Regulation of starvation-mediated hyperactivity and triglyceride metabolism by Short neuropeptide F and Insulin Like Peptide in *Drosophila*

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Running title: sNPF and DILP2 govern starvation-mediated hyperactivity and triglyceride metabolism in fruit flies.

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Abstract:

Circadian clock regulates various behavioral, metabolic and physiological processes to occur at the most suitable time of the day. The most apparent behavioral outputs of the clock are activity-rest rhythm and feeding.   While clock regulates these two behaviors through interconnected neuronal circuits, the precise pathway through which the clock coordinates metabolism in accordance with the behavioral rhythms largely remains to be elucidated. This study was aimed to elucidate the role of two circadian relevant metabolic neuropeptides, short neuropeptide F (sNPF) and *Drosophila* Insulin Like Peptide 2 (DILP2) in triglyceride metabolism, starvation-mediated hyperactivity, and food intake in *Drosophila*. The results showed that *snpf* transcripts exhibit significant rhythmicity under 12:12 hour light-dark cycles (LD) and constant darkness (DD). Knockdown of sNPF in sNPF producing neurons enhanced the starvation-mediated hyperactivity in flies compared to the control. Further studies showed that sNPF suppresses starvation-induced hyperactivity partly through the sNPF receptors (sNPFR) expressed in insulin producing cells (IPC). Knockdown of *Drosophila* insulin like peptide 2 (*dilp2*) in IPCs and reduced expression of the insulin receptors (InR) in the fat body altered the starvation-mediated hyperactivity, lipid storage, and increased the food intake after starvation. These results suggest a role for sNPF in modulating locomotor activity in accordance with the nutrient availability and a novel role for circadian output molecule DILP2 in regulating triglyceride metabolism and starvation-mediated behavioral changes in *Drosophila*.

Keywords: circadian, short neuropeptide F, *Drosophila* Insulin Like Peptide 2, starvation, triglyceride metabolism

Introduction

The circadian clock drives daily rhythms in a wide array of physiological and behavioral processes by scheduling it at the appropriate time of the day. This inherent timekeeping system coordinates the phase of two fundamental behavioral rhythms such as feeding and sleep/wake cycles and it is believed to aid the organism to adapt with the cyclic external environmental changes. The growing body of evidence indicates that the circadian clock not only mediates optimal phasing of behavioral rhythmicity during 24 hour of a day but also has intimate ties with metabolism [1]. While the clock orchestrates numerous metabolic pathways, the nutrient availability and metabolic status can in turn feed back to impinge on the functioning of the circadian clock [2], [3], [4], [5]. A large body of evidence accumulated over the past two decades remarkably improved our understanding of the vital role of the clock in metabolism, feeding behavior, and sleep wake cycle [6]. However, little is known about the precise underlying pathways by which the circadian clock aids the organism to attain temporal harmonics between behavior and metabolism.

The association between the circadian clock and metabolism was further substantiated by the findings that the circadian timing system drives rhythmicity of various peptides involved in metabolism that also play key role in the regulation of sleep wake cycles [7]. For instance, peptides involved in metabolism such as leptin, ghrelin and cholecystokinin display circadian rhythmicity in expression [8], [9], [10], [11]. Another appetite stimulating hormone orexin promotes wakefulness while stimulated by ghrelin and enhances sleep when inhibited by leptin [12] and orexigenic neuron activation is believed to be under circadian control [13]. In addition

to regulating the metabolic endocrine rhythms through the anatomical connections that interlink

with the centers of sleep wake cycles, the cross talk between the master clock in the brain and the

peripheral clocks located in metabolically active tissues is believed to harmonize the metabolic

homeostasis [14]. In *Drosophila*, the peripheral clocks in the fat body mediates optimal phase of

feeding rhythm, regulates the starvation sensitivity, triglyceride and glycogen levels, drive

rhythmicity in the expression of genes involved in metabolism and detoxification to achieve

energy homeostasis [5] [15] [16]. Although it was reported that central clock neurons oppose the

effect of the fat body peripheral clock on energy storage [15], the potential mediators by which

the master clock communicates the time of the day information to the fat body to achieve energy

homeostasis remains to be largely achieved.

A recent study showed that central clock neurons regulate rhythmic gene expression in

the fat body through neuropeptide F (NPF) in *Drosophila* [17]. Moreover, pars intercerebralis

(PI) has been identified as an important clock output region that regulates activity rest rhythms

[18] and the dorsal clock neurons (DN1) are physically and functionally connected to the insulin

producing cells (IPCs) of the PI. *Drosophila* insulin like peptide 2 (DILP2) produced in IPCs is

an identified clock output-signaling molecule that drives rhythmicity in the expression of lipase

transcript in the fat body [19]. Furthermore, DILPs and insulin signaling play a pivotal role in

feeding behavior [20], [21], [22]. Feeding behavior displays a circadian rhythm and its phase is

most likely regulated by the peripheral clock present in the fat body [16]. Thus, it is conceivable

that the cross talk between the master clock and the peripheral clock in the fat body may

optimize the phase of both feeding and lipid metabolism to occur at a specific part of the day.

However, the precise interdependent and independent pathways/networks by which the circadian

clock regulates feeding behavior and lipid metabolism remain to be elucidated. It is likely that

the circadian regulation of metabolism and feeding behavior are intertwined to some degree and

the insulin signaling could be one of the primary clock output peptidergic signals involved in this

cross modulation.

