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Investigation

Ion transport peptide regulates energy intake, expenditure, and metabolic homeostasis in *Drosophila*

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Abstract

In mammals, energy homeostasis is regulated by the antagonistic action of hormones insulin and glucagon. However, in contrast to the highly conserved insulin, glucagon is absent in most invertebrates. Although there are several endocrine regulators of energy expenditure and catabolism (such as the adipokinetic hormone), no single invertebrate hormone with all of the functions of glucagon has been described so far. Here, we used genetic gain- and loss-of-function experiments to show that the *Drosophila* gene *Ion transport peptide* (ITP) codes for a novel catabolic regulator that increases energy expenditure, lowers fat and glycogen reserves, and increases glucose and trehalose. Intriguingly, Ion transport peptide has additional functions reminiscent of glucagon, such as inhibition of feeding and transit of the meal throughout the digestive tract. Furthermore, Ion transport peptide interacts with the well-known signaling via the Adipokinetic hormone; Ion transport peptide promotes the pathway by stimulating Adipokinetic hormone secretion and transcription of the receptor *AkhR*. The genetic manipulations of Ion transport peptide on standard and Adipokinetic hormone-deficient backgrounds showed that the Adipokinetic hormone peptide mediates the hyperglycemic and hypertrehalosemic effects of Ion transport peptide, while the other metabolic functions of Ion transport peptide seem to be Adipokinetic hormone independent. In addition, Ion transport peptide is necessary for critical processes such as development, starvation-induced foraging, reproduction, and average lifespan. Altogether, our work describes a novel master regulator of fly physiology with functions closely resembling mammalian glucagon.

Keywords: *Drosophila*; ITP; metabolism; fat; glycogen; trehalose; development; energy homeostasis; insect hormones; crustacean hyperglycemic hormone; Adipokinetic hormone

Introduction

The fine control of energy catabolism and anabolism is crucial for organismal survival and growth. Energy metabolism needs to be adjusted to changing environmental conditions (such as temperature and water and food availability) and intrinsic processes (such as development, reproduction, and other energy-demanding activities) (Gáliková and Klepsatel 2018; Gillette *et al.* 2021). The key endocrine factors that regulate energy balance are often pleiotropic, i.e. integrate several aspects of animal physiology. Such pleiotropic hormones are also insulin and glucagon. While insulin is a hypoglycemic hormone-promoting anabolism, glucagon is a hyperglycemic peptide that increases catabolism and energy expenditure and inhibits feeding. These 2 antagonistic hormones regulate numerous metabolic processes, including satiety, digestion, energy metabolism of stored reserves, and basal metabolic rate (Guo 2014; Al-Massadi *et al.* 2019; Kleinert *et al.* 2019). Thus, both insulin (Das and Arur 2017) and glucagon (Yu *et al.* 2012; Charron and Vuguin 2015) signaling are essential for processes such as development, reproduction, and average lifespan. While insulin signaling is highly evolutionarily conserved (Das and Arur 2017), insects, including the popular model *Drosophila melanogaster*, do not seem to have a true homolog of glucagon. Instead, the hyperglycemic functions are governed by the Adipokinetic hormone (AKH) (Kim and Rulifson 2004; Lee and Park 2004; Gáliková *et al.* 2015) and, during the immune response, by extracellular adenosine (Zuberova *et al.* 2010; Bajgar *et al.* 2015). AKH is often considered a functional analog of glucagon (e.g. Kim and Rulifson 2004; Ahmad *et al.* 2020; Hughson 2021). The AKH peptide controls a plethora of processes including, for example, fat storage (Gáliková *et al.* 2015; Gáliková, Klepsatel, Xu, *et al.* 2017), levels of circulating sugars (Kim and Rulifson 2004; Lee and Park 2004; Gáliková *et al.* 2015), food intake (Jourjine *et al.* 2016; Gáliková, Klepsatel, Xu, *et al.* 2017), oxidative stress response (Bednářová *et al.* 2015; Zemanova *et al.* 2016), and others. However, apart from the hyperglycemic action, AKH deficiency does not copy phenotypes of mutants in glucagon signaling. For example, glucagon signaling is required for development, reproduction, and lifespan. The mutations in the glucagon receptor (Gcgr) lead to altered placentation, poor fetal growth, and increased fetal–neonatal death (Vuguin *et al.* 2006; Ouhilal *et al.* 2012). In adulthood, Gcgr deficiency causes Mahvash disease, which leads to progressive cachexia, severe hypoglycemia, and early death (Yu *et al.* 2012). In contrast, mutants in the *Akh* signaling are fully viable (Gáliková *et al.* 2015; Sajwan *et al.* 2015).

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Fecundity analysis of *Akh* and *AkhR* mutants carried out during the first 10 days of their lives did not reveal any decrease in reproductive success (Gáliková *et al.* 2015). Furthermore, when the reproduction of *Akh*-deficient and w^{1118} females was compared after a 24-h mating, the *Akh* mutants even performed better than the controls (Wat *et al.* 2021). In males, the AKH pathway modulates pheromone production and courtship (Lebreton *et al.* 2016). The Akh^{A} mutant males have reduced reproductive success when their mating is restricted to a 1-h-long trial (Wat *et al.* 2021), but the effect disappears when they have access to females over a longer period (Gáliková *et al.* 2015; Bednářová *et al.* 2018). In contrast to the detrimental consequences of increased or decreased glucagon (Janah *et al.* 2019), the AKH manipulations seem to have milder effects on general physiology and lifespan. Some studies showed that males deficient in the peptide have normal lifespan (Bednářová *et al.* 2018), while other works reported a lifespan-shortening effect of the *Akh* mutation (Hofbauer *et al.* 2021) and silencing of the AKH-producing cells (Wat *et al.* 2021). Interestingly, ubiquitous overexpression of *Akh* (Katewa *et al.* 2012) or activation of the AKH-producing cells (Waterson *et al.* 2014) even increased life expectancy. The differences between AKH and glucagon also extend to their roles in regulating energy intake. Glucagon represses feeding (Schulman *et al.* 1957; Geary *et al.* 1993), while AKH has the opposite function (Jourjine *et al.* 2016; Gáliková, Klepsatel, Xu, *et al.* 2017). Finally, in contrast to glucagon triggering mobilization of lipid and carbohydrate reserves (Rui 2014; Campbell and Drucker 2015), AKH induces lipolysis but not mobilization of glycogen (Gáliková *et al.* 2015; Gáliková, Klepsatel, Xu, *et al.* 2017; Yamada *et al.* 2018). Moreover, mice deficient in the Gcgr have reduced adipose tissue and triglycerides (Gelling *et al.* 2003), while the *Akh* and AKH receptor (*AkhR*) mutant flies are obese (Grönke *et al.* 2007; Gáliková *et al.* 2015). Altogether, the phenotypes of *Akh* or *AkhR* deficiencies do not fully recapitulate the role of glucagon signaling in mammals. Given the conservation of the insulin pathway between flies and mammals, it is natural to expect its antagonistic hormone in both groups. However, the *Drosophila* genome seems to lack a gene with sequence similarity to glucagon, suggesting that other hormones fulfill its function and regulate fly physiology alongside AKH. Intriguingly, insects, including *Drosophila*, carry homologs of a gene called Crustacean hyperglycemic hormone (CHH), known for its developmental, reproduction-related, and hyperglycemic functions in crustaceans (Chung *et al.* 2010; Webster *et al.* 2012; Chen *et al.* 2020). The CHH-family peptides have a broad expression pattern that includes tissues such as the eyestalk, pericardial organ, gill, stomach, intestine, and others (Chen *et al.* 2020). The CHH peptides control numerous processes, including osmoregulation, digestion, and carbohydrate and lipid metabolism (Webster *et al.* 2012; Chen *et al.* 2020).

The fly homolog of CHH, Ion transport peptide (ITP), is a relatively unknown hormone expressed mainly in the brain and peripheral nervous system. The brain expression includes ipc-1 and ipc-2 neurosecretory cells and the ipc-3 and ipc-4 interneurons. In the periphery, ITP is expressed in the abdominal ganglion cells and the lateral bipolar dendrite neurons of the abdominal segments A7/A8 (Dircksen *et al.* 2008; Gáliková *et al.* 2018). The production of ITP outside of the nervous system has not been fully investigated in the fruit flies, but the FlyAtlas (Chintapalli *et al.* 2007) and FlyAtlas 2 (Leader *et al.* 2018; Krause *et al.* 2022) data suggest expression of the gene in various organs including gut, trachea, heart, and others. The extraneuronal expression of the *ITP* gene has been confirmed in other insect species. For example, the *ITP* gene is expressed in the midgut and carcass of the red flower beetle *Tribolium castaneum* (Begum *et al.* 2009) and in the male reproductive tract and integument of the brown planthopper, *Nilaparvata lugens* (Yu, Li, *et al.* 2016).

The *ITP* gene codes for 3 peptides—the ITP and the so-called ITP-like peptides 1 and 2. However, only ITP has an amidation signal at the N-terminus, which is crucial for the activity of the hormone. Thus, only ITP is considered an active hormone in *Drosophila* (Dircksen *et al.* 2008; Dircksen 2009). Receptor(s) for *Drosophila* ITP is currently unknown. The existence of multiple receptors for CHH was predicted in crustaceans (Nery *et al.* 1993). Remarkably, 3 different promiscuous receptors bind ITP in *Bombyx mori* (Nagai *et al.* 2014). It is thus possible that more receptors also exist in *Drosophila*.

The biological functions of ITP are relatively understudied. The first described role of this peptide has been its stimulatory effect on the evening and nocturnal activity (Hermann-Luibl *et al.* 2014). Later, it was shown that ITP is an antidiuretic hormone that antagonistically regulates water and food intake (Gáliková *et al.* 2018). ITP also protects from diarrhea by repressing the speed of transit throughout the digestive tract (Gáliková *et al.* 2018). These functions probably cannot be attributed to specific ITP neurons; it seems that the neurosecretory cells synthesize the hormone redundantly and compensate for a partial *ITP* knock-down (Gáliková *et al.* 2018).

In contrast to the relatively well-investigated glucagon-like functions of CHHs, not much is known about the roles of ITP in metabolic homeostasis. However, the roles of ITP in the repression of hunger and the speed of transit throughout the digestive tract (Gáliková *et al.* 2018) are intriguingly reminiscent of the role of glucagon in the inhibition of feeding (Schulman *et al.* 1957; Geary *et al.* 1993) and peristalsis (Paul and Freyschmidt 1976). Therefore, we investigated here the potential roles of ITP in the key glucagon-regulated processes, such as glycemia control, basal metabolic rate, homeostasis of stored energy reserves, development, and reproduction. The functional analyses of ITP are complicated by the complexity (Dircksen *et al.* 2008; Gáliková *et al.* 2018) and redundancy (Gáliková *et al.* 2018) in the ITP expressing neurons, the lethality of the *ITP* mutants and ubiquitous RNAi treatment (Gáliková *et al.* 2018), and, most of all, unknown receptors and downstream components of the pathway. Therefore, we focused mainly on ubiquitous adulthood-specific manipulations using the GeneSwitch system, which previously turned out to be useful for discovering the antidiuretic roles of ITP (Gáliková *et al.* 2018). Intriguingly, our gain- and loss-of-function manipulations of ITP resulted in phenotypes reminiscent of dysregulations of mammalian glucagon signaling. In addition, we found that the well-known AKH signaling is partially under the control of ITP and mediates its hyperglycemic functions.

Materials and methods

Fly lines and husbandry

Flies for the standard UAS–GAL4 and GeneSwitch experiments were reared under a 12 h light–12 h dark cycle at 25°C. The *Drosophila* medium consisted of 6 g agar, 50 g yeast, 100 g sugar, 5.43 ml propionic acid, and 1.3 g methyl 4-hydroxybenzoate per 1 l of medium. Adult flies were collected within 24 h after eclosion and housed in groups of approximately 30 females and 30 males per vial. Flies for the GeneSwitch experiments were transferred to the medium supplemented with RU-486 (200 μM) or ethanol vehicle control on the third day after eclosion. Flies for the c929-GAL4- and *Impl2*-GAL4-based TARGET (temporal and

regional gene expression targeting) experiments developed at 18°C and on the third day after eclosion were transferred to 29°C for the transgene induction. After induction of the transgene expression, flies were flipped every second day to fresh media. If not stated otherwise, male flies were used for experiments 6–7 days after the induction of the transgene expression. Controls for the non-GeneSwitch experiments were generated by crossing the UAS and GAL4 lines to the w^{1118} strain. The list of the fly stocks used in the study is available in Supplementary File 1.

