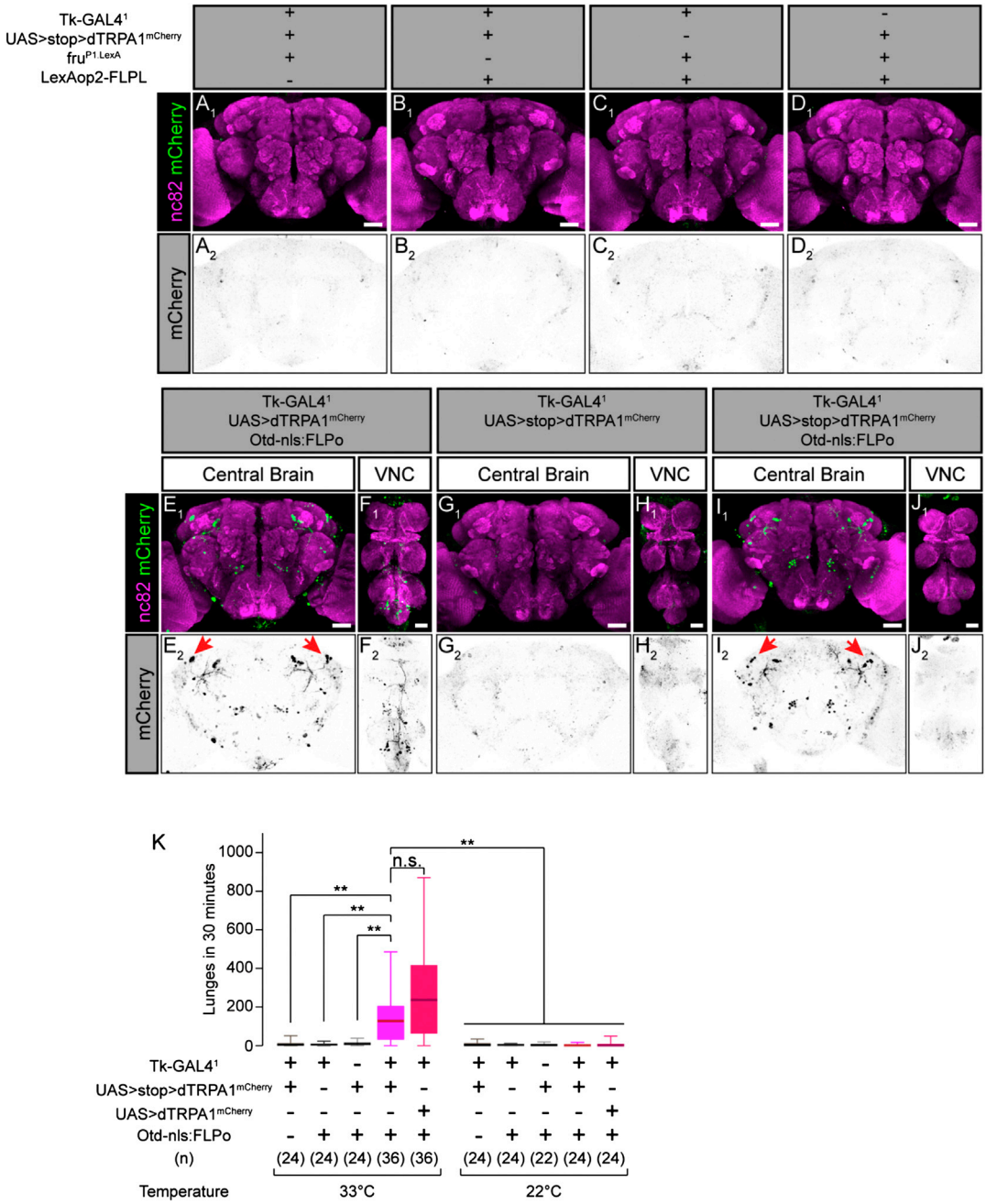

Figure S2. The Central Brain Contains the Aggression-Promoting Tk-GAL4^{FruM} Neurons, Related to Figure 2

(A–D) Male brains of 4 genetic control strains for the Tk-GAL4^{FruM} visualization and thermogenetic activation, immunostained with anti-DsRed antibody (green) and nc82 antibody (magenta) (A_1- D_1). Genotypes are indicated above images. A_2- D_2: anti-DsRed only.