Previous studies showed that short neuropeptide F (sNPF) and sNPF receptor (sNPFR)

signaling in IPCs regulate the expression of *dilps* [23]. In addition, sNPF regulates circadian

rhythm, sleep and feeding behavior in *Drosophila* [24], [25]. Feeding and sleep wake cycles are

interconnected behaviors and the optimum balance between them is disrupted under nutrient

deprivation. Starvation suppresses sleep by promoting hyperactivity in *Drosophila* and this

behavioral change in response to nutrient unavailability is governed by diverse neuropeptides

such as allatostatin, octopamine, adipokinetic hormone, glucagon and insulin [26], [27], [28],

[29]. While significant progress has been made in understanding the circadian and neuronal

basis of starvation-mediated sleep changes in *Drosophila* [30], it remains unclear whether the

circadian clock conveys the time of the day information to coherently coordinate this starvation-

mediated behavioral change with metabolism through interlinked neuronal pathways or via

independent neural circuitry. In the present study, we aimed to elucidate the role of circadian

clock in regulating the metabolic neuropeptides and the specific role of neuropeptides sNPF and

DILP2 in triglyceride metabolism, starvation-mediated hyperactivity, and feeding behavior in

Drosophila. The results showed that the circadian clock regulates the transcription of *snpf* gene

and sNPF suppresses starvation-mediated hyperactivity in *Drosophila*. Further studies

deciphered the vital role of DILP2 in IPCs and Insulin receptors (*InR*) expressed in the fat body

in mediating the starvation-mediated hyperactivity, triglyceride metabolism and hunger induced

food intake in *Drosophila*.

Materials and Methods

Fly stocks and maintenance

Following fly lines were used in this experiment: w^{1118} (BDSC #5905), *dilp2-Gal4/CyOGFP* (Stephen Cohen Laboratory), *tim-Gal4* (Sheeba Vasu Laboratory), *CG-Gal4* (BDSC #7011), *snpf-Gal4, dilp2-Gal4 GFP*, UAS-*snpf* ^{RNAi} (Kweon Yu Laboratory), *UAS-dilp2* ^{RNAi} (VDRC #102158), *UAS-InR* ^{DN} (II) (BDSC #8252) were obtained from Bloomington Drosophila Stock Center (Bloomington, IN). All fly stocks were maintained in the *Drosophila* growth chamber (MIR-154, Panasonic, Japan) at 25º C temperature, 75 ± 5% humidity and 12:12 hour (h) light-dark (LD) cycle where lights came on at Zeitgeber Time 00 (ZT 00) and went off at ZT12.

Measurement of mRNA levels

To assess the mRNA transcription profiles of *snpf*, three replicates were used for each time point. Each replicate contained 30 heads from w^{1118} flies. Total RNA was extracted using QIAGEN RNAeasy Plus Mini kit; DNAase digestion was performed using QIAGEN RNase–free DNase. cDNA was synthesized using SuperScript™ III First-Strand Synthesis System (Invitrogen, Cat. No. 18080051) and real-time PCR was performed using Bio-Rad CFX96TM with the cDNA template, Power SYBR® Green PCR Master Mix (Cat. # 4368702) from ThermoFisher Scientific to check temporal transcription profile of *snpf*. mRNA levels were measured under LD cycle and constant darkness (DD). The molecular oscillation of mRNA levels was determined by normalizing the mRNA level of the gene of interest with the mRNA level of *rp49* at each time point. Knockdown efficiency of *snpf-Gal4 > UAS snpf*^{RNAi} (*snpf-Gal4 >snpf*^{RNAi}) and *dilp2-Gal4 > dilp2*^{RNAi} was validated by qPCR. Primers used for qPCR are listed in Table 1.

Table 1. PCR primers used for quantitative RT-PCR.

Primers	Forward	Reverse
dilp2	5'- GGCCAGCTCCACAGTGAAGT -3'	5'- TCGCTGTCGGCACCGGGCAT -3'
Snpf	5'- CCCGAAAACTTTTAGACTCA-3'	5'- TTTTCAAACATTTCCATCG -3´
rp49	5'- GCTAAGCTGTCGCACAAA-3'	5'- TCCGGTGGGCAGCATGTG-3'

Activity/rest assay

For activity/rest assay, freshly emerged male flies were pre-entrained for 2 days in the LD

cycle at 25°C and at a humidity of 75 ± 5% inside the incubator (MIR-154, Panasonic, Japan).

Two-day-old virgin males were individually introduced into activity tubes. Flies were provided

with standard cornmeal medium and their activity/rest behavior was recorded using Drosophila

Activity Monitors (DAM, Trikinetics, USA). The infrared beam that passes through the middle

of the activity tubes count the movement of the flies. Following acclimatization in activity tubes

for 12 hours, flies were transferred to 1% agar medium or to standard cornmeal medium in glass

tubes at ZT12 to provide starved or fed conditions, respectively, and locomotor activity was

recorded for 36 hours. Each experiment was performed at least three times with 32 flies for each

genotype.

