Interaction between sleep and metabolism in *Drosophila* with altered octopamine signaling

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Running Title: *Independent regulation of sleep and metabolism by octopamine*

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**Background:** Octopamine acts through the insulin-producing cells in the *Drosophila* brain to promote wake.

**Results:** Insulin-like peptides do not mediate effects of octopamine on wake, but contribute to an effect of octopamine on triglyceride levels.

**Conclusion:** Although sleep and metabolic circuitry overlap, octopamine regulates sleep and metabolism independently.

**Significance:** These findings provide insight into the connection between sleep and metabolism.

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SUMMARY

Sleep length and metabolic dysfunction are correlated, but the causal relationship between these processes is unclear. Octopamine promotes wakefulness in the fly by acting through the insulin-producing cells (IPCs) in the fly brain. To determine if insulin signaling mediates the effects of octopamine on sleep:wake behavior, we assayed flies in which insulin signaling activity was genetically altered. We found that increasing insulin signaling does not promote wake, nor does insulin appear to mediate the wake-promoting effects of octopamine. Octopamine also affects metabolism in invertebrate species, including, as we show here, *Drosophila melanogaster.* Triglycerides are decreased in mutants with compromised octopamine signaling and elevated in flies with increased activity of octopaminergic neurons. Interestingly, this effect is mediated at least partially by insulin, suggesting that effects of octopamine on metabolism are independent of its effects on sleep. We further investigated the relative contribution of metabolic and sleep phenotypes to the starvation response of flies with altered octopamine signaling. Hyperactivity (indicative of foraging) induced by starvation was elevated in octopamine receptor mutants, despite their high propensity for sleep, indicating that their metabolic state dictates their behavioral response under these conditions. Moreover, flies with increased octopamine signaling do not suppress sleep in response to starvation, even though they are normally hyper-aroused, most likely because of their high triglyceride levels. Together, these data suggest that observed correlations between sleep and metabolic phenotypes can result from shared molecular pathways rather than causality, and environmental conditions can lead to the dominance of one phenotype over the other.

While trying to address why we sleep, we must also consider the question of what keeps us awake. Many neurotransmitters and neuropeptides are necessary for maintaining normal wakefulness in humans; these include but are not limited to dopamine, histamine, norepinephrine and orexin (1). Since maintaining a waking state involves all the major neurotransmitters and many neuropeptides, it is likely that there is overlap...
between sleep and other biological functions. Understanding this overlap will provide insight into the consequences of sleep deprivation, which is a common feature of modern society.

*Drosophila melanogaster* uses many of the same sleep-regulating neurotransmitters as mammals and has emerged as an excellent model for understanding the molecular control of sleep (2). We recently identified octopamine, the invertebrate homolog of norepinephrine, as a wake-promoting molecule necessary to maintain normal arousal (3,4). Arousal in response to octopamine is mediated largely by neurons in the Pars Intercerebralis (PI), specifically by the neurosecretory cells that produce insulin-like peptides 2 and 3 (*ilp2-3*), the fly homologs of human insulin (5). Since octopamine acts on the *Drosophila* insulin-producing cells to promote wake, and insulin is a major metabolic hormone in both mammals and insects (6,7), it is possible that octopamine functions to control both sleep and metabolism by interacting with the insulin pathway. Indeed, there is considerable evidence indicating a link between sleep and metabolic activity, most notably with respect to sleep times and obesity (8-10). In addition, octopamine signaling modulates metabolic function in other insects (11). The fortuitous finding that octopamine signals through metabolic cells in *Drosophila* provided us with a model we could use to study the relationship between these processes.

