Regulation of aggression by obesity-linked genes TfAP-2 and Twz through octopamine signalling in Drosophila

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ABSTRACT

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

Aggression is an important behavioural trait enabling animals to fight for food, shelter and mates, or over territories where these resources can be found. The behavioural decision to be aggressive is in part controlled by systems that also regulate metabolism, such as the monoamine system (ALEKSEYENKO et al. 2010; DIERICK and GREENSPAN 2007; HOYER et al. 2008; ZHOU et al. 2008). In the fruit fly, Drosophila melanogaster, it was determined that a major regulator of male aggressive and mating behaviour is the noradrenaline analogue, octopamine (BAIER et al. 2002; CERTEL et al. 2010; ERION et al. 2012; HOYER et al. 2008; ZHOU et al. 2012; ZHOU et al. 2008). In addition, the monoamines dopamine and serotonin have been linked to the regulation of aggressive behaviour (ALEKSEYENKO et al. 2013; ALEKSEYENKO et al. 2010; BAIER et al. 2002; BELSARE et al. 2010; LUCKI 1998). Yet the molecular mechanisms underlying aggression are still not fully understood.

The human genes 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), though 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, in order to restrict neural crest formation, though 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 behaviour through octopamine signalling, a central controller of aggression in *Drosophila* (Hoyer et al. 2008; Zhou et al. 2008). Octopaminergic neurons are known to innervate the insulin-producing cells located in the Par intercerebralis of the *Drosophila* brain (Crock&er 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 cholecystokinin (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 (Chen and Ganetzky 2012; Chen et al. 2012; Koon et al. 2011), though it is not known if they interact. From these previous studies we hypothesised that octopamine signalling 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 behaviour. 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* cholecystokinin (CCK) homologue Drosulfakinin (Dsk).
MATERIALS AND METHODS

Fly stocks and maintenance

\( w^{*}, \ P[w^{+mW.hs}=GawB]elav[C155], \ w^{*}; \ P[w^{+mC}=Tdc2-GAL4.C2], \ y^{1} \ w^{*}; \ P[w^{+mC}=UAS-AP.2.PB]a4-2 \) and \( w^{*}, \ P[w^{+mC}=UAS-GFP.S65T] \) were received from the Bloomington Stock Center. \( TfAP-2 (y^{1}w^{3}; P[KK109052]VIE-260B) \) and \( CG10440 (y^{1}w^{3}; P[KK107922]VIE-260B) \) RNAi flies were obtained from the Vienna Drosophila RNAi Centre (Vienna, Austria) (Table 1). \( w^{*}; \ Dilp2-GAL4 \) was a gift from Dr. Eric Rulifson (WANG et al. 2007), and \( w^{*}, \ UAS-Dsk \) flies were a gift from Professor Barry Ganetzky (CHEN et al. 2012). All flies were maintained on enriched Jazz mix standard fly food (Fisher Scientific), and maintained at 25°C, 60% humidity, on a 12:12 light:dark cycle. To inhibit the GAL4 driver flies crossed to a GAL4 driver were kept at 18°C, once the progeny eclosed they were shifted to 29°C 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).

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

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Aggression assay

Cylindrical behavioural chamber dimensions were 2 cm by 2.5 cm (height x diameter), and filled with 1% agarose to 1.5cm in height to maintain proper humidity. Newly emerged male
flies were collected and isolated for 5 to 7 days at 29°C, 60% humidity, on a 12:12 light:dark cycle. Behavioural 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 behavioural chamber. After a recovery period of at least 3 minutes, a camera (Panasonic HDC-SD90), positioned above the chamber, was used to record activity for a minimum of 30 minutes. After the 3 minute recovery period the behavioural interactions between the males was scored for 20 minutes. Distinct stereotypic aggressive interactions were scored as described by Nilsen et al. 2004 (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 frontal lunging (lunging) or boxing face-to-face with the two front legs (fencing), holding the wings at a 30° angle (wing threat), as well as chasing one another (chasing). Courtship behaviour (CB) 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 towards the other fly (abdomen bending). At least 10 replicates were conducted for each genotype.