Triglyceride assay

This assay comprised of three replicates and each replicate containing 5 male flies were

homogenized in homogenization buffer (0.05% Tween 20). The homogenate was incubated at

70° C for 5 minutes to inactivate the enzymes and centrifuged at 14000 rpm for 3 minutes and

the supernatant was collected into a sample tube. Sigma serum triglyceride determination kit

TR0100 was used to estimate the triglyceride level in the sample by measuring absorbance at

540 nm using TECAN Infinite M200 pro-multimode plate reader. The triglyceride content was

normalized to the total protein content of the flies estimated by Quick Start™ Bradford 1X Dye Reagent (BioRad, Cat. # 500-0205). Assays were carried out at ZT 12 in freshly emerged (Day 0), 2 day old (Day 2) and 5 day old (Day 5) flies.

Starvation resistance Assay

Freshly emerged male flies (Day 0) were kept in the 1% agar medium vials. Six replicates with each vial containing 10-15 flies were used for each genotype. The number of dead flies was recorded at every two-hour interval until all the flies died.

Feeding assay

Two-day-old male flies were used for feeding assay. This assay comprised of three replicates and each replicate containing 8 male flies were maintained under fed (corn medium) and starved (1% agar medium) conditions for 24 hours starting from ZT00. After 24 hours of fed and starved condition treatment, flies were provided with yeast paste mixed with Orange G (Sigma) at ZT00 and allowed to feed for the next 30 minutes. In order to assess the amount of food intake, flies were homogenized with 0.05% Tween 20 homogenization buffer and zirconium oxide beads. The supernatant obtained after the centrifugation at 5000 rpm was used for the calorimetric measurement at 478 nm using TECAN Infinite M200 pro multi-mode plate reader. The absorbance measured from flies fed with normal food was subtracted from the absorbance measured from the flies fed with orange G. The net absorbance reflected the amount of food ingested.

Statistical analysis

Cosinor analysis was implemented in MATLAB-R2016a to test for rhythmicity, to estimate the peak phase and the amplitude (peak/trough ratio) of relative mRNA expression of

snpf [31]. The waveform of activity/rest rhythm, total activity, day time activity, nighttime

activity and food intake was analyzed by using ANOVA followed by Tukey's post hoc multiple

comparisons. TGA levels and time taken for 50% of the fly death under starvation were

analyzed by Student's t-test. The statistical analyses were implemented on Statistica for

Windows Release 5.0 B [32]. Error bars in all the graphs represent standard error of the mean

(SEM).

Results

snpf transcript exhibit circadian oscillation

To assess whether the circadian clock regulates *snpf* transcript oscillation, the

transcription profile of *snpf* was estimated in w^{1118} flies under LD and under constant darkness

(DD). The *snpf* mRNA level increased during light phase and decreased during the dark phase

resulting in a significant daily oscillation with a peak at ZT 8.1h under LD (cosinor analysis,

p<0.001) (Fig 1A). The *snpf* transcriptional oscillation persisted under DD with a peak at CT

8.5h (cosinor analysis, *p*<0.002). However, the amplitude (peak/trough ratio) of the oscillation

decreased under DD compared to that obtained under LD (Fig 1B). These results suggest that

the transcriptional oscillation of *snpf* mRNA is endogenously controlled by the clock.

sNPF suppress starvation-mediated hyperactivity

To assess the role of sNPF in locomotor activity, we expressed *snpf*^{RNAi} under *snpf-Gal4*

driver (*snpf-Gal4 > snpf*^{RNAi}) and recorded the locomotor activity under fed LD conditions for 36

h. The *snpf-Gal4 > snpf*^{RNAi} flies exhibited increased activity around lights-on and after lights-

off compared to the control (*snpf-Gal4 > w*^{1118}) flies (ANOVA, *p*<0.0001) (Fig 1C). This

resulted in enhanced total activity during day 1 (24 h) (ANOVA, $p<0.0001$) (Fig 1G). To test the

role of sNPF in starvation-mediated hyperactivity, we analyzed the locomotor activity of *snpf-*

Gal4 > snpf ^{RNAi} flies under fed and starved conditions and compared it with that of the control

flies. Both the control flies and the *snpf-Gal4 > snpf* ^{RNAi} flies exhibited an increase in total

activity on day 1 and during dark phase of day 2 (ZT 13-24) under starved condition compared to

what was observed under fed condition (*snpf-Gal4 > w^{1118}* flies; ANOVA, $p<0.0001$ for day1,

$p<0.0001$ for day2: ZT 13-24) (*snpf-Gal4 > snpf* ^{RNAi} flies; ANOVA, $p<0.0001$ for day1,

$p<0.0001$ for day2: ZT 13-24) (Fig 1D, E, G, H). Comparison of activity between control and

snpf-Gal4 > snpf ^{RNAi} flies under starved condition revealed that *snpf-Gal4 > snpf* ^{RNAi} flies

exhibited greater morning and evening activity peaks than control (Fig 1F), resulting in greater

total activity on Day1 (ANOVA, $p<0.01$) and during the dark phase of Day 2 (ZT 13-24)

(ANOVA, $p<0.0001$) (Fig 1 G, H). These results suggest that down regulation of sNPF in sNPF

expressing tissues enhances starvation-mediated hyperactivity in *Drosophila.*

We further assessed the role of sNPF in regulating the triglyceride levels and feeding

behavior. *Snpf-Gal4 > snpf* ^{RNAi} flies did not exhibit any significant difference in the starvation

resistance and triglyceride levels compared to the control flies (ANOVA, $p>0.05$) (Fig 1I, J). To

test whether sNPF play any role in hunger induced food intake after starvation, we examined the

food intake in both the control and the *snpf-Gal4 > snpf* ^{RNAi} flies under both fed and starvation.

