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Expression of a constitutively active insulin receptor in Drosulfakinin (Dsk) neurons regulates metabolism and sleep in *Drosophila*

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ABSTRACT

The ability of organisms to sense their nutritional environment and adjust their behavior accordingly is critical for survival. Insulin-like peptides (ilps) play major roles in controlling behavior and metabolism; however, the tissues and cells that insulin acts on to regulate these processes are not fully understood. In the fruit fly, *Drosophila melanogaster*, insulin signaling has been shown to function in the fat body to regulate lipid storage, but whether ilps act on the fly brain to regulate nutrient storage is not known. In this study, we manipulate insulin signaling in defined populations of neurons in *Drosophila* and measure glycogen and triglyceride storage. Expressing a constitutively active form of the insulin receptor (*dInR*) in the insulin-producing cells had no effect on glycogen or triglyceride levels. However, activating insulin signaling in the Drosulfakinin (*Dsk*)-producing neurons led to triglyceride accumulation and increased food consumption. The expression of *ilp2*, *ilp3* and *ilp5* was increased in flies with activated insulin signaling in the Dsk neurons, which along with the feeding phenotype, may cause the triglyceride storage phenotypes observed in these flies. In addition, expressing a constitutively active *dInR* in Dsk neurons resulted in decreased sleep in the fed state and less starvation-induced sleep suppression suggesting a role for insulin signaling in regulating nutrient-responsive behaviors. Together, these data support a role for insulin signaling in the *Dsk*-producing neurons for regulating behavior and maintaining metabolic homeostasis.

1. Introduction

Animals must balance their nutritional state with their energy expenditure and activity to achieve homeostasis [1]. Feeding behavior and metabolism are modulated by several pathways that originate in both the brain as well as peripheral tissues such as the muscle, liver, adipose tissue, and the gastrointestinal tract [2], and endocrine hormones such as insulin, cholecystokinin (CCK) and leptin seem to mediate these effects in mammals [3,4]. Alterations in many of these hormonal signals that originate in the periphery lead to metabolic diseases such as obesity and diabetes [5,6]; therefore, investigating the role of the brain in regulating feeding and metabolism is essential for understanding these metabolic disorders. In addition, metabolic diseases like diabetes and obesity have been linked to excessive sleep loss [7,8], but the molecular mechanisms and hormonal signals linking sleep and metabolism are not fully understood.

Interactions between behavior, metabolism and nutrient status have been shown in many organisms [9–11]. Mutation of the *Drosophila* fat body lipase gene *brummer*, or the mammalian adipose hormone gene *leptin* results in animals that are obese and have altered sleep regulation, supporting a notion for interactions between the adipose tissue and sleep regulation [12,13]. In addition, when animals are starved, their sleep is suppressed to forage for food [14,15]. Moreover, insulin signaling in *Drosophila* has been shown to promote food consumption during starvation by targeting the insulin-producing cells (IPCs) and neurons that secrete short NPF [16], suggesting that sleep and feeding are altered when nutrients are limiting. Nutrient storage and metabolism as well as behaviors like sleep and feeding have been well characterized in *Drosophila* [14,15,17] making it an excellent system to study the interactions between metabolism and behavior.

In *Drosophila*, several hormones and neuropeptides including the insulin-like peptides (ilps), the glucagon-like adipokinetic hormone (AKH) and drosulfakinin (Dsk), a cholecystokinin (CCK)-homolog, have been implicated in feeding, metabolism, sleep and altered activity in response to changes in food availability [15,18–21]. Decreasing insulin signaling by deleting *ilp1-5* results in blunted triglyceride storage [22],

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while increasing insulin action by overexpression of *ilp2* inhibits food consumption [23]. The 8 insulin-like peptides produced in flies signal through a single receptor (dInR; [24]) and activating dInR in the fat body promotes lipid storage [25]. However, whether insulin acts in the fly brain to regulate nutrient storage and sleep and feeding is not known.