Mating behaviour assay
Newly eclosed males were collected and aged in isolation for 5 to 7 days, at 29°C, 60% humidity, on a 12:12 light:dark cycle. Individual males and 3-4 day old virgin wild type CSORC females were then transferred to a behavioural chamber, using ice-water anesthetization. After a recovery period of at least 3 minutes, a camera (Panasonic HDC-SD90), positioned above the chamber, was used to record activity for a minimum of 30 minutes. After the 3 minute recovery period the behavioural interactions between the males was scored for 20 minutes 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 behaviours was performed as described by Becnel et al. (BECNEL et al. 2011). Latency, courtship index as well as the frequency of mating behaviours were measured. Latency was calculated by counting the time it took a male to initiate mating and courtship index is calculated as the percentage of time a male spends actively courting a female over a 20 minute period (Seconds spent actively courting/(1200 seconds – Latency seconds). At least 10 replicates per genotype were conducted.

**Activity assay**

Cylindrical behavioural chamber dimensions were 2 cm by 2.5 cm (height x diameter), and filled with 1% agarose to 1.5cm in height to maintain proper humidity. Newly emerged male flies were collected and isolated for 5 to 7 days at 29°C, 50% humidity, on a 12:12 light:dark cycle. Behavioural 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 behavioural chamber. After a recovery period of at least 3 minutes, a camera (Panasonic HDC-SD90), positioned above the chamber, was used to record activity for a minimum of 30 minutes. Activity was determined at the percentage of time the male spent activity walking over the 30 minute period, preening activity was ignored for this assay. Ctrax and Matlab were used to determine speed.

**Antagonist assays**

Newly eclosed *TfAP-2* overexpressing males were collected and isolated on normal food for 3 days. They were then fed by Capillary feeding assay method for 2-3 days with 1, 3 and 5mM of octopamine antagonists Phentolamine (DUDAI 1982), Sigma-Aldrich) or Epinastine (STEVenson et al. 2005; UNOKI et al. 2005), or 1 mM CCK antagonist (SR27897 (HARPER
et al. 1999), Sigma-Aldrich). Each fly was transferred to a transparent plastic cylindrical vial 9 cm by 2 cm (height X diameter), containing 1% agarose (5cm high) to provide moisture and humidity for the flies, the opening of the vial was covered with paraffin film. A calibrated capillary glass tube (5μl, VWR) was filled with liquid food (5% of sucrose and 5% yeast extract) containing the appropriate antagonist, 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 behavioural chamber and activity was videotaped and scored as before.

**Immunohistochemistry**

Male flies were anesthetized, decapitated and the proboscis removed. Heads were placed into a staining glass bowl containing 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (PBS), and left to fixate in the dark for two hours 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 15 minutes each with 0.1 M PBS. Tissues were blocked in 10 % Normal Goat Serum (NGS) for one hour. The NGS was then discarded and tissues were incubated with primary antibody (Abcam® rabbit polyclonal AP-2γ), diluted 1:5000 in 0.01M PBS containing 0.25% Triton X-100 (PBX) for two days at 4°C. Bowls were sealed with parafilm and aluminum foil. Following 48 hour incubation, brains were washed four times 15 minutes 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, USA), 200 µl Chloroform (Sigma-Aldrich) was added and samples were centrifuged at 12000 rpm for 15 minutes at 4°C. The aqueous layer, which contained RNA, was separated and 500 µl isopropanol (Solvaco AB, Sweden) was added. The RNA was precipitated by storing the samples at -32°C for 2 hours. Samples were centrifuged at 12000 rpm for 10 minutes at 4°C, to collect the RNA pellets, which were then washed with 75% ethanol (Solvaco AB, Sweden) 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 GmBH, Germany) and 2.6 µl of DNAse incubation buffer (Roche GmBH, Germany). The samples were incubated at 75°C for 15 minutes to ensure complete dissolution of RNA-pellets. 2 µl of DNAse I (10 U/µl, Roche GmBH, Germany) was added to each sample, and incubated at 37°C for 3 hr to remove DNA contamination. DNAse was deactivated by incubating the samples at 75°C for 15 minutes. Removal of DNA was confirmed by PCR using Taq polymerase (5U/µl, Biotools B & M Labs, Spain), followed by agarose gel electrophoresis. The RNA concentration was measured using a nanodrop ND 1000 spectrophotometer (Saveen Werner).

