Drosophila Insulin-Producing Cells Are Differentially Modulated by Serotonin and Octopamine Receptors and Affect Social Behavior

Jiangnan Luo1, Oleh V. Lushchak1, Philip Goergen2, Michael J. Williams2, Dick R. Nässel1

1 Department of Zoology, Stockholm University, Stockholm, Sweden, 2 Department of Neuroscience, Uppsala University, Uppsala, Sweden

Abstract

A set of 14 insulin-producing cells (IPCs) in the Drosophila brain produces three insulin-like peptides (DILP2, 3 and 5). Activity in IPCs and release of DILPs is nutrient dependent and controlled by multiple factors such as fat body-derived proteins, neuropeptides, and neurotransmitters. Two monoamine receptors, the octopamine receptor OAMB and the serotonin receptor 5-HT1A, are expressed by the IPCs. These receptors may act antagonistically on adenylate cyclase. Here we investigate the action of the two receptors on activity in and output from the IPCs. Knockdown of OAMB by targeted RNAi led to elevated Dlp3 transcript levels in the brain, whereas 5-HT1A knockdown resulted in increases of Dlp2 and 5. OAMB-RNAi in IPCs leads to extended survival of starved flies and increased food intake, whereas 5-HT1A-RNAi produces the opposite phenotypes. However, knockdown of either OAMB or 5-HT1A in IPCs both lead to increased resistance to oxidative stress. In assays of carbohydrate levels we found that 5-HT1A knockdown in IPCs resulted in elevated hemolymph glucose, body glycogen and body trehalose levels, while no effects were seen after OAMB knockdown. We also found that manipulations of the two receptors in IPCs affected male aggressive behavior in different ways and 5-HT1A-RNAi reduced courtship latency. Our observations suggest that activation of 5-HT1A and OAMB signaling in IPCs generates differential effects on Dlp transcription, fly physiology, metabolism and social interactions. However the findings do not support an antagonistic action of the two monoamines and their receptors in this particular system.

Introduction

Insulin and insulin-like growth factors (IGFs) are evolutionary conserved peptides that regulate development, growth and aspects of physiology in a broad range of animals [1–9]. In Drosophila, eight insulin-like peptides (DILP1–8) have been identified as likely ligands of a single insulin tyrosine kinase receptor [5,6,8,10,11]. In adult Drosophila, the different DILPs, and thus insulin/IGF signaling (IIS), are of vital importance in the regulation of reproduction, metabolic homeostasis, resistance to stress and life span [11–15]. Additionally, attraction to food odors and feeding behavior are modulated by DILPs [16–18]. A cluster of 14 insulin-producing cells (IPCs) in the pars intercerebralis of the brain express DILP2, 3 and 5, which are secreted into the circulation via axon terminations in the corpora cardiaca, anterior aorta, foregut and anterior midgut as well as the crop [11,12,19].

In adult flies the activity in IPCs and thus production and release of DILPs is controlled by fat body-derived diffusible molecules such as DILP6 and the leptin-like cytokine Unpaired 2 (Upd2) [20,21]. Systemic release of these factors from the fat body is nutrient-dependent. Hence, when the fly feeds the increased levels of circulating carbohydrate and amino acids are sensed by adipocytes in the fat body, which induces signaling to the IPCs. In addition several neurotransmitters such as GABA and serotonin, as well as the neuropeptides corazonin, short neuropeptide F and Drosophila tachykinin [22–27] act on the brain IPCs. Except for the inhibitory transmitter GABA it is, however, not known what triggers the signaling by these substances to the IPCs. A portion of the GABergic system in the pars intercerebralis seems to be inactivated by circulating Upd2 after feeding and thereby tonic inhibition of the IPCs is lifted (via the action of Jak/Stat signaling) which facilitates DILP release [20].

Another neurotransmitter implicated in the regulation of IPC activity in Drosophila is the biogenic amine octopamine [28]. Activation of an octopamine receptor, OAMB (OAMB-K3 splice form), in IPCs was found to promote sleep in Drosophila by stimulating adenylate cyclase and production of cyclic AMP (cAMP) [28,29]. However, there is no evidence that sleep modulation is caused by release of DILPs from the IPCs. In fact, a later paper showed that insulin signaling has no effect on the sleep/wake state, whereas increased octopamine signaling to IPCs lead to increased circulating triglyceride levels which is DILP dependent [30]. Thus, octopamine and OAMB seem to play a role in activating IPCs, and this activation produces responses in sleep and metabolism, but only the latter is insulin-dependent. Here we decided to further investigate the role of OAMB in IPC activation and subsequent insulin signaling using metabolism and behavior as readouts.
Previously we demonstrated a role of one of the serotonin receptors, 5-HT1A, in regulation of Drosophila IPCs [22]. This receptor commonly inhibits adenylate cyclase (AC), and thus decreases levels of cyclic AMP (cAMP) and thereby diminishes activity of protein kinase A (PKA) (See reviews [31–33]). The OAMB receptor (K3 splice form) can both increase intracellular Ca2+ and activate adenylate cyclase and thus elevate cAMP and activate PKA [28,34,35]. The possible convergence of the two monoamine receptors on adenylate cyclase signal transduction lead us to compare the action of OAMB and 5-HT1A on IPCs. Do the two receptors mediate antagonistic activity in IPCs via opposite actions on adenylate cyclase or do they act on independent intracellular systems?

To test this we employed the Gal4-UAS system [36] to direct OAMB and 5-HT1A-RNAi to IPCs and analyzed the effect on transcript levels of Dilp2, 3 and 5 and on carbohydrate metabolism and stress responses. We found that manipulations of the two receptors had differential effects on Dilp transcription, and mostly also in the other assays. Since both serotonin and octopamine are known to regulate social behavior in flies [37–42] we furthermore investigated the role of IPCs on aggressive and courtship behaviors by manipulating OAMB and 5-HT1A in IPCs.

Our results do not support that octopamine and serotonin act antagonistically on the IPCs but suggest that activation of OAMB and 5-HT1A in these cells induce differential effects on Dilp transcription, metabolism, stress resistance as well as male-male and male-female interactions.