In this study, we set out to assay effects of octopamine on metabolism in *Drosophila* and to determine if metabolic signals mediate effects on sleep: wake. We show that altering the activity of the insulin signaling pathway has little effect on total fly sleep and moreover, flies lacking insulin-like peptides 2 and 3 (*ilp2-3* mutants), show normal increases in wake in response to octopamine. These data suggest that ILPs do not mediate the wake-promoting effects of octopamine. Nevertheless, octopamine does play a role in metabolism because decreasing octopamine signaling reduces triglycerides while activating octopaminergic cells increases triglycerides. An *ilp2-3* mutant background largely abrogates the metabolic effects of octopamine, which indicates that octopamine interacts with the insulin signaling pathway to alter metabolism in the fly. This metabolic phenotype is specific for octopamine; other short sleeping flies do not show consistent changes in their triglyceride levels, implying that loss of sleep per se does not lead to increased triglycerides. We also examined the activity of octopamine mutants in response to starvation, since this response typically depends upon metabolic status and involves changes in sleep (12). Surprisingly, animals with decreased octopamine signaling that sleep more under normal conditions are hyperactive while starved, suggesting that metabolic needs outweigh the need for sleep. The behavioral response of flies with increased octopamine signaling also supports the idea that the metabolic phenotype dominates under starvation conditions. These findings suggest a role for octopamine signaling in the control of sleep and metabolism and in the coordination of these processes to achieve overall homeostasis.

**EXPERIMENTAL PROCEDURES**

*Fly Genetics*- The following fly strains were used in this study: UAS-dInR<sup>UAS</sup> (Bloomington #8263), UAS-myrt (13), UAS-AktRNAi (VDRC #2902), UAS-ilp2 (also known as UAS-dilp2) (14), yolk-Gal4 (15), oamb<sup>307</sup> (16), ss<sup>spl</sup> (17); Fumin (18,19), ilp2-3 (Bloomington #30888) and Iso31 (isogenic *w1118* stock; Bloomington #5905). The following lines were outcrossed into Iso31: Tdc2-Gal4 (Bloomington #9313), Ilp2-Gal4 (also known as Dilp2-Gal4) (5), UAS-B16b (NaChBac channel) (Bloomington #9466), UAS-dTrpA1 (20), Elav-Gal4, Elav-GeneSwitch, MB-GeneSwitch, and UAS-<i>me</i><sup>*</sup> (constitutively active PKA) (the last four were reported previously in (21)).

Flies were grown on standard cornmeal-molasses medium as described previously, and maintained at 25°C unless otherwise noted.

*Triglyceride and protein assays*—Triglyceride and protein measurements were performed as described (6). Briefly, individual 4-7 day old mated female flies, entrained in a 12h:12h light:dark (LD) cycle at 25°C for at least 3 days, were collected on ice and homogenized in lysis buffer containing 140 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.1% Triton X and 1X protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). For TrpA1 triglyceride experiments, flies were raised at 18-21°C, shifted to 28°C when they were 0-3 days old, and then tested after 4 days at 28°C to activate the TrpA1 channel (20).
For experiments utilizing the GeneSwitch system, animals were placed on 500 mM RU486 in 1% EtOH in standard medium for 4 days to activate GeneSwitch activity as previously described (21). Triglyceride and protein measurements were made using the Triglyceride LiquiColor kit (Stanbio Laboratory, Boerne, TX) and bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA), respectively, according to manufacturer’s instructions. Thin layer chromatography (TLC) assays were performed as described in (22) with the slight modification that 5 female flies were used instead of 10 male flies.

RNA isolation, cDNA synthesis and quantitative PCR – Total RNA was isolated from abdomens of 3-8d old female flies using Trizol Reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, NY) and random hexamers. Quantitative PCR was performed on an ABI Prism 7000 using Sybr Green (ABI, Life Technologies, Grand Island, NY). Primer sequences used for QPCR were:

- dFAS (sense 5’ CTGGCTGACAAAGATTGTGTG 3’ and antisense 5’ TCGCACAACCAGAGCGTAGTA 3’),
- dACC (sense 5’ AGATGCAGAACGATGTCCGC 3’ and antisense 5’ CTCTTTGTGAAGCAGCTCCG 3’),
- dATPCL (sense 5’ CACGACAGATGTCCAAGCTC 3’ and antisense 5’ CTTGCTCTTCACGTCGGCTAAC 3’),
- bmm (sense 5’ ACGTGAATCTCGGAGTTTG 3’ and antisense 5’ ATGGTGTTCTCGTCCAGAATG 3’),
- whd/dCPTI (sense 5’ GCCAATGTGATTTCCCTGCTTC 3’ and antisense 5’ CTTTGCCCCCTCAGATTTCTCT 3’),
- and rp49 (sense 5’ GACGTTCAGGGACAGATCTCG 3’ and antisense 5’ AAACCGCGTCTGATGAG 3’).

Genes were normalized to rp49 for analysis.

Behavior and starvation experiments – All flies were kept on a 12:12 LD cycle at 25°C except for flies associated with TrpA1 sleep experiments, which were measured for baseline sleep at 21°C and shifted to 28°C at lights on to activate the TrpA1 channel. For these experiments nighttime sleep was plotted instead of total sleep because the increase in temperature affected daytime sleep in control and experimental animals. The wildtype flies used in this experiment (Fig. 1B) contained an ilp2-mcherry construct in their background (to be discussed elsewhere). For starvation experiments, 1-7 day old female flies were placed in 65 X 5 mm tubes containing 5% sucrose and 2% agar and maintained in LD cycles for ~3 days prior to the initiation of starvation and/or high temperature treatment. Locomotor activity was monitored using the Trikinetics DAM system (Trikinetics, Waltham, MA) for one baseline day followed by the entire duration of the starvation protocol. Flies were starved by placing them in tubes containing 2% agar only. Activity levels under starvation were defined as the total number of beam crossings over a specified period of time (1, 3, 6, 12, 24 hrs) following the start of food deprivation (2% agar tubes). Baseline activity was defined as the total number of beam crossings over a period of time the day prior to starvation and corresponds to the same time of day as assessed under starvation. Statistical tests were done as described in figure legends.

RESULTS

Wake promoting effects of octopamine are not mediated by insulin. Since octopamine acts through the insulin-producing neurons of the fly brain to regulate sleep, we asked whether alterations in insulin signaling lead to the changes in sleep seen with octopamine modulation. In *Drosophila*, there are 7 insulin-like peptides (ilps) but only 3 (ilps 2, 3 and 5) are expressed in the octopamine-sensitive PI neurons in the adult fly brain (14,23,24). However, all ILPs signal through a single insulin receptor (dInR) (25,26). Whether this receptor is expressed in the adult brain is controversial, but it is known to be highly expressed in the fat body, the fly equivalent of liver and adipose tissue (27,28). The main action of ILPs in flies is to regulate growth throughout development, although the pathway has also been shown to regulate aging, reproduction and metabolism in adults (6,29,30). However, the contribution of this pathway to the control of sleep is unclear.

To determine whether the wake-promoting effects of increased octopamine are due to increased insulin signaling, we first overexpressed ilp2 in the PI neurons. *ilp2* was chosen since it has the highest homology to human insulin and its
expression can rescue the growth phenotype observed by partially ablating PI neurons (5). Overexpression of ilp2 in the PI neurons had very little effect on total sleep (Fig. 1A). In addition, flies with increased octopamine signaling in either a heterozygous or homozygous ilp2-3 mutant background showed decreases in nighttime sleep comparable to those seen in a wildtype background (Fig. 1B). Consistent with these findings, activating the insulin signaling pathway by expressing a constitutively active form of dInR (dInRΔ1235Δ) or a myristylated form of the kinase dAkt (myrAKT) in either the adult fly fat body or in the brain led to only minor effects on total sleep (Fig. 1C and D). Moreover, any effects observed were in the opposite direction from those seen with increased octopamine signaling; in other words, there was an increase in sleep rather than wake. However, the small increase in sleep may not be physiological as it occurred even when insulin signaling was decreased through expression of Akt RNAi in the fat body (Fig. 1C). Together, these data suggest that the insulin signaling pathway is not directly responsible for the sleep effects observed when octopamine signaling is altered.