(E and F) *Tk-GAL4^{1}*; *UAS > dTRPA1^{mCherry}*; *Otd-nls:FLPo* male brain (E) and VNC (F) immunostained with anti-DsRed antibody (green) and nc82 antibody (magenta) (E_1, F_1). E_2, F_2: anti-DsRed only. Arrows: the lateral cluster neurons.

(G and H) *Tk-GAL4^{1}*; *UAS > stop > dTRPA1^{mCherry}* male brain (G) and VNC (H) immunostained with anti-DsRed antibody (green) and nc82 antibody (magenta) (G_1, H_1). G_2, H_2: anti-DsRed only.

(I and J) *Tk-GAL4^{1}*; *UAS > stop > dTRPA1^{mCherry}*; *Otd-nls:FLPo* male brain (I) and VNC (J) immunostained with anti-DsRed antibody (green) and nc82 antibody (magenta) (I_1, J_1). I_2, J_2: anti-DsRed only. Arrows: the lateral cluster neurons.

(K) Number of lunges during thermogenetic activation of *Tk-GAL4^{1}* central brain neurons using *Otd-nls:FLPo*. **p < 0.01 (Kruskal-Wallis test and *post-hoc* Mann-Whitney U-test)

A

	Manually scored	Detected	False positives	False negatives	Recall (%)	Precision (%)
Bouts	113	101	15	12	89.4	87.1
Duration (s)	38	35.6	2.9	2.4	93.7	92.4