Although starvation enhanced food intake (ANOVA, $p<0.0001$ for *snpf-Gal4 > w^{1118}*; ANOVA,

$p<0.0001$ for *snpf-Gal4 > snpf* ^{RNAi}) (Fig S1A), there was no significant difference in food intake

between the control and the *snpf-Gal4 > snpf* ^{RNAi} flies subjected to starvation, suggesting that

sNPF play no significant role in regulation of triglyceride level, starvation resistance and

enhanced food intake after starvation.

Fig 1. Role of sNPF in starvation-mediated hyperactivity. (A) Transcription profile of *snpf* mRNA in w^{1118} flies under 12:12 h light-dark cycle (LD). White and grey shaded bars represent light and dark phase, respectively, under LD. (B) Transcription profile of *snpf* mRNA in w^{1118} flies constant darkness (DD). *snpf* transcript exhibited a significant daily oscillation with a peak at ZT 8.1h under LD and the oscillation persisted under DD with a peak at CT 8.5h. (C) Average locomotor activity counts/hour of *snpf-Gal4 > w^{1118}* and *snpf-Gal4 > UAS-snpf^{RNAi}* (*snpf-Gal4 > snpf^{RNAi}*) flies assayed with ad libitum food under LD for 36 h. Crossings per 1h are averaged using 32 flies for each genotype. (D) Exposition of starvation-mediated hyperactivity by *snpf-Gal4 > w^{1118}* flies under starvation condition. Flies were transferred to fresh food or to 1% agar medium at ZT12 to provide the fed and starved condition respectively. Gray shaded bars indicate dark phase under LD for 36 h time window. Flies exhibited enhanced locomotor activity under starvation condition compared to fed controls. (E) Average activity counts/hour of *snpf-Gal4 > snpf^{RNAi}* flies assayed under fed and starved condition. (F) Exposition of starvation-mediated hyperactivity by *snpf-Gal4 > w^{1118}* and *snpf-Gal4 > snpf^{RNAi}* flies assayed in the absence of food. (G, H) Total activity of *snpf-Gal4 > w^{1118}* and *snpf-Gal4 > snpf^{RNAi}* flies on Day1 (24h) and Day 2 (ZT13-24) under fed and starved conditions. Total activity (24h) is the sum of activity exhibited under 12 hours of darkness and 12 hours of light. Starvation enhances the activity in both control and experimental flies. Experimental flies exhibited a significant increase in total activity compared to controls. (I) Percentage survivorship of freshly emerged (Day 0) *snpf-Gal4 > w^{1118}* and *snpf-Gal4 > snpf^{RNAi}* flies under starvation condition. (J) Normalized triglyceride levels in Day 0, Day 2 and Day 5 old *snpf-Gal4 > w^{1118}* and *snpf Gal4 > snpf^{RNAi}* flies. No significant difference was observed in starvation resistance and triglyceride

242 levels. The asterisks on locomotor activity waveform and different lower case letters on the

243 vertical bar graphs indicate that the values are significantly different. Error bars represent mean ±

244 SEM. For further explanations see text.

sNPFR expression in IPC is required for starvation-mediated hyperactivity

To test the role of sNPFR expressed in IPCs on starvation-mediated hyperactivity,

we expressed the *UAS-snpfR* ^{RNAi} under *dilp2-Gal4 GFP* driver (*dilp2-Gal4 GFP > snpfR* ^{RNAi}).

We recorded the locomotor activity of these flies under fed condition for 36 h and observed that

flies expressing *snpfR* ^{RNAi} in IPCs show an enhanced evening peak compared to the control flies

(ANOVA, $p<0.03$) (Fig 2A). We analyzed the effect of starvation on locomotor activity in the

control and the *dilp2-Gal4 GFP > snpfR* ^{RNAi} flies. When starved, the control flies exhibited an

increase in locomotor activity during the light phase of Day 1 (ANOVA, $p<0.001$) and during the

dark phase of Day 2 (ZT 13-24) (ANOVA, $p<0.003$) compared to those under fed condition (Fig

2B, F, G). We next examined whether sNPFR expressed in IPCs play any role in starvation-

mediated hyperactivity by comparing the locomotor activity of the *dilp2-Gal4 GFP > snpfR* ^{RNAi}

flies between fed and starved conditions. ANOVA revealed that starved *dilp2-Gal4 GFP >*

snpfR ^{RNAi} flies showed an enhanced evening peak and a prolonged morning peak compared to

the fed flies (ANOVA, $p<0.0001$) (Fig. 2C). This resulted in enhanced total activity during the

dark phase of Day 1 (ANOVA, $p<0.0001$) (Fig. 2E) and Day 2 (ZT 13-24) (ANOVA, $p<0.0001$)

(Fig. 2G). When compared the locomotor activity waveform between the control (*dilp2-Gal4*