**Octopamine signaling regulates fat levels independent of its effects on sleep.** Since octopamine has actions on metabolism in other invertebrates and its wake-promoting actions are mediated through the insulin-producing neurons of the *Drosophila* brain (4,11), we hypothesized that octopamine regulates metabolic function in *Drosophila*. To test this hypothesis, we first measured ilp2 levels in animals with increased or decreased octopamine signaling. Unfortunately, the ilp2 mRNA and protein measurements, by qPCR and immunohistochemistry respectively, were very variable (data not shown). Similarly, the phosphorylation status of dAKT, a downstream mediator of insulin signaling, varied in the fat bodies of animals with altered octopamine signaling (data not shown). While phosphorylated dAKT levels were consistently low in flies carrying a null mutation in the *oamb* receptor, they were highly variable when octopamine signaling was increased. Since insulin signaling regulates triglyceride levels (6), we measured triglycerides in the fat bodies of flies with increased or decreased octopamine signaling. We found that activation of octopamine-producing neurons, through the use of a bacterial Na⁺ channel (NaChBac) driven by Tdc2-Gal4 (Gal4 expressed in octopamine and tyramine-producing neurons), increased triglycerides (Fig. 2). To address whether the effect on triglycerides was developmental, we also activated these neurons in adult flies by inducing expression of a heat-sensitive *TrpA1* channel. As with NaChBac, triglyceride levels increased with *TrpA1* activation (Fig. 2B). Most of our triglyceride measurements used a colorimetric assay (6,31), but we also verified effects of altered octopamine levels on lipids through thin layer chromatography assays (Fig. 2C-E) (22). While the *oamb* mutants had robust effects on triglycerides regardless of nutrient conditions, we found that the metabolic phenotype of flies with increased octopamine, particularly the ones that had increased octopamine throughout development (NaChBac), was more evident under low nutrient conditions (Fig. 2E).

**Effects of increased octopamine on triglycerides were not due to increased feeding as feeding assays indicated that these animals actually eat less (data not shown).** As octopaminergic neurons also produce tyramine, we considered the possibility that the effect on triglycerides was mediated by tyramine. However, decreased triglycerides in flies mutant for the octopamine receptor, *oamb* (Fig. 2B), support a role for octopamine in increasing triglyceride levels.

To determine if insulin signaling mediates the metabolic effects of octopamine, we increased octopamine signaling in an *ilp2-3* mutant background and found that the effect on triglycerides was reduced (Fig. 2B). These data suggest that octopamine acts, at least in part, through the insulin signaling pathway to alter triglyceride levels.

Changes in overall triglyceride storage can occur by modifications in fatty acid and triglyceride synthesis, breakdown or a combination of both. To further understand the mechanism whereby octopamine promotes fat storage, we measured the expression of the *Drosophila* homologs of enzymes important for the synthesis of fatty acids (fatty acid synthase (dFAS), acetyl-CoA carboxylase (dACC), and ATP citrate lyase (dATPCL), as well as the lipase brummer (bmm), and the enzyme responsible for
mitochondrial transport and subsequent oxidation of fatty acids, carnitine palmitoyltransferase (dCPTI) (32,33). oamb mutants displayed decreased dFAS mRNA levels, suggesting a decrease in the synthesis of fatty acids in these mutants (Fig. 3). Conversely, bmm and dCPTI mRNA levels were augmented in the oamb mutants, indicating an increase in fat breakdown also (Fig. 3). Surprisingly, dATPCL expression was also increased in oamb mutants (Fig. 3), which is perhaps a compensatory response to the decreased dFAS levels. Together, these data suggest that octopamine controls triglyceride homeostasis by promoting fatty acid synthesis and inhibiting lipid breakdown; alterations in the expression of enzymes important for these processes may account for the triglyceride phenotypes observed in flies with altered octopamine signaling.