Developmental lethality

Approximately 4-day-old parental flies were reared on a standard medium with sprinkled yeast for 3 days. The food was exchanged daily to prevent egg retention. Afterward, flies were transferred on standard medium, and eggs were collected over 4 h. These eggs were used for the analysis of developmental lethality. Embryonic, larval, and pupal lethality was expressed as a proportion of animals that died during the given developmental stage. Viability was examined in at least 3 replicates per genotype. Fisher's exact test was conducted on pooled data. Altogether, the viability of at least 370 eggs was tested per each genotype/treatment.

Developmental timing

Approximately 4-day-old parental flies were reared for 3 days on a standard medium with sprinkled yeast. Food was exchanged daily to prevent egg retention. The flies were then transferred to a new medium, and eggs for the analysis of the developmental timing were collected over 4 h. Developmental timing was measured as the time from the egg deposition until the eclosion of the adult fly (Fig. 1b) and as the time from the egg deposition until the pupation (Supplementary Fig. 1b). All experiments were conducted in 3 replicates.

Body size measurement

Body size was estimated as the pupal volume, using a previously described method (Delanoue *et al.* 2010), based on the formula $4/3\pi\ (L/2)(l/2)^2$ (L = length; l = diameter of the pupae). Photographs were taken by Leica WILD M32 stereomicroscope and analyzed by ImageJ.

Lifespan determination

Freshly eclosed flies (24-h cohorts) were collected, transferred to standard food, and allowed to mate for 3 days. Afterward, male and female flies were separated and randomly distributed on the GeneSwitch food (200 μM RU-486 or ethanol as vehicle control). Flies were flipped on fresh media every other day. At least 5 replicates (altogether, at least 106 animals) were tested per sex/genotype/treatment.

Fecundity assays

Fecundity was measured in 5 replicates, each consisting of 3 females and 3 males. The number of eggs laid was determined on days 7 and 8 after the induction of the transgene expression (overexpression experiments) and on days 7, 8, and 10 after the induction of the transgene expression (RNAi experiments).

Fertility assay

Fertility was estimated as embryonic viability, i.e. as the proportion of eggs that hatched into the 1st larval instar. The experiment was conducted on the eggs from the fecundity assay. At least 291 eggs were tested per genotype/treatment.

Determination of dry weight

Flies were desiccated for 2 days at 65°C and weighed on a microbalance. At least 5 replicates (each consisting of 5 flies) were tested per genotype/treatment.

Determination of the whole body fat, glycogen, protein, and trehalose content

Lipids, proteins, glycogen, trehalose, and glucose were quantified by methods adapted from Tennessen *et al.* (2014) with modifications described previously (Gáliková *et al.* 2015; Gáliková, Klepsatel, Münch, *et al.* 2017). Briefly, 5 flies per replicate were homogenized in 600 ml of 0.05% Tween-20 in PBS and heat-inactivated at 70°C. After centrifugation, the supernatant was transferred into deep-well plates and further used for the colorimetric assays. Lipids were measured by the Triglycerides (liquid) assay (Randox, TR1697), proteins were measured by the Pierce BCA Protein assay kit (Thermo Fisher Scientific), and glycogen was determined by the Glucose assay kit (GO, Sigma) supplemented with 3 U of amyloglucosidase (Sigma) per 1 ml of the reagent. Glycogen content was calculated as the difference between the total glucose after the incubation with amyloglucosidase and the free glucose before the incubation with the enzyme. Trehalose was measured by the GO kit supplemented with 3 μl of porcine trehalase (Sigma, T8778-1U) per 1 ml of the reagent. Trehalose content was calculated as the difference between the total glucose after the incubation with trehalase and the free glucose before the incubation with the enzyme. The homogenates for the trehalose, glucose, and glycogen measurements were diluted 1:3 in PBS prior to the experiments.

Determination of circulating sugars

Circulating trehalose was measured by a method adapted from Tennessen *et al.* (2014) with modifications described before (Gáliková *et al.* 2015). Briefly, the hemolymph was collected by centrifugation (6 min, 9,000 rcf at 4°C) of decapitated flies. These flies were placed in a holder tube with bottom holes, which was inserted in a 0.5-ml collection tube. Each sample contained hemolymph collected from 50 flies. A total of 1 μl of the collected hemolymph was diluted with 99 μl of 0.05% Tween-20 in PBS, heat-inactivated, further diluted 1:6 in PBS, and used for the measurements using the same method as in the case of the whole body homogenates.

Quantitative PCR

RNA was extracted using the Zymo Research QuickRNA MicroPrep kit. cDNA was synthesized by the QuantiTect Reverse Transcription Kit (Qiagen) using 1 μg of the total RNA. Quantitative PCR was performed in the StepOne Real-Time PCR System (Applied Biosystems) in the SensiFAST SYBR Hi-ROX Kit (Bioline) reaction mix. Expression levels were normalized to the *Actin 5C* (*Act5C*) and *Ribosomal protein L32* (*Rpl32*, data in the main text) or to *β-Tubulin at 60D* (*βTub60D*, confirmatory data in the Supplementary material). Primer sequences are found in Supplementary File 1.

Metabolic rate measurements

The basal metabolic rate was estimated by manometric respirometry as the volume of consumed O_2 according to the protocol described by Yatsenko *et al.* (2014). Six replicates (each consisting of 5 male flies) were tested per each genotype/treatment. The metabolic rate was measured over 4 h.

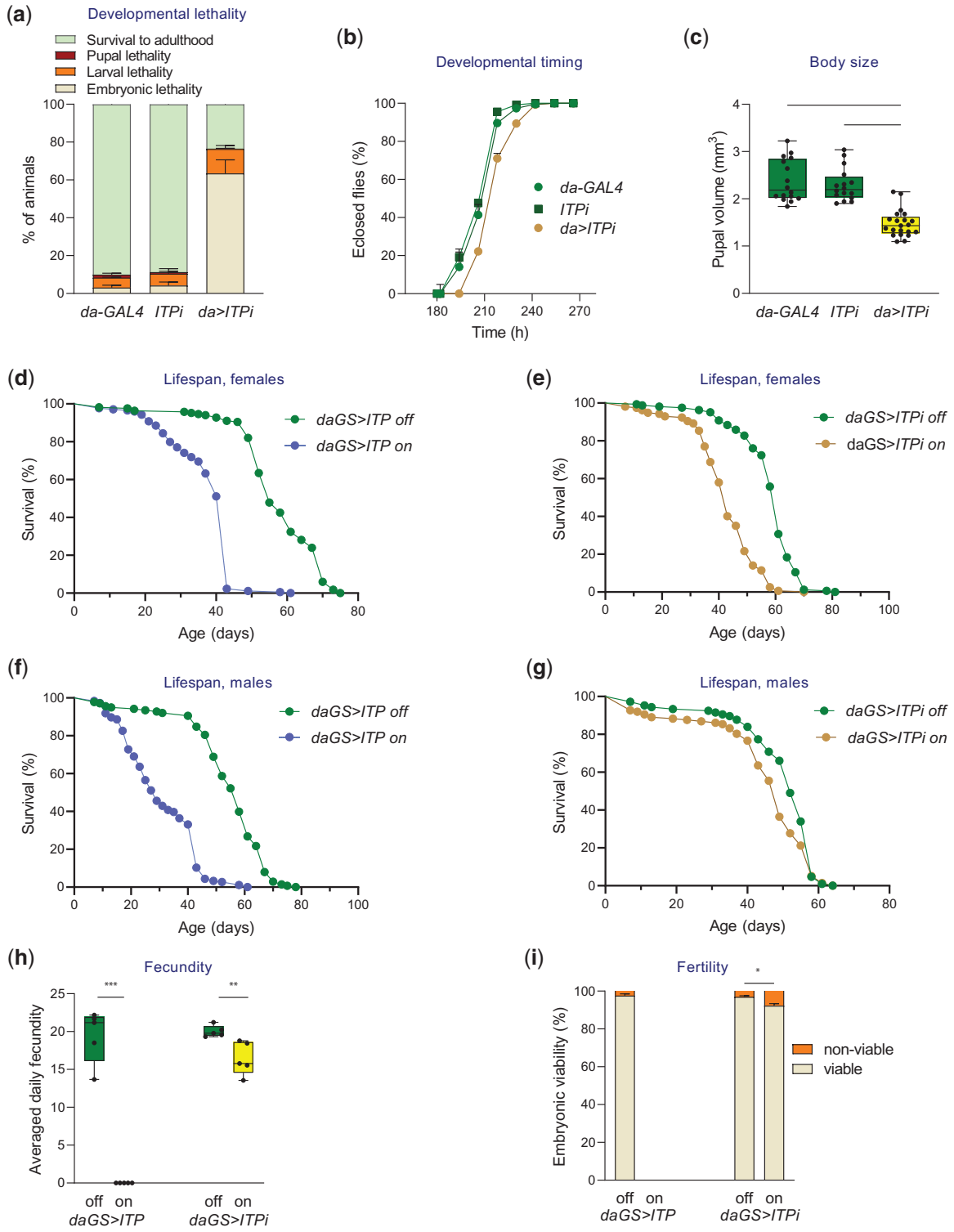

Fig. 1. ITP is required for development, reproduction, and lifespan. a) ITP is required for the development. *Daughterless*-GAL4-driven *ITP* RNAi (*da>ITPi*) results in increased embryonic and larval lethality when compared to controls (UAS and GAL4 lines crossed to w^{1118} background). Fischer's exact test: $P < 0.001$ for comparison with each control for both embryonic and larval survival. Animals were analyzed in 3 replicates; Fischer's exact test was performed on pooled data. Sample size: *da-GAL4*, $n = 382$; *ITPi*, $n = 381$; *da>ITPi*, $n = 370$. Error bars represent SEM. b) A decrease in ITP via *da>ITPi* results in a prolonged development. Gehan–Breslow–Wilcoxon test: $P < 0.001$ for comparison with each control. Sample size: *da-GAL4*, $n = 167$; *ITPi*, $n = 140$; *da>ITPi*, $n = 110$. c) A decrease in ITP via *da>ITPi* results in a smaller body size (estimated as pupal volume). Two-tailed Student's *t*-test: $P < 0.001$ for comparison with each control. Sample size: *da-GAL4*, $n = 16$; *ITPi*, $n = 16$; *da>ITPi*, $n = 21$. d–g) ITP is required for a normal lifespan. Adulthood-specific upregulation and downregulation of ITP via *daughterless*-GeneSwitch (*daGS*) decrease lifespan in males and females. d) Effect of *ITP* overexpression in females: log-rank test $P < 0.001$. Sample size *daGS>ITP* off $n = 164$, *daGS>ITP* on $n = 174$. e) Effect of *ITPi* in females: log-rank test $P < 0.001$. Sample size *daGS>ITPi* off $n = 163$, *daGS>ITPi* on $n = 157$. f) Effect of *ITP* overexpression in males: log-rank test $P < 0.001$. Sample size *daGS>ITP* off $n = 138$, *daGS>ITP* on $n = 184$. g) Effect of *ITPi* in males: log-rank test $P < 0.01$. Sample size *daGS>ITPi* off $n = 106$, *daGS>ITPi* on $n = 137$. h) An increase in ITP signaling via *daGS>ITP* inhibits egg production. Two-tailed Student's *t*-test: $P < 0.001$. A decrease in ITP signaling via *daGS>ITPi* reduces egg production. Two-tailed Student's *t*-test: $P < 0.01$. i) A decrease in ITP signaling via *daGS>ITPi* reduces embryonic viability (estimated as the

Determination of the spontaneous locomotor activity

Spontaneous activity of individual males was tested using the *Drosophila* Activity Monitor 2 system (TriKinetics). Monitoring tubes contained the standard medium supplemented with RU-486 or the vehicle control (ethanol). Spontaneous locomotion was determined as the total number of midline crossings during the tested period of time (3 days). At least 22 flies were tested per genotype/treatment.

Determination of starvation resistance

Starvation resistance was measured as the time survived on a medium containing 0.6% agarose cooked in distilled water. At least 3 replicates (each with a minimum of 22 flies) were tested per sex/genotype/treatment.

Analysis of the starvation-induced hyperactivity

The starvation-induced hyperactivity was determined as the total activity during the last 12 h of life under starvation as described previously (Gáliková *et al.* 2015). After 6 days of the transgene induction in standard vials, males were loaded individually into the monitor tubes for the *Drosophila* Activity Monitor 2 system. The tubes contained 0.3% agarose as a water source. The starvation-induced hyperactivity was analyzed by visual inspection of individual locomotion patterns (Fig. 3g) and by statistical analysis of midline crossings during the last 12 h premortem (Fig. 3h). At least 23 flies were tested per genotype/treatment.