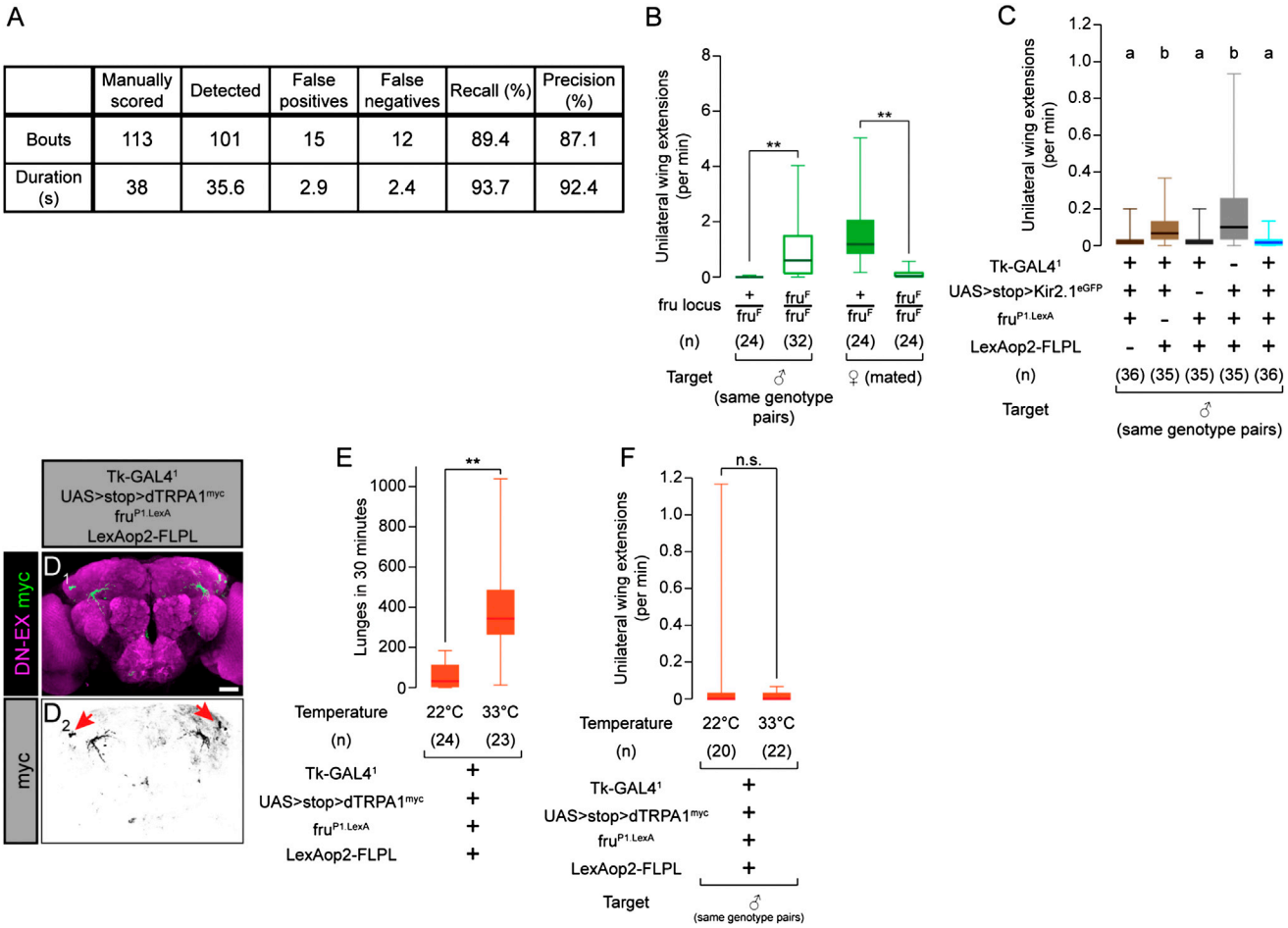

Figure S3. Thermogenetic Activation or Silencing of Tk-GAL4^{FruM} Neurons Does Not Affect Male-Male Courtship Behavior, Related to Figure 3

(A) Summary of the performance of the automated wing extension classifier. Twenty-four movies of male-male pairs were used for the evaluation.

(B) Unilateral wing extension frequencies of *fruF* mutants (Demir and Dickson, 2005) and its heterozygous controls toward each other (left) and toward mated females (right).

(C) Unilateral, male-male wing extension frequency during silencing of Tk-GAL4^{FruM} neurons.

(D) Tk-GAL4^{FruM} neurons in the brain visualized by the *UAS > stop > dTRPA1^{myc}* reporter, immunostained with anti-myc antibody (green) and a neuropil marker DN-EX antisera (magenta) (D_1). D_2; anti-myc only. Arrows: the Tk-GAL4^{FruM} neurons.

(E) Number of lunges during thermogenetic activation of Tk-GAL4^{FruM} neurons with the dTRPA1^{myc} effector.