Given that sleep deprivation is implicated in obesity (8) and flies with increased neuronal octopamine signaling sleep significantly less, it is possible that the sleep loss itself contributes to the increased triglycerides. In order to address this issue, we measured triglycerides in other short-sleeping flies. We examined three different genotypes previously shown to have decreased sleep: sleepless (sss) and fumin (fmm) mutant flies, and flies expressing a constitutively active protein kinase A (PKA) transgene in the mushroom body (17,18,21). In contrast to flies with increased octopamine signaling, the fumin mutants show no differences in triglyceride levels compared to controls (Fig. 4). Moreover, the sss mutants and the flies that express constitutively active PKA in the mushroom body have decreased triglycerides (Fig. 4). Together, these data suggest that the triglyceride phenotypes observed in animals with altered octopamine signaling are due to specific effects of the octopamine pathway and are not secondary to the changes in sleep observed in these animals.

Coordination of sleep and metabolic needs during starvation. States of long-term sleep deprivation stimulate hunger and promote feeding while starvation suppresses sleep in mammals as well as flies (12,34,35). Since the response to starvation also depends upon metabolic status, it provides a good example of a situation where metabolic and sleep needs must be balanced (12,36). Because octopamine affects both of these physiological processes, we asked how flies with altered octopamine respond to starvation. Thus, we subjected flies with increased (Tdc2-Gal4<UAS-NaChBac) and decreased (oamb mutants) octopamine activity to starvation medium (2% agar) and assayed their survival on this medium as well as their activity during this time. Consistent with their higher triglyceride levels, flies with increased octopamine signaling live longer on starvation medium than controls (Fig. 5A). Conversely, flies with decreased octopamine signaling are more sensitive to starvation, which is consistent with their lower triglyceride levels (Fig. 5A).

Starvation was previously shown to induce hyperactivity and also suppress sleep in Drosophila (12,37). Both of these effects probably reflect the activation of a foraging or food-seeking response. We hypothesized that under starvation conditions flies with increased sleep pressure due to decreased octopamine signaling (oamb mutants) would be less active relative to controls. We found, however, that although both oamb mutants and background controls suppress sleep by a similar amount relative to their baseline levels, oamb mutants become hyperactive prior to controls (Fig. 5B). This response probably results from the decreased triglycerides in these flies. Investigation of flies with increased octopamine signaling (Tdc2-Gal4<UAS-NaChBac) revealed a similar dominance of the metabolic phenotype. We found that while these flies increase their activity in response to starvation, the increase reflects enhanced activity specifically during wake. In fact, these flies fail to suppress sleep under starvation conditions (Fig. 5C). These data indicate that sleep and activity during starvation are based on an animal’s metabolic demands, rather than its sleep drive, supporting the idea that the need for nutrients is dominant over an animal’s need for sleep.

DISCUSSION

In this study, we addressed the role of the biogenic amine octopamine in controlling both sleep and metabolism and the relationship between these processes in the context of octopamine signaling. While the PI neurons are activated by octopamine to promote wakefulness (4), the effect is not mediated by the insulin-like peptide, ILP2,
secreted by these neurons, as overexpressing ILP2 in the PI neurons and manipulating downstream insulin signaling in the brain or the fat body does not increase wake (Fig. 1). Furthermore, increasing octopamine signaling in an ilp2-3 mutant background does not prevent a reduction in nighttime sleep (Fig. 1B). This leaves open the question of what in these insulin-producing neurons is the wake-promoting signal. Although we show here that it is not ilp2 or ilp3, we cannot rule out ilp5, which is also expressed in these neurons. We believe, however, that this peptide is also an unlikely signal as it would act through the same downstream insulin signaling pathway, which we show is dispensable for proper sleep regulation with respect to octopamine (Fig. 1C and D). It is possible that other unidentified peptides are produced in the PI neurons and are released in response to octopamine stimulation to control sleep. Alternatively, octopamine may stimulate synaptic transmission from the PI neurons to increase wakefulness. Regardless, a more in depth characterization of these neurons will be needed to determine the mechanism whereby octopamine promotes wake.