Measurement of the startle-induced climbing

The startle-induced climbing (negative geotaxis) was analyzed using the Rapid iterative negative geotaxis assay (Gargano *et al.* 2005) modified after Liao *et al.* (2021). Briefly, male flies were housed in empty transparent vials (25 mm × 95 mm) placed in a holder with an attached ruler. The holder accommodated 3 replicates for each treatment (GeneSwitch on vs off). After a brief recovery (around 15 min), the flies were tapped to the bottom of the tube. Four seconds later, photographs were taken to record the position of individual flies. Subsequently, we measured the distance that flies managed to climb using ImageJ software. At least 22 flies were tested per genotype/treatment.

Immunohistochemistry

Male flies were dissected in ice-cold *Drosophila* Ca^{2+}-free saline. The brain-thoracic/abdominal ganglia complexes were fixed overnight in Zamboni's fixative at room temperature, washed, and treated as described before (Dircksen *et al.* 2008). Primary antibodies were a polyclonal rabbit anti-ITP diluted 1:10,000 (Hermann-Luibl *et al.* 2014), polyclonal rabbit anti-AKH diluted 1:1,000 (Kerafast EGA261, Mark R. Brown, University of Georgia), and monoclonal mouse anti-GFP (Invitrogen) diluted 1:1,000. Secondary antibodies were goat antirabbit Alexa 546 and goat antimouse Alexa 488 (Invitrogen), both diluted 1:1,000. Samples were mounted in Mowiol supplemented with 2.5% 1,4-diazabicyclo-[2.2.2]-octane (DABCO, Sigma, D2522) and imaged under a Zeiss LSM 780 confocal microscope. Images were processed using the Zeiss ZEN software for maximum intensity projections of z-stacks. Cell fluorescence was measured using standard procedures. Briefly, the area of the CC cells was selected and their area, integrated density, and mean gray values were measured. For each sample, the background values for 5 adjacent regions were measured and averaged. The corrected total cell fluorescence (CTCF) was calculated using the standard equation: CTCF = integrated density − (the area of selected cell × the mean fluorescence of the background readings).

Food intake measurement by the Capillary Feeder assay

Food intake was measured by a modification of the Capillary Feeder assay (Ja *et al.* 2007) in a device constructed out of 24-well plates as described before (Gáliková, Klepsatel, Xu, *et al.* 2017). Capillaries with food (Hirschmann minicaps, 5μ) were exchanged daily. The volume of ingested food was measured over 5 days in at least 18 males per genotype/treatment and corrected for the evaporation rate. The liquid food contained the same concentration of RU-486 (200 μM) or vehicle control (ethanol) as during the prefeeding period.

Measurement of the speed of the food transit throughout the digestive tract

The speed of food transit was measured as the time from the intake of the blue-dyed food until the excretion of the dye in feces, as described in detail previously (Gáliková *et al.* 2018). Flies were housed in vials with a drop (approximately 0.2 ml) of food medium containing 0.5% Brilliant Blue (Sigma) and allowed to feed continuously. The cumulative numbers of feces that contained the blue dye were counted every hour until 5 h after the switch to the blue-dyed medium. Four replicates were tested per genotype/treatment, each consisting of 22 male flies.

Statistical analyses

We used a 2-tailed Student's *t*-test to analyze the pupal volume, dry weight, fecundity, lipid, glycogen, protein, trehalose, and glucose contents, metabolic rate, locomotor activity, starvation-induced activity, food intake, differences in the AKH fluorescence, and expression differences. For experiments conducted in both standard and Akh^{A} genetic backgrounds, we performed, in addition, a 2-way ANOVA with *Akh* background and *ITP* manipulation as fixed effects. The details of these analyses are included in Supplementary File 3. Differences in developmental timing were evaluated by the Gehan–Breslow–Wilcoxon test. Data on developmental lethality and fertility were analyzed using Fisher's exact test. Differences in lifespan and starvation survival were assessed using the log-rank test. To analyze the defecation rate, we performed a 2-way ANOVA with 2 fixed factors, genotype and time, and their interaction. *P* values are indicated by asterisk symbols ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Error bars represent SEM. Data were analyzed using Excel (Microsoft) and GraphPad Prism 8 (GraphPad Software Inc.). Raw data are included in Supplementary File 2.

Results

ITP is essential for development, reproduction, and lifespan

Previous works have shown that the *ITP* mutation is embryonically lethal (Park 2004), and its partial knock-down reduces

Fig. 1. Continued
percentage of eggs that hatch into the 1st larval instar). Fischer's exact test: $P < 0.05$. Animals were analyzed in 5 replicates; Fischer's exact test was performed on pooled data. Sample size: *daGS>ITP* off $n = 306$; *daGS>ITP* on females were completely sterile, no embryos were produced; *daGS>ITPi* off $n = 291$; *daGS>ITPi* on $n = 223$. Error bars represent SEM.

survival to adulthood (Gáliková *et al.* 2018). Here, we further examined the roles of *ITP* in preadult development, testing the developmental consequences of its genetic downregulation. The expression pattern of *ITP* involves multiple central and peripheral neurons, which probably synthesize the hormone redundantly (Gáliková *et al.* 2018). Therefore, we knocked down the hormone production ubiquitously, using the *daughterless*-GAL4 driver and 2 RNAi lines that have been previously shown to efficiently reduce *ITP* levels (Gáliková *et al.* 2018). We found that *ITP* is required for embryonic and larval development, as RNAi increased lethality during these stages but not during the pupal phase (Fig. 1a). Escapers that survived to adulthood had delayed development (Fig. 1b) and reduced body size (Fig. 1c). These developmental roles of *ITP* were also confirmed using an alternative RNAi line and ubiquitous *daughterless*-GeneSwitch (*daGS*) system (Supplementary Fig. 1), which allowed us to compare genetically identical animals (Tricoire *et al.* 2009).

Subsequently, we analyzed the effects of *ITP* gain of function and loss of function during the adult stage.

We used the same manipulations that led to the discovery of the roles of ITP in water balance (Gáliková *et al.* 2018)—the *daGS*-driven RNAi to reduce the ITP signaling and the overexpression of the *ITP* transcript to increase the pathway. To avoid the confounding effects of the developmental roles of ITP, we induced the GeneSwitch 3 days after eclosion. Both up- and downregulation of *ITP* shortened the lifespan of females (Fig. 1, d and e and Supplementary Fig. 2a) and males (Fig. 1, f and g and Supplementary Fig. 2b). These manipulations also reduced fecundity; overexpression of *ITP* blocked egg production completely, whereas *ITPi* reduced the egg-laying to a considerable extent (Fig. 1h). The fertility of these flies (estimated as the viability of embryos produced by parents with reduced ITP signaling) was mildly decreased as well (Fig. 1i).

Altogether, our data show that similar to mammalian glucagon signaling (Vuguin *et al.* 2006; Ouhilal *et al.* 2012; Yu *et al.* 2012), *ITP* is necessary for development, normal body size, reproduction, and lifespan.

ITP is a hyperglycemic hormone necessary for the maintenance of glycogen and lipid reserves

It has been shown that ITP inhibits both feeding and the speed of transit throughout the digestive tract (Gáliková *et al.* 2018). Thus, individuals with *ITP* overexpression gain fewer nutrients due to reduced feeding, while flies with downregulated *ITP* suffer from a diarrhea-like phenotype (Gáliková *et al.* 2018). Consistent with these results, we observed that the dry weight of flies significantly decreased upon both *ITP* manipulations in males (Fig. 2a) and females (Supplementary Fig. 3). Both sex and ITP manipulation had a significant effect on the dry weight. The effect of *ITP* seems to be more pronounced in females (2-way ANOVA for both *ITP* treatments, sex and genetic manipulations as fixed factors; the effect of *ITP*: $P < 0.001$, the effect of sex: $P < 0.001$). The weight-decreasing effect of *ITPi* was confirmed by an independent RNAi line (Supplementary Fig. 4a). Both RNAi lines have shown that the reduction in the dry weight was caused by the depletion of lipids (Fig. 2b and Supplementary Fig. 4b) and glycogen (Fig. 2c and Supplementary Fig. 4c). Moreover, *ITP* overexpression increased the catabolism of proteins (Fig. 2d). Our work also showed that the fly ITP is a hypertrehalosemic hormone; overexpression of *ITP* elevated the whole body trehalose, while *ITPi* caused its reduction (Fig. 2e and Supplementary Fig. 4d). Like CHH (Webster, Keller, and Dircksen 2012), also ITP is a hyperglycemic hormone; overexpression of *ITP* increased, while *ITPi* reduced the body glucose (Fig. 2f). The hyper- and hypotrehalosemic phenotypes driven by the genetic manipulations of *ITP* were accompanied by corresponding changes in the expression of *Trehalose-6-phosphate synthase* (*Tps1*) (Fig. 2g), which codes for the key enzyme in the synthesis of trehalose (Yoshida *et al.* 2016). *ITP* overexpression increased, while *ITPi* decreased expression of this gene (Fig. 2g). The *ITP* overexpressing flies had likely increased the turnover of trehalose suggested by the elevated expression of *cytoplasmic trehalase* (*cTreh*, Fig. 2h), which codes for a trehalose-degrading enzyme. These expression changes were confirmed by normalization to different housekeeping genes (*Act5C* and *Rpl32* in Fig. 2, g and h and *βTub60D* in Supplementary Fig. 5).

Since trehalose and glucose are often determined in the hemolymph samples, their concentration depends on the amount of body water. Previous work has shown that ITP is an antidiuretic hormone; its overexpression increases while its downregulation decreases water content (Gáliková *et al.* 2018). Consistently with the dilution of sugars by increased body fluids, the hemolymph of the *ITP* overexpressing flies does not contain a higher concentration of sugars (Supplementary Fig. 6), despite the increased trehalose and glucose in the whole body samples (Fig. 2, e and f).

It is important to note that to exclude potential confounding developmental effects on body size, the above-mentioned GeneSwitch manipulations were initiated in adult flies. Thus, the differences in body weight, protein, and energy reserves can be directly attributed to the metabolic functions of ITP. However, we cannot differentiate whether the changes in energy reserves are caused solely by the roles of *ITP* in feeding and digestion or whether they are also contributed by some direct effects of ITP on the fat body tissue.

Altogether, our results show that similar to glucagon (Bürger and Brandt 1935) and AKH (Kim and Rulifson 2004; Lee and Park 2004; Gáliková *et al.* 2015; Sajwan *et al.* 2015), ITP regulates trehalose and glucose levels. Unlike AKH (Gáliková *et al.* 2015; Gáliková, Klepsatel, Xu, *et al.* 2017; Yamada *et al.* 2018), but like glucagon (Rui 2014; Campbell and Drucker 2015), ITP reduces both lipid and glycogen stores. Moreover, in contrast to the obese *Akh* mutants (Gáliková *et al.* 2015), flies with reduced ITP suffer from reduced lipid reserves, resembling the cachectic mutants in glucagon signaling (Gelling *et al.* 2003; Yu *et al.* 2012).

ITP increases basal metabolic rate

Like glucagon, ITP diminishes energy intake (Gáliková *et al.* 2018). Therefore, we tested whether the analogy between glucagon and ITP extends to the regulation of energy expenditure. By using manometric respirometry to measure O_2 consumption, we found that ITP increases basal metabolic rate. Overexpression of ITP elevated O_2 consumption already after 24 h of the GeneSwitch induction (Fig. 3a). Consistently, *ITPi* via both tested RNAi lines reduced O_2 consumption (Fig. 3a and Supplementary Fig. 7). However, long-term overexpression of *ITP* decreased the metabolic rate (Supplementary Fig. 8), probably due to the depletion of energy reserves (Fig. 2, a–c).

Altogether, our data suggest that, similar to glucagon (Breton *et al.* 1983), ITP controls energy expenditure by increasing metabolic rate.