Results
Processes from octopaminergic neurons superimpose IPC branches

In a recent study it was shown that the IPCs express the OAMB-K3 receptor splice form, as determined by RT-PCR on RNA extracted from single neurons, and that a small set of octopamine-producing neurons, designated ASM, send axon processes to the IPCs [28]. The ASMs are a subpopulation of the Tdc2-Gal4 expressing neurons [28]. However, the octopamine distribution in relation to the presumed dendrites of IPCs was not revealed in detail. With application of Dilp2 antiserum to brains with octopaminergic and tyraminergic neurons marked by Tdc2-Gal4 driven GFP, we found that some GFP-labeled branches superimpose those of the IPC dendrites in the pars intercerebralis of the brain (Fig 1). In the following we rely on published data [28] that the octopaminergic ASM neurons mediate activation of IPCs. Expression of the 5-HT1A receptor in IPCs, and the possible intervention of IPCs by serotoninergic neurons were shown previously [22].

Knockdown of 5HT1A or OAMB receptors in IPCs does not change cell size

To study the roles of the 5HT1A and OAMB receptors in IPCs, we targeted RNAi for the two receptors to these cells using the Gal4-UAS technique [36]. First we determined the efficiency of the UAS-5-HT1A-RNAi (referred to as 5HT1A-RNAi) and the UAS-OAMB-RNAi GD lines (referred to as OAMB-RNAi), by crossing these flies to the pan-neuronal elav-Gal4 driver, and performing qPCR to measure the level of 5-HT1A and OAMB transcript (Fig. 1D, E). 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 to obtain maximal expression from the Gal4-UAS system [36]. Compared with elav-Gal4 heterozygous controls (SEM±0.05), 5HT1A-RNAi flies had 0.36-fold (SEM±0.06, P<0.005) of the normal 5HT1A RNA expression levels, while OAMB-RNAi flies had 0.32-fold (SEM±0.06, P<0.005) of the normal OAMB RNA levels (Fig. 1D, E).

We next used a Dilp2-Gal4 driver to specifically target receptor RNAi to the IPCs, using the same two RNAi lines. Since manipulations of IPC activity can lead to alterations of cell growth [43] we determined the effects of receptor-RNAi on the size of cell bodies of IPCs. No changes in cell size were observed after knockdown or either receptor (Fig. S1A,B). Therefore, we can exclude the possibility that physiological or behavioral phenotypes observed in subsequent experiments are due to gross developmental effects on cell morphology.

Figure 1. Branches of octopaminergic neurons superimpose those of insulin producing cells (IPCs). A–C Dissected adult brains were stained with rabbit anti-DILP2 (magenta) and octopaminergic and tyraminergic neurons marked by Tdc2-Gal4 driving GFP (green). Cb, cell bodies of IPCs; Dendr1 and dendr2, presumed dendrites of IPCs. Scale bar = 20 μm. A1–C1 Magnification of regions of interest in A–C. D and E The efficiencies of the UAS-5-HT1A and UAS-OAMB-RNAi (UAS-OAMB-RNAi GD) were determined by using the pan-neuronal Elav-Gal4 driver and monitoring extracted RNA by qPCR. The transcript value for Elav-Gal4>elav-Ga4 was set as 1 in both assays. A significant knockdown (about 60–70% reduction) of 5-HT1A and OAMB transcript was seen (***p<0.001 to both parental controls, One-way ANOVA, n = 2 samples for each genotye).

doi:10.1371/journal.pone.0099732.g001
Knockdown of 5-HT1A or OAMB affects Dilp transcription in IPCs

To test for effects of 5-HT1A and OAMB knockdown on IPCs function we utilized qPCR to analyze Dilp2, 3 and 5 brain transcript levels after targeted receptor RNAi in IPCs. Knockdown of 5-HT1A in IPCs resulted in a significant increase in brain Dilp2 and Dilp5, but not Dilp3 transcript levels (Fig. 2). This is partly consistent with our earlier data where Dilp2 immunoreactivity increased after 5-HT1A knockdown both in fed and starved flies. On the other hand, knockdown of OAMB in IPCs (using OAMB-RNAi GD line) caused a significant increase in Dilp3, but not Dilp2 and Dilp5 (Fig. 2). These data indicate that each receptor would upon activation reduce specific Dilp transcript levels. However, it is important to note that the targeted RNAi does not mimic inactivation of the IPCs (or the two receptors), but induces a partial downregulation of receptor expression levels, which probably renders the IPCs slightly less responsive to octopamine or serotonin. The differential actions of serotonin and octopamine on IPCs in Dilp transcription might explain the different effects of OAMB and 5-HT1A knockdown in some assays described in the following sections. It should be noted that we have no data to show effects on DILP release and therefore no clear readouts for alterations of IIS. Furthermore, these findings do not suggest clear-cut antagonistic actions of the two receptors since no decrease in Dilp RNA was detected for either receptor.

Knockdown of OAMB in IPCs causes increased resistance to starvation

Since previous evidence [22] suggests that modulation of activity in IPCs results in changes in stress tolerance, we measured the starvation resistance in flies with diminished IPC OAMB receptor levels. Compared to controls, the flies with reduced OAMB expression displayed a significant increase in survival when exposed to starvation (Fig. 3A). Two independent UAS-OAMB-RNAi fly lines were tested in this assay and the same phenotypes were obtained. One of these RNAi lines (UAS-OAMB-RNAi GD), used in all experiments described above, produced a more prominent effect when crossed with the Dilp2-Ga4, and was therefore used also in subsequent experiments. Previously similar experiments were performed after 5-HT1A RNAi in IPCs and resulted in decreased starvation resistance [22]. Thus OAMB and 5-HT1A knockdowns in IPCs produce opposite effects on starvation resistance.