(F) Unilateral, male-male wing extension frequency during thermogenetic activation of Tk-GAL4^{FruM} neurons with the dTRPA1^{myc} effector.

For (B), (E), and (F), **$p < 0.01$, n.s.: $p > 0.05$ by Mann-Whitney U-test. For (C), Alphabets denote statistically significant groups (**$p < 0.01$) by Kruskal-Wallis and *post-hoc* Mann Whitney U-tests.

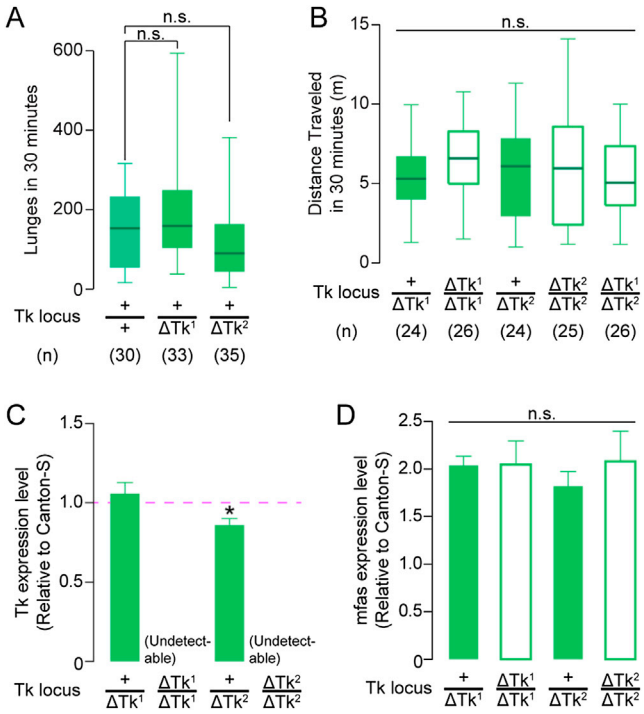

Figure S4. Additional Behavioral and Molecular Characterization of *ΔTk¹* and *ΔTk²* Alleles, Related to Figure 4

(A) Number of lunges performed by pairs of Canton-S (+/+ for *Tk* locus), +/*ΔTk¹* and +/*ΔTk²* males. n.s.: $p > 0.05$ (Kruskal Wallis and *post-hoc* Mann-Whitney U-tests)

(B) Total distance traveled by single *Tk* deletion mutants. Distance traveled was measured in the same manner as in Figure S1B and C. n.s.: $p > 0.05$ (Kruskal-Wallis test). The absence of a locomotion phenotype by the *Tk* deletion is in contrast to the previously reported locomotion increase by the RNAi-mediated knockdown of *Tk* (Winther et al., 2006).