ITP does not seem to be required for spontaneous walking but is necessary for the startle-induced locomotion

In the next step, we analyzed whether ITP contributes to energy balance by regulating spontaneous locomotion. We measured the spontaneous walking activity of individual flies using the

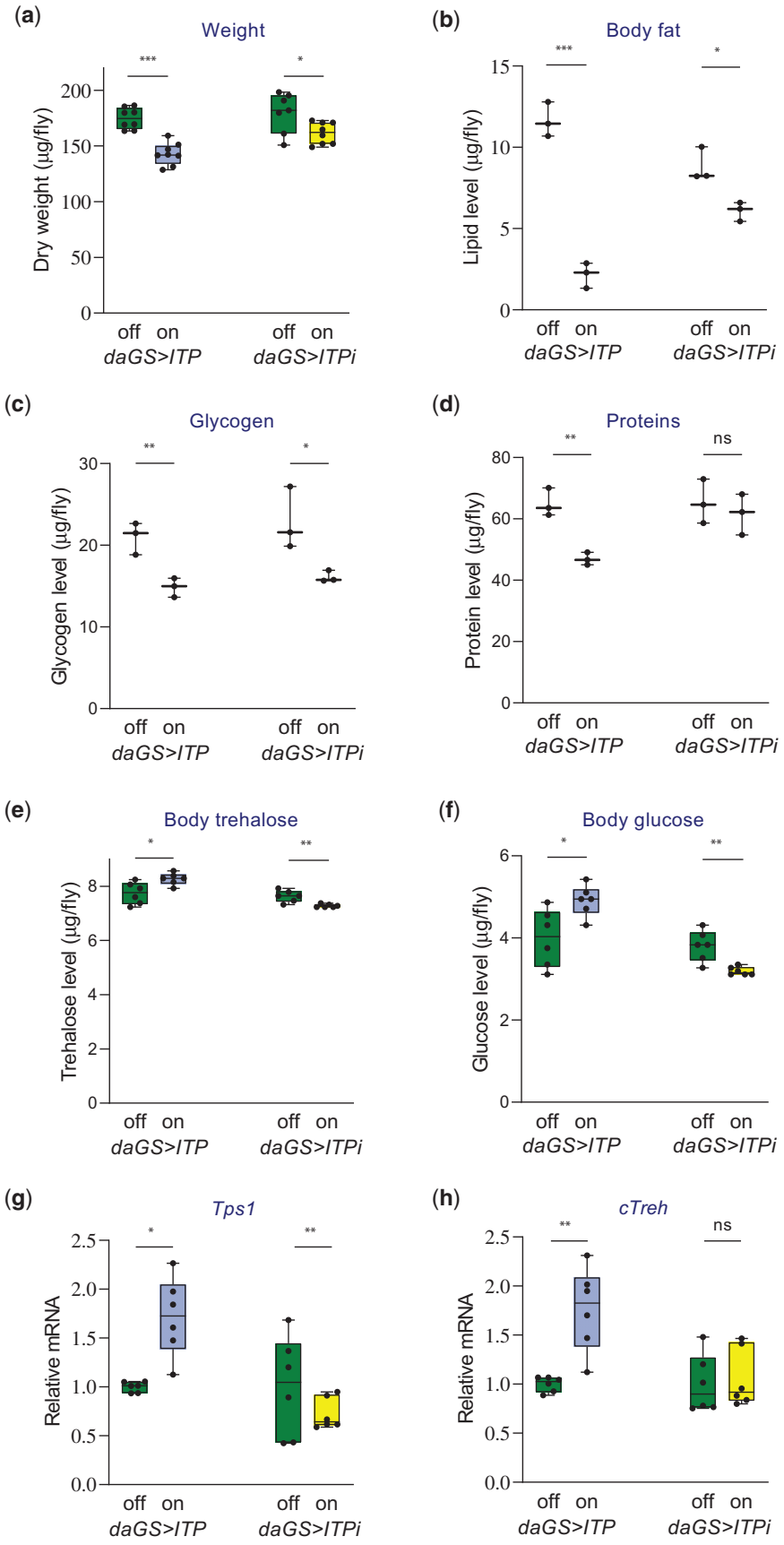

Fig. 2. ITP is a hyperglycemic hormone required for the homeostasis of energy reserves. a) Both upregulation (*daGS>ITP*) and downregulation of ITP (*daGS>ITPi*) results in reduced dry weight. Two-tailed Student's *t*-test: $P < 0.001$ for the *ITP* overexpression; $P < 0.05$ for the *ITPi*. b) Both upregulation (*daGS>ITP*) and downregulation of ITP (*daGS>ITPi*) result in reduced lipid reserves. Two-tailed Student's *t*-test: $P < 0.001$ for the *ITP* overexpression; $P < 0.05$ for the *ITPi*. c) Both upregulation (*daGS>ITP*) and downregulation of ITP (*daGS>ITPi*) results in reduced glycogen reserves. Two-tailed Student's

Drosophila activity monitoring system. ITP manipulations did not affect the total activity (Fig. 3b), although the analyses of locomotor behavior showed that overexpression of *ITP* significantly reduced spontaneous diurnal activity (Fig. 3b). *ITP* RNAi did not have any statistically significant effect on locomotor activity (Fig. 3c). Thus, these results suggest that the manipulations of *ITP* do not alter energy balance by changing spontaneous locomotor activity.

Subsequently, we examined the potential roles of ITP in startle-induced locomotion, which can be considered an escape reaction. We measured the vertical distance flies walked upon a startle impulse (tapping to the bottom of a vial). Both *ITP* overexpression and RNAi reduced the climbing distance (Fig. 3d). Thus, although ITP is not required for spontaneous locomotion under normal conditions, this peptide is essential for startle-induced vertical climbing (negative geotaxis).

ITP inhibits the starvation-induced hyperactivity

The locomotion of flies depends on their nutritional balance. Under nutritional shortage, flies increase their foraging activity (Lee and Park 2004). This process is independent of circadian rhythms and becomes particularly pronounced during the terminal phase of starvation (Lee and Park 2004). The starvation-induced hyperactivity depends on the previous diet (Huang *et al.* 2020) and is regulated by the main nutritional pathways such as insulin (Yu, Huang, *et al.* 2016) and AKH signaling (Lee and Park 2004; Isabel *et al.* 2005; Braco *et al.* 2012; Gáliková *et al.* 2015; Huang *et al.* 2020). Therefore, we tested wherever ITP regulates this behavior as well. Consistently with the reduced energy reserves (Fig. 2, a–c), both *ITP* overexpression and downregulation dramatically decreased starvation resistance (Fig. 3, e and f). Visual inspection of the locomotor activity patterns of individual flies under starvation revealed that the *ITP* overexpressing flies often failed to increase their activity, while downregulation of *ITP* enhanced the starvation-induced locomotion (Fig. 3g). Consistently, a quantitative analysis of the locomotor activity toward the end of starvation proved that ITP indeed inhibits this process—overexpression of *ITP* diminished while *ITPi* RNAi increased the total activity during the last 12 h of life (Fig. 3h).

ITP activates the AKH pathway by stimulating the secretion of AKH

Several of the biological functions affected by ITP manipulations are also controlled by AKH (reviewed in e.g. Koyama *et al.* 2020). Therefore, we aimed to test whether ITP regulates these processes via AKH or independently of this pathway. First, we examined whether ITP manipulations alter AKH signaling. We did not find a significant effect of ITP manipulations on the expression of *Akh* (Fig. 4a) when the expression data were normalized to *Act5C* and *Rpl32*. However, when we used *βTub60D* as a reference gene, the *ITP* overexpression increased while *ITP* RNAi reduced *Akh* mRNA (Supplementary Fig. 9a). In addition, both normalization approaches confirmed that ITP increases the mRNA of the AKH receptor *AkhR* (Fig. 4b and Supplementary Fig. 9b). These data thus suggest that ITP may regulate the AKH pathway at the transcriptional level.

However, the AKH signaling is regulated primarily at the level of hormone secretion (Van der Horst *et al.* 2001), and we have thus investigated whether ITP manipulations also affect this process. AKH is produced by a neuroendocrine organ called corpora cardiaca (CC) (Kim and Rulifson 2004; Lee and Park 2004), and the secretion of the peptide is inversely correlated with the intensity of its immunolabeling in these cells. Therefore, immunostainings are frequently used to study the release of this hormone (e.g. Braco *et al.* 2012; Kim and Neufeld 2015). We used this approach to investigate the potential effects of ITP on the secretion of AKH. Overexpression of *ITP* increased secretion of AKH, while *ITPi* inhibited the release of the peptide (Fig. 4, d and e). This supports the conclusion that ITP regulates the AKH pathway at the level of AKH secretion.

Interestingly, a small subset of ITP-producing cells called ipc-1 and ipc-2a send their axons toward the AKH-producing cells of CCs (Gáliková *et al.* 2018; Fig. 4c). This suggests that they may directly regulate AKH release. Intriguingly, these cells have already been shown to regulate metabolism (Kahsai *et al.* 2010). Thus, we have tested their potential roles in sugar homeostasis. However, the *ITPi* in the pattern of these neurosecretory cells [covered by the previously tested *Impl2* (Gáliková *et al.* 2018) and *c929* (Kahsai *et al.* 2010) GAL4 drivers] did not recapitulate the effects obtained by the ubiquitous RNAi, such as reduced trehalose levels (Supplementary Fig. 10). Accordingly, this observation suggests that the AKH release from CCs is likely regulated by ITP-producing cells other than ipc-1 and ipc-2a or that the ITP-producing cells act redundantly [as shown previously for their role in the water balance (Gáliková *et al.* 2018)].

Altogether, our results indicate that the ITP pathway interacts with the AKH signaling; ITP increases the expression of the AKH receptor and triggers the secretion of AKH from the CC cells.

The ITP-dependent regulation of energy intake and expenditure does not require AKH

Because the ITP manipulations affect the AKH release, we next explored whether the functions of ITP are mediated via the AKH peptide or whether ITP controls *Drosophila* physiology independently of this hormone. We reasoned that if ITP modulates the tested processes via AKH, the effects of ITP manipulations will disappear in the absence of the AKH peptide. Therefore, we analyzed the consequences of *ITP* up- and downregulations in flies with the previously described functional null mutation of *Akh*, Akh^{A} (Gáliková *et al.* 2015). We focused on the biological processes that we discovered as ITP regulated and that were previously shown to be under AKH control. First, we examined the role of ITP in energy intake and expenditure. Overexpression of *ITP* reduced food intake in both standard and AKH-deficient backgrounds (Fig. 5a), and RNAi increased feeding independently of the presence of AKH (Fig. 5b). Thus, the anorexigenic effect of ITP seems to be AKH independent. Similarly, the regulation of the speed of transit throughout the digestive tract via ITP does not require AKH. The experimental increase in ITP decreased the

Fig. 2. Continued
t-test: $P < 0.01$ for the *ITP* overexpression; $P < 0.05$ for the *ITPi*. d) Overexpression of *ITP* results in the catabolism of proteins. Two-tailed Student's *t*-test: $P < 0.01$. e) ITP increases trehalose levels. Overexpression of *ITP* increases, while *ITPi* reduces trehalose content. Two-tailed Student's *t*-test for the effect of overexpression: $P < 0.05$, Student's *t*-test for the effect of RNAi: $P < 0.01$. f) ITP increases glucose levels. Overexpression of *ITP* increases, while *ITPi* reduces glucose content. Two-tailed Student's *t*-test for the effect of overexpression: $P < 0.05$, Student's *t*-test for the effect of RNAi: $P < 0.01$. g) ITP regulates the expression of *Trehalose-6-phosphate synthase* (*Tps1*). Overexpression of *ITP* increases, while *ITPi* reduces *Tps1* mRNA. Two-tailed Student's *t*-test for the effect of overexpression: $P < 0.05$, Student's *t*-test for the effect of RNAi: $P < 0.01$. h) Overexpression of *ITP* increases mRNA of the trehalose-degrading enzyme, cytoplasmic *Trehalase* (*cTreh*). Two-tailed Student's *t*-test $P < 0.01$.

