# Regulation of Aggression by Obesity-Linked Genes *TfAP-2* and *Twz* Through Octopamine Signaling in *Drosophila*

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**ABSTRACT** In *Drosophila*, the monoamine octopamine, through mechanisms that are not completely understood, regulates both aggression and mating behavior. Interestingly, our study demonstrates that the *Drosophila* obesity-linked homologs *Transcription factor AP-2* (*TfAP-2*; *TFAP2B* in humans) and *Tiwaz* (*Twz*; *KCTD15* in humans) interact to modify male behavior by controlling the expression of *Tyramine β-hydroxylase* and *Vesicular monanime transporter*, genes necessary for octopamine production and secretion. Furthermore, we reveal that octopamine in turn regulates aggression through the *Drosophila cholecystokinin* satiation hormone homolog *Drosulfakinin* (*Dsk*). Finally, we establish that TfAP-2 is expressed in octopaminergic neurons known to control aggressive behavior and that TfAP-2 requires functional Twz for its activity. We conclude that genetically manipulating the obesity-linked homologs *TfAP-2* and *Twz* is sufficient to affect octopamine signaling, which in turn modulates *Drosophila* male behavior through the regulation of the satiation hormone *Dsk*.

AGGRESSION is an important behavioral trait enabling animals to fight for food, shelter, and mates or over territories where these resources can be found. The behavioral decision to be aggressive is in part controlled by systems that also regulate metabolism, such as the monoamine system (Dierick and Greenspan 2007; Hoyer *et al.* 2008; Zhou *et al.* 2008; Alekseyenko *et al.* 2010). In the fruit fly *Drosophila melanogaster*, it was determined that a major regulator of male aggressive and mating behavior is the noradrenaline analog, octopamine (Baier *et al.* 2002; Hoyer *et al.* 2008; Zhou *et al.* 2008, 2012; Certel *et al.* 2010; Erion *et al.* 2012). In addition, the monoamines dopamine and serotonin have been linked to the regulation of aggressive behavior (Lucki 1998; Baier *et al.* 2002; Alekseyenko *et al.* 2010, 2013; Belsare *et al.* 2010). Yet the molecular mechanisms underlying aggression are still not fully understood.

The human genes *Transcription factor AP-2* (*TFAP2B*) (encoding AP-2β) and *KCTD15* have been identified as novel loci associated with obesity (Bauer *et al.* 2009; Renstrom *et al.* 2009; Willer *et al.* 2009; Zhao *et al.* 2011), although it is still not known how they regulate obesity at the molecular level. *TFAP2B* is a member of the AP-2 family of transcription factors, key regulators of various developmental processes (Eckert *et al.* 2005; Meng *et al.* 2010; Wenke and Bosserhoff 2010), and in mice it was demonstrated that *TFAP2B* is necessary for the proper development of peripheral and central nervous system noradrenergic neurons (Hong *et al.* 2008; Schmidt *et al.* 2011). *KCTD15* belongs to a family of potassium-channel tetramerization domain-containing proteins. In zebrafish embryos, *Kctd15* functions to inhibit the activity of AP-2α to restrict neural crest formation, although the exact mechanism of this inhibition is unknown (Dutta and Dawid 2010; Zarelli and Dawid 2013).

In *Drosophila*, *TFAP2B* is highly conserved, encoded by the gene *TfAP-2*. There is preliminary evidence from a yeast two-hybrid screen that *TfAP-2* associates with the *Drosophila KCTD15* homolog *CG10440*, which we have named *Tiwaz* (*Twz*; see *Materials and Methods*) (Giot *et al.* 2003). Furthermore, both genes are highly expressed in the central nervous system (Chintapalli *et al.* 2007). In mice, TFAP2B regulates the noradrenergic system, and we asked if *TfAP-2* and *Twz* could be involved in regulating behavior through octopamine signaling, a central controller of aggression in *Drosophila* (Hoyer *et al.* 2008; Zhou *et al.* 2008). Octopaminergic


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neurons are known to innervate the insulin-producing cells located in the *Par intercerebralis* of the *Drosophila* brain (Crocker *et al.* 2010). Intriguingly, it was recently discovered that these insulin-producing cells also produce the *Drosophila* homolog of *cholecystokinin* (*CCK*), known as *Drosulfakinin* (*Dsk*) (Söderberg *et al.* 2012), and in rodents levels of the satiation hormone *CCK* are correlated with aggression (Zwanzger *et al.* 2012). Furthermore, it has been reported that in *Drosophila* both octopamine and Dsk are involved in regulating muscle contractions necessary for the control of locomotory behavior (Koon *et al.* 2011; Chen and Ganetzky 2012; Chen *et al.* 2012), although it is not known if they interact. From these previous studies, we hypothesized that octopamine signaling could be modulating aggressiveness by regulating the expression of *Dsk*.

In the current study, we show that *TfAP-2* and *Twz* genetically interact in Tdc2 octopaminergic neurons to modulate male behavior. Furthermore, we demonstrate that *TfAP-2* and *Twz* are required for the proper expression of two genes necessary for the production and secretion of octopamine. Finally, we have evidence that octopamine regulates aggression by controlling the expression of the *Drosophila CCK* homolog *Dsk*.

## Materials and Methods

### *Fly stocks and maintenance*

w*, *P{w[+mW.hs]=GawB}elav[C155]*, *w**; *P{w[+mC]=Tdc2-GAL4.C}2*, $y^1$ *w[*]*; *P{w[+mC]=UAS-AP-2.PB}a4-2* and *w**, *P{w[+mC]=UAS-GFP.S65T}* were received from the Bloomington Stock Center. *TfAP-2* ($y^1w^3$; *P{KK109052}VIE-260B*) and *CG10440* ($y^1w^3$; *P{KK107922}VIE-260B*) RNA interference (RNAi) flies were obtained from the Vienna *Drosophila* RNAi Centre (Table 1). *w**; *Dilp2-GAL4* was a gift from Eric Rulifson (Wang *et al.* 2007), and *w**, *UAS-Dsk* flies were a gift from Barry Ganetzky (Chen *et al.* 2012). All flies were maintained on enriched Jazz mix standard fly food (Fisher Scientific) and maintained at 25°, 60% humidity, on a 12:12 light:dark cycle. To inhibit the GAL4 driver, flies crossed to a GAL4 driver were kept at 18° and, once the progeny had eclosed, were shifted to 29° for at least 5–7 days before any assays were performed. Due to its involvement in male aggression, we have decided to name the *Drosophila KCTD15* homolog *CG10440* after the Nordic god of single-combat, *Tiwaz* (*Twz*).

### *Aggression assay*

Cylindrical behavioral chamber dimensions were 2 × 2.5 cm (height × diameter) and filled with 1% agarose to 1.5 cm in height to maintain proper humidity. Newly emerged male flies were collected and isolated for 5–7 days at 29°, 60% humidity, on a 12:12 light:dark cycle. Behavioral tests were carried out at room temperature with 60% humidity. Two male flies were anesthetized using an ice-water bath before being transferred to a behavioral chamber. After a recovery period of at least 3 min, a camera (Panasonic HDC-SD90), positioned above the chamber, was used to record activity for a minimum of 30 min. After the 3-min recovery period, the behavioral interactions between the males were scored for 20 min. Distinct stereotypic aggressive interactions were scored as described by Nilsen *et al.* (2004). Aggressive interactions were further scored as either low- or high-intensity engagements. Low-intensity fighting (LIF) was scored as side-by-side pushing with a leg ("shoving") or quick wing flicking ("wing flick"); high-intensity fighting (HIF) was graded as lunging ("lunging") or boxing face-to-face with the two front legs ("boxing"), holding the wings at a 30° angle ("wing threat"), as well as chasing one another ("chasing"). Courtship behavior was marked as one wing extended at a 90° angle ("singing"), circling to the posterior ("circling"), tapping the abdomen ("tapping"), licking the genitalia ("licking"), or bending the abdomen toward the other fly ("abdomen bending"). At least 10 replicates were conducted for each genotype.

### *Mating behavior assay*

Newly eclosed males were collected and aged in isolation for 5–7 days at 29°, 60% humidity, on a 12:12 light:dark cycle. Individual males and 3- to 4-day-old virgin wild-type *CSORC* females were then transferred to a behavioral chamber, using ice-water anesthetization. After a recovery period of at least 3 min, a camera (Panasonic HDC-SD90), positioned above the chamber, was used to record activity for a minimum of 30 min. After the 3-min recovery period, the behavioral interactions of the males were scored for 20 min or until copulation occurred. *CSORC* is a lab wild-type strain created by crossing *Canton-S* and *Oregon-R* wild-type strains. Scoring of the courtship behaviors was performed as described by Becnel *et al.* (2011). Latency, courtship index, and the frequency of mating behaviors were measured. Latency was calculated by counting the time that it took a male to initiate mating, and courtship index was calculated as the percentage of time that a male spends actively courting a female over a 20-min period (seconds spent actively courting/(1200 sec − latency seconds). At least 10 replicates per genotype were conducted.

### *Activity assay*

Cylindrical behavioral chamber dimensions were 2 × 2.5 cm (height × diameter) and filled with 1% agarose to 1.5 cm in height to maintain proper humidity. Newly emerged male flies were collected and isolated for 5–7 days at 29°, 50% humidity, on a 12:12 light:dark cycle. Behavioral tests were carried out at room temperature with 60% humidity. The male fly to be analyzed was anesthetized using an ice-water bath before being transferred to a behavioral chamber. After a recovery period of at least 3 min, a camera (Panasonic HDC-SD90), positioned above the chamber, was used to record activity for a minimum of 30 min. Activity was determined at the percentage of time that the male spent actively walking over the 30-min period; preening activity was ignored for this assay. Ctrax and Matlab were used to determine speed.