cDNA synthesis:
cDNA was synthesized from RNA template using dNTP 20 mM (Fermentas Life Science), random hexamer primers and M-MLV Reverse Transcriptase (200 U/µl, Invitrogen, USA) by following manufactures instructions. cDNA synthesis was confirmed by PCR followed by agarose gel electrophoresis.
qRT-PCR:
Relative expression levels of three housekeeping genes (EF-1, Rp49 & 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 MgCl₂, 0.2 mM dNTP, DMSO (1:20) and SYBR Green (1:50000). 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 duplicates; 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, Sweden) under the following conditions: initial denaturation at 95°C for 3 min, 50 cycles of denaturing at 95°C for 15 sec, annealing at 52.8–60.1°C for 15 sec and extension at 72°C 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. (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 PLSD test where appropriate. P<0.05 was used as the criterion of statistical significance. The following primers were used: EF-1 F: 5´-CGTGTTTGTGTGATCATGTT-3´, R: 5´-GATCTTTCTCTGCCCATCC-3´; Rp49 F: 5´-CACACCAAATCTTACAAAATGTGTGA-3´, R: 5´-AATCCGCGCCTTGACAT-3´; RpL11 F: 5´-CCATCGGTATCTATGGTCTGGA-3´, R: 5´-CATCGTATTTCGGTGAACCA-3´; TfAP-2 F: 5´-CTAAGAGCAAGAACGGAG-3´, R: 5´-AACCAAGGATGTCAGTAG-3´; Tiwaz F: 5´-GCCACATTCTGAACTTTATG-3´, R:
5´-GCCACTACCTCGTAATTG-3´; Mur89F F: 5´-GGAGTCCAATCGGTACGG-3´, R: 5´-GAACTTTGATGCTGCCAGA-3´; Psi F: 5´-AACTACGGCTATGGGTACGG-3´, R: 5´-TGGAGGAAGTGTTCGAGTG-3´; sens-2 F: 5´-CGCAGTAGTTGCAGGGATAA-3´, R: 5´-ATGGTCGTACGTTGGTGTCC-3´; Tdc2 F: 5´-GGCACTCCCAAGCTCTCAAT-3´, R: 5´-TGAAAGCATTCTGCAAGTGG-3´; Tbh F: 5´-TTATGCCAGTGATGCTGCTC-3´, R: 5´-TGAAAGCATTCTGCAAGTGG-3´; Vmat F: 5´-CGTGACCTTCCGGGACGATAG-3´, R: 5´-ACTAGAGCGGGAAAACCAGC-3´

Statistical analysis:
Mean and standard error from all replicates of each experiment was calculated. All analysis was performed with GraphPad Prism 4, and used 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 signalling (HONG et al. 2008; SCHMIDT et al. 2011), and in Drosophila TFAP2B is encoded by the homologue TfAP-2 (MONGE et al. 2001). Since octopamine, the Drosophila analogue of noradrenaline, is known to control aggression and mating behaviour 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 Experimental procedures). 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 1B bracket marked SOG), an area of the brain known to regulate aggressive behaviour (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 behaviour**

Before beginning the behavioural 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} (Monge et al. 2001), referred to as TfAP-2^{OE}) were functioning properly, we first crossed these flies to the Tdc2-GAL4 driver, and performed quantitative RT-PCR (qPCR) to measure the level of TfAP-2 and Twz transcript (Figure 2). These flies were raised at 18°C until they eclosed, at which point the newly eclosed flies were collected and kept at 29°C for 5-7 days. The flies were put at 29°C to get maximal expression from the GAL4/UAS system (Brand and Perrimon 1993). Since raising the flies at 18°C inhibits, but does not completely block GAL4 activity, to make sure the effects we observed were not due to a developmental phenotype, we also collected and kept newly eclosed flies at 18°C 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 in such a way so the F1 males would 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°C had only 0.36-fold (SE ± 0.02, P < 0.005) of the normal TfAP-2
RNA expression levels, while males maintained at 18°C had 0.88-fold (SE ± 0.05, P = 0.182) of normal TfAP-2 expression (Tdc2-GAL4 18°C SE ± 0.04). TfAP-2RNAi males raised at 29°C had 0.88-fold (SE ± 0.07, P < 0.322) of normal Twz RNA expression levels. On the other hand, expressing TwzRNAi with the Tdc2-GAL4 driver affected both TfAP-2 and Twz expression. TwzRNAi males maintained at 29°C had only 0.33-fold (SE ± 0.08, P < 0.005) of the normal Twz RNA expression levels, while males at 18°C had 0.85-fold (SE ± 0.06, P = 0.132) of normal expression (Tdc2-GAL4 18°C SE ± 0.03). Furthermore, TwzRNAi males maintained at 29°C 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 manuscript 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-2OE was expressed in a TwzRNAi background (AP-2OE;TwzRNAi, 1.1-fold, SE ± 0.05, P = 0.433). Similar to TfAP-2, there was an increase in Twz transcript levels in AP-2OE 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 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 TwzRNAi males kept for 5-7 days at 29°C (Figure 2B). Expressing TwzRNAi 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; Certel et al. 2010; Erion et al. 2012; Hoyer et al. 2008; Zhou et al. 2012; Zhou et al. 2008), to establish if TfAP-2 and Twz were required for octopamine regulated male behaviour...
we performed an aggression assay. To do this, Tdc2-GAL4, UAS-Tfap2RNAi, UAS-TwzRNAi and UAS-Tfap2OE were all crossed to w1118 and the heterozygous progeny were used as controls. Since there is debate on how w influences behaviour we also used w1118 hemizygous males as controls (ANAKA et al. 2008; ZHANG and ODENWALD 1995).