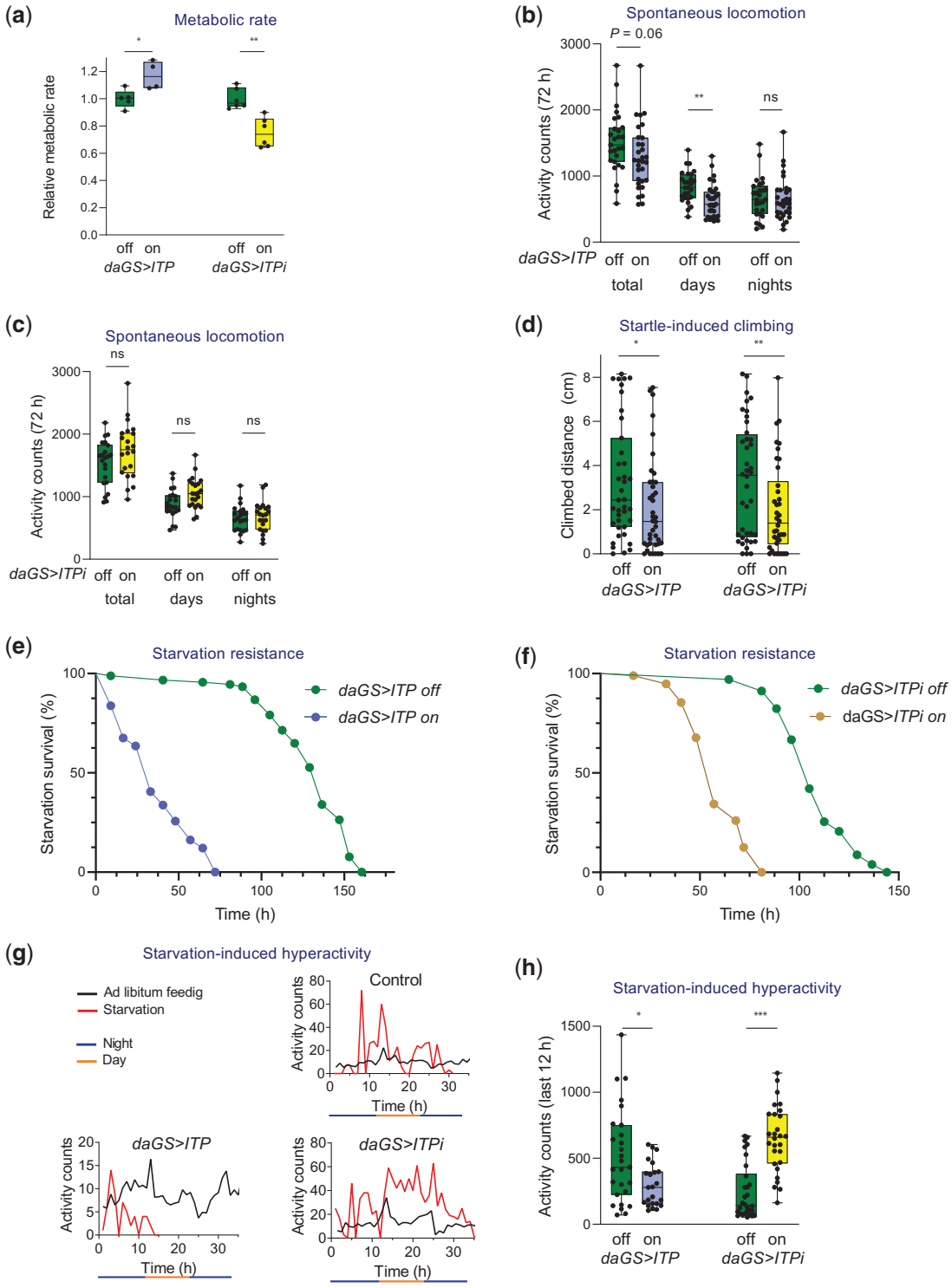

Fig. 3. ITP regulates metabolic rate, startle-induced climbing, starvation resistance, and starvation-induced hyperactivity. a) ITP regulates metabolic rate. Short-term overexpression of *ITP* results in increased metabolic rate. Two-tailed Student's *t*-test: $P < 0.05$. *ITPi* decreases metabolic rate. Two-tailed Student's *t*-test: $P < 0.001$. Data are normalized to their controls. For raw data, see Supplementary Fig. 7a. b) Overexpression of *ITP* affects spontaneous locomotor activity. ITP marginally nonsignificantly reduces total activity tested over 3 days (2-tailed Student's *t*-test: $P = 0.06$), significantly decreases diurnal activity (2-tailed Student's *t*-test: $P < 0.01$), but does not change nocturnal activity. c) *ITPi* does not affect spontaneous locomotion during the day or during the night. d) Both *ITP* overexpression and *ITPi* reduce the startle-induced climbing (negative geotaxis assay). Two-tailed Student's *t*-test for the effect of overexpression: $P < 0.05$, for the effect of RNAi: $P < 0.01$. e) Overexpression of *ITP* reduces starvation resistance. Log-rank test: $P < 0.001$. Sample size: *daGS>ITP* off $n = 91$; *daGS>ITP* on $n = 74$. f) Reduction of *ITP* via RNAi reduces starvation resistance. Log-rank test: $P < 0.001$. Sample size: *daGS>ITPi* off $n = 102$; *daGS>ITPi* on $n = 96$. g) Representative patterns of locomotor activity of individual flies upon starvation vs ad libitum feeding. Depicted are control flies, flies overexpressing *ITP* (*daGS>ITP*), and flies with *ITP RNAi* (*daGS>ITPi*). Note that starvation increases activity, which is particularly pronounced shortly before death. Overexpression of *ITP* abolishes this increase in activity, while *ITPi* enhances this behavior. h) Quantification and statistical analysis of starvation-induced hyperactivity during the last 12 h. Overexpression of *ITP* reduces (2-tailed Student's *t*-test: $P < 0.05$) while *ITPi* increased this activity (2-tailed Student's *t*-test: $P < 0.001$).

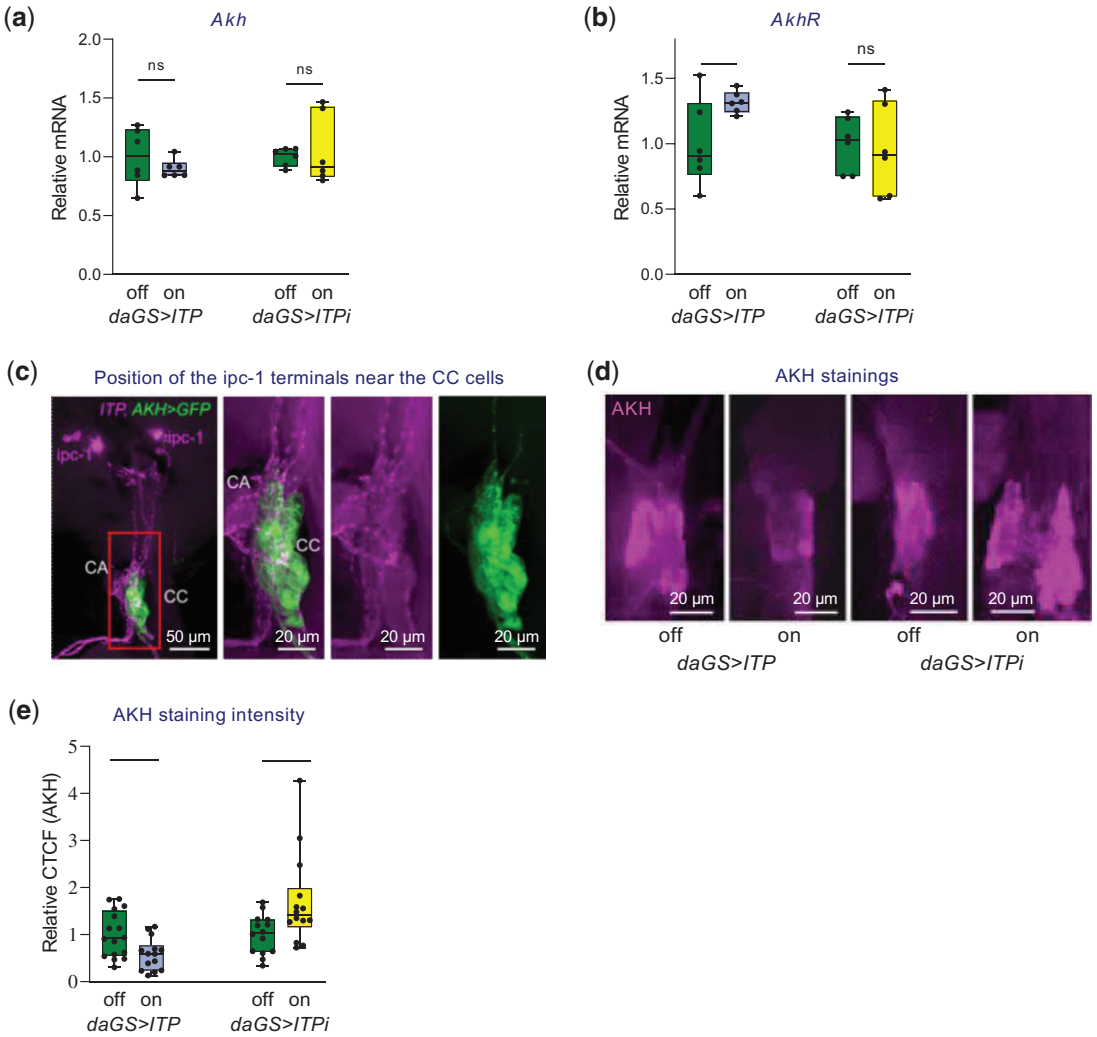

Fig. 4. ITP regulates AKH signaling. a) Manipulations of ITP do not change the expression of *Akh* when expression data are normalized to *Act5C* and *Rpl32*. Two-tailed Student's *t*-test: $P > 0.05$ for both overexpression of *ITP* and *ITPi*. b) ITP regulates the expression of the AKH receptor *AkhR*. Overexpression of *ITP* increases levels of *AkhR* (2-tailed Student's *t*-test: $P < 0.05$). c) Anatomy of the ITP-producing ipc neurons. The ITP-producing ipc neurons send their axons toward the neurohemal release sites, which are located nearby the corpora allata (CA) and corpora cardiaca (CC). The ipc neurons are stained with the ITP antibody (in magenta), and the CCs are visualized via AKH-GAL4-driven GFP (in green). d) Representative staining of CC (the AKH-producing organs) with the AKH antibody. Overexpression of *ITP* reduces the AKH levels in the CCs, which is indicative of increased secretion of AKH. *ITPi* increases the AKH in CCs, suggesting a reduced release of AKH. e) Quantification of AKH staining in CCs of flies with genetically manipulated *ITP* signaling. Overexpression of *ITP* increases AKH secretion (2-tailed Student's *t*-test: $P < 0.05$), while *ITP RNAi* reduces it (2-tailed Student's *t*-test: $P < 0.05$). Plotted is the CTCF normalized to the values of the genetically-matching controls.

defecation rate (Fig. 5c), while the loss of ITP led to a diarrhea-like phenotype in standard and in the Akh^A background (Fig. 5d). The hypermetabolic effect of ITP appears to be AKH independent as well; overexpression of *ITP* increased (Fig. 6a), while *ITPi* decreased (Fig. 6b) the O_2 consumption in the absence of AKH.

Taken together, under standard feeding conditions, ITP regulates energy intake and energy expenditure independently of the AKH pathway.

ITP inhibits starvation-induced locomotion only in the presence of a functional AKH peptide

Both startle-induced vertical climbing (Bednářová *et al.* 2018) and starvation-induced locomotion (Gáliková *et al.* 2015) are dependent on functional AKH peptide. Our results presented in Fig. 3 show that these processes also require ITP. We have therefore tested whether these ITP phenotypes might be mediated by AKH, i.e. whether the effect of ITP persists in the AKH-deficient background. Overexpression of ITP reduced the distance that flies climbed upon the startle in the standard as well as in the AKH-deficient background (Fig. 6c). Similarly, *ITPi* impaired the startle-induced climbing irrespective of AKH presence (Fig. 6d). Consistently, overexpression of *Akh* did not revert the impaired climbing of the *ITPi*-treated flies (Supplementary Fig. 11). Thus, ITP is necessary alongside AKH for the standard startle-induced locomotion.

In contrast to vertical climbing, the role of ITP in inhibiting hunger-driven locomotion seems to depend on AKH. *ITP* overexpression reduces (Fig. 6e) and *ITPi* elevates (Fig. 6f) this phenotype only in the presence of the functional AKH peptide.