**Table 1 Information on the RNAi constructs from the Vienna *Drosophila* RNAi Center**

| Transformant ID | Construct ID | Library | Gene no. | Gene | On targets | Off targets | s19 | CAN repeats |
|---|---|---|---|---|---|---|---|---|
| 101552 | 109052 | KK | CG7807 | *TfAP-2* | 1 | 0 | 1 | 2 |
| 110265 | 107922 | KK | CG10440 | *Tiwaz* | 1 | 3 | 0.99 | 3 |

KK = the phiC31 RNAi library, CAN repeats = defined as greater than or equal to 6 repeats of the sequence CAN (where N = any nucleotide), which can lead to off-target effects.

### *Antagonist assays*

Newly eclosed *TfAP-2* overexpressing males were collected and isolated on normal food for 3 days. They were then fed by the capillary feeding assay method for 2–3 days with 1, 3, and 5 mM of the octopamine antagonists phentolamine (Dudai 1982), Sigma-Aldrich) or epinastine (Stevenson *et al.* 2005; Unoki *et al.* 2005) or with 1 mM CCK antagonist (SR27897, Sigma-Aldrich) (Harper *et al.* 1999). Each fly was transferred to a transparent plastic cylindrical vial (9 × 2 cm) (height × diameter), containing 1% agarose (5 cm high) to provide moisture and humidity for the flies, and the opening of the vial was covered with paraffin film. A calibrated capillary glass tube (5 μl, VWR International) was filled with liquid food (5% sucrose and 5% yeast extract) containing the appropriate antagonist, and a mineral oil layer was used to prevent evaporation from the capillary tube. Feeding tubes were inserted through the paraffin film into the chambers. After 2–3 days of feeding, two male flies were transferred into a behavioral chamber, and activity was videotaped and scored as before.

### *Immunohistochemistry*

Male flies were anesthetized and decapitated, and the proboscis was removed. Heads were placed into a staining glass bowl containing 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS) and left to fixate in the dark for 2 hr on ice. After fixation, heads were placed in a petri dish containing 4% agarose, and brains were dissected under a light microscope using fine forceps. Brains were washed four times for 15 min each with 0.1 M PBS. Tissues were blocked in 10% normal goat serum (NGS) for 1 hr. The NGS was then discarded, and tissues were incubated with primary antibody (Abcam rabbit polyclonal AP-2γ), diluted 1:5000 in 0.01 M PBS containing 0.25% Triton X-100 (PBX) for 2 days at 4°. Bowls were sealed with parafilm and aluminum foil. Following 48 hr of incubation, brains were washed four times for 15 min each with 0.01 M PBX and incubated with secondary antibody (Alexa Fluor 594 goat anti-rabbit), diluted 1:1000 in 0.01 M PBX, overnight. Bowls were sealed with parafilm and aluminum foil. Tissues were washed once with 0.01 M PBX for 15 min and twice with 0.01 M PBS for 15 min. Samples were mounted with 60% glycerol containing 1.6% propyl gallate.

### *RNA extraction*

The phenol–chloroform method was used for RNA extraction from tissue samples (Chomczynski and Sacchi 1987). Fifty fly heads were homogenized with 800 μl TRIzol (Invitrogen), 200 μl chloroform (Sigma-Aldrich) was added, and samples were centrifuged at 12,000 × *g* for 15 min at 4°. The aqueous layer, which contained RNA, was separated and 500 μl isopropanol (Solvaco AB) was added.The RNA was precipitated by storing the samples at −32° for 2 hr. Samples were centrifuged at 12,000 × *g* for 10 min at 4° to collect the RNA pellets, which were then washed with 75% ethanol (Solvaco AB) to remove the organic impurities. Samples were allowed to air dry to remove any traces of ethanol. Dried RNA pellets were dissolved in 21.4 μl of RNAse free water (Qiagen) and 2.6 μl of DNAse incubation buffer (Roche). The samples were incubated at 75° for 15 min to ensure complete dissolution of RNA pellets. Two microliters of DNAse I (10 U/μl, Roche) was added to each sample and incubated at 37° for 3 hr to remove DNA contamination. DNAse was deactivated by incubating the samples at 75° for 15 min. Removal of DNA was confirmed by PCR using Taq polymerase (5 U/μl, Biotools B & M Labs), followed by agarose gel electrophoresis. The RNA concentration was measured using a nanodrop ND 1000 spectrophotometer (Saveen Werner).

### *Complementary DNA synthesis*

Complementary DNA (cDNA) was synthesized from RNA template by using dNTP 20 mM (Fermentas Life Science), random hexamer primers, and M-MLV Reverse Transcriptase (200 U/μl, Invitrogen) and by following manufacturer instructions. cDNA synthesis was confirmed by PCR followed by agarose gel electrophoresis.

### *Quantitative RT-PCR*

Relative expression levels of three housekeeping genes (*EF-1*, *Rp49*, and *RpL11*) and of the genes of interest were determined with quantitative RT-PCR (qPCR). Each reaction, with a total volume of 20 μl, contained 20 mM Tris–HCl, pH 9.0, 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM dNTP, DMSO (1:20), and SYBR Green (1:50,000). Template concentration was 5 ng/μl, and the concentration of each primer was 2 pmol/μl. Primers were designed with Beacon Designer (Premier Biosoft) using the SYBR Green settings. All qPCR experiments were performed in duplicate; for each primer pair, a negative control with water and a positive control with 5 ng/μl of genomic DNA were included on each plate. Amplifications were performed with 0.02 μg/ml Taq DNA polymerase (Biotools) under the following conditions: initial denaturation at 95° for 3 min, 50 cycles of denaturing at 95° for 15 sec, annealing at 52.8°–60.1° for 15 sec and extension at 72° for 30 sec. Analysis of qPCR data was performed using MyIQ 1.0 software

(Bio-Rad) as previously reported (Lindblom *et al.* 2006). Primer efficiencies were calculated using LinRegPCR (Ramakers *et al.* 2003), and samples were corrected for differences in primer efficiencies. The GeNorm protocol described by Vandesompele *et al.* (2002) was used to calculate normalization factors from the expression levels of the housekeeping genes. Grubbs' test was performed to remove outliers. Differences in gene expression between groups were analyzed with ANOVA followed by Fisher's protected least significant difference (PLSD) test where appropriate. $P < 0.05$ was used as the criterion of statistical significance. The following primers were used: *EF-1* (forward—5′-GCGTGGGTTTGTGATCAGTT-3′; reverse—5′-GATCTTCTCCTTGCCCATCC-3′); *Rp49* (forward—5′-CACAC CAAATCTTACAAAATGTGTGA-3′; reverse—5′-AATCCGGCCT TGCACATG-3′); *RpL11* (forward—5′-CCATCGGTATCTATGG TCTGGA-3′; reverse—5′-CATCGTATTTCTGCTGGAACCA-3′); *TfAP-2* (forward—5′-CTAAGAGCAAGAACGGAG-3′; reverse—5′-AACCAAGGATGTCAGTAG-3′); *Tiwaz* (forward—5′-GCCA CATTCTGAACTTTATG-3′; reverse—5′-GCCACTACCTCGTAA TTG-3′); *Mur89F* (forward—5′-GGAGTCCAATTCGGGATCTA-3′; reverse—5′-GAACTTTGATTGCTGCCAGA-3′); *Psi* (forward—5′-AACTACGGCTATGGGTACGG-3′; reverse—5′-TGGTTGATCA GCTTGATGGT-3′); *sens-2* (forward—5′-TGGAGAAAGTGTTCG AGTGC-3′; reverse—5′-CGCAGTAGTTGCAGGGATAA-3′); *Tdc2* (forward—5′-GGCACTCCCAAGCTCTCAAT-3′; reverse—5′-ATGGTCGTACGTTGGTGTCC-3′); *Tyramine β hydroxyase* (*Tbh*) (forward—5′-TTATGCCAGTGATGCTGCTC-3′; reverse—5′-TGAAAGCATTCTGCAAGTGG-3′); and *Vesicular momoamine transporter* (*Vmat*) (forward—5′-CGTGACCTTCGGGACGA TAG-3′; reverse—5′-ACTAGAGCGGGAAAACCAGC-3′).

### Statistical analysis

Mean and standard error from all replicates of each experiment were calculated. All analysis was performed with GraphPad Prism 4, using ANOVA with appropriate *post hoc* analysis for multiple comparisons.