In this study, we activated insulin signaling in the *Drosophila* brain by overexpressing a constitutively active form of *dInR* (*dInR-CA*). While expressing *dInR-CA* in the IPCs has no effect on nutrient storage, activating *dInR* in the *Dsk*-expressing neurons promotes triglyceride storage. Flies with active insulin signaling in the Dsk neurons also have increased expression of *ilp2, ilp3* and *ilp5*, and increased feeding, which may account for the triglyceride accumulation phenotype in these animals. In addition, expressing *dInR-CA* in the *Dsk*-expressing neurons decreases sleep in the fed state, but increases sleep during starvation, suggesting a role for insulin signaling in regulating hunger-induced behaviors. Together, these data support a model where insulin acts in the Dsk neurons in the fly brain to regulate metabolic homeostasis and behavior.

2. Materials and methods

2.1. Fly strains

The following fly lines were used in this study: *Dsk-Gal4* [26], *ilp2-Gal4* [20] and *UAS-dInR-R418P* (Bloomington #8250; referred to here as *UAS-dInR-CA*). All strains were outcrossed into the w^{1118} (Bloomington #5905) genetic background. Flies were grown on standard yeast-cornmeal-sugar medium at 25 °C under 12:12h light:dark conditions.

2.2. Triglyceride, glycogen, and protein measurements

Single 4–7 day old adult females were homogenized in lysis buffer (140 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% Triton-X and 1X complete, EDTA-free protease inhibitor cocktail (Roche Life Science). Triglyceride, glycogen, glucose, and protein levels were measured as described previously [27].

2.3. CAFÉ assay

Food consumption over a 24-h period was measured using a modified version of the Capillary Feeder (CAFÉ) Assay as described previously [28]. Briefly, three adult females were placed in a vial with 1% agar and 5% sucrose in a 5-μl glass micropipette (ThermoFisher Scientific) as the sole food source. After 24 h, the amount of liquid consumed by the flies was measured and corrected for any evaporation that occurred during the experiment.

2.4. Sleep measurements

Measurements of sleep and arousal threshold were measured in female flies over the course of three days starting at ZT0 using the *Drosophila* Locomotor Activity Monitor (DAM) System (Trikinetics, Waltham, MA, USA), as previously described [29–31]. For each individual fly, the DAM system measures activity by counting the number of infrared beam crossings over time. These activity data were then used to calculate sleep, defined as bouts of immobility of 5 min or more, using the *Drosophila* Sleep Counting Macro [32], from which sleep traits were then extracted. Waking activity was quantified as the average number of beam crossings per waking minute, as previously described [32].

2.5. RNA isolation and qPCR

Total RNA was isolated from the heads of 5–7 day old adult female flies using Ribozol reagent (Amresco). The DNA free kit (Ambion) was used to remove any remaining genomic DNA and cDNA was generated from 0.25 μg of total RNA using qScript cDNA Supermix (Quanta Biosciences) according to manufacturer's instructions. Quantitative PCR was performed using Power SYBR Green Master Mix (Life Technologies). The relative quantity of each mRNA was normalized to the quantity of *rp49* mRNA levels. Primer sequences used were:

Dsk F 5′ CCGATCCCAGCGCAGACGAC 3'; *Dsk* R 5′ TGGCACTCTGCGACCGAAGC 3' [21]

ilp2 F 5′ TCTGCAGTGAAAAGCTCAACGA 3'; *ilp2* R 5′ TCGGCACCGGGCATG 3' [33]

ilp3 F 5′ AGAGAACTTTGGACCCCGTGAA 3'; *ilp3* R 5′ TGAACCGAACTATCACTCAACAGTCT 3' [33]

ilp5 F 5′ GAGGCACCTTGGGCCTATTC 3'; *ilp5* R 5′ CATGTGGTGAGATTCGGAGCTA 3' [33]

rp49 F 5′ GACGCTTCAAGGGACAGTATCTG 3'; *rp49* R 5′ AAACGCGGTTCTGCATGAG 3'