Aggression analysis experiments were executed by placing pairs of 5-7 day old males, raised in isolation, in a behavioural assay chamber, containing 1% agarose, and their interactions were monitored over a 20 minute period. The total number of interactions for each fly was recorded, whether it involved aggressive or courtship behaviour. The assayed male-male interactions consisted of eight distinct behaviours. Aggressive interactions were 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), 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 behaviour was marked as one-wing extended at a 90° angle (singing), circling to the posterior (circling), or bending the abdomen towards the other fly (abdomen bending).

When it came to HIF behaviours there was no significant difference between controls, TfAP-2 and Twz knockdown males. However, there was a significant difference between controls and TfAP-2OE males. Tdc2-GAL4/+ controls males performed 0.6 (SE ± 0.3) boxing behaviours per bout and TfAP-2OE+/ control males performed 0.3 (SE ± 0.08), while TfAP-2 overexpressing males performed 4.8 (SE ± 0.7, P < 0.005) boxing behaviours 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-2OE+/ (2.1, SE ± 0.08) control males (Figure 3A). TfAP-2OE induced HIF behaviours could be rescued by co-expressing TwzRNAi (Tdc2-GAL4;TfAP-2OE/TwzRNAi: Boxing 1.2, SE ± 0.5; Lunges 1.0, SE ± 0.5) (Figure 3A). When LIF behaviours were compared, TwzRNAi 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 minute fighting bout than either Tdc-GAL4+/− (Wing flicks 10.9, SE ± 3.2; Shoves 6.6, SE ± 1.8) or TwzRNAi+/− (Wing flicks 14.3, SE ± 2.5; Shoves 6.3, SE ± 1.3) control males. On the other hand, TfAP-2OE 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 behaviours. Compared to Tdc2-GAL4+/− (3.1, SE ± 1.9), TfAP-2RNAi+/− (3.3, SE ± 0.6) and TwzRNAi+/− (2.4, SE ± 1.4) controls, TfAP-2RNAi (25.2, SE ± 5.8, P < 0.005) and TwzRNAi (20.6, SE ± 3.4, P < 0.005) knockdown males performed significantly more singing behaviours (Figure 3A). TfAP-2RNAi and TwzRNAi knockdown males also performed more circling manoeuvres than controls, and unlike controls they performed abdomen bends towards other males (Figure 3A).