## Results

### TfAP-2 is expressed in octopaminergic neurons

In mice the transcription factor *TFAP2B* is known to regulate noradrenaline signaling (Hong *et al.* 2008; Schmidt *et al.* 2011), and in *Drosophila TFAP2B* is encoded by the homolog *TfAP-2* (Monge *et al.* 2001). Since octopamine, the *Drosophila* analog of noradrenaline, is known to control aggression and mating behavior in males, we first wanted to determine if TfAP-2 was expressed in octopaminergic neurons. TfAP-2 expression in octopaminergic neurons was verified by staining *Drosophila* adult male brains with human anti-AP-2γ (see *Materials and Methods*). To do this, *Tdc2-GAL4* flies were crossed with *UAS-GFP* flies. *Tyrosine decarboxylase 2* (*Tdc2*) is specifically expressed in octopaminergic neurons, where it is necessary to produce the monoamine tyramine from tyrosine (Cole *et al.* 2005). TfAP-2 was expressed in Tdc2-GFP-positive neurons found in the subesophageal ganglion (SOG) (Figure 1A, shaded region, and Figure 1B bracket marked "SOG"), an area of the brain known to regulate aggressive behavior (Zhou *et al.* 2008). TfAP-2 protein was also observed in Tdc2-GFP-negative neurons on either side of the brain near the SOG (Figure 1A, arrows). Staining was severely reduced in brains from flies expressing *TfAP-2* RNAi in all neurons using the pan-neuronal *Elav-GAL4* driver (Figure 1C) (Lin and Goodman 1994).

### AP-2 and Twz regulate male behavior

Before beginning the behavioral assays, to clarify if *UAS-Tfap2$^{RNAi}$* (referred to as *Tfap2$^{RNAi}$*), *UAS-Twz$^{RNAi}$* (referred to as *Twz$^{RNAi}$*), and *UAS-Tfap2$^{OE}$* [*yw*; *UAS-AP-2$^{PB}$*, referred to as *TfAP-2$^{OE}$* (Monge *et al.* 2001)] were functioning properly, we first crossed these flies to the *Tdc2-GAL4* driver and performed qPCR to measure the level of *TfAP-2* and *Twz* transcript (Figure 2). These flies were raised at 18° until they eclosed, at which point the newly eclosed flies were collected and kept at 29° for 5–7 days. The flies were put at 29° to get maximal expression from the GAL4/UAS system (Brand and Perrimon 1993). Since raising the flies at 18° inhibits, but does not completely block, GAL4 activity, to make sure that the effects that we observed were not due to a developmental phenotype, we also collected and kept newly eclosed flies at 18° for 5–7 days before preparing them for qPCR analysis. *Tdc2-GAL4*, *UAS-Tfap2$^{RNAi}$*, *UAS-Twz$^{RNAi}$*, and *UAS-TfAP-2$^{OE}$* were all crossed to the *white* (*w*) allele *w$^{1118}$*, and the heterozygous progeny were used as controls. The *w$^{1118}$* allele was used because we always set up our experimental crosses such that the $F_1$ males will be in a *w* mutant background. The level of *TfAP-2* and *Twz* expression in *Tdc2-GAL4* heterozygous controls were set as 100%, represented as 1 on the graph (Figure 2A). Compared with *Tdc2-GAL4* heterozygous controls (SE ± 0.05), *TfAP-2$^{RNAi}$* males kept at 29° had only 0.36-fold (SE ± 0.02, $P < 0.005$) of the normal *TfAP-2* RNA expression levels, while males maintained at 18° had 0.88-fold (SE ± 0.05, $P = 0.182$) of normal *TfAP-2* expression (*Tdc2-GAL4* at 18°, SE ± 0.04). *TfAP-2$^{RNAi}$* males raised at 29° had 0.88-fold (SE ± 0.07, $P < 0.322$) of the normal *Twz* RNA expression levels. On the other hand, expressing *Twz$^{RNAi}$* with the *Tdc2-GAL4* driver affected both *TfAP-2* and *Twz* expression. *Twz$^{RNAi}$* males maintained at 29° had only 0.33-fold (SE ± 0.08, $P < 0.005$) of the normal *Twz* RNA expression levels, while males at 18° had 0.85-fold (SE ± 0.06, $P = 0.132$) of normal expression (*Tdc2-GAL4* at 18°, SE ± 0.03). Furthermore, *Twz$^{RNAi}$* males maintained at 29° had only 0.42-fold (SE ± 0.05, $P < 0.005$) of normal *TfAP-2* expression (Figure 2A). Due to this result, for the rest of the article when we refer to "*Twz* knockdown males" we actually mean "*Twz* and *Tfap-2* double knockdowns," whereas when we refer to "*Tfap-2* knockdowns," we mean "flies where only *Tfap-2* transcript levels were lowered." Overexpressing *TfAP-2* in Tdc2 neurons induced a strong increase in *TfAP-2* transcript levels (1.7-fold, SE ± 0.09, $P < 0.005$); this expression was decreased when *AP-2$^{OE}$* was expressed in a *Twz$^{RNAi}$* background (*AP-2$^{OE}$;Twz$^{RNAi}$*, 1.1-fold, SE ± 0.05, $P = 0.433$). Similar to

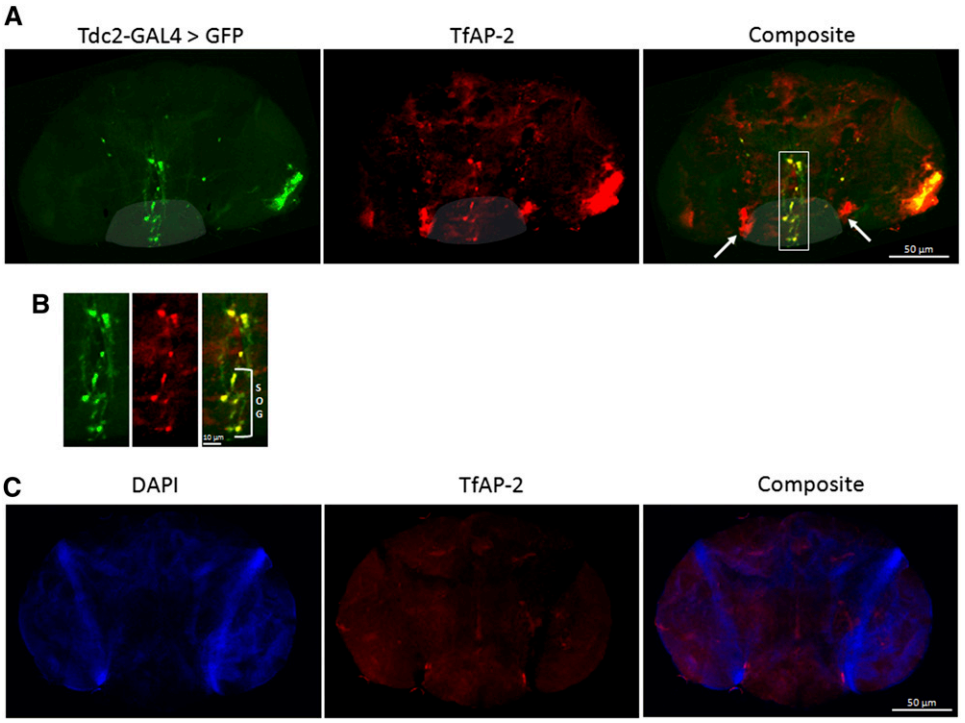


**Figure 1** TfAP-2 is expressed in octopaminergic neurons. (A) Immunofluorescence of whole *Drosophila* male brain to visualize TfAP-2 expression (red) in Tdc2 neurons (GFP, green). Shaded area indicates approximate region of the subesophageal ganglia. Box represents section of brain shown in B. Arrows indicate two neurons that are TfAP-2 positive but GFP negative. (B) Extensive TfAP-2 (red) expression in Tdc2 neurons (GFP, green) overlap appears as yellow in composite picture (SOG). (C) *TfAP-2* RNAi was expressed in the entire CNS using the Elav-GAL4 driver, DAPI staining (blue), and TfAP-2 expression (red). The picture was overexposed to reveal any possible TfAP-2 staining, and some residual staining was observed in the two neurons that were TfAP-2 positive but GFP negative in A.

*TfAP-2*, there was an increase in *Twz* transcript levels in *AP-2*$^{OE}$ males (1.45-fold, SE ± 0.09, $P < 0.05$) (Figure 2A).

The RNAi line used to knock down *Twz* possibly affects the expression of three off-target genes, *Mucin related 89F* (*Mur89F*), *P-element somatic inhibitor* (*Psi*), and *senseless-2* (*sens-2*) (Table 1). To make sure that the phenotypes we observe are actually due to the knocking down of *Twz*, we also performed qPCR to study *Mur89F*, *Psi*, and *sens-2* expression in *Twz*$^{RNAi}$ males kept for 5–7 days at 29° (Figure 2B). Expressing *Twz*$^{RNAi}$ in octopaminergic neurons had no significant affect on the expression levels of *Mur89F*, *Psi*, and *sens-2* (Figure 2B).

Since octopamine is known to control aggression in *Drosophila* males (Baier *et al.* 2002; Hoyer *et al.* 2008; Zhou *et al.* 2008, 2012; Certel *et al.* 2010; Erion *et al.* 2012), to establish if *TfAP-2* and *Twz* were required for octopamine-regulated male behavior, we performed an aggression assay. To do this, *Tdc2-GAL4*, *UAS-Tfap2*$^{RNAi}$, *UAS-Twz*$^{RNAi}$, and *UAS-Tfap2*$^{OE}$ were all crossed to $w^{1118}$, and the heterozygous progeny were used as controls. Since there is debate on how *w* influences behavior, we also used $w^{1118}$ hemizygous males as controls (Zhang and Odenwald 1995; Anaka *et al.* 2008).