2.6. Statistical analysis

Statistics were performed using R. All data were first tested for normality using the Shapiro Wilk test. Data that was normally distributed was analyzed with one-way or two-way ANOVA and Tukey *post hoc* test and data that was not normally distributed was analyzed with a Kruskal Wallis test and pairwise Wilcoxon rank sum test. Each experimental genotype was compared to two controls, specifically the genetic background stocks crossed to both the Gal4 and UAS transgenes. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Insulin signaling in Dsk neurons promotes triglyceride storage and food consumption

Ablating the IPCs as well as inhibiting the *Dsk*-producing neurons or inducing RNAi towards *Dsk* have been shown to decrease food intake in *Drosophila* [21,34]. Therefore, it is possible that Dsk and insulin signaling interact to regulate nutrient storage and nutrient-responsive behaviors such as feeding and sleep. To test this connection between behavior and metabolism, we drove *UAS-dInR-R418P* specifically in either IPCs or Dsk neurons using *ilp2-Gal4* and *Dsk-Gal4*, respectively. *dInR-R418P* is a mutation like that discovered in human insulin which was shown to impair insulin binding but cause constitutive activation of glucose transport [35]. Thus, we used this mutated form of the *Drosophila* insulin receptor (referred to here as dInR-CA) in combination with the Gal4/UAS system to activate insulin signaling in defined classes of cells. While ilps have been previously shown to be necessary for proper lipid storage [22], expressing *dInR-CA* in insulin-producing cells (IPCs) in the fly brain had no effect on triglyceride or glycogen levels (Fig. 1A and B), suggesting that ilps do not act on the IPCs to regulate nutrient storage. However, activating insulin signaling in Dsk neurons results in an increase in total triglyceride and a trend for increased glycogen storage (Fig. 1C and D). These results suggest that activating insulin signaling in Dsk neurons, but not the IPCs, promotes the accumulation of triglycerides.

One mechanism to account for an increase in triglycerides can be more food intake. Both ilps and Dsk have been shown to regulate food consumption [21,23,33]; therefore, it is possible that activating insulin signaling in Dsk neurons may increase feeding leading to changes in lipid storage. To test this hypothesis, we measured food consumption in female flies with active insulin signaling in the Dsk neurons. Interestingly, these flies consume more food over a 24-h period than controls (Fig. 2). These results suggest that activating insulin signaling in the Dsk neurons promotes feeding behavior, which may contribute to the increased triglyceride storage in these flies.