Next we wanted to determine if TfAP-2 and Twz were also regulating male behaviour towards 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 behaviours, latency and courtship index (see Materials and Methods). When TfAP-2 (114.1, SE ± 31.1, P < 0.05) or Twz (113.8, SE ± 21.1, P < 0.05) were knocked down in octopaminergic neurons, males performed significantly more courtship behaviours than Tdc2-GAL4+/− (48.6, SE ± 5.4), Tfap2RNAi+/− (43.0, SE ± 4.0), TwzRNAi+/− (47.3 SE ± 5.8) or w1118 (46.5, 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), Tfap2RNAi+/− controls CI was 55.3 (SE ± 4.0), TwzRNAi+/− controls had a similar CI (48.6% SE ± 5.2) and w1118 males CI was 55.2% (SE ± 5.3), while TfAP-2RNAi males CI was significantly higher at 80.7% (SE ± 3.9, P < 0.005) and TwzRNAi males CI was 79.5% (SE ± 5.9, P < 0.005). TfAP-2OE males had a significant decrease in the number of courtship behaviours they performed (10.9, SE ± 7.2, P < 0.005) (Figure 3B), as well as a substantial increase in latency (422.3 seconds, SE ± 78.2, P < 0.005)
compared to either Tdc2-GAL4\textsuperscript{+/−} (125.3 seconds, SE ± 20.3), TfAP-2\textsuperscript{OE+/−} (112.3 seconds, SE ± 18.7) or w\textsuperscript{1118} (157.2 seconds, SE ± 33.5) controls (Figure 3C). TfAP-2\textsuperscript{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 signalling**

One possibility for a change in behaviour could be an overall change in activity. To ascertain if 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\textsuperscript{+/−} 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\textsuperscript{RNAi} and Twz\textsuperscript{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, where TfAP-2\textsuperscript{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\textsuperscript{OE};Twz\textsuperscript{Ri} males were significantly less active than TFAP-2\textsuperscript{OE} males (1.63-fold compared to controls, SE ± 0.21, P < 0.05). Furthermore, TfAP-2\textsuperscript{OE};Twz\textsuperscript{RNAi} males also walked significantly slower than TfAP-2\textsuperscript{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 signalling, 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\textsuperscript{OE} males, after which an activity assay was performed. All manipulations were compared to TfAP-2\textsuperscript{OE} flies not fed antagonist (0 mM). Compared to TfAP-2\textsuperscript{OE} controls (80.4\%, SE ± 4.3) a significant
A 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).

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

The transcription levels of a select number of genes, *Tdc2, Tyramine β hydroxyase (Tbh)* and *Vesicular monoamine transporter (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 5B-D). The transcript level of *Tdc2*, necessary to convert tyrosine to tyramine, was not affected in TfAP-2^RNAi (0.98-fold, SE ± 0.03, P = 0.98), *Twz^RNAi* (0.78-fold, SE ± 0.13, P = 0.76) or TfAP-2^OE males (0.87-fold, SE ± 0.05, P = 0.75), compared to controls (Figure 5B). *Tyramine β hydroxyase (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 more similar to *Twz^RNAi* males (0.47-fold, SE ± 0.07, P < 0.005) (Figure 5D).
The CCK homologue Dsk regulates aggression downstream of octopamine

So far we have determined that TfAP-2 and Twz genetically interact to regulate male behaviour. Furthermore, we show that TfAP-2 and Twz regulate the expression of genes involved in controlling octopamine production and signalling. Yet, we still haven’t determined how TfAP-2 and Twz control of octopamine production may regulate behaviour. Although it is well known that octopamine regulates aggressive behaviour in *Drosophila* (Ceruel et al. 2010; Ceruel et al. 2007; Hoyer et al. 2008; Zhou et al. 2008), it is not currently known how octopamine exerts its effects. In rodents, levels of the satiation hormone cholecystokinin (CCK) are correlated with aggressiveness (Zwanzger et al. 2012) and the *Drosophila* homolog of CCK is Drosulfakinin (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 behaviour, 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 insulin producing cells (IPCs) in the brain (Söderberg et al. 2012), the IPC specific driver Dilp2-GAL4 was used to overexpress UAS-Dsk (referred to as DskOE) and an aggression assay was performed on these males, or DskOE males fed CCK antagonist. Males overexpressing Dsk in the insulin-producing cells (IPCs) performed significantly more lunges (6.7, SE ± 1.1, P < 0.005) than controls. Intriguingly, feeding DskOE males 1mM SR27897 induced severe intermittent jump behaviours and no interactions were observed. Similar to what was observed before, overexpressing TfAP-2 in Tdc2 neurons significantly increased the number of HIF behaviours (Figure 6A). Unlike DskOE males, feeding TfAP-2OE males 1 mM SR27897 did not induce a tick-like phenotype, but did inhibit the number of HIF behaviours they performed to control levels (Figure 6A).
Feeding 1 mM SR27897 to TfAP-2^{OE} males significantly induced more courtship behaviour (Figure 6A). Whereas TfAP-2^{OE} males performed 3.1 (SE ± 0.7) singing behaviours, TfAP-2^{OE} males fed 1 mM SR27897 performed 10.0 (SE ± 3.6, P < 0.005). Furthermore, while TfAP-2^{OE} males never performed circling or abdomen bending behaviours, feeding 1 mM SR27897 to TfAP-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-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-2^{OE} males (2.42-fold, SE ± 0.56, P < 0.005) (Figure 6B). Finally, feeding TfAP-2^{OE} males the octopamine antagonist phentolamine blocked Dsk induction (0.15-fold, SE ± 0.02, P < 0.005), and TfAP-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, overexpressing Dsk in the IPCs, 1mM of the CCK antagonist
SR 27897 reduced their activity to 45% (SE ± 9.2, P = 0.51 compared to controls). As observed before, TfAP-2\textsuperscript{OE} males were much more active than controls (Tdc2-Gal4\textsuperscript{+/1}), 80.4% (SE ± 4.3, P < 0.005) for TfAP-2\textsuperscript{OE}, compared to 32.2% (SE ± 5.6) for controls. Feeding TfAP-2\textsuperscript{OE} males the CCK antagonist reduced their activity to control levels (25.6%, SE ± 3.7, P < 0.34) (Figure 7).