Aggression analysis experiments were executed by placing pairs of 5- to 7-day-old males, raised in isolation, in a behavioral assay chamber containing 1% agarose, and their interactions were monitored over a 20-min period. The total number of interactions for each fly was recorded, whether it involved aggressive or courtship behavior. The assayed male–male interactions consisted of eight distinct behaviors. Aggressive interactions were scored as either low- or high-intensity engagements. LIF was scored as side-by-side pushing with a leg (shoving), or quick wing flicking (wing flick); HIF was graded as lunging (lunging), boxing face-to-face with the two front legs (boxing), as well as holding the wings up at a 30–45° angle (wing threat). Courtship behavior was marked as one-wing extended at a 90° angle (singing), circling to the posterior (circling), or bending the abdomen toward the other fly (abdomen bending).

When HIF behaviors are considered, there was no significant difference between controls, *TfAP-2*, and *Twz* knockdown males. However, there was a significant difference between controls and *TfAP-2*$^{OE}$ males. *Tdc2-GAL4*$^{+/-}$ control males performed 0.6 (SE ± 0.3) boxing behaviors per bout and *TfAP-2*$^{OE+/-}$ control males performed 0.3 (SE ± 0.08), while *TfAP-2*-overexpressing males performed 4.8 (SE ± 0.7, $P < 0.005$) boxing behaviors per bout. Also, *TfAP-2*-overexpressing males performed significantly more lunges (3.2, SE ± 0.07, $P < 0.05$) than either *Tdc2-GAL4*$^{+/-}$ (0.2, SE ± 0.02) or *TfAP-2*$^{OE+/-}$ (2.1, SE ± 0.08) control males (Figure 3A). *TfAP-2*$^{OE}$-induced HIF behaviors could be rescued by co-expressing *Twz*$^{RNAi}$ (*Tdc2-GAL4;TfAP-2*$^{OE}$/*Twz*$^{RNAi}$—boxing: 1.2, SE ± 0.5; lunges: 1.0, SE ± 0.5) (Figure 3A). When LIF behaviors were compared, *Twz*$^{RNAi}$ performed significantly more wing flicks (57.3, SE ± 11.4, $P < 0.005$) and shoves (14.4, SE ± 3.1, $P < 0.05$) over a 20-min fighting bout than either *Tdc-GAL4*$^{+/-}$ (wing flicks: 10.9, SE ± 3.2; shoves: 6.6, SE ± 1.8) or *Twz*$^{RNAi+/-}$ (wing flicks: 14.3, SE ± 2.5; shoves: 6.3, SE ± 1.3) control males. On the other hand, *TfAP-2*$^{OE}$ males performed significantly fewer wing flicks than controls (6.0, SE ± 2.0, $P < 0.05$). Finally, knocking down either *TfAP-2* or *Twz* had a significant effect on all scored mating behaviors. Compared to *Tdc2-GAL4*$^{+/-}$ (3.1, SE ± 1.9), *TfAP-2*$^{RNAi+/-}$ (3.3, SE ± 0.6), and *Twz*$^{RNAi+/-}$ (2.4, SE ± 1.4) controls, *TfAP-2*$^{RNAi}$ (25.2,

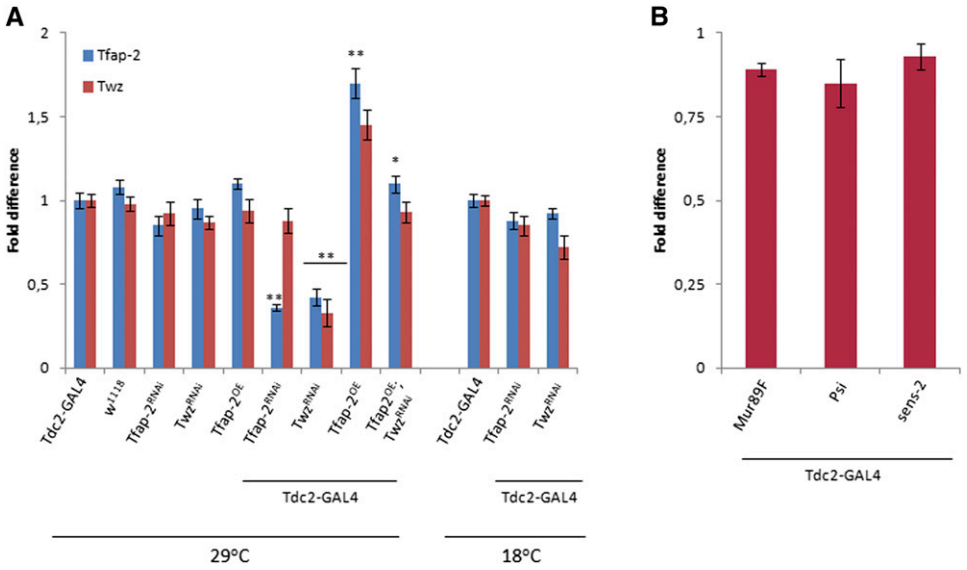


**Figure 2** Relative transcript levels of *TfAP-2* and *Twz*. (A) Relative level of *TfAP-2* and *Twz* expression in octopaminergic neurons in males kept at either 29° or 18° to verify the efficiency of the various UAS constructs. (B) Relative expression of possible *UAS-Twz$^{RNAi}$* line off-target genes *Mur89F*, *Psi*, and *sens-2* (see *Materials and Methods*). ($n$ = 10 qPCR runs; $^{*}P < 0.05$ $^{**}P < 0.005$ compared with controls, two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons).

SE ± 5.8, $P < 0.005$) and *Twz$^{RNAi}$* (20.6, SE ± 3.4, $P < 0.005$) knockdown males performed significantly more singing behaviors (Figure 3A). *TfAP-2$^{RNAi}$* and *Twz$^{RNAi}$* knockdown males also performed more circling maneuvers than controls, and unlike controls they performed abdomen bends toward other males (Figure 3A).

Next we wanted to determine if *TfAP-2* and *Twz* also regulate male behavior toward virgin females. To do this, males were paired with wild-type *CSORC* virgin females, and three aspects of male–female courtship were measured: number of behaviors, latency, and courtship index (see *Materials and Methods*). When *TfAP-2* (114.1 behaviors, SE ± 31.1, $P <$ 0.05) or *Twz* (113.8 behaviors, SE ± 21.1, $P < 0.05$) were knocked down in octopaminergic neurons, males performed significantly more courtship behaviors than *Tdc2-GAL4$^{+/-}$* (48.6 behaviors, SE ± 5.4), *Tfap2$^{RNAi+/-}$* (43.0 behaviors, SE ± 4.0), *Twz$^{RNAi+/-}$* (47.3 SE ± 5.8), or *w$^{1118}$* (46.5 behaviors, SE ± 4.0) controls (Figure 3B), and there was a substantial increase in the courtship index (CI) (Figure 3D). *Tdc2-GAL4$^{+/-}$* males had a CI of 56.6% (SE ± 5.2), *Tfap2$^{RNAi+/-}$*controls' CI was 55.3 (SE ± 4.0), *Twz$^{RNAi+/-}$* controls had a similar CI (48.6% SE ± 5.2), and *w$^{1118}$* males' CI was 55.2% (SE ± 5.3), while *TfAP-2$^{RNAi}$* males CI was significantly higher at 80.7% (SE ± 3.9, $P < 0.005$) and *Twz$^{RNAi}$* males CI was 79.5% (SE ± 5.9, $P < 0.005$). *TfAP-2$^{OE}$* males had a significant decrease in the number of courtship behaviors that they performed (10.9 behaviors, SE ± 7.2, $P < 0.005$) (Figure 3B), as well as a substantial increase in latency (422.3 sec, SE ± 78.2, $P < 0.005$) compared to *Tdc2-GAL4$^{+/-}$* (125.3 sec, SE ± 20.3), *TfAP-2$^{OE+/-}$* (112.3 sec, SE ± 18.7), or *w$^{1118}$* (157.2 sec, SE ± 33.5) controls (Figure 3C). *TfAP-2$^{OE}$* males had little interest in mating (CI = 12.3%, SE ± 6.2, $P < 0.005$) (Figure 3D).

### *TfAP-2 regulates activity levels via octopamine signaling*

One possibility for a change in behavior could be an overall change in activity. To ascertain the general movement of flies where *TfAP-2* or *Twz* expression is disrupted, the activity and speed of the various genotypes were measured. *Tdc2-Gal4$^{+/-}$* control males were used as a reference, represented as 1 (activity: SE ± 0.17; speed: SE ± 0.21) on the graph (Figure 4A). No change in overall activity was observed when *TfAP-2* or *Twz* were knocked down specifically in octopaminergic neurons. Although overall activity was not affected, compared to controls, *TFAP-2$^{RNAi}$* and *Twz$^{RNAi}$* males walked at a significantly slower pace—0.36-fold (SE ± 0.04, $P < 0.005$) and 0.59-fold (SE ± 0.04, $P <$ 0.05), respectively. Overexpression of *TfAP-2* in octopaminergic neurons induced a hyperactive phenotype, whereas *TfAP-2$^{OE}$* males were both significantly more active (2.5-fold, SE ± 0.13, $P < 0.005$) and walked considerably faster (2.1-fold, SE ± 0.32, $P < 0.005$) than controls (Figure 4A). Finally, *TfAP-2$^{OE}$;Twz$^{Ri}$* males were significantly less active than *TFAP-2$^{OE}$* males (1.63-fold compared to controls, SE ± 0.21, $P < 0.05$). Furthermore, *TfAP-2$^{OE}$;Twz$^{RNAi}$* males also walked significantly slower than *TfAP-2$^{OE}$* males ($P <$ 0.005), but not significantly slower than controls (0.65-fold, SE ± 0.08, $P = 0.082$).