(C) Relative quantity of *Tk* mRNA (mean ± SD) in *Tk* deletion mutants head RNA, compared to the ratio of *Tk* mRNA to *α-Tubulin at 84B* in Canton-S males (see Experimental Procedures for details; n = 3). *$p < 0.05$ by Student's t test (relative to Canton-S ratio i.e., 1.0)

(D) Relative quantity of *mfas* mRNA (mean ± SD) in *Tk* deletion mutants head RNA, compared to the ratio of *mfas* mRNA to *α-Tubulin at 84B* in Canton-S males (n = 3). n.s.: Kruskal-Wallis test, $p > 0.05$.

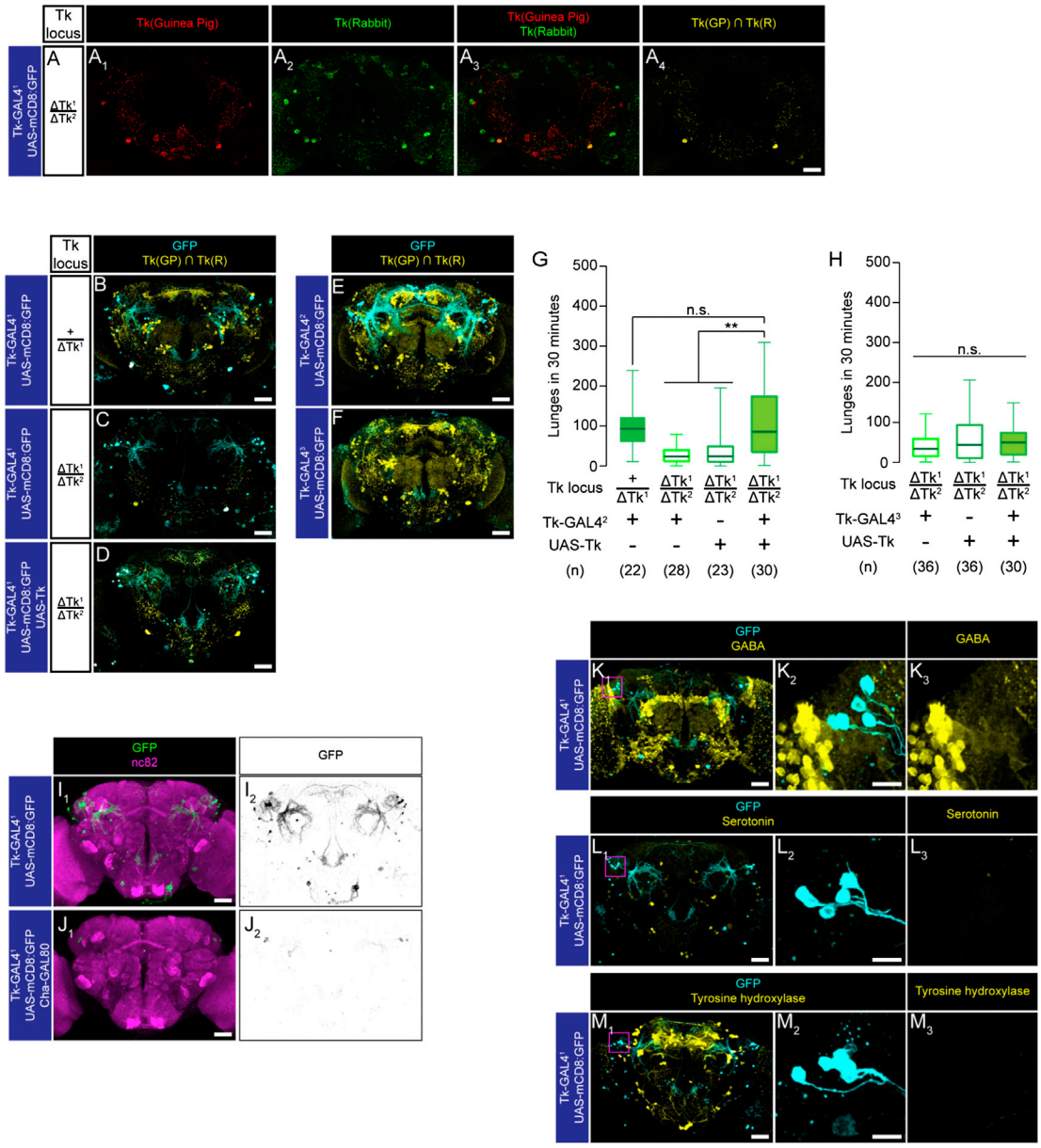

Figure S5. Tk-GAL4^{FruM} Neurons Promote Male-Male Aggression with DTK Neuropeptide Together with the Excitatory Neurotransmitter Acetylcholine, Related to Figure 5

(A) *Tk-GAL4^{1}*; *UAS-mCD8:GFP, ΔTk^{2}/ΔTk^{1}* male brain immunostained with anti-DTK guinea pig antiserum (red, A_1 and A_3) and anti-DTK rabbit antiserum (green, A_2 and A_3). The overlap of the guinea pig antiserum and rabbit antiserum is absent except a pair of large neurons on the ventral side of the brain (A_4).

(B–D) *Tk-GAL4^{1}* neurons in brains in *+/ΔTk^{1}* (B), *ΔTk^{2}/ΔTk^{1}* (C), *ΔTk^{2}/ΔTk^{1}* plus *UAS-Tk* (D) backgrounds immunostained with anti-GFP antibody (cyan), anti-DTK guinea pig antiserum and anti-DTK rabbit antiserum (shown as the overlap in yellow).

(E and F) *Tk-GAL4^{2}*; *UAS-mCD8:GFP* (E) and *Tk-GAL4^{3}*; *UAS-mCD8:GFP* (F) male brains immunostained with anti-GFP antibody (cyan), anti-DTK guinea pig antiserum and anti-DTK rabbit antiserum (shown as the overlap in yellow).

(G and H) Number of lunges in *ΔTk^{1}/ ΔTk^{2}* rescued by *Tk-GAL4^{2}* (G) or *Tk-GAL4^{3}* (H) driving *UAS-Tk*. **$p < 0.01$, n.s.: $p > 0.05$ (Kruskal-Wallis test or Kruskal-Wallis and *post-hoc* Mann-Whitney U-tests).

(I and J) *Tk-GAL4^{1}*; *UAS-mCD8:GFP* (I) and *Tk-GAL4^{1}*; *Cha-GAL80*; *UAS-mCD8:GFP* (J) male brains immunostained with anti-GFP antibody (green) and nc82 (magenta).

(K–M) *Tk-GAL4^{1}*; *UAS-mCD8:GFP* male brains immunostained with anti-GABA antibody (K), anti-5HT antibody (L) and anti-Tyrosine hydroxylase antibody (M), all shown in yellow, together with anti-GFP antibody (cyan).