GFP > w ^{1118}) and the *dilp2-Gal4 GFP > snpfR* ^{RNAi} flies under starved condition, *dilp2-Gal4 >*

snpfR ^{RNAi} flies exhibited a greater evening peak on Day 1 and 2 (ANOVA, $p<0.001$) (Fig 2D),

showing greater total activity during the dark phase of Day 1 (ANOVA, *p<0.0001*) (Fig 2E) than

the control flies. In addition, the *dilp2-Gal4 GFP > snpfR ^{RNAi}* flies exhibited a marked decrease

in activity during the middle of the light phase on Day 1 (ANOVA, *p<0.001*) (Fig 2D, F),

suggesting that sNPF suppresses starvation-induced hyperactivity partly through the sNPFR

expressed in IPCs.

sNPF expression in circadian clock neurons is not required for starvation-mediated hyperactivity

To test the role of sNPF expressed in circadian clock neurons on starvation-

mediated hyperactivity, we used flies expressing *UAS- snpf ^{RNAi}* under *tim-Gal4* driver (*tim-Gal4*

> snpf ^{RNAi}). Flies expressing *snpf ^{RNAi}* in clock neurons exhibited no significant differences in

starvation-mediated hyperactivity compared to the control flies (*tim-Gal4 > w^{1118}*) (Fig 2H),

suggesting that sNPF expressed in clock neurons has no significant effect on starvation-mediated

hyperactivity.

Fig 2. Role of SNPF receptors expressed in IPC in regulating starvation-mediated

hyperactivity. (A) Average activity counts/hour of *dilp2-Gal4 GFP > w^{1118}* and *dilp2-Gal4*

GFP > UAS-*snpfR ^{RNAi}* (*dilp2-Gal4* GFP > *snpfR ^{RNAi}*) flies assayed under the fed condition. (B)

Exposition of starvation-mediated hyperactivity by *dilp2-Gal4 GFP > w^{1118}* flies assayed in the

absence of food and compared it with their activity under fed condition. (C) Exposition of

starvation-mediated hyperactivity by *dilp2-Gal4 GFP > snpfR^{RNAi}* flies assayed in the absence of

food and compared it with their activity under the fed condition. (D) Starvation-mediated

hyperactivity of *dilp2-Gal4 GFP > w^{1118}* flies and *dilp2-Gal4 GFP > snpfR^{RNAi}* flies assayed in

the absence of food. Experimental flies exhibited enhanced evening peak of activity compared

to the control flies under starved condition. (E, F) Total activity of *dilp2-Gal4 GFP > w^{1118}* and

dilp2-Gal4 GFP > snpfR^{RNAi} flies during the dark phase and light phase of Day 1 under both fed

and starved conditions. (G) Total activity of *dilp2-Gal4 GFP > w^{1118}* and *dilp2-Gal4 GFP >*

snpfR^{RNAi} flies during the dark phase of Day 2 under fed and starved conditions. (H) Exposition

of starvation-mediated hyperactivity by *tim-Gal4 > w^{1118}* and *tim-Gal4 > snpfR^{RNAi}* flies assayed

in the absence of food. Other details are the same as in Fig 1.

Role of *dilp2* in starvation-mediated hyperactivity

As it is already known that sNPF regulates *dilp2* expression in IPCs, we further

studied the role of *dilp2* in starvation-mediated hyperactivity. To examine the effect of tissue-

specific knockdown of *dilp2* in IPCs on starvation-mediated hyperactivity, we expressed the

UAS-dilp2^{RNAi} under *dilp2-Gal4* driver (*dilp2-Gal4 > dilp2^{RNAi}*). We recorded the locomotor

activity of the *dilp2-Gal4 > dilp2^{RNAi}* flies expressing *dilp2^{RNAi}* in IPCs under the fed condition.

These flies exhibited a reduction in the light phase activity of Day 1 compared to controls

(ANOVA, *p*<0.0001) (Fig 3A). To test the effect of starvation on the locomotor activity, we

analyzed the locomotor activity of control (*dilp2-Gal4 > w^{1118}*) flies under fed and starved

conditions. The starved control flies exhibited an increased activity during the light phase of

Day 1 (ANOVA, *p*<0.0001) (Fig 3B), resulting in enhanced total activity on Day 1 (24 h)

(ANOVA, *p*<0.001) (Fig 3E) compared to fed flies. We next examined whether *dilp2* plays any

role in starvation-mediated hyperactivity by recording the locomotor activity of the *dilp2-Gal4 >*

dilp2^{RNAi} flies under fed and starved conditions. Although the starved *dilp2-Gal4 > dilp2^{RNAi}*

flies showed enhanced daytime activity during the first day of starvation (Fig 3B), there was no

significant changes in daily total activity on Day 1 compared to the fed flies (ANOVA, $p>0.05$)

(Fig 3E). During the 2nd dark phase, however, the starved flies exhibited an aberrant and

enhanced activity (ANOVA, $p<0.0001$) (Fig 3C, F). Comparison between the starved control

and $dilp2$-$Gal4 > dilp2$ ^{RNAi} flies revealed that $dilp2$-$Gal4 > dilp2$ ^{RNAi} flies exhibited less total

activity than control flies on Day 1 (ANOVA, $p<0.0001$) (Fig 3D, E). However, there were no

significant differences in total activity on Day 1 between the $dilp2$-$Gal4 > dilp2$ ^{RNAi} flies under

both fed and starved conditions (Fig 3E). These results suggest that down regulation of $dilp2$ in

IPCs reduces the starvation-mediated hyperactivity only during the first 24 h of starvation.