To confirm that *TfAP-2* and *Twz* were actually regulating octopamine signaling, varying concentrations of two different octopamine antagonists, epinastine and phentolamine (Dudai 1982; Stevenson *et al.* 2005; Unoki *et al.* 2005), were fed to 3-day-old *TfAP-2$^{OE}$* males, after which an activity assay was performed. All manipulations were compared to *TfAP-2$^{OE}$* flies not fed antagonist (0 mM). Compared to *TfAP-2$^{OE}$* controls (80.4%, SE ± 4.3) a significant reduction in activity was observed when *TfAP-2$^{OE}$* males were fed 3 mM of either epinastine (54%, SE ± 3.6, $P < 0.005$) (Figure 4B) or phentolamine (47.2%, SE ± 6.7, $P < 0.005$) (Figure 4C). Feeding *TfAP-2$^{OE}$* males 5 mM of epinastine was lethal (Figure 4B), while 5 mM of phentolamine reduced activity even further (10%, SE ± 4.2, $P <$ 0.005) (Figure 4C).

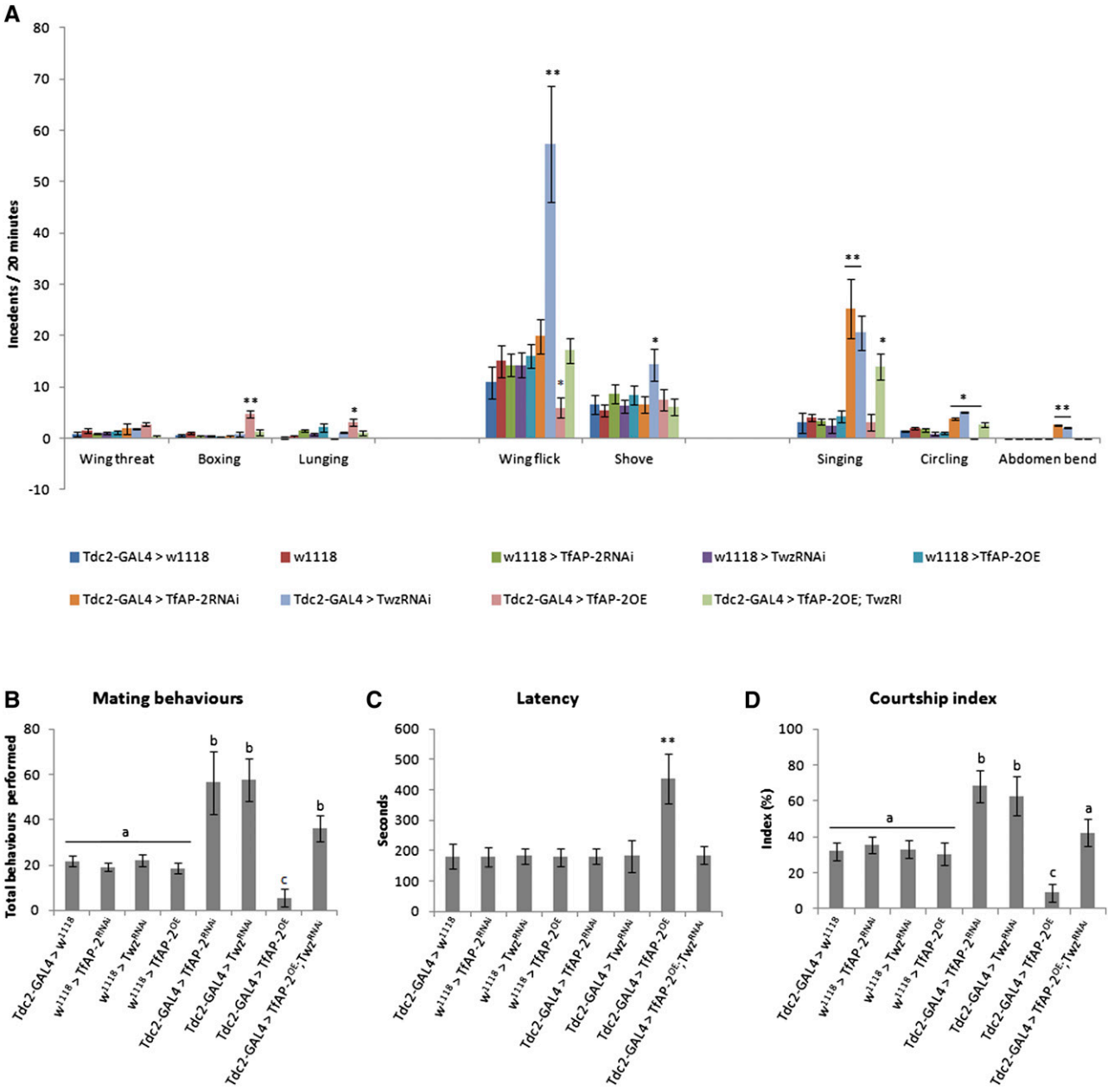


**Figure 3** Disrupting *TfAP-2* or *Twz* expression in Tdc2-specific octopaminergic neurons affects male behavior. *TfAP-2* and *Twz* RNAi were expressed specifically in octopaminergic neurons using the *Tdc2-GAL4* driver. (A) Total interactions that were either high- or low-intensity aggression or courtship behavior for all genotypes were determined. All males were between 5 and 7 days old. The types of behaviors were distributed into three catagories—HIF, LIF, and courtship behavior—and the number of each type of behavior performed is represented. In all instances, the assay was repeated at least 10 times ($n$ = 20 males/treatment; $^*P < 0.05$, $^{**}P < 0.005$ compared with controls, two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons). (B–D) Mating behavior of 5- to 7-day-old males toward 3- to 4-day-old *Csorc* wild-type virgin female was recorded over a 10-min period or until copulation occurred. In all instances the assay was repeated at least 10 times. (B) Male courtship behavior toward a virgin female. (C) Total number of behaviors a male performed. (D) Amount of time, in seconds, before the first courtship behavior, or latency, was determined. Percentage of time a male spent actively courting (courtship index) was determined. In B and D, different letters indicate similar groups (*i.e.*, "a" is significantly different than "b" or "c" and so on). $n$ = 20 males per aggression or mating assay; $^*P<0.05$ $^{**}P < 0.005$ compared with controls, one-way ANOVA with Bonferroni *post hoc* test for multiple comparisons.

### *TfAP-2 and Twz regulate genes involved in octopamine synthesis*

The transcription levels of a select number of genes—*Tdc2*, *Tbh*, and *Vmat*—known to influence octopamine production or release were examined (Figure 5A). Transcription levels of the *Tdc2-GAL4* heterozygous controls were set at 100%, shown as 1 on the various graphs (Figure 5, B–D). The transcript level of *Tdc2*, necessary to convert tyrosine to tyramine, was not affected in $TfAP\text{-}2^{RNAi}$ (0.98-fold, SE ± 0.03, $P = 0.98$), $Twz^{RNAi}$ (0.78-fold, SE ± 0.13, $P = 0.76$), or