Knockdown of OAMB or 5-HT1A in IPCs both cause increased resistance to oxidative stress

It was shown that insulin signaling plays a role in resistance to oxidative stress; flies with ablated IPCs are more stress resistant [14]. We found that flies with diminished OAMB in IPCs were more resistant to oxidative stress induced by feeding 20mM paraquat in standard food (Fig. 3B). Also flies where 5-HT1A was knocked down in IPCs displayed increased resistance to oxidative stress (Fig. 3B). Based on earlier findings [14] these effects on oxidative stress resistance suggest that both OAMB and 5-HT1A activation in IPCs stimulate insulin signaling, or that the stress resistance phenotype is caused by other mechanisms mediated by the IPCs.

Knockdown of OAMB or 5-HT1A in IPCs results in opposite effects on food intake

Insulin signaling is known to modulate food search and feeding behavior in flies [16–18,44,45]. We determined whether food intake is affected by diminishment of OAMB or 5-HT1A in IPCs by testing flies in a capillary feeding (CAFE) assay over 96 h. After 5-HT1A-RNAi a slight, but significant, decrease in food consumption was observed the third day (Fig. 4A). On the other hand OAMB knockdown resulted in increased food intake days 2–4 (Fig. 4B).

Knockdown of 5-HT1A or OAMB in IPCs affect circulating and stored carbohydrate levels

Previous studies have shown that DILPs produced by the brain IPCs are important in the regulation of carbohydrate and lipid homeostasis [12,14,46]. Therefore we assayed carbohydrate levels to determine whether interference with OAMB and 5-HT1A in IPCs affects systemic insulin signaling and metabolism. We measured the hemolymph levels of glucose and trehalose in ad lib fed male flies of different genotypes. Flies with 5-HT1A knocked down in the IPCs displayed significantly increased hemolymph glucose levels, but not trehalose compared to parental controls (Fig. 5A, B). Knocking down OAMB did not influence circulating glucose or trehalose levels (Fig. 5A, B). Next we

Figure 2. Dilp transcript levels in the brain are affected by 5-HT1A and OAMB knockdown in IPCs. A–C Relative expression of Dilp2, 3 and 5 transcripts after 5-HT1A-RNAi and OAMB-RNAi (UAS-OAMB-RNAi GD) in IPCs was measured from head extracts of fed flies. The value for w1118>5-HT1A-RNAi was set to 1. The Dilp2 and 5 transcripts increase significantly after 5-HT1A knockdown, and Dilp3 transcript increases after OAMB knockdown (one way ANOVA; **p<0.01, *p<0.05, ns, not significant; n = 3 samples for each genotype). doi:10.1371/journal.pone.0099732.g002
measured whole body trehalose and glycogen in the same experimental flies (normally fed) and found that 5-HT1A knockdown in IPCs increase both stored carbohydrates, but OAMB-RNAi had no effect (Fig. 5C, D). Thus OAMB knockdown in IPCs does not seem to affect carbohydrate homeostasis in fed flies.

Knockdown of 5HT1A or OAMB in IPCs has no effect on body weight

Insulin signaling was shown to regulate larval development and growth in Drosophila [11,12]. We therefore tested whether knocking down 5-HT1A or OAMB in IPCs affected the body weight as measured in adult male flies. No effect was seen on body weight (Fig. S1C), similar to effects of GABAB receptor knockdown in IPCs [27]. For the serotonin receptor this could be explained by the finding that 5-HT1A appears to be absent in larval IPCs when growth occurs [22]. It is not known when OAMB starts to be expressed in IPCs.

5-HT1A and OAMB regulate social behavior

It is known that serotonin and octopamine regulate aggression and mating behaviors in Drosophila [37–41,47]. It was furthermore suggested that octopamine could modulate aggressive behavior via neurons releasing the cholecystokinin-like peptide drosulfakinin (DSK) [40]. One set of neurons that express DSK are the brain IPCs [45]. Thus, we tested the effects of 5-HT1A and OAMB knockdown in IPCs on social behavior in male flies.

Aggression analysis experiments were executed by placing pairs of 5–7 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. Low intensity fighting (LIF) was scored as side-by-side pushing with a leg (side-fencing), face-to-face pushing with one leg (fencing), 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

![Figure 3. Stress responses after knockdown of octopamine receptor OAMB and serotonin receptor 5-HT1A in IPCs.](image_url)

**A** Reduction of octopamine receptor OAMB level in IPCs increases resistance to starvation (seen by extended survival). OAMB was diminished in IPCs by driving two different OAMB-RNAi lines with Dilp2-Gal4. The Dilp2-Gal4->OAMB-RNAi GD flies showed about 50% percent increase of median lifespan compared with controls (***p<0.001 to both parental controls; Log rank test, Mantel-Cox; n = 120 for each genotype). We used the OAMB-RNAi GD lines hereafter for all other experiments. In an earlier study it was shown that 5-HT1A knockdown decreased starvation resistance [22]. **B** Reduction of octopamine receptor OAMB or serotonin receptor 5-HT1A levels in IPCs increases resistance to oxidative stress induced by feeding male flies 20mM paraquat in standard food. Both receptor knockdowns result in flies that display significantly increased survival under paraquat exposure (**p<0.001 to both parental controls, Log rank test, Mantel-Cox; n = 60–90 for each genotype).

doi:10.1371/journal.pone.0099732.g003

![Figure 4. Knockdown of OAMB or 5-HT1A in IPCs resulted in opposite phenotypes in a capillary feeding assay (CAFE).](image_url)

**A** Diminishing 5-HT1A in IPCs (Dilp2-Gal4->5-HT1A-RNAi) significantly decreases food intake only during day 2. The units in the Y-axis are mm food consumed from the calibrated capillary tube (total volume 5 μL; see methods). The graph shows cumulative intake of food over 4 days (**p<0.05, n = 5–10 flies for each genotype in 3 replicates, One-way ANOVA). **B** Reduction of OAMB level in IPCs (Dilp2-Gal4->OAMB-RNAi) significantly increases food intake from the second day onwards (**p<0.001, ***p<0.0001, n = 5–10 flies for each genotype in 3 replicates, One-way ANOVA).

doi:10.1371/journal.pone.0099732.g004
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 towards the other fly (abdomen bending).