3.2. Insulin signaling in Dsk neurons increases ilp expression

Dsk neurons produce both Dsk and ilp2, ilp3 and ilp5, all of which

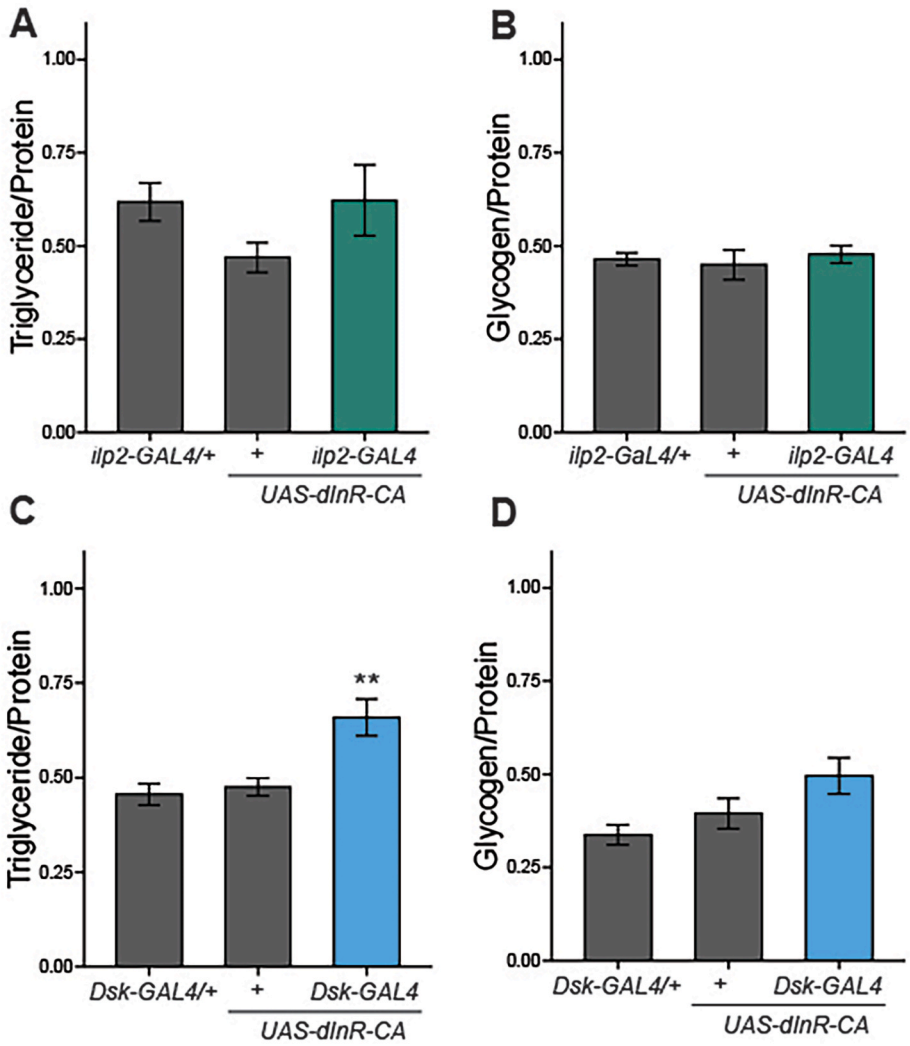

Fig. 1. Active insulin signaling in Dsk neurons leads to increased storage of triglyceride. Triglyceride/protein **(A)** and glycogen/protein **(B)** ratios of 4–7 day old *ilp2-Gal4 > dInR-CA* females compared to *UAS-dInR-CA/+* and *ilp2-Gal4/+* controls (n = 31–32). Triglyceride/protein **(C)** and glycogen/protein **(D)** ratios of 4–7 day old *Dsk-Gal4>dInR-CA* females compared to *UAS-dInR-CA/+* and *Dsk-Gal4/+* controls (n = 40). Bars indicate the mean and error bars represent ±SEM. *, $p < 0.05$, **0.01 by Kruskal-Wallis test with Wilcoxon rank sum tests comparing the *Dsk-Gal4>dInR-CA* flies to both controls.

have been shown to inhibit feeding [21,23,33]. The ilps have also been shown to regulate the storage of lipids and glycogen [22,33,36]; therefore, they may be involved in controlling the triglyceride accumulation phenotype seen in *Dsk-Gal4>dInR-CA* flies. To determine whether these neuropeptides were being altered when insulin signaling was activated in the Dsk neurons, we measured the expression of *ilp2*, *ilp3* and *ilp5* and *Dsk* in fly heads by quantitative PCR. Activating insulin signaling in the Dsk neurons had no effect on *Dsk* transcript levels in fly heads; however, the levels of all three *ilps* were increased in flies with active insulin signaling in the Dsk neurons (Fig. 3A). This change in *ilp* expression is not due to insulin signaling in the IPCs as activating insulin signaling in the IPCs has no effect on *ilp* expression (Fig. 3B). These data suggest that active insulin signaling in the Dsk neurons leads to an increase in *ilp* expression, which could in turn lead to increased storage of triglycerides.