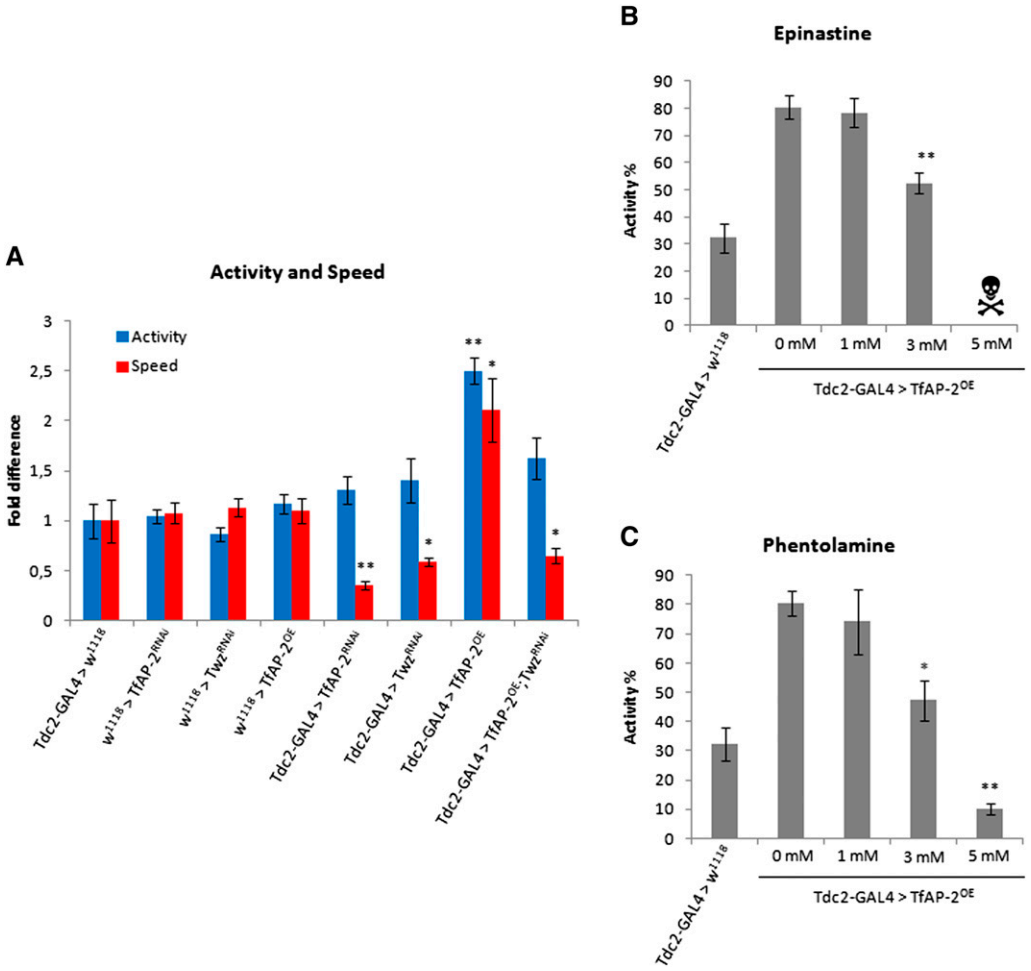


**Figure 4** Inhibiting octopamine signaling affects TfAP-2 induced hyperactivity. (A) Ctrax and Matlab were used to measure both activity and speed of walking of 5- to 7-day-old males for each genotype. Males were put individually into a behavioral assay chamber and monitored for 30 min ($n$ = 20 males; $^{*}P < 0.05$ $^{**}P < 0.005$ compared with controls, one-way ANOVA with Bonferroni *post hoc* test for multiple comparisons). (B-C) Five- to 7-day-old *Tdc2-GAL4; TfAP-2$^{OE}$* males were fed varying concentrations of the octopamine antagonists phentolamine or epinastine for 24 hr before an activity test was performed. (B) Epinastine (C) Phentolamine ($n$ = 20 males per concentration; $^{*}P < 0.05$ $^{**}P < 0.005$ compared with controls, one-way ANOVA with Bonferroni *post hoc* test for multiple comparisons).

*TfAP-2$^{OE}$* males (0.87-fold, SE ± 0.05, $P = 0.75$), compared to controls (Figure 5B). *Tbh*, necessary to convert tyramine to octopamine, was decreased significantly in *TfAP-2$^{RNAi}$* and *Twz$^{RNAi}$* males, 0.17-fold (SE ± 0.05, $P < 0.005$) and 0.28-fold (SE ± 0.07, $P < 0.005$), compared to controls, while in *TfAP-2$^{OE}$* males *Tbh* expression was significantly increased (1.61-fold, SE ± 0.43, $P < 0.05$). *TfAP-2$^{OE}$;Twz$^{RNAi}$* males had significantly lower levels of *Tbh* expression (0.31-fold, SE ± 0.09, $P < 0.005$) (Figure 5C). Finally, the transcript level of *Vmat*, involved in the transportation of monoamines such as octopamine into the synaptic vesicles, was also significantly reduced in *TfAP-2$^{RNAi}$* (0.33-fold, SE ± 0.08, $P <$ 0,005) and *Twz$^{RNAi}$* (0.42-fold, SE ± 0.07, $P <$ 0,005) males, compared to controls. In *TfAP-2$^{OE}$* males, *Vmat* expression was significantly increased (2.1-fold, SE ± 0.32, $P < 0.005$) (Figure 5D). *TfAP-2$^{OE}$;Twz$^{RNAi}$* males had significantly increased *Vmat* expression levels, which is more similar to *Twz$^{RNAi}$* males (0.47-fold, SE ± 0.07, $P < 0.005$) (Figure 5D).

### *CCK homolog Dsk regulates aggression downstream of octopamine*

So far we have determined that *TfAP-2* and *Twz* genetically interact to regulate male behavior. Furthermore, we show that *TfAP-2* and *Twz* regulate the expression of genes involved in controlling octopamine production and signaling. Yet we still have not determined how *TfAP-2* and *Twz* control of octopamine production may regulate behavior. Although it is well known that octopamine regulates aggressive behavior in *Drosophila* (Certel *et al.* 2007, 2010; Hoyer *et al.* 2008; Zhou *et al.* 2008), it is not known how octopamine exerts is affects. In rodents, levels of the satiation hormone *CCK* are correlated with aggressiveness (Zwanzger *et al.* 2012), and the *Drosophila* homolog of *CCK* is *Dsk* (Chen and Ganetzky 2012; Chen *et al.* 2012; Söderberg *et al.* 2012). Previously, it was shown that octopaminergic neurons innervate the insulin-producing cells (IPCs) and that the IPCs produce Dsk (Crocker *et al.* 2010; Söderberg *et al.* 2012). To begin to determine if Dsk could be regulating male behavior, we performed an aggression assay. Initially, we determined that 1 mM of the CCK antagonist SR27897 was insufficient to cause a phenotype in control heterozygous males (Figure 6A). Since *Dsk* is expressed by IPCs in the brain (Söderberg *et al.* 2012), the IPC-specific driver *Dilp2-GAL4* was used to overexpress *UAS-Dsk* (referred to as *Dsk$^{OE}$*), and an aggression assay was performed on these males or on *Dsk$^{OE}$* males fed CCK antagonist. Males overexpressing *Dsk* in the IPCs performed significantly more lunges (6.7, SE ± 1.1, $P < 0.005$) than controls. Intriguingly, feeding

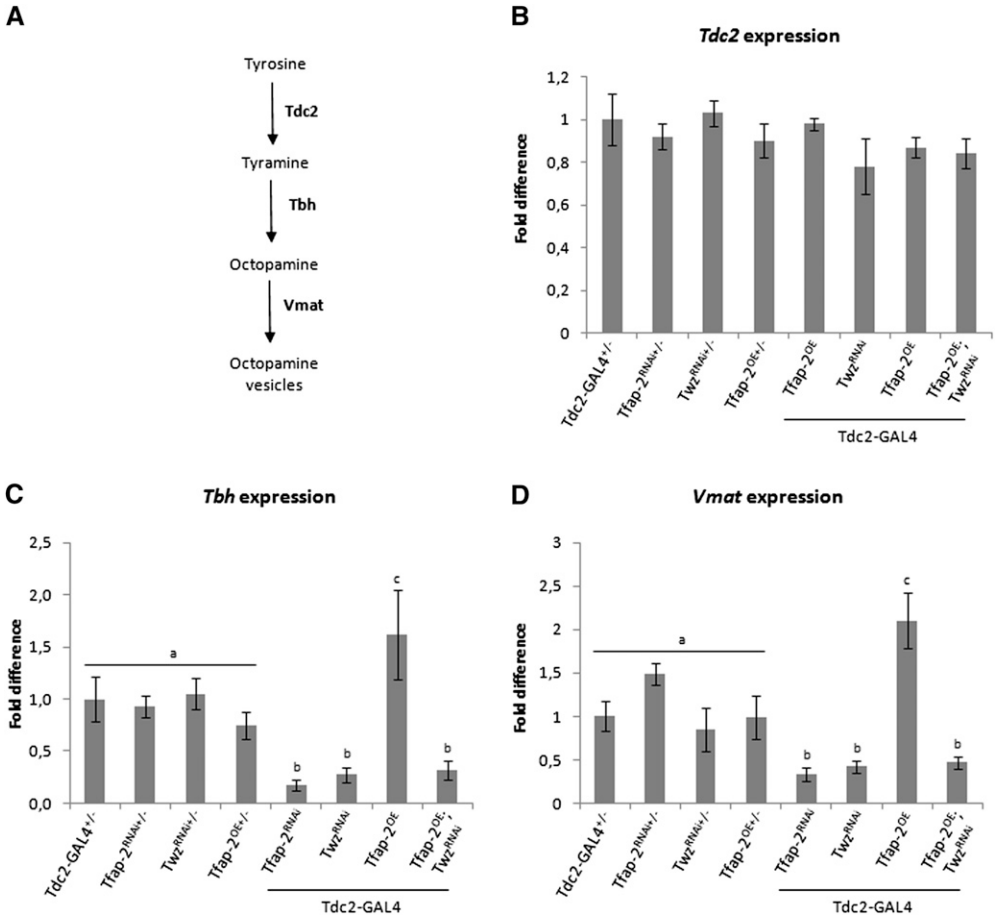


**Figure 5** TfAP-2 and Twz regulate genes involved in octopamine signaling. (A) Simplified schematic diagram of the pathway involved in octopamine production and secretion. (B–D) The transcript levels of genes (A) *Tdc2*, (B) *Tbh*, and (C) *Vmat*. RNA was collected from the heads of 5- to 7-day-old males for each genotype. qPCR was repeated at least seven times for each transcript. *Tbh* and *Vmat* transcript levels are significantly lower in flies where *TfAP-2* or *Twz* are knocked down in octopaminergic neurons (one-way ANOVA, $P < 0.005$), while their transcript levels are significantly increased when *TfAP-2* is overexpressed in these same neurons ($n$ = 7 qPCR runs; $^{*}P < 0.05$ $^{**}P < 0.005$ compared with controls, one-way ANOVA with Bonferroni *post hoc* test for multiple comparisons). In C and D different letters indicate similar groups (i.e., 'a' is significantly different than 'b' or 'c' and so on).