For the HIF behaviors there was significant difference between controls, 5-HT1A and OAMB knockdown males (P<0.05). Unlike controls, OAMB-RNAi males did not perform any wing threats (Table S1), while 5HT1A-RNAi males performed significantly fewer lunging behaviors than controls (Table S1). Interestingly, when LIF behaviors were compared, 5HT1A-RNAi males performed significantly more wing flicks and side fencing over a 20 min fighting bout than control males (Table S1). While the percentage of LIF behaviors performed by OAMB-RNAi males was significantly lower than controls (Fig. 6A), this was most likely due to the significant increase observed in courtship behaviors, since the actual number of LIF behaviors was not significantly different from controls (Table S1). Finally, knocking down OAMB had a significant effect on all scored mating behaviors, especially singing (Fig. 6A and Table S1).

Next we determined whether 5-HT1A and OAMB also regulate male behavior towards virgin females. To test this, males were paired with wild-type virgin females and two aspects of male-female courtship were measured: latency and courtship index (see Materials and Methods). No effect was observed on courtship index for either receptor knockdown (Fig. 6B). When 5-HT1A was knocked down there was a substantial decrease in latency (45.4 seconds, SEM±6.4) compared to either Dilp2-GAL4/+ (179.9 seconds, SEM±29.4) or 5-HT1A-RNAi/+ (172.8 seconds, SEM±16.4) controls (Fig. 6C). No significant effect was observed when OAMB was knocked down.
Discussion

Our study shows that octopamine and serotonin differentially modulate the activity of brain IPCs in Drosophila via the receptors OAMB and 5-HT₁A. The physiological readout of diminishing expression of either receptor in IPCs is complex and does not suggest convergence of the two receptors on the same downstream signaling cascade and also indicates involvement of IPC outputs additional to systemic IIS. Also the effects of targeted receptor-knockdown on social behavior may suggest that the two receptors act on independent pathways. We first discuss the effects of diminishment of OAMB and 5-HT₁A on physiology that may be regulated by IIS.

We find that knockdown of OAMB in IPCs affects some aspects of physiology that may be diagnostic of decreased systemic IIS, such as increased starvation and oxidative stress resistance and increased food intake (see Table 1). Surprisingly, however, OAMB-RNAi induced increased Dilp3 transcription (and no effects on Dilp2 and 5). These findings would suggest that the activation of OAMB in IPCs in normally fed flies decreases Dilp3 production, but stimulates some aspects of systemic IIS or produces phenotypes reminiscent of increased IIS. We did not detect any effect of OAMB knockdown on carbohydrate metabolism. We did not test triacylglyceride (TAG) levels here, but using a crude technique [22] to estimate of lipid levels in flies with OAMB knocked down in IPCs, we recorded a significant lipid decrease both in fed flies and flies starved for 24 h (not shown). A previous study found that OAMB mutants display decreased TAG levels, and activation of octopamine producing neurons increases TAG [30]. These authors also propose that the effect on TAG levels is via the IPCs since activation of octopamine neurons in a Dilp2, 3 mutant background leads to less increase in TAG. In our earlier study we found that 5-HT₁A knockdown in IPCs results in decreased levels of stored lipid in fed and starved flies; the same was found in 5-HT₁A mutant flies [22]. Although the lipid assays performed in the two studies were different, it seems that knockdown of both OAMB and 5-HT₁A lead to decreased lipid levels. Possibly this suggests that activation of these receptors triggers increases stored lipids.

Although the role of OAMB in regulation of IIS remains somewhat unclear, our present data from manipulations of the 5-HT₁A in IPCs, combined with those of an earlier report [22], are more consistent with effects on systemic IIS. We show that 5-HT₁A knockdown increases Dilp2 and 5 transcripts, reduces resistance to starvation, increases oxidative stress resistance, diminishes food intake and elevates levels of circulating and stored carbohydrates (Table 1). Taken together these findings indicate that targeted 5-HT₁A knockdown increases systemic IIS, and thus the activation of this receptor should decrease IIS. However, as seen in Table 1 our data from physiological readouts may suggest more complex effects of the receptor manipulations in IPCs.

One question raised in the present study was whether octopamine and serotonin act antagonistically on a shared downstream signal cascade to regulate IPC function. This was prompted by the possibility that both OAMB and 5-HT₁A act on adenylate cyclase, cAMP and PKA [28,31–35]. We therefore compared the effects of receptor RNAi in several assays. In three of these we saw distinct effects of knocking down the two receptors: starvation resistance, food intake and transcription of Dilp2, 3 and 5 (Table 1). In assays of carbohydrates only 5-HT₁A knockdown increased levels and no effect was seen with OAMB. Actually only in two assays, food intake and starvation resistance, did we observe opposite phenotypes after diminishing the two receptors. None of the receptor knockdowns diminished Dilp transcript levels, instead they increased RNAs of mutually exclusive Dilps (Table 1). Thus it is not possible to assign clear antagonistic effects of the two receptors in IPCs. One dilemma is that for each receptor we obtained incomplete or conflicting results. The effects of 5-HT₁A-RNAi on metabolism suggest decreased IIS, which indicates that activation of the receptor should stimulate DILP release and increase IIS. However, the starvation resistance assay produced a phenotype opposite to the expected one after diminishment of 5-HT₁A [22]. In this context it should be noted that starvation resistance in the experimental flies could be caused by factors other than diminished IIS, such as increased locomotion or altered feeding [48,49].