3.3. Insulin signaling in Dsk neurons regulates sleep

When starved, wild-type flies have been shown to suppress their sleep and lipid levels seem to correlate with sleep in *Drosophila* [14,15,37]. Since flies with activated insulin signaling in Dsk neurons have augmented triglyceride storage, it is possible that these flies also have increased sleep. To test this hypothesis, we measured sleep in *Dsk-Gal4>dInR-CA* flies under both fed conditions and during a 24-h starvation. In contrast to our hypothesis, activating insulin signaling in the Dsk neurons resulted in less sleep under fed conditions, but waking activity was not changed (Fig. 4A-C). However, *Dsk-Gal4>dInR-CA* flies starved for 24 h did not suppress their sleep like control flies did consistent with our hypothesis (Fig. 4C and D). While waking activity is still induced in *Dsk-Gal4>dInR-CA* flies, we observed lower waking activity in the starved state when compared to control flies indicating that starvation-induced hyperactivity is dampened rather than abolished when insulin signaling is chronically active in the Dsk neurons. Together, these data suggest that insulin acts on the Dsk neurons to regulate both nutrient storage as well as metabolic behaviors such as feeding and sleep.

4. Discussion

Here we show that activating insulin signaling in the *Drosophila Dsk*-producing neurons can control sleep and promote triglyceride storage. Triglycerides are stored mostly in the *Drosophila* fat body; therefore, it is possible that the Dsk neurons communicate with the fat body to induce these changes. The Dsk neurons are known to secrete Dsk as well as ilp1, ilp2, ilp3, and ilp5 as several *Dsk*-producing cells are also IPCs [21]. Moreover, the ilps have been shown to regulate macromolecule storage [34,36] and may be responsible for the triglyceride storage phenotype observed in *Dsk-Gal4>dInR-CA* flies. For example, flies deficient for *ilps 1–5* have lower triglyceride levels when compared to controls [22]. In addition, activating insulin signaling in the fat body promotes fat storage [25]. This suggests that increasing *ilp* levels or secretion results in augmented nutrient storage. Interestingly, *ilp2*, *ilp3* and *ilp5* mRNA

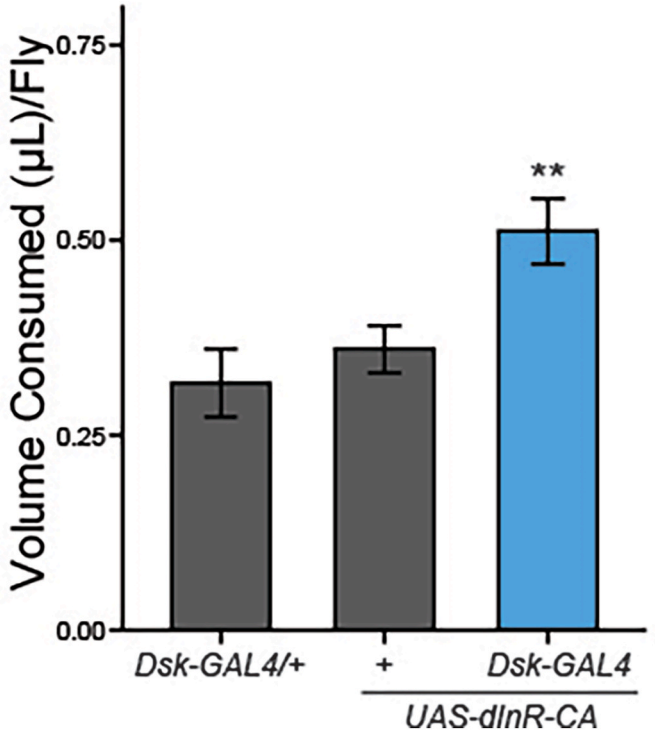

Fig. 2. Active insulin signaling in Dsk neurons leads to increased food consumption. Food consumption of 5–7 day old *Dsk-Gal4>dInR-CA* females compared to *UAS-dInR-CA/+* and *Dsk-Gal4/+* controls as measured using the CAFÉ Assay over a 24 h period (n = 19–24). Error bars represent ±SEM. **0.01 by Kruskal-Wallis test with Wilcoxon rank sum tests comparing the *Dsk-Gal4>dInR-CA* flies to both controls.