316 *dilp2* governs starvation resistance and triglyceride metabolism

317    We further assessed the starvation resistance of freshly emerged $dilp2$-$Gal4 > dilp2^{RNAi}$

flies and compared it with that of the control ($dilp2$-$Gal4 > w^{1118}$) flies. The $dilp2$-$Gal4 > dilp2$

^{RNAi} flies showed reduced starvation resistance compared to the control flies (t-test, $p<0.03$) (Fig

3G, S1B). We quantified triglyceride levels on Day 0, Day 2 and Day 5 in $dilp2$-$Gal4 > w^{1118}$

and $dilp2$-$Gal4 > dilp2^{RNAi}$ flies and found that the triglyceride levels were significantly reduced

in $dilp2$-$Gal4 > dilp2$ ^{RNAi} flies on these days (t-test, $p<0.0001$ for Day 0, t-test, $p<0.02$ for Day 2,

t-test, $p<0.0001$ for Day 5) (Fig 3H). These results suggest that $dilp2$ regulates starvation

resistance by maintaining the triglyceride metabolism.

Fig 3. Role of *dilp2* in starvation-mediated hyperactivity and triglyceride metabolism. (A)

Average locomotor activity counts/hour of $dilp2$-$Gal4 > w^{1118}$ and $dilp2$-$Gal4 > UAS$-$dilp2$ ^{RNAi}

(dilp2-Gal4 > dilp2 ^{RNAi}) flies assayed under the fed condition. (B) Exposition of starvation-

mediated hyperactivity by *dilp2-Gal4 > w^{1118}* flies assayed in the absence of food and compared

it with their activity under the fed condition. (C) Exposition of starvation-mediated hyperactivity

by *dilp2-Gal4 > dilp2 ^{RNAi}* flies assayed under starvation condition and compared it with their

activity under the fed condition. (D) Starvation-mediated hyperactivity of *dilp2-Gal4* control

flies and *dilp2-Gal4 > dilp2^{RNAi}* flies assayed in the absence of food. Experimental flies

exhibited a reduction in activity during the light phase of day 1 compared to controls under

starvation condition. (E, F) Total activity (24 h) of *dilp2-Gal4 > w^{1118}* and *dilp2-Gal4 > dilp2*

^{RNAi} flies on Day1 and during the dark phase (ZT 13-24 hr) of Day 2 under fed and starved

conditions. *dilp2-Gal4 > dilp2 ^{RNAi}* flies exhibited a reduction in total activity on Day 1

compared to *dilp2-Gal4 > w^{1118}* flies under starvation condition, whereas *dilp2-Gal4 > dilp2 ^{RNAi}*

flies did not exhibit any significant difference in total activity on Day 1 under both fed and

starved conditions. (G) Percentage survivorship of freshly emerged flies expressing *dilp2^{RNAi}* in

IPC under *dilp2-Gal4* control under starvation condition. Flies expressing *dilp2^{RNAi}* in IPCs

exhibit reduced starvation resistance compared to the control flies. (H) Normalized triglyceride

levels of Day 0, Day 2 and Day 5 old *dilp2-Gal4 > w^{1118}* and *dilp2-Gal4 > dilp2 ^{RNAi}* flies. Flies

expressing *dilp2 ^{RNAi}* in IPCs exhibit reduced triglyceride levels compared to the control flies.

Other details are the same as in Fig 1.

Role of insulin receptors expressed in the fat body in regulating starvation-mediated hyperactivity.

Since *dilp2* is shown to effect fat metabolism, we checked for the effects by disrupting

the insulin signalling in the fat body, the tissue responsible for the storage of lipid droplets. To

assess whether insulin receptors (*InR*) expressed in the fat body play any role in regulating the

starvation-mediated hyperactivity, we overexpressed *InR* dominant negative (*InR* ^{DN}) under the

CG-Gal4 driver (*CG-Gal4 > InR* ^{DN}) and recorded the locomotor activity of the *CG Gal4 > InR*

^{DN} flies. The *CG Gal4 > InR* ^{DN} flies overexpressing *InR* ^{DN} in fat body exhibited reduced activity

during the middle of the light phase under the fed condition compared to the control (*CG-Gal4 >*

w ^{1118}) flies (ANOVA, $p<0.0001$) (Fig 4A). To test the effect of insulin receptors (*InR*) expressed

in the fat body in starvation-mediated hyperactivity, we recorded the locomotor activity of

control and *CG-Gal4 > InR* ^{DN} flies under fed and starved conditions. The starved control flies

exhibited an increase in locomotion during the light phase on Day 1 (ANOVA, $p<0.006$) (Fig

4B, F) and during the dark phase of Day 2 (ZT 13-24) compared to fed flies (ANOVA,

$p<0.0001$) (Fig 4B, G). Starved *CG-Gal4 > InR* ^{DN} flies showed enhanced daytime activity

compared to the fed flies on Day1 (ANOVA, $p<0.0001$) (Fig 4C). The starved flies exhibited

aberrant and enhanced activity during the 2nd dark phase (ANOVA, $p<0.0001$) (Fig 4C, G).