In the CAFE assay we noted opposite effects on food intake for the different receptor knockdowns. The effects of IIS on feeding

Table 1. Effects of OAMB and 5-HT₁A knockdown in various assays.

| Assays with adult flies | Effect | Likely effect on insulin signaling¹ (output) | Different effects |
|------------------------|--------|-------------------------------------------|------------------|
|                        | OAMB-Ri | 5-HT₁A-Ri | OAMB-Ri | 5-HT₁A-Ri |                           |
| Starvation resistance  | Increase | Decrease | Decrease | Increase | Yes*                  |
| Food intake (CAFE)     | Increase | Decrease | Increase | Decrease | Yes*                  |
| Glucose (in circulation) | NE | Increase | NE | Decrease | Yes                  |
| Trehalose (in circulation) | NE | NE | NE | NE | No                  |
| Glycogen (stored)      | NE | Increase | NE | Decrease | Yes                  |
| Trehalose (stored)     | NE | Increase | NE | Decrease | Yes                  |
| Dilp2 transcript       | NE | Increase | NE | Decrease | Yes                  |
| Dilp3 transcript       | Up | NE | Increase | NE | Yes                  |
| Dilp5 transcript       | NE | Up | NE | Increase | Yes                  |
| Oxidative stress resistance | Increase | Increase | Decrease | Decrease | No                  |
| IPC cell body size     | NE | NE | NE | NE | No                  |
| Body weight            | NE | NE | NE | NE | No                  |

NOTES: ¹ This assumes that targeted receptor RNAi is similar to inactivating the receptor, which of course is not entirely correct. NE, no effect; * indicates opposite effect for the two knockdowns.

doi:10.1371/journal.pone.0099732.t001
are not clearly established in *Drosophila*. Some experiments suggest that silencing of the IPCs diminishes appetite and food intake when food has low calorie content [44,40]. We showed in a recent study that silencing IPCs by expression of a constitutively active hyperpolarizing K-channel (Otk) increased intake of caffeine-spiked or sugar-free food [45], and conversely conditional activation of the IPCs with a temperature inducible TrpA1 channel diminishes food intake [J. Luo, Y. Liu and Nasell, in prep]. In our present experiments 5-HT1A knockdown in IPCs decreased food intake slightly. Thus, our data might indicate that serotonin receptor knockdown increases IIS and thereby diminishes feeding, and OAMB deficiency causes the opposite phenotype.

The effects on social behavior of OAMB and 5-HT1A knockdown in IPCs are partly differential. Among the male interactions high intensity fighting is reduced by both knockdowns, low intensity fighting is up-regulated after 5-HT1A-RNAi and male-male courtship is up-regulated by OAMB-RNAi. In male-female courtship we only recorded a decrease in courtship latency for 5-HT1A-RNAi. There is no direct evidence that IIS affects aggression or courtship behaviors, although sexual receptivity and sexual attraction in female flies was shown to involve IPCs and IIS, respectively [50,51], and aggression may depend on activity in neurons of the pars intercerebralis [52]. However octopamine was shown to regulate aggression in *Drosophila* via the cholecystokinin-like peptide drosulfakinin (DSK) [40]. DSK was detected in a subpopulation of the larval and adult IPCs [45,53]. Thus, it is possible that the effects of manipulating OAMB in IPCs are caused by changes in DSK signaling rather than IIS, both in social behavior and physiology.

The action of OAMB and 5-HT1A on IPCs may be complicated by the fact that OAMB-K3 is known to also activate intracellular Ca2+ in *Drosophila* [34,35]. Thus, OAMB activation may cause more complex responses in IPCs including both Ca2+ and adenylyl cyclase. Another factor that induces additional complexity is the role of OAMB in IPCs in regulation of sleep/wake activity, independent of IIS [30]. Also serotonin is implicated in sleep [54,55] and the possible role of IPCs in mediating this action has not been investigated. Thus, if the IPCs play roles in regulatory activities other than systemic IIS, we might expect that phenotypes obtained after manipulating the two monoamine receptors are complex.

There are several important questions for the future. What are the triggers of octopamine and serotonin action on IPCs and how are the IPCs integrated into octopaminergic and serotonergic modulatory pathways? Octopamine has been extensively investigated in insects and crustaceans and is known to act as a neuromodulator and neurohormone with pleotropic functions, including roles in modulation of muscle and neurons, appetite, ovulation, aggression, sleep, and learning and memory [27,34,39,56,57,58]. Octopamine is also involved in modulation of flight and escape jumping in *Drosophila* [56,59]. Since octopamine seems central in modulation of energy demanding activities a role in regulation of IPCs and IIS is not surprising. Serotonin is also known to play multiple roles in *Drosophila* physiology and behavior: visual and olfactory learning [60,61], courtship and mating [38,62], aggression [37,47], sleep and circadian activity [34,55], olfaction [63] and feeding [64]. It is therefore important to design experiments to reveal which octopaminergic and serotonergic pathways that act on the IPCs and under what conditions they do so.

In summary, we established differential effects of OAMB and 5-HT1A actions on IPCs in flies that suggest that octopamine and serotonin play distinct roles in modulation of these important neurosecretory cells. However, our data indicate that each receptor may trigger complex activity in the IPCs, some of which seem not to affect IIS.

### Materials and Methods

#### Fly strains and husbandry

All flies were reared at 25°C on standard yeast, corn meal agar medium [according to Bloomington *Drosophila* Stock Center (BDSC), Bloomington, IN] under 12:12 h light:dark conditions. Flies with other original genetic backgrounds were backcrossed into w1118 background for four generations before experiments, and w1118 flies were used as controls in crosses in all experiments. Fly stocks for behavior experiments were maintained on a DJ mix (M. D. Fisher Scientific) containing sucrose, corn meal, 10% yeast, agar, benzoic acid, methyl paraben and propionic acid, and maintained at 25°C, 60% humidity, on a 12:12 light:dark cycle. To delay Gal4 expression flies crossed to a Gal4 driver were kept at 18°C, and once the progeny eclosed they were shifted to 29°C for at least 5–7 days before any behavior assays were performed.

The following Gal4 lines were used: Ttk2-Gal4 [65] (J. Hirsh, Charlottesville, VA), Dlp2-Gal4 [12] (E. Rulifson, Stanford, CA) and elsal-Gal4 (BDSC). We used the UAS lines: UAS-OAMB-RNAi [2 different lines with stock ID 2861 and 106511] and UAS-5-HT1A-RNAi [stock ID 106994; see [22]] from the Vienna *Drosophila* RNAi Center (VDRC), Vienna, Austria, and UAS-mcd8-gfp are from BDSC. For mating behavior females of a CSORC strain was used. This is a lab wild type strain created by crossing Canton-S and Oregon-R wild type strains (BDSC).