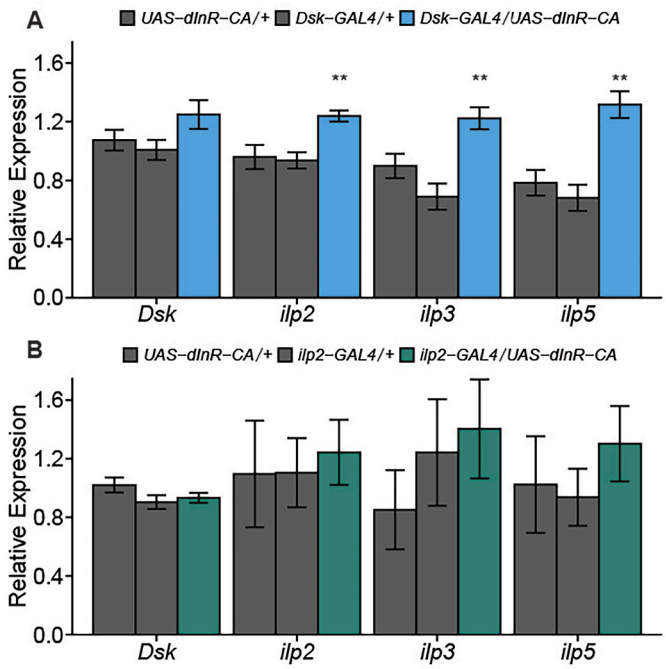

Fig. 3. Expression of *ilp2, ilp3 and ilp5* is increased in flies with activated insulin signaling in Dsk neurons. (A) qPCR for *Dsk, ilp2, ilp3* and *ilp5* was performed on heads from 4 to 7 day old *Dsk-Gal4>dInR-CA* females compared to *UAS-dInR-CA/+* and *Dsk-Gal4/+* controls (n = 8). **(B)** qPCR for *Dsk, ilp2, ilp3* and *ilp5* was performed on heads from 4 to 7 day old *ilp2-Gal4>dInR-CA* females compared to *UAS-dInR-CA/+* and *ilp2-Gal4/+* controls (n = 8). Expression of each *ilp* was normalized to *rp49* expression. Bars indicate mean expression and error bars represent ±SEM. **, $p < 0.01$ by one-way ANOVA with post hoc Tukey Test comparing the *Dsk-Gal4>dInR-CA* flies to both controls.

levels all rise in fly heads when insulin signaling is activated in Dsk neurons, potentially contributing to the increased triglycerides observed in these flies; however, it is also possible that activating the Dsk neurons may also contribute to this phenotype. It remains to be seen which ilp endogenously acts on the Dsk neurons to induce changes in nutrient storage and specifically which neurons normally express dInR. It is also possible that a novel, unidentified protein could be produced in the Dsk neurons that when secreted promotes nutrient storage and transcriptional profiling of these neurons would help us identify any such proteins.

In addition to increases in triglyceride storage, flies expressing a constitutively active insulin receptor in the Dsk neurons exhibited more food intake, which could also contribute to the increased triglyceride phenotype in these flies. Dsk has been demonstrated to decrease feeding in *Drosophila* [21]. Therefore, it is possible that Dsk production, secretion or action is inhibited when insulin signaling is activated in Dsk neurons, thereby increasing food consumption. While we have shown that *Dsk* mRNA levels were not significantly different in *Dsk-Gal4>dInR-CA* flies when compared to controls, it is possible that activating insulin signaling in the Dsk neurons affects Dsk secretion. Measuring Dsk levels in the hemolymph in *Dsk-Gal4>dInR-CA* flies would address whether activating insulin signaling in the Dsk neurons controls Dsk secretion.