Comparison the starvation induced hyperactivity between control and *CG-Gal4 > InR* ^{DN} flies

revealed that *CG-Gal4 > InR* ^{DN} flies exhibited a greater evening and morning peaks but less

activity during the middle of dark and light phase on day 1 (ANOVA, $p<0.04$) (Fig 4D) and that

they showed greater activity during the 2nd dark phase. These results suggest that down

regulation of *InR* in the fat body alters the starvation-mediated hyperactivity.

Involvement of *InR* in starvation resistance and triglyceride metabolism

We further compared the starvation resistance between freshly emerged (Day 0) *CG-*

Gal4 > InR ^{DN} flies and *CG-Gal4 > w* ^{1118} control flies. The *CG-Gal4 > InR* ^{DN} flies showed

reduced starvation resistance compared to the control flies (t-test, $p<0.04$) (Fig 4H, I).

Consistent with the starvation resistance phenotype, they showed decreased triglyceride levels (t-

test, $p<0.003$) (Fig 4J), suggesting involvement of *InR* expressed in the fat body in starvation

resistance and triglyceride metabolism.

InR expressed in fat body governs enhanced food intake after starvation

We next compared the food intake between $CG\text{-}Gal4 > InR^{DN}$ flies and control (*CG-*

$Gal4 > w^{1118}$) flies. The results showed that 24h starvation enhances food intake in control flies

(ANOVA, $p< 0.03$). Food intake was more enhanced in starved $CG\text{-}Gal4 > InR^{DN}$ flies

compared to the starved control flies (ANOVA, $p<0.01$) (Fig S1C), suggesting that insulin

signaling in fat body suppresses food intake under starvation.

Fig 4. Role of insulin receptors (*InR*) expressed in the fat body in regulating starvation-

mediated hyperactivity and triglyceride metabolism. (A) Average activity counts/hour of

$CG\text{-}Gal4 > w^{1118}$ and $CG\text{-}Gal4 > UAS\text{-}InR^{DN}$ ($CG\text{-}Gal4 > InR^{DN}$) flies assayed under fed

condition. (B) Exposition of starvation-mediated hyperactivity by $CG\text{-}Gal4 > w^{1118}$ flies assayed

under starvation condition and compared it with their activity under the fed condition. (C)

Exposition of starvation-mediated hyperactivity by $CG\text{-}Gal4 > InR^{DN}$ flies assayed in the

absence of food and compared it with their activity under fed condition. (D) Locomotor activity

waveform of $CG\text{-}Gal4 > w^{1118}$ control flies and $CG\text{-}Gal4 > InR^{DN}$ flies assayed under starvation

condition. Flies expressing InR^{DN} in the fat body alter starvation-mediated hyperactivity

compared to control flies. (E, F) Total activity of *CG-Gal4 > w[1118]* and *CG-Gal4 > InR [DN]* flies

during the dark phase and light phase of Day 1 under fed and starved conditions. (G) Total

activity of *CG- Gal4 > w[1118]* and *CG-Gal4 > InR [DN]* flies during the dark phase of Day 2 under

fed and starved conditions. (H) Percentage survivorship of freshly emerged *CG- Gal4 > InR [DN]*

and control flies under starvation condition. (I) Time taken for 50% of death in *CG-Gal4 > w[1118]*

control and *CG-Gal4 > InR [DN]* flies under starvation condition. Expressing *InR [DN]* in the fat body

reduced the starvation resistance in freshly emerged flies. (J) Triglyceride content of freshly

emerged (Day 0) *CG*-Gal4 > *w[1118]* control and *CG-Gal4 > InR [DN]* flies. Expressing *InR [DN]* in the

fat body reduced the triglyceride levels in freshly emerged flies. Other details are the same as in

Fig 1.

Discussion

Behavior and metabolism are functionally intertwined processes that modulate each

other. However, it remains elusive how the circadian clock governs behaviors, including feeding

and locomotor activity, in synchrony with the metabolic status of an organism. In the present

study, we aimed to investigate the specific role of two circadian relevant metabolic

neuropeptides, i.e., sNPF and DILP2, in lipid metabolism and starvation-mediated behavioral

changes in *Drosophila*. The results of the present study showed that *snpf* transcript exhibited a

circadian oscillation in the fly head. sNPF protein is expressed in a diverse subset of neurons in

the adult fly brain including olfactory receptor neurons, various small interneurons, kenyon cells

of the mushroom bodies and in the circadian clock neurons [33]. Among the ~150 clock neurons

clustered in the fly brain, two dorsal lateral neurons (LNds) and PDF expressing small ventral

lateral neurons (s-LNvs) express sNPF [34]. Based on the previous findings, mRNA cycling is

essential for the circadian clock neuron function including the neuronal firing or electrical

excitability [25], [35]. Hence it is quite likely that the *snpf* transcript oscillation is more apparent

in circadian clock neurons than other sNPF expressing diverse neuronal subsets. Most of the

previously identified cycling head transcripts are tissue or cell-specific in the mammalian system

[36]. Ubiquitous mRNA cycling is relatively rare. Further studies are required to confirm

whether sNPF oscillation is indeed restricted to clock neurons or if it also oscillates in non-clock

neurons.

sNPF expression in a large population of diverse neurons indicates the multiple discrete

functions of this neuropeptide [33]. So far studies demonstrated that sNPF regulates feeding and

its expression in fan shaped body might be involved in fine-tuning the locomotor activity level