#### Antisera and immunocytochemistry

For immunocytochemistry adult *Drosophila* heads were dissected in 0.1 M sodium phosphate buffer (PB), pH 7.4 and fixed in ice-cold 4% paraformaldehyde in 0.1 M PB for 2-4 h and dissected adult brains were used for whole mount immunocytochemistry.

Incubation with primary antisera for whole mount tissues was performed for 48 h at 4°C. The following primary antisera were used: rabbit antiserum to *Drosophila* insulin-like peptide 2 (anti-DIPE2) at 1:4000 (gift from M. Brown, Athens, GA), rabbit and mouse anti-GFP (Invitrogen) were used at 1:1000. For detection of primary antiserum Cy3-tagged goat anti-rabbit antiserum (Jackson Immuno Research) and Alexa-488 tagged goat anti-mouse (Invitrogen) were used at 1:1000.

#### Body weight

For each genotype at least three groups of 10 male flies were weighed on a Mettler MT5 Microbalance (Mettler Toledo, Switzerland) to obtain wet weight, and the average weight was calculated.

#### Stress assays

Male and female flies (4–6 d old) were collected for starvation experiments. Flies were placed individually in 2 ml glass vials with 500 µl of 0.5% aqueous agarose and dead flies were monitored every 12 h. These starvation experiments were run in three replicates with at least 30 flies of each genotype per replicate.

For the oxidative stress assay, 20–30 flies were placed into vials with 5 ml of standard food containing 20 mM paraquat (methyl viologen, Sigma, St Louis). Dead flies were recorded every 4 h or 12 h. These oxidative stress assays were run in two replicates. In all the above experiments survival curves and statistics (Log rank test; Mantel-Cox) were made using Prism GraphPad 5.0.
Assays of carbohydrates

Male flies (4–6 days old) were used to measure concentrations of circulating glucose and trehalose together with stored glycogen and whole body trehalose. Pre-weighted flies were decapitated and hemolymph was collected by centrifugation (3000 g, 6 min). Hemolymph was used to measure circulating glucose and trehalose whereas whole bodies were used for determination of glycogen and stored trehalose. All parameters were measured with a glucose assay kit involving glucose oxidase and peroxidase (Liquick Cor-Glucose diagnostic kit, Cormay, Poland). Trehalose was converted to glucose by porcine kidney trehalase (Sigma T9770) and glycogen by amyloglucosidase from Aspergillus niger (Sigma 10115). Glucose and trehalose are expressed as concentration in hemolymph whereas glycogen and body trehalose are given as amount per wet weight. All genotypes were tested in 3 independent replicates (15–20 flies of each genotype in each sample) and one-way ANOVA was used to compare differences between genotypes.

Capillary feeding (CAFE) assay

The capillary feeding (CAFE) assay was conducted according to Ja and others [66] with slight changes. Male flies were placed into 1.5 ml Eppendorf tubes with an inserted 5 μl capillary tube with 5% sucrose, 2% yeast extract and 0.1% propionic acid. Three food-filled capillaries were inserted as controls in identical tubes without flies. The final consumption of food was determined as the diminished food level (in mm) minus the average diminishment in control capillaries (due to evaporation). Daily food consumption was measured every 24 h and calculated cumulatively over 4 consecutive days. These experiments were run in three replicates with 10 flies of each genotype for each replicate.

Quantitative real-time PCR (qPCR)

Relative amounts of Dilp 2, 3 and 5 RNA in heads of male flies were measured by qPCR. RNA was isolated from 4 biological replicates of each genotype tested. One μg of total RNA was used for cDNA synthesis. cDNA was synthesized in triplicates, which were subsequently pooled and diluted for qPCR. Expression of genes of interests was measured relative to that of the housekeeping gene Actin88 (Act) using an ABI Prism 7000 instrument (Applied Biosystems) and a SensiFAST SYBR Hi-ROX Kit (Bioline) under conditions recommended by manufacturer. Each analytical and standard reaction was performed in three technical replicates. The levels of Dilp2, 3 and 5 and Act were measured with the following primer pairs (all 5’ to 3’): Dilp2F: AGCAAGGCTTTGTCCTCATTCTC and Dilp2R: ACACCATATCGACCTGGTTG; Dilp3F: TGTGTGATGGCTTCACGCAATG and Dilp3R: CACTCAACAGTCTTTCAAGGG; Dilp5F: GAGGCACCTTGGGCCTATTC and Dilp5R: CATGTGGTGAGATTCGGAGC; Act88F: AGGGTGTGATGGTGGGTATG and Act88R: CATGTGGTGAGATTCGGAGC; TAPR1F: CATGTGGTGAGATTCGGAGC; TAPR1R: CATGTGGTGAGATTCGGAGC.

For analysis of the efficiency of the two RNAi lines by qPCR we used a slightly different protocol. Relative expression levels of three housekeeping genes (EF-1, Rp49 & RpL11) and of the genes of interest were determined with qPCR. Each reaction, with a total volume of 20 μl, contained 20 m Tris/HC1 pH 9.0, 50 mM KC1, 4 mM MgCl2, 0.2 mM dNTP, DMSO (1:20) and SYBR Green (1:50000). 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 was included on each plate. Analysis of qPCR data was performed using MyIQ 1.0 software (Bio-Rad) as previously reported [67]. Differences in gene expression between groups were analyzed with ANOVA followed by Fisher’s LSD test where appropriate. P<0.05 was used as the criterion of statistical significance. The following primers were used to amplify reference housekeeping genes: EF-1F: 5'-GGCTTGGTTTGATGACAGT-3', EF-1R: 5'-GATCTTCTCCTGTCGCCAATC-3'; Rp49F: CACACAAAACTTACAAAAATGTTGGA-3', Rp49R: 5'-AATCCGGGCTTTGCACATG-3'; RpL11F: 5'-CCATCGGTATCTATGGTCGTTGA-3', RpL11R: 5'-CATGATATTCCTGCGTGGAAACCA-3'. To amplify 5-HT1A the primers were as follows: 5'-GTGGCCCAATACC-3', 5'-ATCTGGTTGCGCAAGTTGCTT-3'. To amplify OAMB the primers were as follows: 5'-TTGCGCGTCTGCTACCGTCT-3', 5'-CGGTCCGATGTGATGGCAGAC-3'.