We also present data that octopamine increases fat levels and it does so by regulating the expression of enzymes important for fat synthesis and breakdown (Fig. 2,3). Effects on lipids appear to be independent of changes in sleep as other short sleeping flies do not show the same increases in triglycerides (Fig. 4). In addition, unlike the sleep phenotype, effects of octopamine on metabolism are mediated by ILPs, which is consistent with known effects of insulin signaling on lipid accumulation in flies and mammals (6,7). Although analysis of ILP2 expression in PI neurons and phospho-dAKT levels in the fat bodies of animals with altered octopamine signaling yielded variable results, we found that increases in triglycerides in flies with increased octopamine signaling were partially suppressed in an ilp2-3 mutant background (Fig. 2B). The lack of complete suppression may be due to compensation by ilp5, which is known to be increased in ilp2-3 mutants and may be further increased upon octopamine stimulation (38). Changes in the transcript levels of metabolic enzymes involved in lipid synthesis and breakdown correspond with decreased triglyceride storage in oamb mutants (Fig. 3). In mammals, some of these metabolic enzymes, particularly fatty acid synthase, have been shown to be transcriptionally regulated by insulin (39-41). These data suggest that the insulin signaling pathway contributes to the metabolic effects of octopamine.

An interesting condition where sleep and metabolism interact is during times of starvation. Periods of starvation suppress sleep in flies and humans, most likely due to the need for the animal to search for food (12,34). We show here that despite their large drive to sleep, oamb mutants increase their activity in response to starvation even before controls do (Fig. 5B). Thus, these animals rapidly initiate their foraging response, which is consistent with their decreased triglycerides sensitizing them to metabolic stress, and indicates that metabolic need is dominant over the drive to sleep. This is supported by the response of flies that have increased octopamine signaling through activation of octopaminergic neurons. These flies fail to suppress sleep when starved. Thus, while they show an increase in activity, the activity is restricted to times when they are normally awake. Given their heightened arousal, the inability of these animals to curtail sleep again indicates that the metabolic status, which in this case is that of high triglycerides, dictates the response. One question that still needs to be addressed is how the sleep system of the animal senses changes in nutrient conditions and alters its activity accordingly. Keene et al. (2010) showed that the suppression of sleep during starvation is indeed due to a caloric deficit, raising the possibility that the activity of neurons that control sleep depends upon nutrient flux through metabolic pathways (12,34).

It was shown previously that two circadian mutants with short-sleeping phenotypes, Clk

\(^{exk}\)

and cyc

\(^{01}\)

, have enhanced sleep suppression under starvation conditions. Nevertheless, this is not a general characteristic of short-sleeping flies. The short-sleeping mutants fmn and shaker

\(^{minisleep}\)

reduce sleep similar to controls (12), and we report here that the short sleepers produced by increased octopamine signaling do not suppress sleep at all. The relevant Clk-expressing neurons were localized by Keene et al to circadian clock neurons, specifically to a subpopulation of the dorsal neurons (DN1s) and the dorsally located lateral neurons (LNds). The authors showed that
driving TrpA1 in these neurons abolished sleep suppression during starvation. It was previously suggested that oscillatory signals received by the DNs from central clock neurons are subsequently propagated to the PI (42,43). We found that stimulation of octopaminergic neurons, whose projections include the ILP-producing PI neurons, results in an abnormal response to starvation in that while they increase their activity they do not suppress their sleep (Fig. 5C). We propose that sleep suppression during starvation ultimately depends on the activity of cells within the PI. Thus, stimulating these neurons by activating inputs to them from either clock neurons or octopaminergic neurons prevents sleep suppression. Further studies are needed to address this hypothesis.