$Dsk^{OE}$ males 1 mM SR27897 induced severe intermittent jump behaviors, and no interactions were observed. Similar to what was observed before, overexpressing *TfAP-2* in Tdc2 neurons significantly increased the number of HIF behaviors (Figure 6A). Unlike $Dsk^{OE}$ males, feeding $TfAP\text{-}2^{OE}$ males 1 mM SR27897 did not induce a tick-like phenotype, but did inhibit the number of HIF behaviors that flies performed (Figure 6A). Feeding 1 mM SR27897 to $TfAP\text{-}2^{OE}$ males significantly induced more courtship behavior (Figure 6A). Whereas $TfAP\text{-}2^{OE}$ males performed 3.1 (SE ± 0.7) singing behaviors, $TfAP\text{-}2^{OE}$ males fed 1 mM SR27897 performed 10.0 (SE ± 3.6, $P < 0.005$). Furthermore, while $TfAP\text{-}2^{OE}$ males never performed circling or abdomen-bending behaviors, feeding 1 mM SR27897 to $TfAP\text{-}2^{OE}$ males induced significant circling (2, SE ± 0.4, $P < 0.05$) and abdomen bending (2, SE ± 0.8, $P < 0.005$).

Analysis by qPCR demonstrated that *Dsk* transcript decreased significantly in the brain when control flies were fed 3mM of the octopamine antagonist phentolamine (0.05-fold, SE ± 0.004, $P < 0.005$), (Figure 6B) (Evans and Robb 1993). Next, flies were fed varying concentrations (0.002, 0.01, and 0.05 mg·ml$^{-1}$) of chlordimeform (CDM), an octopamine agonist (Stevenson *et al.* 2005). The lowest concentration of CDM did not induce *Dsk* expression, but feeding flies 0.01 mg·ml$^{-1}$ (1.75-fold, SE ± 0.11, $P < 0.05$) or 0.05 mg·ml$^{-1}$ (2.06-fold, SE ± 0,21, $P < 0.005$) CDM significantly induced *Dsk* expression. Interestingly, *Dsk* transcript levels decreased significantly in $TfAP\text{-}2^{RNAi}$ (0.15-fold, SE ± 0.04, $P <$ 0,005) and $Twz^{RNAi}$ (0.02-fold, SE ± 0.01, $P < 0.005$) males and significantly increased in $TfAP\text{-}2^{OE}$ males (2.42-fold, SE ± 0.56, $P < 0.005$) (Figure 6B). Finally, feeding $TfAP\text{-}2^{OE}$ males the octopamine antagonist phentolamine blocked *Dsk* induction (0.15-fold, SE ± 0.02, $P < 0.005$), and $TfAP\text{-}2^{OE}$;$Twz^{RNAi}$ males had wild-type levels of *Dsk* expression (0.90-fold, SE ± 0.41, $P = 0.82$) (Dudai 1982; Evans and Robb 1993; Stevenson *et al.* 2005).

### *Dsk regulates adult male activity*

Interestingly, similar to *TfAP-2*, overexpressing *Dsk* in IPCs induced a hyperactive phenotype in adult males (Figure 7). While control males (*Dilp2-Gal4*) were active on average 53% of the time (SE ± 7.9), males overexpressing *Dsk* in the IPCs were active 92% of the time (SE ± 2.3, $P <$ 0.005). Feeding males 1mM of the CCK antagonist SR27897, where *Dsk* was overexpressed in the IPCs, reduced their activity to 45% (SE ± 9.2, $P = 0.51$ compared to controls). As observed before, $TfAP\text{-}2^{OE}$ males were much more active than controls ($Tdc2\text{-}Gal4^{+/-}$)—80.4% (SE ± 4.3, $P <$ 0.005) for $TfAP\text{-}2^{OE}$ compared to 32.2% (SE ± 5.6) for controls. Feeding $TfAP\text{-}2^{OE}$ males the CCK antagonist reduced their activity to control levels (25.6%, SE ± 3.7, $P < 0.34$) (Figure 7).

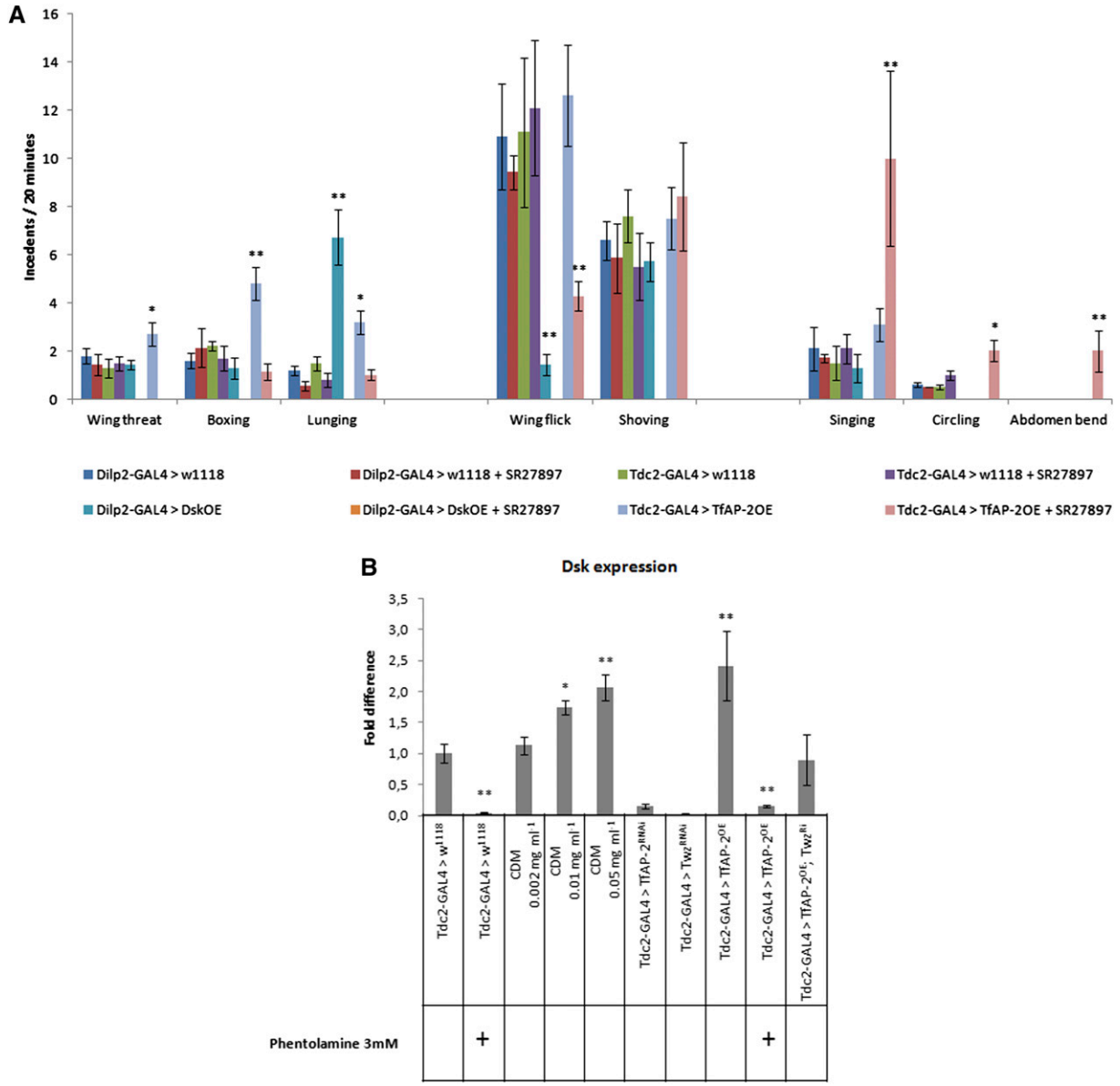


**Figure 6** Disrupting *Dsk* signaling affects male behavior. (A) Total interactions that were either high- or low-intensity aggression or courtship behavior for all genotypes were determined. All males were between 5 and 7 days old. The types of behaviors were distributed into three catagories—HIF, LIF, and courtship behavior—and the number of times that each type of behavior was performed is represented. In all instances the assay was repeated at least 10 times ($n$ = 20 males/treatment; $^*P < 0.05$, $^{**}P < 0.005$ compared with controls, two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons). (B) Relative levels of *Dsk* transcript in flies where *TfAP-2* and *Twz* expression has been disrupted in octopaminergic neurons. RNA was collected from the heads of 5- to 7-day-old males for each genotype ($n$ = 7 qPCR runs; $^*P < 0.05$ $^{**}P < 0.005$ compared with controls, two-way ANOVA with Bonferroni *post hoc* test for multiple comparisons).

## Discussion

Our data reveal that the *Drosophila* homologs for *TFAP2B* and *KCTD15*, *TfAP-2* and *Twaz*, respectively regulate at least two genes, *Tbh* and *Vmat*, known to be involved in the production and secretion of octopamine in *Tdc2* octopaminergic neurons. Furthermore, we demonstrate that octopamine regulates aggression and mating in *Drosophila* by controlling the expression in IPCs of the *CCK* homolog *Dsk*, a neuropeptide known to influence feeding behavior (Söderberg *et al.* 2012) (Figure 8). Of notable interest, the CCK inhibitor SR27897 was able to rescue *TfAP-2*-induced hyperactivity, indicating that TfAP-2 is signaling through a CCK-like pathway.