ITP regulates glycemia via AKH, but the requirement of ITP for homeostasis of lipids and glycogen is AKH independent

Consistently with the AKH-independent roles in energy intake and expenditure, ITP is required for the maintenance of proper energy reserves irrespective of the AKH presence. Manipulations

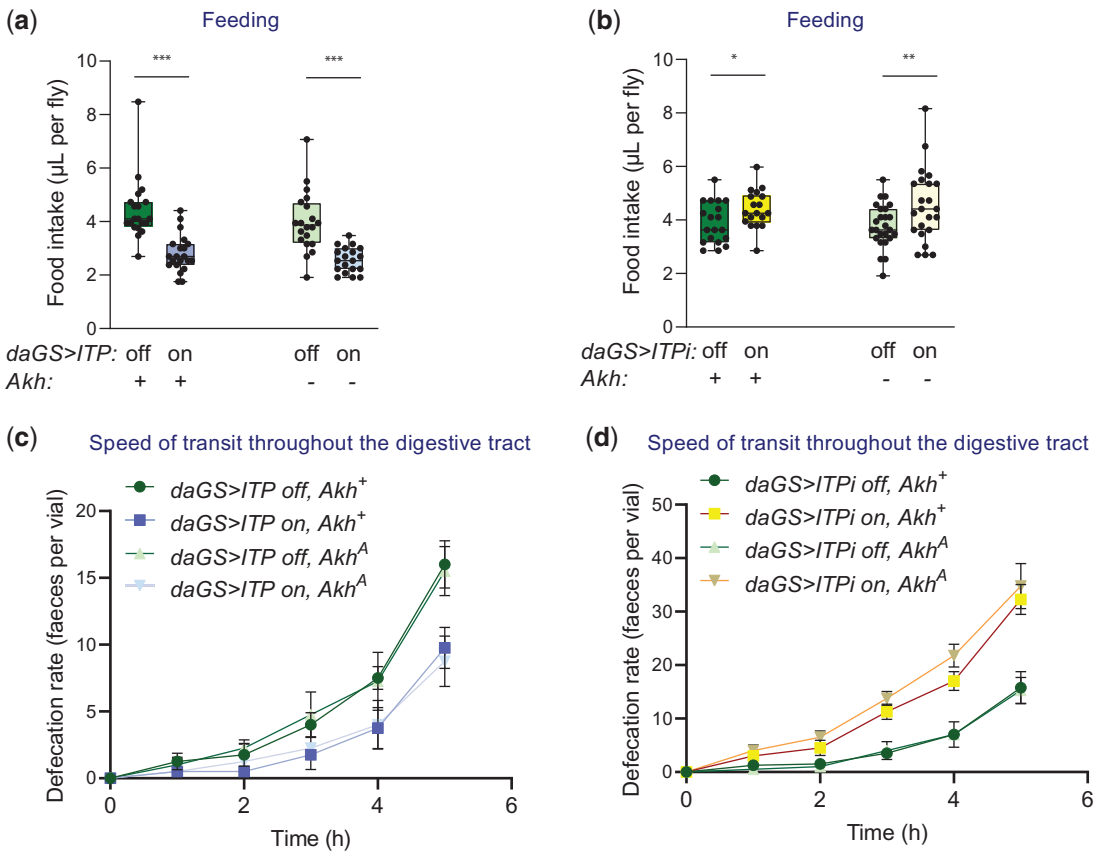

Fig. 5. ITP regulates energy intake independently of AKH signaling. a, b) ITP represses feeding independently of AKH. a) Overexpression of *ITP* reduces food intake in the standard genetic background (2-tailed Student's *t*-test: $P < 0.001$), as well as in the AKH-deficient background (2-tailed Student's *t*-test: $P < 0.001$). The background does not alter the effect of ITP (2-way ANOVA with the *Akh* background and *ITP* overexpression as fixed effects; the effect of ITP: $P < 0.001$, the effect of interaction: $P > 0.05$). b) Reduction of *ITP* via RNAi increases food intake in a standard (2-tailed Student's *t*-test: $P < 0.05$), as well as in the AKH-deficient background (2-tailed Student's *t*-test: $P < 0.01$). The background does not alter the effect of ITP (2-way ANOVA with the *Akh* background and *ITP* RNAi as fixed effects; the effect of *ITPi*: $P < 0.01$, the effect of interaction: $P > 0.05$). c, d) The antidiarrheal effect of ITP does not require AKH. c) Overexpression of *ITP* reduces the pace of transit through the digestive tract irrespective of AKH. Two-way ANOVA for the standard as well as in the Akh^A background: *ITP* and time as fixed factors; the effect of *ITP*: $P < 0.05$, the effect of time: $P < 0.001$, the effect of the interaction: $P > 0.05$. Error bars represent SEM. d) Reduction of ITP via RNAi increases the pace of transit through the digestive tract in the presence as well as in the absence of AKH. Two-way ANOVA for the standard genetic background: *ITP* and time as fixed factors; the effect of *ITPi*: $P < 0.01$, the effect of time: $P < 0.001$, the effect of the interaction: $P > 0.05$. Two-way ANOVA for the Akh^A genetic background: *ITPi* and time as fixed factors; the effect of *ITPi*: $P < 0.05$, the effect of time: $P < 0.001$, the effect of the interaction: $P > 0.05$. Error bars represent SEM.

of *ITP* reduce lipid (Fig. 7, a and b) and glycogen (Fig. 7, c and d) reserves in both standard and AKH-deficient backgrounds. In contrast to stored energy reserves, circulating energy sources seem to be ITP-regulated via AKH. *ITP* upregulation increased trehalose levels in control but not in the Akh^A background (Fig. 7e). Consistently, *ITP* RNAi reduced trehalose levels in a standard genetic background but not in the absence of AKH (Fig. 7f). Similar to the regulation of trehalose, glucose levels are also controlled via the ITP–AKH axis. Overexpression of *ITP* increased glucose content in a standard but not in the AKH-deficient background (Fig. 7g), and *ITPi* reduced levels of this saccharide in a standard, but not in the Akh^A background (Fig. 7h).

In summary, ITP has metabolic functions intriguingly similar to mammalian glucagon—it inhibits energy intake while increasing metabolic rate. The peptide also increases trehalose and glucose and reduces stored energy reserves. Our work describes a novel hormonal axis, where ITP acts upstream of the well-known AKH peptide. ITP activates the AKH pathway by triggering the secretion of AKH and by inducing the transcription of its receptor *AkhR*. The hyperglycemic function of ITP is mediated by the AKH pathway, while its roles in energy intake, expenditure, and homeostasis of stored energy reserves are AKH independent (Fig. 8).

Discussion

Hormonal regulation of energy catabolism

Despite the growing popularity of *Drosophila* as an invertebrate model for metabolism, the regulation of energy catabolism is a relatively understudied topic. In mammals, the hormone glucagon induces catabolic processes at several levels—it reduces energy intake, increases energy expenditure via enhanced metabolic rate, and induces catabolism of lipids, glycogen, and proteins. The situation in invertebrates seems to be more complicated. To our best knowledge, no single hormone with all these functions has been described so far. The AKH hormone mobilizes fat (Kim and Rulifson 2004; Lee and Park 2004; Gáliková *et al.* 2015; Gáliková, Klepsatel, Xu, *et al.* 2017), but not glycogen (Gáliková *et al.* 2015; Gáliková, Klepsatel, Xu, *et al.* 2017; Yamada *et al.* 2018) or proteins (Mochanová *et al.* 2018). In contrast to glucagon, AKH does not reduce food intake but, on the contrary, enhances appetite (Jourjine *et al.* 2016; Gáliková, Klepsatel, Xu, *et al.* 2017). Its role in energy expenditure is unclear. While some studies found increased metabolic rate upon application of AKH in insects such as the firebug *Pyrrhocoris apterus* (Gautam *et al.* 2020), others described normal oxygen consumption in flies

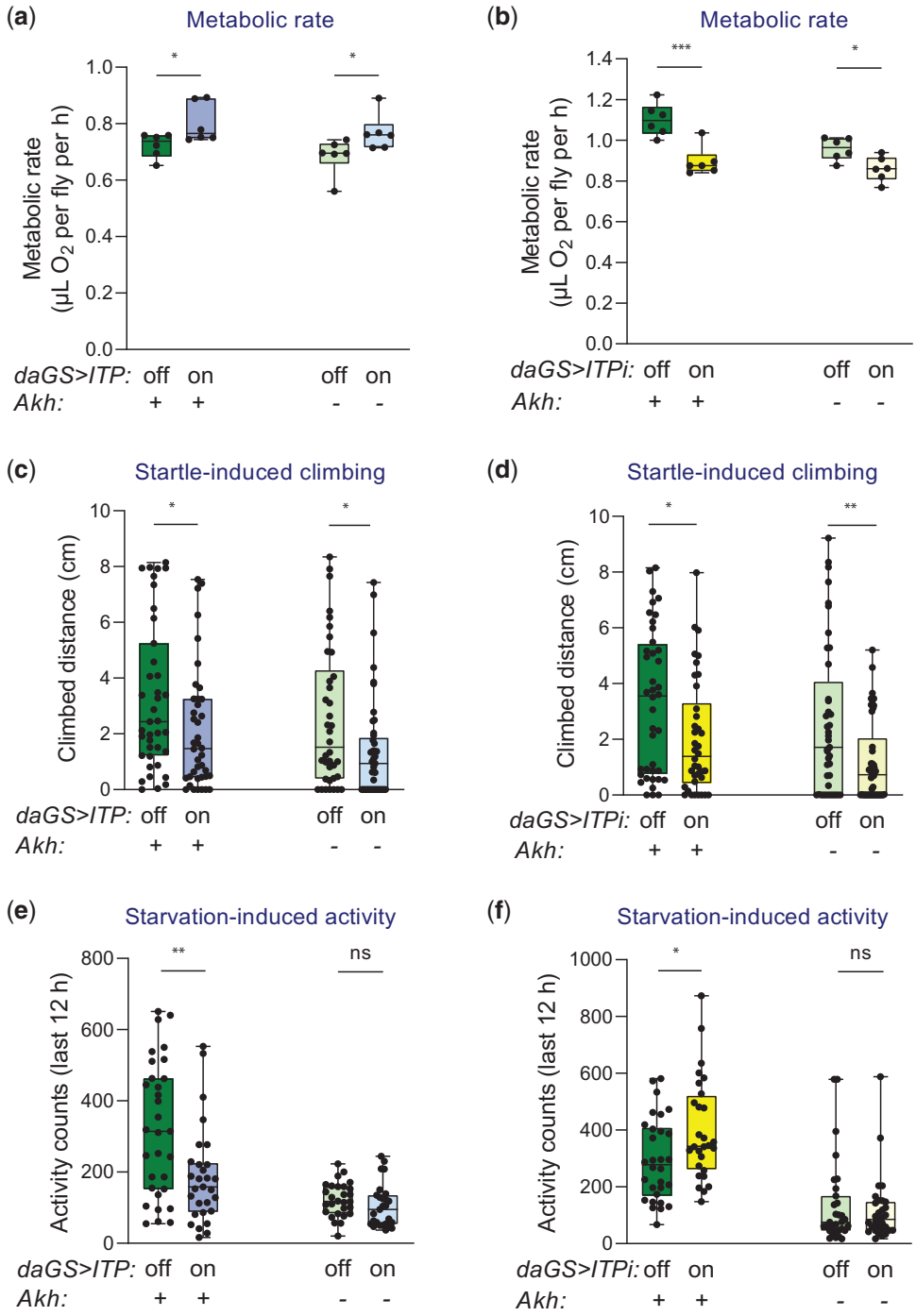

Fig. 6. ITP regulates the metabolic rate and startle-induced climbing independently of AKH, but its effects on starvation-induced hyperactivity disappear in the absence of AKH. a, b) ITP increases metabolic rate independently of AKH. a) Overexpression of ITP increases basal metabolic rate in the standard genetic background (2-tailed Student's *t*-test: $P < 0.05$) as well as in the Akh^A background (2-tailed Student's *t*-test: $P < 0.05$). The background does not alter the effect of ITP (2-way ANOVA with the *Akh* background and *ITP* overexpression as fixed effects; the effect of *ITP*: $P < 0.01$, the effect of interaction: $P > 0.05$). b) *ITPi* decreases basal metabolic rate in the standard genetic background (2-tailed Student's *t*-test: $P < 0.001$) as well as in the Akh^A background (2-tailed Student's *t*-test: $P < 0.05$). The background modulates the effect of ITP (2-way ANOVA with the *Akh* background and *ITP* RNAi as fixed effects; the effect of *ITPi*: $P < 0.001$, the effect of interaction: $P < 0.001$). c, d) Proper ITP levels are required for the startle-induced vertical climbing independently of AKH. c) Overexpression of *ITP* impairs vertical climbing in both standard and Akh^A backgrounds (2-tailed Student's *t*-test: $P < 0.05$. The background does not alter the effect of ITP (2-way ANOVA with the *Akh* background and *ITP* overexpression as fixed effects; the effect of *ITP*: $P < 0.05$, the effect of interaction: $P > 0.05$). d) *ITPi* impairs vertical climbing in both standard genetic background (2-tailed Student's *t*-test: $P < 0.05$) as well as in the Akh^A background (2-tailed Student's *t*-test: $P < 0.01$). The background does not alter the effect of *ITPi* (2-way ANOVA with the *Akh* background and *ITPi* as fixed effects; the effect of *ITPi*: $P < 0.001$, the effect of interaction: $P > 0.05$). e, f) The ITP-dependent starvation-induced hyperactivity requires AKH. e) Overexpression of *ITP* reduces the starvation-induced foraging in the standard genetic background (2-tailed Student's *t*-test: $P < 0.01$), but not in the already hypoactive Akh^A background (2-tailed Student's *t*-test: $P > 0.05$). f) Reduction of ITP via RNAi enhances the starvation-induced foraging in the standard genetic background (2-tailed Student's *t*-test: $P < 0.05$), but not in the Akh^A background (2-tailed Student's *t*-test: $P > 0.05$).