Recently much attention has been paid to interactions between sleep and metabolism in organisms from the fly to humans (10,12,44). While the mechanisms underlying these interactions are still being elucidated, the data presented in this study favor a model where the octopamine signaling pathway controls both sleep and metabolism independently and so one is not affected as a consequence of the other. This is similar to the effects of norepinephrine, the mammalian homolog of octopamine, on biological processes that use overlapping circuitry. In mammals, the norepinephrine-producing neurons in the LC increase their firing in response to heightened arousal (45). The LC is also known to activate the stress pathway and coordinate the brain’s response to stress (46). Thus, it is likely that in human sleep deprivation studies norepinephrine affects not only arousal, but also the stress pathway, which in turn can affect metabolism (47). The parallels between these regulatory processes in mammals and flies indicate that Drosophila is a powerful model to understand the mechanisms underlying the coordination between sleep and metabolism.

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FIGURE LEGENDS

FIGURE 1. The insulin pathway does not mediate wake-promoting effects of octopamine.
(a): Total sleep over a 24-hour period in female flies overexpressing ILP2 in ilp2-producing neurons (ilp2-Gal4<UAS-ilp2), compared to control flies that contain the ilp2-Gal4 or UAS-ilp2 transgene only. Total sleep is shown as mean ± SEM. n=18 for each genotype. (b): Nighttime sleep in flies with increased octopamine signaling in the presence or absence of ilps 2 and 3 is shown as mean ± SEM. n≥12 for each genotype. *=p<0.01 as compared to 21°C baseline using Student’s t-test. (c,d): Total sleep in flies with altered insulin signaling. Either a fat body driver (yolk-Gal4) (c), or a pan-neuronal driver (Elav-Gal4 or an RU486 inducible Elav-GS-Gal4) (d), was used to express UAS-dAkt RNAi, UAS-myrAKT or UAS-dInR(1325D) (constitutively active). Elav-GS-Gal4 was activated by RU486 in 1% ethanol (EtOH) for 4 days and sleep in these flies was compared to that in flies given 1% EtOH without RU486. For other manipulations, flies containing both the Gal4 and UAS transgenes (Gal4 and UAS alone) are compared to their Gal4 and UAS transgene alone controls. Total sleep is shown as mean ± SEM. n=32 for each genotype. *=p<0.01 as compared to each control line (Gal4 and UAS alone) using one-way ANOVA followed by Tukey-HSD post hoc test.

Elav= Elav Gene Switch; myrAKT= myristylated AKT; dInR= Drosophila Insulin Receptor.

FIGURE 2. Triglyceride levels are changed in flies with altered octopamine signaling.
(a) Image shows a control fly (UAS-NaChBac+/+) on the left and a fly with increased octopamine signaling, through activation of octopaminergic neurons (Tdc2-Gal4<UAS-NaChBac), on the right. Note the enlarged abdomen of the Tdc2-Gal4<UAS-NaChBac flies. (b) Octopamine signaling was increased either throughout development (Tdc2-Gal4<UAS-NaChBac) or conditionally during adulthood (Tdc2-Gal4<UAS-TrpA1) and triglyceride/total protein ratios were compared to those in control flies possessing only the Gal4 or UAS transgene. The effect of octopamine on triglycerides was reduced in an ilp2-3 mutant background. Octopamine signaling was decreased using an octopamine receptor mutant, oamb286, and triglyceride levels were compared to those of their background control (w1118). Each experiment was performed 3 times and values represent mean ± SEM of the pooled data. n≥12 for each genotype. *=p<0.05 relative to the control lines carrying Gal4 and UAS transgenes alone, as determined by one-way ANOVA followed by Tukey-HSD post hoc test. Ψ= p<0.01 by Student’s t-test. Triglycerides were also analyzed using Thin Layer Chromatography in (c) oamb286 mutants, (d) flies with increased octopaminergic signaling (Tdc2-Gal4<UAS-TrpA1) on standard food and (e) flies with increased octopaminergic signaling (Tdc2-Gal4<UAS-NaChBac) on low nutrient food (2% agar, 5% sucrose). Arrows point to the butter standard. Each duplicate spot (indicated by a line below the two spots) on the plate represents a technical replicate for that genotype. Each experiment was replicated two or more times and a representative image is shown.