Our initial question was to determine if the *Drosophila* obesity-linked homologs could be linked to a pathway(s) known to regulate behavior. In mice, *Tfap2b* regulates noradrenaline signaling (Hong *et al.* 2008, 2011), and thus we addressed the octopaminergic system, the *Drosophila* equivalent (Roeder 2005). When *Drosophila* males are first introduced, they perform aggressive behaviors to establish a dominance hierarchy (Vrontou *et al.* 2006). Similar to what

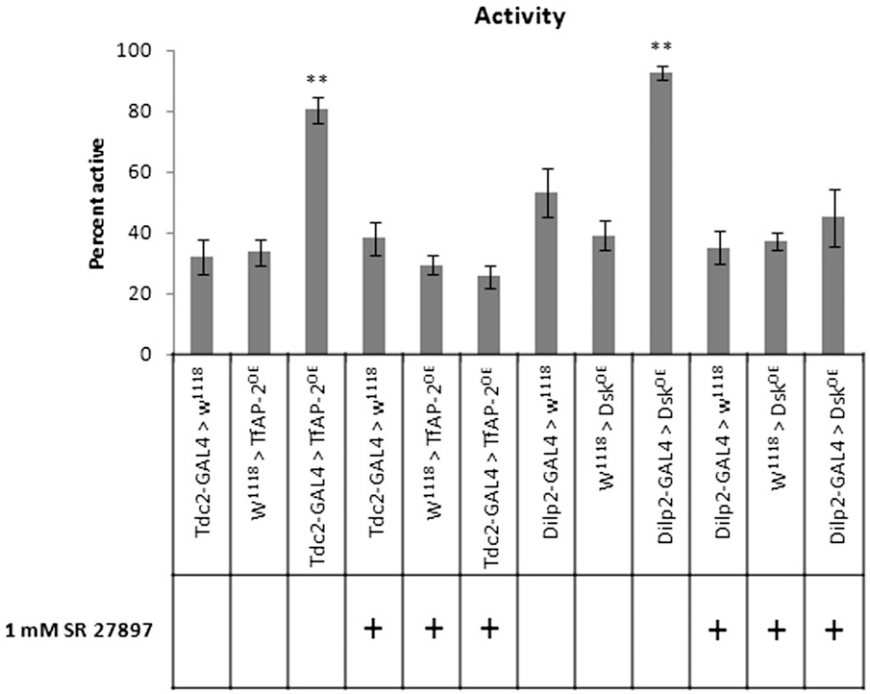


**Figure 7** Overexpressing Dsk makes males hyperactive. (A) Ctrax and Matlab were used to measure both activity and speed of walking of 5- to 7-day-old males for each genotype. Males were put individually into a behavioral assay chamber and monitored for 30 min. $n = 20$ males per concentration; $^{*}P < 0.05$ $^{**}P < 0.005$ compared with controls, one-way ANOVA with Bonferroni *post hoc* test for multiple comparisons.

was observed when the enzyme Tbh, necessary to convert tyramine into octopamine, was mutated to inhibit its function, *TfAP-2* and *Twz* knockdown males display reduced stereotypical high-intensity male aggressive behaviors (Zhou *et al.* 2008). Interestingly, we observed that *Tbh* expression was regulated by *TfAP-2* and *Twz* (see Figure 5C). Also, overexpression in octopaminergic neurons of NaCHBac, a bacterially derived voltage-sensitive sodium channel used to lower the activation threshold, induces male aggression (Zhou *et al.* 2008), and overexpression of *TfAP-2* in these same neurons induces aggressive behavior. All of these phenotypes lead us to suggest that TfAP-2 and Twz regulate octopaminergic neuronal signaling, perhaps similar to what was observed for AP-2β in the mouse noradrenergic system (Hoyer *et al.* 2008; Zhou *et al.* 2008).

CCK, a gastrointestinal hormone secreted by the gut when nutrients enter the lumen in mammals, binds to the cholecystokinin A receptor (CCKAR) located on vagal sensory terminals, which in turn delivers satiation signals to the nucleus of the solitary tract (NTS) (Wank *et al.* 1994; Mönnikes *et al.* 1997). CCK signaling within the brain to the cholecystokinin B receptor (CCKBR) induces hyperactivity and aggression in rodents (Bellier *et al.* 2004; Li *et al.* 2007). *TfAP-2* overexpression in octopaminergic neurons induces the expression of

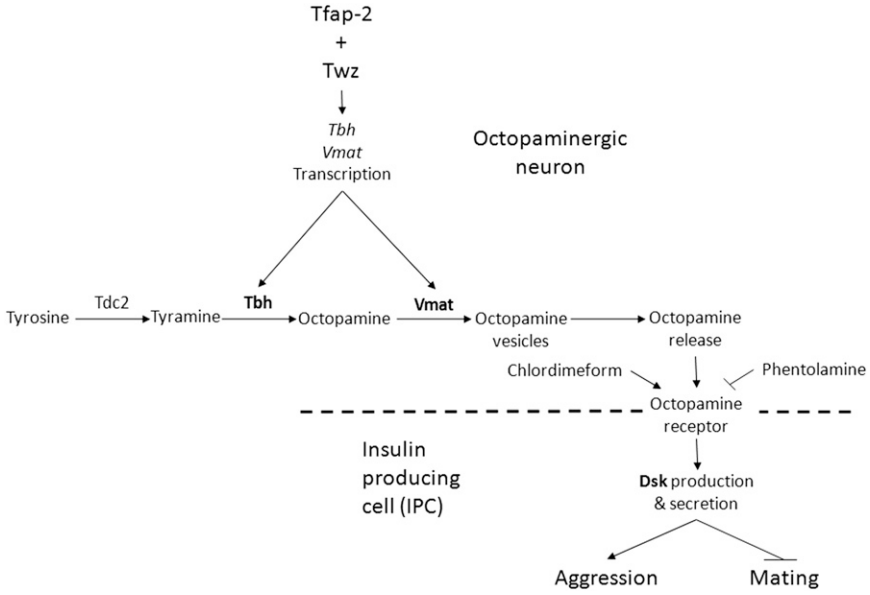


**Figure 8** Model for possible modulation of aggressive behavior in *Drosophila*. Twz and TfAP-2 interact in octopaminergic neurons, possibly to regulate TfAP-2 activity. TfAP-2 induces the expression of *Tbh* and *Vmat*, which in turn regulate octopamine production and release from octopaminergic neurons. Octopamine signals to the IPCs to induce *Dsk*. Dsk signals to induce male aggressive behavior, while inhibiting mating behavior in males.

the *Drosophila CCK* homolog, *Dsk*. This induction could be blocked by feeding *TfAP-2*-overexpressing males an octopamine antagonist and induced by feeding wild-type flies the octopamine agonist chlordimeform, revealing that *TfAP-2* induces *Dsk* expression via octopamine signaling (Figure 6). *Dsk* is expressed in the *Drosophila* IPCs within a structure homologous to the hypothalamus, known as the *Pars intercerebralis* (Söderberg *et al.* 2012). We observed that overexpressing *Dsk* in the IPCs, similar to CCK signaling in mice, induced hyperactivity and aggressive behavior. The *Drosophila* genome encodes two different *Dsk* receptors, *CCK-like receptor at 17D1* (*CCKLR-17D1*) and *CCK-like receptor at 17D3* (*CCKLR-17D3*), with *CCKLR-17D1* with slightly higher amino acid identity with *CCKBR*. Interestingly, in larvae, CCKLR-17D1 signaling is necessary to promote larval body-wall muscle contractions involved in stress-induced locomotory escape behavior (Chen *et al.* 2012). It was also shown that *Dsk* and *CCKLR-17DI* are required for proper neuromuscular junction formation in the developing *Drosophila* (Chen and Ganetzky 2012). Intriguingly, we could rescue aggressive behavior induced by *TfAP-2* overexpression with the CCK antagonist SR27897, meaning that octopamine-induced aggression, downstream of TfAP-2 and Twz, is probably due to an increase in Dsk signaling.

Another intriguing result was that feeding *Dsk*-overexpressing flies the CCK receptor antagonist SR27897 induced severe intermittent jump behavior. Previously, it was observed that feeding flies the $GABA_B$ agonist 3-aminopropyl-(methyl)phosphinic acid (3-APMPA) induced intermittent jumps, a phenotype similar to what we observed (Dzitoyeva *et al.* 2003). In mammals, there are two CCK receptors, CCKAR and CCKBR. CCK activation of the CCKAR was shown to inhibit GABA release, while CCKBR activation induced GABA release (Ferraro *et al.* 1996; Lee and Soltesz 2011). In *Drosophila*, there are also two *Dsk* receptors, CCKLR-17D1 and CCKLR-17D3. The CCK receptor antagonist SR27897 is a much more potent antagonist for CCKAR than CCKBR (Gully *et al.* 1993; Poncelet *et al.* 1993). It could be that the Dsk pathway in *Drosophila* has a similar effect on GABA signaling. Overepxression of Dsk could activate both receptors, having no overall effect on GABA signaling. Furthermore, feeding the flies 1 mM SR27897 may have increased GABA release, but not over a threshold level necessary to cause the intermittent phenotype. Finally, overexpressing *Dsk* in adult males, while feeding them SR27897, may have induced the release of enough GABA to cause the intermittent jump phenotype that we observed. This is very speculative and will need to be tested in the future.

An intriguing task for the future will be to understand how neuropeptides such as CCK and Dsk, known to modify feeding behavior (Ritter *et al.* 1999; Söderberg *et al.* 2012), regulate disparate behaviors, including aggression. Of notable interest is the fact that anorexic patients, who have a higher propensity to be aggressive, also have higher levels of circulating CCK levels (Sturm *et al.* 2003; Zalar *et al.* 2011).

## Acknowledgments

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