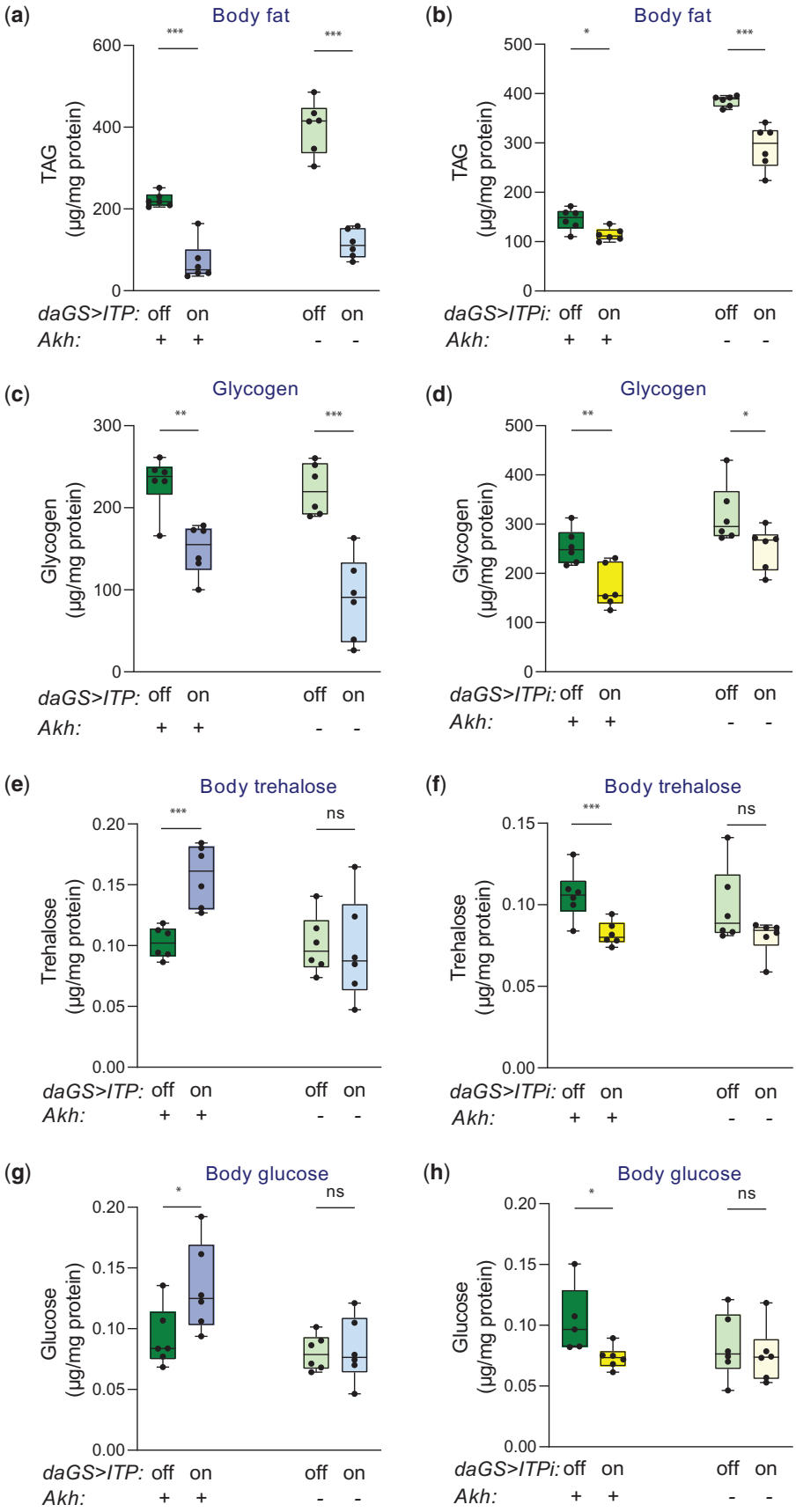

Fig. 7. ITP regulates energy reserves independently of AKH signaling, while the hypertrehalosemic and hyperglycemic effects of ITP are AKH dependent. a, b) ITP is required for proper fat storage independently of AKH. Overexpression of *ITP* reduces fat content in the standard genetic background, as well as in the Akh^{A} background (2-tailed Student's *t*-test: $P < 0.001$ for both comparisons). The background modulates the effect of ITP (2-way ANOVA with the *Akh* background and *ITP* overexpression as fixed effects; the effect of *ITP*: $P < 0.001$, the effect of interaction: $P < 0.01$). *ITPi* reduces fat content in the

overexpressing this peptide (Gáliková, Klepsatel, Xu, *et al.* 2017) and slight reduction of metabolic rate in the *Akh* mutants (Sajwan *et al.* 2015; Gáliková, Klepsatel, Xu, *et al.* 2017). Another peptide with possible catabolic functions is the neuropeptide corazonin. Corazonin depletes glycogen but does not alter fat, trehalose, or glucose levels (Kubrak *et al.* 2016). Similar to AKH (Jourjine *et al.* 2016; Gáliková, Klepsatel, Xu, *et al.* 2017), corazonin increases food intake (Kubrak *et al.* 2016). Metabolic rate and fat accumulation are also regulated by the fly counterpart of norepinephrine—octopamine (Li *et al.* 2016). Like AKH, octopamine induces lipolysis (Socha *et al.* 2008) and increases food intake in insects (Youn *et al.* 2018). Our work shows that ITP is another peptide that regulates fly metabolism alongside AKH and the above-mentioned catabolic hormones.

The CHH/ITP family and its functional similarity to mammalian glucagon

The CHH/ITP hormone family is evolutionarily conserved in ecdysozoan invertebrates. These peptides were described in arthropods (crustaceans, insects, myriapods, and arachnids) and nematode worms (Dircksen 2009; Webster *et al.* 2012). Physiological functions of CHH have been first characterized in crustaceans. The name originates from its hyperglycemic effect studied in blue crabs *Callinectes sapidus* and fiddler crabs *Uca pugilator* (Abramowitz *et al.* 1944). Interestingly, CHH regulates lipid metabolism in several crustacean species (Santos *et al.* 1997). In addition, several studies described the osmoregulatory, developmental, and reproduction-related functions of CHH, suggesting a general requirement of the hormone for almost all aspects of physiology (Chung *et al.* 2010; Webster *et al.* 2012). Our study shows that the developmental, reproduction- and metabolism-related roles of the CHH family are conserved in *Drosophila*. The fly CHH, encoded by the *ITP* gene, controls energy homeostasis at several key levels, including energy intake, expenditure, and accumulation of energy reserves. ITP is necessary for traits such as development, reproduction, and lifespan. Since ITP is an essential regulator of water and energy balance (Gáliková *et al.* 2018), it remains to be investigated whether the ITP dysregulations decrease energy balance and fitness-related traits indirectly (due to the impairment of energy and water balance) or whether ITP has additional signaling functions.

The ITP gain- and loss-of-function phenotypes are intriguingly similar to the effects observed in mammalian models upon glucagon administration and in the Gcgr mutants, respectively (Vuguin *et al.* 2006; Ouhilal *et al.* 2012; Yu *et al.* 2012; Campbell and Drucker 2015; Müller *et al.* 2017). For example, the Gcgr deficiency lowers body size at birth, impairs reproduction (Vuguin *et al.* 2006; Ouhilal *et al.* 2012), leads to cachexia, and considerably decreases lifespan (Yu *et al.* 2012). These developmental phenotypes are similar to the effects of ITP manipulations; deficiency for ITP is embryonically lethal (Park 2004), while partial knock-down results in increased developmental lethality, viability, smaller body size, and shortened lifespan. The hormones have a similar impact on energy homeostasis; glucagon regulates energy balance by repressing energy intake (feeding, peristalsis, and gastric emptying) and increasing energy expenditure [basal metabolism (Campbell and Drucker 2015; Müller *et al.* 2017)]. ITP seems to have the same functions in *Drosophila*. Previous work showed that ITP decreases appetite and prolongs the transit time throughout the digestive tract (Gáliková *et al.* 2018), and our study reveals that this hormone increases energy expenditure by enhancing basal metabolic rate. Glucagon is known for inducing lipolysis, glycogenolysis, and gluconeogenesis. Consistently, overexpression of *ITP* resulted in the depletion of fat, glycogen reserves, and proteins, suggesting catabolic effects of ITP. Nevertheless, it is unknown whether ITP acts on the fat body directly or if the low energy reserves result only from the reduced energy intake of the flies.

Balanced glucagon signaling is critical for the maintenance of energy reserves. Defects in this pathway (as described in the Gcgr deficient mice) decrease the amount of energy reserves, leading to cachexia (Yu *et al.* 2012). Similarly, we have observed that the reduction of ITP depletes glycogen and fat stores. Thus, both up- and downregulation of ITP result in low energy reserves, although the underlying mechanism differs. The gain of function is connected with reduced feeding (Gáliková *et al.* 2018) and increased metabolic rate, while the loss of function is coupled with diarrhea (Gáliková *et al.* 2018). In addition, it is possible that ITP directly induces catabolism of energy stores and gluconeogenesis. Like glucagon, ITP acts as a hyperglycemic hormone. ITP increases trehalose and glucose levels and stimulates the expression of the key enzyme for the synthesis of trehalose, *Tps1* (Yoshida *et al.* 2016). The exact mechanism behind the hyperglycemic and hypertrehalosemic actions of ITP is unclear, but it seems to rely on the AKH signaling because the effects of *ITP* overexpression and RNAi disappear in an *Akh*-deficient background.

The ITP–AKH axis

AKH is an important hyperglycemic and lipolytic hormone in *Drosophila* (Kim and Rulifson 2004; Lee and Park 2004; Gáliková *et al.* 2015; Gáliková, Klepsatel, Xu, *et al.* 2017), with pleiotropic functions in the defense against oxidative stress (Bednářová *et al.* 2015; Gáliková *et al.* 2015; Kodrík *et al.* 2015; Zemanova *et al.* 2016), immunity (Ibrahim *et al.* 2017, 2018), promotion of appetite (Jourjine *et al.* 2016; Gáliková, Klepsatel, Xu, *et al.* 2017), starvation-induced foraging (Lee and Park 2004; Isabel *et al.* 2005; Gáliková *et al.* 2015), daily locomotor activity (Pauls *et al.* 2020),

Fig. 7. Continued
standard genetic background, as well as in the Akh^A background (2-tailed Student's *t*-test: $P < 0.05$ for the standard and $P < 0.001$ for the Akh^A background). The background modulates the effect of ITP (2-way ANOVA with the *Akh* background and *ITP*i as fixed effects; the effect of *ITP*i: $P < 0.001$, the effect of interaction: $P < 0.01$). c, d) ITP is required for proper glycogen storage independently of AKH. Overexpression of *ITP* reduces glycogen content in the standard genetic background, as well as in the Akh^A background (2-tailed Student's *t*-test: $P < 0.01$ for the standard and $P < 0.001$ for the Akh^A background). The background does not alter the effect of ITP (2-way ANOVA with the *Akh* background and *ITP* overexpression as fixed effects; the effect of *ITP*: $P < 0.001$, the effect of interaction: $P > 0.05$). *ITP*i reduces glycogen content in the standard genetic background (2-tailed Student's *t*-test: $P < 0.01$), as well as in the Akh^A background (2-tailed Student's *t*-test: $P < 0.05$). The background does not alter the effect of ITP (2-way ANOVA with the *Akh* background and *ITP*i as fixed effects; the effect of *ITP*i: $P < 0.001$, the effect of interaction: $P > 0.05$). e, f) The hypertrehalosemic function of ITP requires functional AKH. Overexpression of *ITP* increases trehalose content in the standard genetic background (2-tailed Student's *t*-test: $P < 0.001$) but not in the absence of AKH (2-tailed Student's *t*-test: $P > 0.05$). *ITP*i decreases trehalose content in the standard genetic background (2-tailed Student's *t*-test: $P < 0.001$) but not in the absence of AKH (2-tailed Student's *t*-test: $P > 0.05$). g, h) The hyperglycemic function of ITP requires functional AKH. Overexpression of *ITP* increases body glucose in the standard genetic background (2-tailed Student's *t*-test: $P < 0.05$) but not in the absence of AKH (2-tailed Student's *t*-test: $P > 0.05$). *ITP*i decreases glucose in the standard genetic background (2-tailed Student's *t*-test: $P < 0.05$) but not in the absence of AKH (2-tailed Student's *t*-test: $P > 0.05$).