**Discussion**

Our data reveals that the *Drosophila* homologues for TFAP2B and KCTD15, TfAP-2 and Tiwaz, 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 insulin-producing cells (IPCs), of the CCK homologue *Dsk*, a neuropeptide known to influence feeding behaviour (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 signalling through a CCK-like pathway.

Our initial question was to determine if the *Drosophila* obesity-linked homologues could be linked to a pathway(s) known to regulate behaviour. In mice, *Tfab2b* regulates noradrenaline signalling (Hong et al. 2008; Hong et al. 2011), thus we addressed the octopaminergic system, the *Drosophila* equivalent (Roeder 2005). When *Drosophila* males are first introduced, they perform aggressive behaviours in order to establish a dominance hierarchy (Vrontou et al. 2006). Similar to what was observed when the enzyme Tyramine β-hydroxylase (*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 behaviours (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 behaviour. All of these phenotypes lead us to suggest that TfAP-2 and Twz regulate octopaminergic neuronal signalling; 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) (MÖNNIKES et al. 1997; WANK et al. 1994). CCK signalling 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 the Drosophila CCK homolog, Drosulfakinin (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 signalling (Figure 6). Dsk is expressed in the Drosophila insulin producing cells (IPCs) within a structure homologous to the hypothalamus, known as the Pars intercerebralis (SÖDERBERG et al. 2012). We observed that overexpressing Dsk in the insulin-like cells (IPCs), similar to CCK signalling in mice, induced hyperactivity and aggressive behaviour. 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 signalling is necessary to promote larval body wall muscle contractions involved in stress-induced locomotory escape behaviour (CHEN et al. 2012). It was also shown that Dsk and CCKLR-17D1 are required for proper
neuromuscular junction (NMJ) formation in developing the Drosophila (Chen and Ganetzky 2012). Intriguingly, we could rescue aggressive behaviour 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 signalling.

Another intriguing result was that feeding Dsk overexpressing flies the CCK receptor antagonist SR27897 induced severe intermittent jump behaviour. Previously it was observed that feeding flies the GABA\textsubscript{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 signalling. Overexpression of Dsk could activate both receptors, having no overall effect on GABA signalling. 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 we observed. This is very speculative and will need to be tested in future.

An intriguing task for the future will be to understand how neuropeptides such as CCK and Dsk, known to modify feeding behaviour (Ritter et al. 1999; Söderberg et al. 2012), regulate disparate behaviours, 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).
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REFERENCES


Li, Q., X. DENG and P. SINGH, 2007 Significant increase in the aggressive behavior of transgenic mice overexpressing peripheral progastrin peptides: associated changes in CCK2 and serotonin receptors in the CNS. Neuropsychopharmacology 32: 1813-1821.