Image analysis

Specimens were imaged with Zeiss LSM 510 META and Zeiss LSM 780 confocal microscopes (Jena, Germany) using 20x, 40x oil or 63x oil immersion objectives. Confocal images were processed with Zeiss LSM software for either projection of z-stacks or single optical sections. Images were edited for contrast and brightness in Adobe Photoshop CS3 Extended version 10.0. For cell size determination, the outline of cell body was delineated manually and its area determined using Image J [from NIH, Bethesda, MD, USA (http://rsb.info.nih.gov/ij/)]. For each genotype neurons of 8–15 male flies from 3 independent crosses were measured.

Aggression assay

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. Behavioral tests were carried out at room temperature with 60% humidity in cylindrical behavioral chambers (2 cm by 2.5 cm; height x diameter), filled with 1% agarose to 1.5 cm in height to maintain proper 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 minutes, a camera (Panasonic HDC-SD90), positioned above the chamber, was used to record activity for a minimum of 30 minutes. After the 3 min recovery period the behavioral interactions between the males was scored for 20 minutes. Distinct stereotypic aggressive interactions were scored as described by Nilsen et al. [15] and Chen et al. [68]. 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 (side-fencing), face-to-face pushing with one leg (fencing) 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 (boxing), holding the wings at a 30° angle (wing thrash), as well as chasing one another (chasing). Courtship behavior (CB) was marked as one-wing extended at a 90° angle (singing), circling to the posterior (circling), tapping the abdomen (tapping). At least 10 replicates were conducted for each genotype.

Mating behavior 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 behavioral 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 min recovery period the behavioral interactions between the males and females was scored for 20 minutes or until copulation occurred. Scoring of the courtship behaviors was performed as described by Becnel et al. [30]. Latency, courtship index as well as the frequency of mating behaviors 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.

**Statistical Analysis**

All statistical analyses were performed using GraphPad Prism 5.0. Survival data were analyzed by Log rank test with Mantel-Cox post test, for quantification of immunofluorescence, lipid values and body weights we used One-way ANOVA with Tukey’s comparison or two way ANOVA depending on analysis (see Figure legends for details). Data are presented as means and standard error of means (SEM). For behavior analysis we used ANOVA with appropriate post hoc analysis for multiple comparisons.

**Supporting Information**

**Figure S1** Knockdown of 5HT1A or OAMB in IPCs does not affect of cell sizes of IPCs, or body weight. A IPCs were visualized by anti-DILP2 labeling after 5-HT1A and OAMB knockdown in IPCs (using Dlp2-Gal4). No difference in immunolabeling intensity or cell body size was noted. Scale bar 10 μm. B Quantification of cell body size of IPCs after 5-HT1A and OAMB knockdown in IPCs. Adult male flies of 4-6 d age were used (ns, not significant; Student’s T-test, n = brains of 7-9 flies for each genotype). C Adult body weight after 5-HT1A and OAMB knockdown in IPCs. Adult 4-6d old male flies were weighed as described in Materials and Methods. (ns, not significant; one-way ANOVA; n = 40 flies for each genotype).

**Table S1** Effects of OAMB- and 5-HT1A-RNAi on social behavior. Experimental conditions and statistics are described in legend of Fig. 6.

**Acknowledgments**

We thank M. Brown, J. Hirsh, E. Rulifson, The Bloomington Drosophila Stock Center and the Vienna Drosophila RNAi Center for providing fly stocks and reagents.