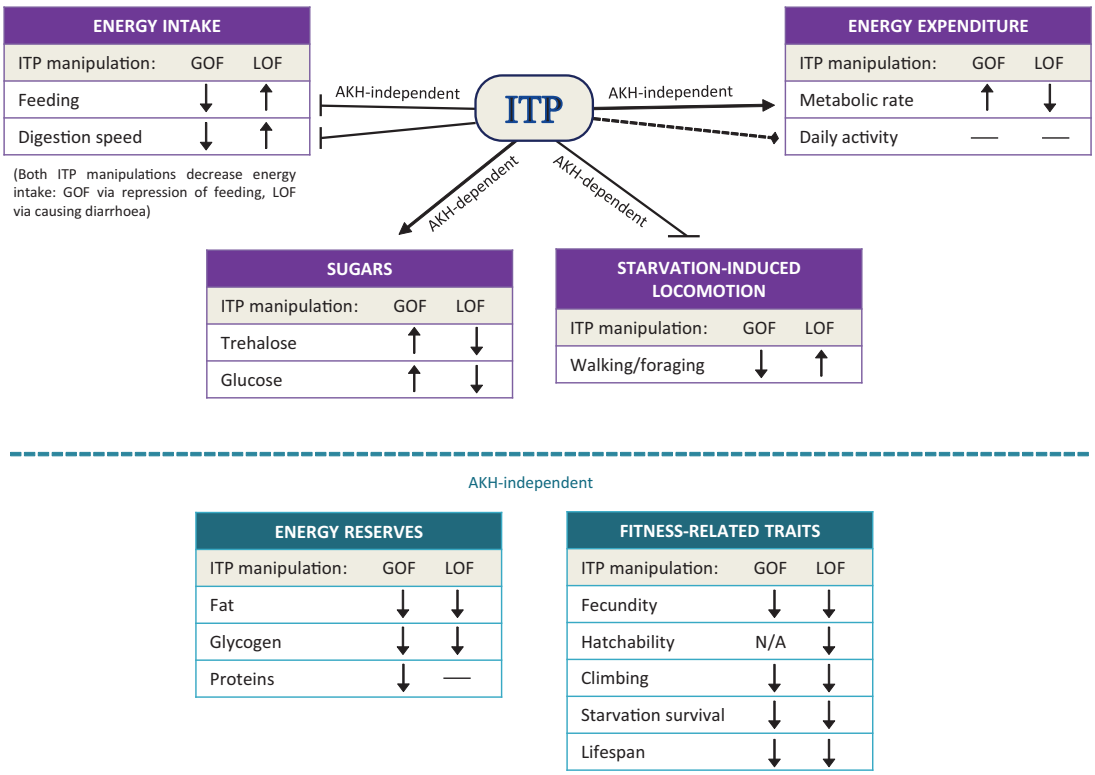

Fig. 8 Summary of ITP functions identified by the *daughterless*-GeneSwitch driven *ITP* overexpression (gain of function, GOF) and RNAi (loss of function, LOF). *ITP* overexpression reduces feeding, the speed of transit throughout the digestive tract (i.e. protects from diarrhea), and increases metabolic rate. The *ITPi* results in the opposite phenotype. Although *ITP* overexpression and *ITPi* have antagonistic effects on the tested traits, both manipulations reduce energy intake: *ITP* overexpression via feeding inhibition, while *ITPi* via diarrhea. Accordingly, we have observed a decline in energy reserves (fat and glycogen) and energy-demanding and fitness-related traits upon both manipulations. Testing the direct vs indirect effects of ITP on these traits requires further studies, including the identification of the peptide receptor(s). We have observed that ITP stimulates the secretion of the catabolic hormone AKH. Subsequently, we have tested whether the metabolic roles of ITP depend on this peptide. Our data showed that the effects of ITP on starvation-induced locomotion and trehalose and glucose levels depend on the presence of the functional AKH. However, the rest of the ITP functions in energy intake, expenditure, and energy reserves are independent of this peptide. Altogether, our study shows that ITP is a novel metabolic regulator that controls energy homeostasis alongside AKH.

courtship (Lebreton *et al.* 2016), mating (Wat *et al.* 2021), and alteration of metabolism in response to nutrition deficiency experienced during larval development (Hughson *et al.* 2021). Thus, the AKH signaling appears to be an important mechanism contributing to the fine-tuning of energy catabolism to various needs. The AKH-producing CC cells constitute a hub regulated by diverse inputs, including circulating sugars (Kim and Neufeld 2015), neuropeptides Unpaired 2 (Zhao and Karpac 2017), Allatostatin A (Hentze *et al.* 2015), octopaminergic/tyraminergic neurons (Pauls *et al.* 2018), glucose sensing CN neurons (Oh *et al.* 2019), and Bursicon-responsive DLgr2 neurons (Scopelliti *et al.* 2018). Our work shows that ITP is another regulator of the CC cells. ITP seems to increase AKH signaling at the level of the hormone secretion and by increasing transcription of the AKH receptor *AkhR*. Interestingly, the ITP-producing ipc-1 and ipc-2a secretory neurons have their branches and release sites nearby the AKH-producing cells of CC. However, knock-down of ITP in the ipcs did not cause changes in the trehalose or glucose levels. This suggests that either ITP-producing cells other than the ipc-1 and ipc-2a regulate the secretion of AKH from the CCs or that the ITP cells act redundantly. In addition, we cannot exclude that the ipc-1 and ipc-2a (or other ITP cells) regulate the release of other CC-produced peptides, such as the decretin Limostatin (Alfa *et al.* 2015).

In addition to triggering the secretion of AKH, ITP seems to control AKH signaling via transcriptional control of *AkhR*. However, it remains to be investigated whether ITP acts on the CCs and fat body (tissue-expressing *AkhR*) directly or indirectly via ITP-regulated metabolites or other factors. Altogether, the hyperglycemic action of ITP depends on the AKH peptide. AKH is one of the most studied insect hormones, and its hyperglycemic and hypertrehalosemic functions are well known (Kim and Rulifson 2004; Lee and Park 2004; Gáliková *et al.* 2015). However, the mechanism behind these roles is not clear. As glycogen is considered to be the major source of trehalose in insects (Becker *et al.* 1996; Van der Horst 2003), AKH has been expected to exert its hyperglycemic functions by inducing glycogenolysis (Van der Horst 2003). Nevertheless, at least in the fly model, this seems not to be the case (Gáliková, Klepsatel, Xu, *et al.* 2017; Yamada *et al.* 2018). Moreover, insects cannot carry out gluconeogenesis from lipid substrates (due to the absence of the glyoxylate cycle). Thus, the hyperglycemic effects of AKH likely do not result from its lipolytic functions. Altogether, the mechanism behind the hyperglycemic and hypertrehalosemic roles of the ITP–AKH axis remains enigmatic.

It also remains to be investigated whether the functions of the ITP–AKH axis are conserved in other ecdysozoan invertebrates. Crustaceans have a structural homolog of AKH, called Red pigment concentrating hormone (RPCH). Interestingly, a recent study showed that it has a hyperglycemic effect (Alexander *et al.* 2018). It has been suggested that RPCH regulates glycemia via an eyestalk-produced factor (Alexander *et al.* 2018), and it is thus tempting to hypothesize that RPCH is under the control of CHH.

The AKH-independent roles of ITP

Our results show that except for the hyperglycemic and hypertrehalosemic effects of ITP, the other metabolic functions of the peptide (such as the control of feeding, metabolic rate, and energy reserves) do not require AKH. Interestingly, hunger-related phenotypes are regulated by AKH and ITP in an antagonistic manner. AKH induces (Jourjine *et al.* 2016; Gáliková, Klepsatel, Xu, *et al.* 2017) while ITP represses feeding (Gáliková *et al.* 2018), and AKH is necessary for starvation-induced foraging (Lee and Park 2004; Isabel *et al.* 2005; Gáliková *et al.* 2015), while ITP inhibits this process.

Altogether, AKH seems to be part of a broader axis that is functionally similar to the glucagon pathway in mammals. Nevertheless, it is unclear how the AKH-independent effects of ITP are mediated. For example, the essential role of ITP in the control of energy intake and expenditure may be responsible for the metabolic and fitness-related functions of the peptide. Alternatively, ITP may also have direct signaling effects. Understanding the molecular mechanism behind these ITP functions will require, first of all, the identification of ITP receptor(s) and downstream targets.

The open questions in ITP expression, splice variants, receptor(s), and downstream targets

Our study was based primarily on ubiquitous manipulations of ITP. Thus, we cannot differentiate whether specific subpopulations of ITP-producing cells are responsible for the tested phenotypes or whether the ITP neurons produce the peptide redundantly. Although the antibody stainings detected the fly ITP only in the nervous system (Dircksen *et al.* 2008; Gáliková *et al.* 2018), we cannot exclude the existence of additional ITP-producing cells. The extraneuronal expression is suggested by the microarray (Chintapalli et al. 2007) and RNA seq (Leader et al. 2018; Krause et al. 2022) data, and the expression of ITP homologs in other insects (Begum *et al.* 2009; Yu, Li, *et al.* 2016) and crustaceans (Chen *et al.* 2020).

ITP is considered the only functional hormone produced by the *ITP* gene, because only this peptide bears the amidation signal crucial for its activity (Dircksen *et al.* 2008; Dircksen 2009). Consistently, mutational analysis of the gene showed that the N-terminus is necessary for the ion transport in the hindgut of *Schistocerca gregaria* (Zhao *et al.* 2005), and only ITP (but not ITPLs) is needed for the development in the brown planthopper *N. lugens* (Yu, Li, *et al.* 2016). Nevertheless, the ITP of the red flower beetle *T. castaneum* lacks the canonical amidation signal, and the ITPL forms are required for development and reproduction (Begum *et al.* 2009). Thus, we cannot exclude the possibility that the 2 long forms of ITP, ITPLs, may also have certain biological functions in the fly. Moreover, our RNAi approach reduced mRNA for all 3 peptides, although the overexpression experiments were ITP specific.

Receptor(s) for *Drosophila* ITP is currently unknown, but flies carry homologs of the ITP receptors identified in silkworm *B. mori* (Nagai *et al.* 2014). The most related fly genes are the *Tachykinin-like receptor at 99D* (*TkR 99D*), *Tachykinin-like receptor at 86C* (*TkR 86C*), *Pyrokinin 2 receptor 2* (*PK2-R2*), and *Pyrokinin 2 receptor 1* (*PK2-R1*) (Nagai *et al.* 2014). However, it is unclear whether any of these GPCRs interact with the ITP peptide in *Drosophila*.

Altogether, although our study brought the first characterization of metabolic roles of ITP in the fly model, it still left many questions open. Further understanding of the exact metabolic actions of ITP requires the identification of the target tissues, receptor(s), and other members of the ITP signaling pathway.

The role of ITP in the integration of water and energy balance

As an antidiuretic hormone (Gáliková *et al.* 2018), ITP seems to fulfill the functions of mammalian renin–angiotensin and vasopressin systems. Interestingly, mammalian glucagon is also involved in water homeostasis; it cooperates with vasopressin to increase glomerular filtration after a protein-rich meal (Ahloulay *et al.* 1992; Bankir *et al.* 2015, 2018). ITP integrates water metabolism with energy homeostasis as well. Briefly, ITP increases body water by promoting water intake while inhibiting its excretion. At the same time, the peptide decreases energy balance by inhibiting energy intake and increasing metabolic rate. This may actually be the primary, overarching function of ITP. In contrast to the protected laboratory conditions with a balanced diet providing sufficient water and energy, animals are often exposed to a shortage of food or water in nature. Thus, animals need to adjust water and food intake to maintain the osmolarity of body fluids. When the food is too dry, ITP increases the ingestion of free water while reducing feeding. At the same time, the hormone promotes water conservation by inhibiting excretion (Gáliková *et al.* 2018). To cope with the reduced energy intake, ITP likely stimulates the catabolism of stored energy reserves, which also releases bound water. Given the natural transcriptional responses of ITP to desiccation and osmotic stress, ITP signaling may be one of the critical factors integrating water and energy homeostasis.

Data availability

All data necessary for confirming the conclusions presented in this article are available in the article and its online supplementary material. Fly stocks and reagents are available upon request.

Supplemental material is available at GENETICS online.

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Conflicts of interest

None declared.

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