While *Dsk*-expressing neurons and insulin-producing cells overlap in the fly brain [21], activating insulin signaling in the IPCs has no effect on triglycerides or *ilp* expression while activating insulin signaling in Dsk neurons increases triglycerides and *ilp* levels; therefore, it is possible that non-IPC Dsk neurons are responsible for the nutrient storage and *ilp* expression phenotypes observed in *Dsk-Gal4>dInR-CA* flies. In addition, the non-IPC Dsk neurons may also be responsible for the increased food consumption phenotype observed in *Dsk-Gal4>dInR-CA* flies as overexpressing *ilp2* in IPCs decreases food consumption [23], a phenotype opposite of what we see in flies with active insulin signaling in Dsk neurons. Alternatively, it has been shown that *ilp* levels rise in response to feeding in *Drosophila* [38]. As a result, the activated insulin receptor in Dsk neurons may be driving the animals to feed and store more nutrients and produce more *ilp* because of this increased feeding. Additional experiments aimed at understanding the functions of subpopulations of neurons, including those expressing *Dsk* and *ilp2*, and how they regulate feeding and nutrient storage, will help further our understanding of the neuronal control of this important interaction between feeding behavior and metabolism.

Activating insulin signaling specifically in the *Dsk*-producing neurons also decreased sleep in the fed state and starvation-induced sleep suppression, suggesting that insulin acts on the *Dsk*-producing neurons to regulate sleep. This blunting of starvation-induced sleep suppression may be due to excess nutrient storage as has been seen previously [13,39] as activating insulin signaling in the Dsk neurons leads to increased triglyceride stores. Interestingly, insulin also regulates fly sleep as mutants in many of the *ilp* genes sleep less and have altered sleep intensity [18,40] while activating insulin signaling in either the fat body or cells in which dInR is expressed results in increased sleep [18,41]. How activating insulin signaling in the Dsk neurons regulates sleep under fed and starved conditions is still not fully understood, but it is possible that the increased expression of *ilp2, ilp3* and *ilp5* or increased food consumption in these flies may be responsible for the observed sleep phenotypes. Experiments aimed to further our understanding of the neuropeptides regulating sleep when insulin signaling is activated in Dsk neurons may help answer these questions.

In summary, our results demonstrate a role for insulin signaling in Dsk neurons to regulate triglyceride storage and the metabolic behaviors feeding and sleep. While much is known about the target tissues of the