[24], [37]. Expression of sNPF and sNPF receptor in the mushroom body and clock neurons

regulate sleep in *Drosophila* [25], [38]. The results of our present studies showed that

knockdown of *snpf* in sNPF expressing neurons enhances starvation-mediated hyperactivity,

suggesting the role of sNPF in suppressing hyperactivity under starvation. sNPF acts as an

inhibitory modulator in neuronal networks that express sNPFR [39]. Knockdown of *snpfR* in

IPCs in an adult stage-specific manner does not affect the starvation-mediated sleep loss in

Drosophila [24], whereas the results of our study showed that constitutive knockdown of *snpfR*

in IPCs partially suppress starvation-mediated hyperactivity in *Drosophila*. sNPF may suppress

the starvation-mediated hyperactivity mainly via IPCs not through the circadian clock neurons in

Drosophila. Our results showed that sNPF play no significant role in regulation of triglyceride

levels and starvation-mediated feeding behavioral changes suggesting that sNPF independently

regulate starvation-induced hyperactivity without an effect on enhanced food intake upon

starvation.

IPCs are metabolic clock output cells and the rhythmic output of IPC is modulated

by both the circadian clock and nutrient uptake [19]. Flies expressing *dilp2* RNAi in IPCs and

flies expressing InR^{DN} in the fat body exhibit differential starvation-induced hyperactivity

phenotypes. Since, in the latter, InR was blocked only in the fat body, InR expression in other

tissues may partly regulate the starvation-mediated hyperactivity phenotype [29]. This could be

the possible reason for the difference observed in the starvation-mediated hyperactivity

phenotypes between the flies expressing *dilp2*-RNAi in IPCs and the flies expressing InR^{DN} in

the fat body. Our results showed for the first time that the clock output molecule DILP2 plays an

important role in starvation resistance, and triglyceride metabolism in *Drosophila*. It has been

documented that the peripheral circadian clock in the *Drosophila* fat body regulates the storage

of lipid and glycogen [5], [16]. However, the potential mediators through which the central

clock communicates with the peripheral clock to regulate the triglyceride metabolism still

remains to be elucidated. In the present study, flies expressing *dilp2* RNAi in IPCs and flies

expressing InR^{DN} in the fat body have reduced starvation resistance and triglyceride levels,

suggesting the role of DILP2 as a potential mediator involved in triglyceride metabolism.

Drosophila insulin like peptide2 (DILP2) drives rhythmicity in the expression of

lipase *sxe2* transcript in the fat body. Rhythmic expression of *sxe2* is disrupted in flies

expressing dominant negative insulin receptor InR^{DN} in the fat body [19]. Hence it is likely that

perturbation on *sxe2* oscillation plays a role in the reducing the triglyceride levels in flies

expressing *dilp2*-RNAi in IPCs and the flies expressing InR^{DN} in the fat body. Further studies are

required to confirm whether DILP2 drive the oscillation of other fat body specific gene

transcript/s to mediate triglyceride metabolism. Reduction in triglyceride level could be either

due to the enhanced energy expenditure or due to reduced energy intake [40]. We also tested

whether reduced triglyceride level is due to the reduced energy intake. Our results showed that

expressing InR^{DN} in the fat body does not have significant impact on food intake under fed

condition whereas these flies exhibit enhanced food intake only under starved condition. It is

thus also likely that the insulin signaling may play a role in suppressing food intake only under

starved conditions not under fed condition. In the present study, since the food intake under

starvation condition was assessed only at ZT00 after 24 h of starvation, we need further evidence

to confirm whether hyperactivity exhibited as a response to starvation is indeed food-seeking

behavior.

Taken together, our present study showed that sNPF suppresses the starvation-

mediated hyperactivity partially through sNPF receptors expressed in the IPCs. The insulin

signaling mediated by the circadian clock output molecule DILP2 in the fat body appears to be a

central pathway in triglyceride metabolism and regulates feeding behavior in accordance with the

nutrient availability in *Drosophila*.

Acknowledgements

We thank Sabira A for the experimental assistance. We thank Jishy Varghese for the valuable

suggestions and for providing the infrastructure facility to carry out some of the experiments.

We thank Kenji Tomioka for the valuable suggestions and Sheeba Vasu for providing the fly

lines. This work was supported by the Wellcome Trust/DBT India Alliance Fellowship

[IA/E/15/1/502329] awarded to NNK and intramural fund from Indian Institute of Science

Education and Research, Thiruvananthapuram.

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Supporting information

S1 Fig. Role of sNPF and DILP2 in feeding behavior after starvation. (A) Food intake in 2-day-old *snpf-Gal4 > w^{1118}* control and *snpf-Gal4 > snpf* ^{RNAi} flies under fed and starved conditions. Both control and experimental flies exhibited enhanced food intake after 24hr of starvation compared to control flies under fed condition. (B) Time taken for 50% death of freshly emerged flies expressing *dilp2^{RNAi}* in IPC under *dilp2-Gal4 > w^{1118}* control under starvation condition. (C) Food intake in 2-day-old *CG-Gal4 > w^{1118}* control and *CG-Gal4 > InR^{DN}* flies under fed and starved conditions. Experimental flies exhibited enhanced food intake after 24h of starvation compared to starved *CG-Gal4 > w^{1118}* flies.