**Author Contributions**

Conceived and designed the experiments: JL OVL PG MJW DRN. Performed the experiments: JL OVL PG. Analyzed the data: JL OVL PG MJW DRN. Wrote the paper: JL DRN. Edited manuscript (wrote behavior parts): MJW.
34. Kim YC, Lee HG, Lim J, Han KA (2013) Appetitive learning requires the alpha-like octopamine receptor OAMB in the Drosophila mushroom body neurons. J Neurosci 33: 1672-1677.
35. Han KA, Müller NS, Davis RL (1998) A novel octopamine receptor with preferential expression in Drosophila mushroom bodies. J Neurosci 18: 3650-3658.
36. Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401-415.
37. Alekseyenko OV, Lee C, Kravitz EA (2010) Targeted manipulation of serotonergic neurotransmission affects the escalation of aggression in adult male Drosophila melanogaster. PLoS One 5: e10800.
38. Becnel J, Johnson O, Luo J, Nåsell DR, Nichols CD (2011) The serotonin 5-HT7/Dro receptor is expressed in the brain of Drosophila, and is essential for normal courtship and mating. PLoS One 6: e20800.
39. Certel SJ, Leung A, Lin CY, Perez P, Chiang AS, et al. (2010) Octopamine preferential expression in Drosophila cell fates and generating dominant phenotypes. Development 118: 401–415.
40. Williams MJ, Goergen P, Rajendran J, Klockars A, Kasagianinis A, et al. (2014) Regulation of aggression by obesity-linked genes TIAP-2 and Twz through octopamine signaling in Drosophila. Genetics 196: 349–362.
41. Zhou C, Huang H, Kim SM, Lin H, Meng X, et al. (2012) Molecular genetic analysis of sexual rejection: roles of octopamine and its receptor OAMB in Drosophila courtship conditioning. J Neurosci 32: 14201–14207.
42. Baier A, Wiiteck B, Brembo B (2002) Drosophila as a new model organism for the neurobiology of aggression? The Journal of experimental biology 205: 1233–1240.
43. Luo J, Liu Y, Nåsell DR (2013) Insulin/IGF-regulated size scaling of neuroendocrine cells expressing the bHLH transcription factor Dimmed in Drosophila. PLoS genetics 9: e1004052.
44. Cognigni P, Bailey AP, Miguel-Aliaga I (2011) Enteric neurons and systemic signals couple nutritional and reproductive status with intestinal homeostasis. Cell Metab 13: 92–104.
45. So¨derberg JA, Carlsson MA, Nåsell DR (2012) Insulin-producing cells in the Drosophila brain also express satiety-inducing cholecystokinin-like peptide, drosulakin. Frontiers Endocrinol 3: 109.
46. Breya T, Galic M, Belawat P, Naiz K, Hafen E (2002) Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. Curr Biol 12: 1293–1300.
47. Johnson O, Becnel J, Nichols CD (2009) Serotonin 5-HT2(1A-like) receptors differentially modulate aggressive behaviors in Drosophila melanogaster. Neuroscience 158: 1292–1300.
48. Broughton SJ, Slack C, Alie N, Metaxakis A, Bass TM, et al. (2010) DILP-producing median neurosecretory cells in the Drosophila brain mediate the response of lifespan to nutrition. Aging Cell 9: 336–346.
49. Mattaleno MD, Montana ES, Parisky KM, Littleton JT, Griffith LC (2007) The Drosophila ARC homolog regulates behavioral responses to starvation. Mol Cell Neurosci 36: 211–223.
50. Kuo TH, Fedina TY, Hansen I, Dreisewerd K, Dierick HA, et al. (2012) Insulin signaling mediates sexual attractiveness in Drosophila. PLoS Genet 8: e1002684.
51. Sakai T, Watanabe K, Ohashi H, Sato S, Inami S, et al. (2014) Insulin-Producing Cells Regulate the Sexual Receptivity through the Painless TRP Channel in Drosophila Virgin Females. PLoS ONE 9(2): e88173.
52. Davis SM, Thomas AL, Nomie KJ, Huang I, Dierick BA (2014) Tailless and Atrophin control Drosophila aggression by regulating neuropeptide signalling in the pars intercerebralis. Nature communications 5: 3177.
53. Park D, Veenstra JA, Park JH, Taghert PH (2008) Mapping peptidergic cells in Drosophila where DMM fits in. PLoS ONE 3: e1896.
54. Yuan Q, Lin F, Zheng X, Sehgal A (2005) Serotonin modulates circadian entrainment in Drosophila. Neuron 47: 115–127.
55. Yuan Q, Joger WJ, Sehgal A (2006) A deep-promoting role for the Drosophila serotonin receptor 1A. Curr Biol 16: 1051–1062.
56. Yuan Q, Joger WJ, Sehgal A (2008) Serotonin is necessary for place memory in Drosophila. J Neurogenetics 26: 233–244.
57. Yuan Q, Joger WJ, Sehgal A (2008) Serotonin is necessary for place memory in Drosophila. J Neurogenetics 26: 233–244.
58. Kim YC, Lee HG, Seong CS, Han KA (2003) Expression of a D1 dopamine receptor dDA1/DmDOP1 in the central nervous system of Drosophila melanogaster. Gene Expr Patterns 3: 237–245.
59. Brembs B, Christiansen F, Pfluger HJ, Duch C (2007) Flight initiation and muscle activation of neuronal and peripheral serotonin synthesis in Drosophila melanogaster. Neuron 47: 121–149.
60. Dacks AM, Green DS, Root CM, Nighorn AJ, Wang JW (2009) Serotonin modulates olfactory processing in the antennal lobe of Drosophila. J Neurogenet 23: 366–377.
61. Neckameyer WS, Coleman CM, Eade A, Goodwin SF (2007) Compartmentalization of neuronal and peripheral serotonin synthesis in Drosophila melanogaster. Genes, brain, and behavior 6: 756–769.
62. Cole SH, Carney GE, McClung CA, Willard SS, Taylor BJ, et al. (2005) Serotonin is necessary for place memory in Drosophila. Proc Natl Acad Sci USA 102: 5579–5584.
63. Lee G, Vilella A, Taylor BJ, Hall JC (2001) New reproductive anomalies in fruitless-mutant Drosophila males: extreme lengthening of mating durations and infertility correlated with defective serotonergic innervation of reproductive organs. J Neurobiol 47: 121–149.
64. Brembs B, Christiansen F, Pfluger HJ, Duch C (2007) Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. J Neurosci 27: 11122–11131.
65. Sitaraman D, LaFerriere H, Birman S, Zars T (2012) Serotonin is critical for rewarded olfactory short-term memory in Drosophila. J Neurogenetics 26: 233–244.
66. Sitaraman D, Zars T, LaFerriere H, Chen YC, Sable-Smith A, et al. (2008) Serotonin is necessary for place memory in Drosophila. Proc Natl Acad Sci USA 105: 5579–5584.
67. Lee G, Vilella A, Taylor BJ, Hall JC (2001) New reproductive anomalies in fruitless-mutant Drosophila males: extreme lengthening of mating durations and infertility correlated with defective serotonergic innervation of reproductive organs. J Neurobiol 47: 121–149.
68. Dacks AM, Green DS, Root CM, Nighorn AJ, Wang JW (2009) Serotonin modulates olfactory processing in the antennal lobe of Drosophila. J Neurogenet 23: 366–377.
69. Neckameyer WS, Coleman CM, Eade A, Goodwin SF (2007) Compartmentalization of neuronal and peripheral serotonin synthesis in Drosophila melanogaster. Genes, brain, and behavior 6: 756–769.
70. Cole SH, Carney GE, McClung CA, Willard SS, Taylor BJ, et al. (2005) Two functional but noncomplementing Drosophila tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J Biol Chem 280: 14948–14955.
71. Ja WW, Carvalho GB, Mak EM, de la Rosa NN, Fang AY, et al. (2007) Serotonin is necessary for place memory in Drosophila. Proc Natl Acad Sci USA 104: 8253–8256.
72. Zumstein N, Forman O, Nongthomba U, Sparrow JC, Elliott CJ (2004) Distance and force production during jumping in wild-type and mutant Drosophila melanogaster. The Journal of experimental biology 207: 3513–3522.