MÖNNIKES, H., G. LAUER and R. ARNOLD, 1997 Peripheral administration of cholecystokinin activates c-fos expression in the locus coeruleus/subcoeruleus nucleus, dorsal vagal complex


FIGURE LEGENDS

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 (subesophageal ganglia: SOG), as well as in two neurons found on either side of the SOG (arrows in A). (C) TfAP-2 RNAi was expressed in the entire CNS using the Elav-GAL4 driver, DAPI staining (Blue) TfAP-2 expression (Red). The picture was overexposed to reveal any possible TfAP-2 staining, some residual staining was observed in the two neurons that were TfAP-2 positive but GFP negative in A.

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°C or 18°C, to verify the efficiency of the various UAS constructs. (B) Relative expression of possible UAS-TwzRNAi 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)

Figure 3. Disrupting TfAP-2 or Twz expression in Tdc2-specific octopaminergic neurons affects male behaviour. 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 behaviour for all genotypes were determined. All males were between 5-7 days old. The types of behaviours were distributed into three categories, high intensity fighting (HIF), low intensity fighting (LIF) and courtship behaviour (CB) and
the number of each type of behaviour 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 behaviour of 5-7 day old males towards 3-4 day old Csorc wild-type virgin female was recorded over a 10 minute period or until copulation occurred. In all instances the assay was repeated at least 10 times. Male courtship behaviour towards a virgin female (B) Total number of behaviours a male performed, (C) Amount of time, in seconds, before the first courtship behaviour, or latency, was determined, (D) Percentage of time a male spent actively courting (courtship index) was determined. In all figures 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. In all figures different letters indicate similar groups (i.e. ‘a’ is significantly different than ‘b’ or ‘c’ and so on).

**Figure 4.** Inhibiting octopamine signalling affects TfAP-2 induces hyperactivity. (A) Ctrax and Matlab were used to measure both activity and speed of walking of 5-7 day old males for each genotype. Males were put individually into a behavioural assay chamber and monitored for 30 minutes. (n = 20 males; * P<0.05 ** P<0.005 compared with controls, one-way ANOVA with Bonferroni post hoc test for multiple comparisons). 5-7 day old Tdc2-GAL4; TfAP-2OE males were feed varying concentrations of the octopamine antagonists phentolamine or epinastine for 24 hours before a activity test was performed. (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).
**Figure 5.** TfAP-2 and Twz regulate genes involved in octopamine signalling. (A) Simplified schematic diagram of the pathway involved octopamine production and secretion. (B-D) The transcript levels of various genes are shown as follows: (A) Tdc2, (B) Tbh, (C) Vmat. RNA was collected from the heads of 5-7 day old males for each genotype. qPCR was repeated at least 7 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)

**Figure 6.** Disrupting Dsk signalling affects male behaviour. (A) Total interactions that were either high or low intensity aggression, or courtship behaviour for all genotypes were determined. All males were between 5-7 days old. The types of behaviours were distributed into three categories, high intensity fighting (HIF), low intensity fighting (LIF) and courtship behaviour (CB) and the number of each type of behaviour 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 levels in flies where TfAP-2 and Twz expression has been disrupted in octopaminergic neurons. RNA was collected from the heads of 5-7 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).

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

**Figure 8.** Model for possible modulation of aggressive behaviour in *Drosophila*. Twz and TfAP-2 interact in octopaminergic neurons, possibly to regulate TfAP-2 activity. TfAP-2 induces the expression of *Tyrosine β-hydroxylase (Tbh)* and *Vesicular monoamine transporter (Vmat)*, which in turn regulate octopamine production and release from octopaminergic neurons. Octopamine signals to the insulin producing cells (IPC) to induce *Drosulfakinin (Dsk)*. Dsk signals to induce male aggressive behaviour, while inhibiting mating behaviour in males.
Figure 1
Figure 2

A

B

Fold difference

29°C

Tdc2-GAL4

Tdc2-GAL4

18°C

Figure 2
Figure 3
Figure 4

A

Activity and Speed

B

Epinastine

C

Phentolamine

T6c2-GAL4 × TAAR-2ΔE

0 mM 1 mM 3 mM 5 mM

T6c2-GAL4 × TAAR-2ΔE

Activity %

0 20 40 60 80 100

0 1 2 3 4

T6c2-GAL4 × TAAR-2ΔE

Activity %

0 20 40 60 80 100

0 1 2 3 4
Figure 5
Figure 6
Figure 7

![Activity graph](image_url)

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Figure 8