FIGURE 3. Expression of enzymes controlling the synthesis and breakdown of lipids is altered in oamb mutants.
Analysis of transcript levels of enzymes involved in either lipid synthesis (dFAS, dACC, dATPCL) or lipid breakdown (bmm, dCPTI) in the fat bodies of oamb mutants and their background control (w1118). The experiment was performed twice with the data pooled to include 6 biological replicates for each genotype. Values represent mean ± SEM. *=<0.05 by Student’s t-test.

**FIGURE 4. Reduced sleep does not cause increased triglycerides.**
Triglyceride/total protein ratios of short sleeping mutants sleepless (sss) and fumin (fmn) were compared to their respective background controls. Triglycerides were also measured in flies where sleep was reduced in a wildtype genetic background by expressing a constitutively active form of PKA (mc*) in the mushroom body using an RU486 inducible driver (MBGS). Controls were given 1% ethanol (EtOH) without RU486. Plotted values equal the average of data pooled from at least three experiments ± SEM. n≥13 for each genotype *=p<0.01 by Student’s t-test compared to each appropriate control.

**FIGURE 5. The response to starvation is dictated by metabolic need, rather than sleep pressure.**
(a) Lifespan of flies with increased (Tdc2-Gal4<UAS-NaChBac) or decreased (oamb286) octopamine signaling on starvation medium. Survival time reflects lifespan following the start of food deprivation. The number of dead flies was determined every 3 hours. The graph summarizes the results of at least two experiments. (b) oamb286 mutants have exaggerated activity in response to starvation although they reduce sleep similar to controls. Total sleep over a 24-hour period was determined for the first day flies were starved on 2% agar (starvation) and the day before starvation in which flies were given sucrose food (baseline). (c) Flies with increased octopamine signaling do not suppress sleep in response to starvation. Flies expressing NaChBac in octopaminergic cells also display increased activity in response to starvation; however, they do not display similar reductions in sleep. Activity is plotted as the average of data pooled from three independent experiments. The activity value equals the average number of beam crossings over a period of time (1, 3, 6, 12, 24 hours) following starvation. Error bars reflect ± SEM. Statistical significance was determined using pooled data. Ψ=p<0.01 and *=p<0.05 correspond to comparisons of activity and sleep following starvation to the baseline values for each genotype by one-way ANOVA followed by Tukey-HSD post hoc test and Student’s t-test respectively.
Figure 1.

A

B

C

D

Total Sleep (min)

Nighttime Sleep (min)

Total Sleep (min)

Total Sleep (min)
Figure 2.

A

B

C

D

E

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Figure 3.
Figure 4.
Figure 5.

A

% Alive

0 200 400 600 800 1000

Time post starvation (hr)

Tdc2-Gal4< UAS-NaChBac

control

Tdc2-Gal4< UAS-NaChBac

oamb<286
control

oamb<286

B

Average Activity Count

0 250 500 750 1000 1250 1500 1750

Time post starvation (hr)

w1118 Baseline

w1118 Starvation

oamb<286 Baseline

oamb<286 Starvation

C

Average Activity Count

0 250 500 750 1000 1500 2000

Time post starvation (hr)

Iso31 Baseline

Iso31 Starvation

Tdc2-Gal4< UAS-NaChBac Baseline

Tdc2-Gal4< UAS-NaChBac Starvation

Total Sleep (min)

0 200 400 600 800 1000

w1118 Baseline

w1118 Starvation

oamb<286 Baseline

oamb<286 Starvation

Iso31 Tdc2-Gal4< UAS-NaChBac

Baseline

Starvation

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