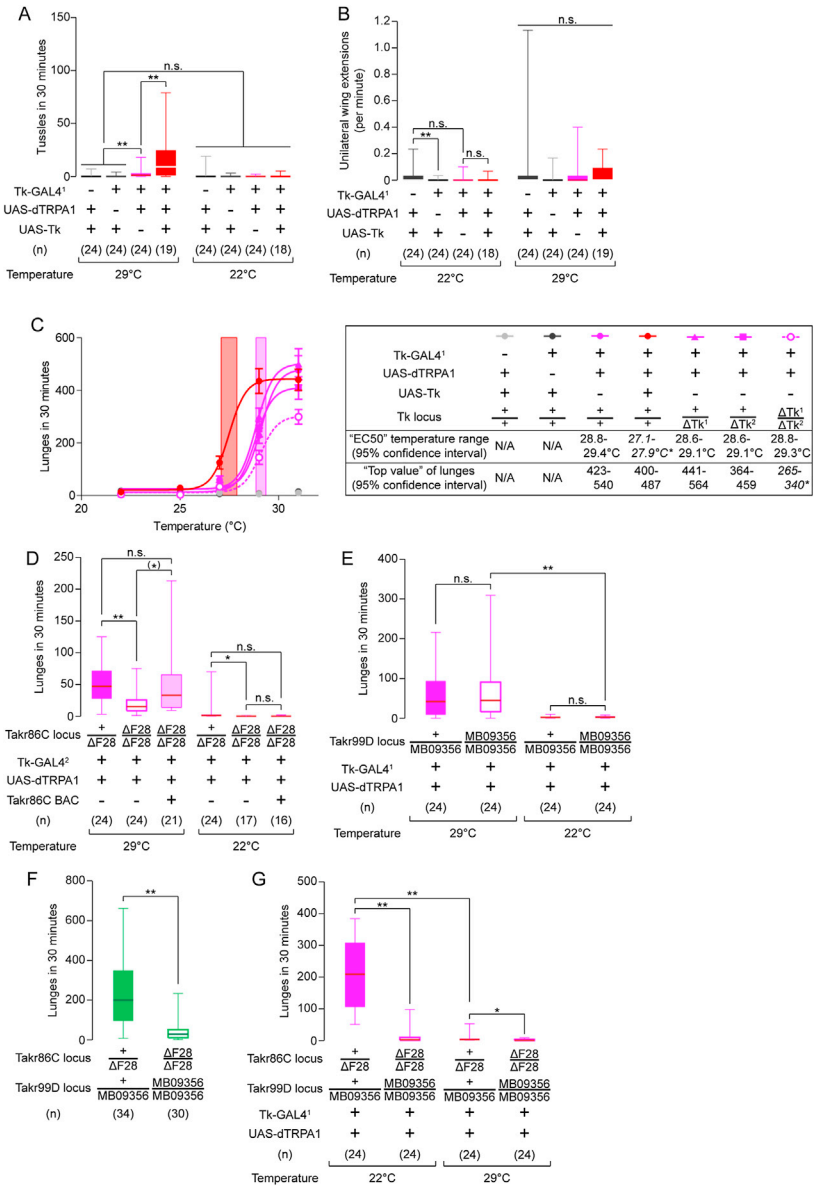

Figure S6. The Level of *Tk* Scales the Aggression-Promoting Effect of Tk-GAL4[1] Neurons Primarily through *Takr86C*, Related to Figure 6

(A) Number of tussles during thermogenetic activation of *Tk-GAL4[1]* neurons with *Tk* overexpression.

(B) Unilateral, male-male wing extension frequency during thermogenetic activation of *Tk-GAL4[1]* neurons with *Tk* overexpression. Genotypes, number of pairs and temperature tested are indicated below.

(C) Temperature-dependent sigmoidal model is fitted to the number of lunges performed by male pairs of genotypes listed (left box) at given temperatures. Each data point (mean ± SEM) contains n = 18-24. Colored boxes represent the 95% confidence intervals of the predicted "EC50" temperatures for *Tk-GAL4[1]*, *UAS-dTRPA1* (pink: [28.84 - 29.35°C]) and *Tk-GAL4[1]*; *UAS-dTRPA1*; *UAS-Tk* (red: [27.08 - 27.88°C]), respectively. * in the box denotes the 95% confidence invervals of the "EC50" value and "top value" that are significantly different from those of the rest of genotypes. The leftward shift of the "EC50" value in *Tk* overexpression genotype (red) is likely caused by higher amount of peptide release at lower temperature, whereas the lower "top value" in the $\Delta Tk^1/\Delta Tk^2$ mutant background (broken magenta line) likely reflects lowered limit of induced aggression in the absence of the peptides.

(D) Number of lunges during thermogenetic activation of *Tk-GAL4[2]* neurons in the $Takr86C^{\Delta F28}$ mutant background.

(E) Number of lunges during thermogenetic activation of *Tk-GAL4[1]* neurons in the $Takr99D^{MB09356}$ background.

(F) Number of lunges $Takr86C^{\Delta F28}$, $Takr99D^{MB09356}$ double mutants.

(G) Number of lunges during thermogenetic activation of *Tk-GAL4[1]* neurons in the $Takr86C^{\Delta F28}$, $Takr99D^{MB09356}$ double mutant background.

For (A), (B), (D), (E), and (G), **p < 0.01, *p < 0.05 (Kruskal-Wallis and *post-hoc* Mann-Whitney U-tests). (*) in (D) indicates p = 0.032, which is significant after pair-wise comparisons but no longer significant after the Bonferroni multiple comparison correction is applied. For (F), **p < 0.01 (Mann-Whitney U-test).