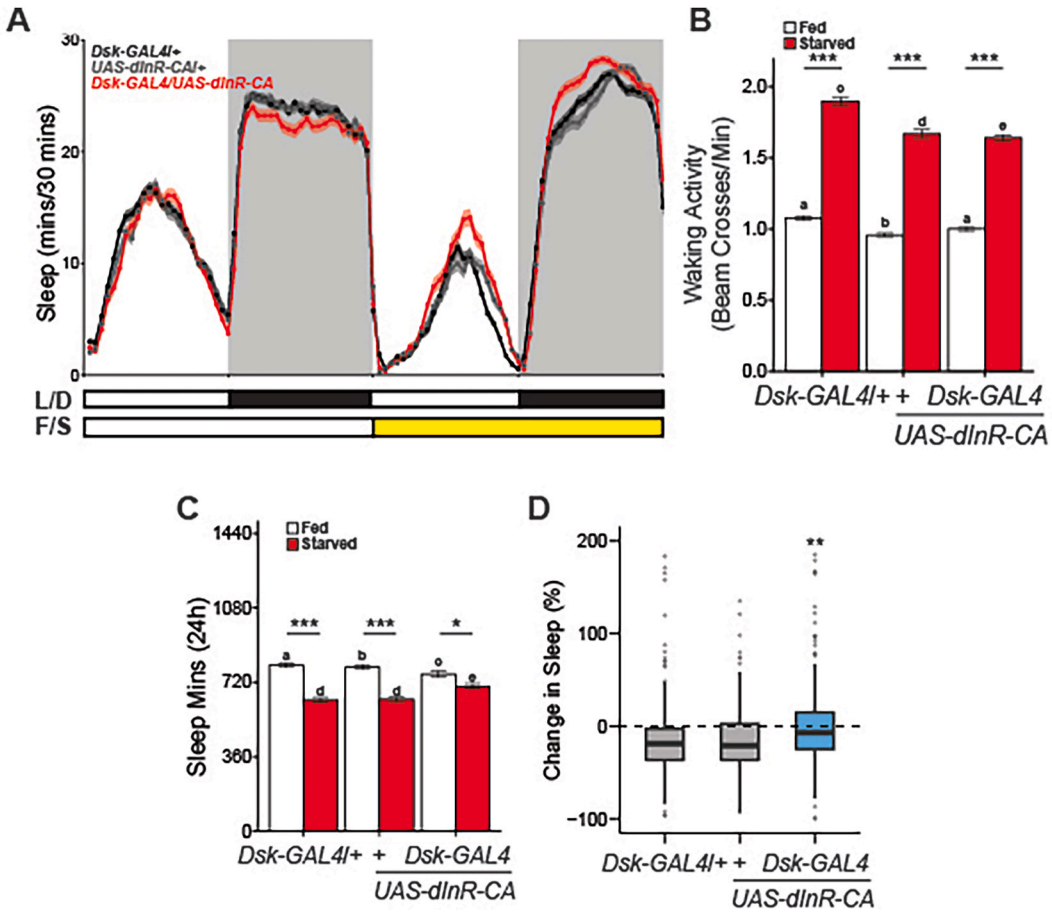

Fig. 4. Activating insulin signaling in Dsk neurons blunts starvation-induced sleep suppression. (A) Sleep profile over 48-h of female *Dsk-Gal4>dInR-CA* flies (red) compared to *Dsk-Gal4/+* (black) and *UAS-dInR-CA/+* (gray) controls. Sleep is calculated over 30-min bins and plotted accordingly for each genotype. Flies were fed for 24 h (white) and then starved (yellow) subsequently for 24 h (for a total of 48 h). **(B)** Waking activity and **(C)** total sleep of female *Dsk-Gal4>dInR-CA* flies compared to *Dsk-Gal4/+* and *UAS-dInR-CA/+* controls (n = 64). Waking activity is calculated as beam crosses divided by the total wake period in minutes. Bars indicate average waking activity or sleep, and error bars represent ±SEM. *p < 0.05, ***p < 0.001 comparing fed and starved flies of each genotype by two-way ANOVA and each letter denotes a statistically unique group as determined by post hoc Tukey tests. **(D)** Change in sleep during the 24-h starvation calculated by determining the percent change in sleep from the fed period to the 24-h starvation period in *Dsk-Gal4>dInR-CA* flies compared to *Dsk-Gal4/+* and *UAS-dInR-CA/+* controls (n = 64). **p < 0.01 using a one-way ANOVA with post hoc Tukey tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

ilps to regulate important biological processes such as growth and energy metabolism [17,36], we have identified an additional cell type in the *Drosophila* brain (the Dsk neurons) as a site of insulin action. CCK, the Dsk homolog in mammals, is produced in specific endocrine cells in the intestine [4] and while insulin signaling has been studied in intestinal epithelial cells [42], the role of insulin signaling in CCK-producing cells has not been studied. This work provides support for our ability to use *Drosophila* as a model system to further understand the role of insulin signaling in controlling sleep and feeding behaviors as well as metabolism in mammalian systems.

Author statement

Justin Palermo: Investigation, Formal analysis, Writing – Original Draft, Writing – Review & Editing, Visualization. **Alex Keene:** Conceptualization, Methodology, Formal analysis, Writing – Review & Editing, Supervision, Project Administration, Funding acquisition **Justin DiAngelo:** Conceptualization, Methodology, Investigation, Formal analysis, Resources, Writing – Review & Editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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