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Regulation of feeding behaviour and locomotor activity by *takeout* in *Drosophila*

Nicolas Meunier*,†, Yesser Hadj Belgacem† and Jean-René Martin‡

Equipe: Bases Neurales des Comportements chez la Drosophile, Laboratoire de Neurobiologie Cellulaire et Moléculaire (NBCM), CNRS, Unité UPR-9040, 1 Avenue de la Terrasse (Bat. 32/33), F-91198 Gif-sur-Yvette Cedex, France

*Present address: INRA, UVSQ, UMR1197, Neurobiologie de l'Olfaction et de la Prise Alimentaire, Récepteurs et Communication Chimique (NOPA), F-78350 Jouy en Josas, France

†These authors contributed equally to this work

‡Author for correspondence (e-mail: jean-rene.martin@inaf.cnrs-gif.fr)

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Summary

The hormonal regulation of feeding behaviour is well known in vertebrates, whereas it remains poorly understood in insects. Here, we report that the *takeout* gene is an essential component of nutritional homeostasis in *Drosophila*. *takeout* encodes a putative juvenile hormone (JH) binding protein and has been described as a link between circadian rhythm and feeding behaviour. However, the physiological role of *takeout* and its putative link to JH remain unknown. In this study, we show that *takeout* (*to^{1}*) flies failed to adapt their food intake according to food availability and that most defects could be genetically rescued. When food is abundant, *to^{1}* are hyperphagic, yielding to hypertrophy of the fat body. When food reappears after a starvation period, *to^{1}* flies do not increase their food intake as much as wild-type flies. This defect in food intake regulation is partly based on the action of Takeout on taste neurons, because the sensitivity of *to^{1}* gustatory neurons to sugars does not increase after starvation, as in wild-type neurons. This lack of regulation is also evident at the locomotor activity, which normally increases during starvation, a behaviour related to food foraging. In addition, *to^{1}* flies lack sexual dimorphism of locomotor activity, which has previously been linked to the JH circulating level. Moreover, application of the JH analog methoprene rescues the phenotype. These results suggest that *takeout* plays a central role as a feeding regulator and may act by modulating the circulating JH level.

Key words: nutritional regulation, taste, Juvenile Hormone, *Drosophila*, fat body, *takeout*.

Introduction

The ability of organisms to adapt to fluctuations in food conditions is crucial for their survival. Thus, food intake is tightly regulated by external factors such as food availability and quality, as well as by internal factors including sex, age, circadian rhythms and most importantly, the hormonal status related to energy homeostasis (Schwartz et al., 2000). The understanding of the physiological basis of feeding behavior regulation is particularly important, as in affluent societies obesity has become a widespread problem (Berthoud, 2004). In vertebrates, the regulatory center of feeding has been mapped to the hypothalamus and is mainly under the control of leptin and insulin (Hillebrand et al., 2002; Horvath and Diano, 2004). Several hypothalamic neuropeptides such as agouti-related protein (AgRP) and neuropeptide Y (NPY) have been shown to be potent feeding stimulants, whereas melanocortins (POMC) and cocaine- and amphetamine-regulated transcript (CART) have been shown to suppress food intake (Stanley et al., 2005).

In invertebrates, numerous studies have described which factors are involved in the regulation of food intake (Barton-Browne, 1975; Dethier, 1976; Thompson, 2003). Recently, important advances have been made to clarify at the molecular level the neuronal circuitry and the hormonal regulation involved, taking particular advantage of the genetic model system of *Drosophila*. Indeed, seven insulin-like genes (dilps) have been identified (Brogiolo et al., 2001) for one insulin receptor gene (InR) (Chen et al., 1996). Among a variety of actions of the insulin-like peptides, a conserved role in the control of the circulating carbohydrate level and fat storage has recently been highlighted (Belgacem and Martin, 2006; Broughton et al., 2005). Another recent study has elegantly demonstrated an implication of DILP in noxious food aversion in *Drosophila* larvae (Wu et al., 2005a). Moreover, the role of DILP in food quality preference has been linked to the receptor of NPF, a NPY analog, underlining a link between the insulin signaling pathway and NPF (Wu et al., 2005b). Together, these results suggest that there are conserved mechanisms for food intake control between insects and mammals, and therefore

demonstrate the relevance of using invertebrate organisms as models.

In *Drosophila*, *takeout* (*to*) has been formerly identified as a circadian clock-regulated output gene. Additional data indicate that *to* could link circadian rhythms and feeding behaviour (Sarov-Blat et al., 2000). Indeed, *to* mRNA is expressed in structures related to feeding such as cardia, crop (a dilatation from the caudal end of the oesophagus, serving as a food reservoir), head fat body and antennae (Dauwalder et al., 2002; Sarov-Blat et al., 2000). Takeout (TO) expression is also induced by starvation (Sarov-Blat et al., 2000) similarly to related genes in *Bombyx mori* (Saito et al., 2006). Finally, *to* mutated flies (to^1) display a reduced resistance to starvation. Although to^1 (a deletion in the 3′ region of *to* genomic DNA) (Sarov-Blat et al., 2000) are still able to express *to*, at a low basal level they lack the ability to regulate its expression by starvation.

In this study, we use complementary approaches to further characterize the physiological role of *to*. First, we show that to^1 mutant flies failed to change their food intake according to food availability, leading to hyperphagic flies. This increased food intake leads to hypertrophy of the fat body, a phenotype that can be related to obesity. Second, using an extracellular electrophysiological recording approach, we found that whereas the sensitivity of taste neurons to glucose increases after starvation in wild-type (WT) flies, it remains constant in to^1 flies. This result suggests that part of the hyperphagia phenotype of to^1 could be based on a deregulation of the peripheral nervous system. Although these results indicate that TO plays a major role in feeding behavior regulation, little is known about its molecular mechanism. It has been previously suggested that TO could act as a juvenile hormone (JH) carrier protein based on its protein sequence similarity with JH binding proteins (JHBPs) (Lerro and Prestwich, 1990). Moreover, we have previously found that JH level is involved in sexual dimorphism of locomotor activity (Belgacem and Martin, 2002). to^1 flies lack such sexual dimorphism and this could be rescued both genetically, by expressing *to* under the *timeless* (*tim*) driver, and pharmacologically, using methoprene, a JH analog. This is the first evidence that TO may act in relation with JH.

Materials and methods

Flies

Drosophila melanogaster lines were maintained at 24°C on the following food medium: 83 g l^{-1} dried yeast, 83 g l^{-1} corn meal, 11 g l^{-1} agar, 10 g l^{-1} sucrose and 1.2% Nipagin (methyl-hydroxy-4-benzoate), prediluted at 10% in ethanol, as anti-fungus. Flies were reared under a tightly controlled L:D cycle (12 h:12 h). Canton Special (CS) flies were used as WT. The ry506, to^1 were kindly provided by B. Dauwalder (University of Houston, TX, USA). The *tim*-GAL4/CyO; ry506, to^1 and the UAS-*to* (141)/CyO lines were kindly provided by M. Rosbash (Brandeis University, MA, USA) (Sarov-Blat et al., 2000). All of these lines have been outcrossed six times with CS to homogenize the genetic background (Cantonization). For starvation condition, flies were kept in a vial containing a water-soaked Kimwipe paper from 16:00 h to 08:00 h.

Quantification of food intake

To quantify the amount of food intake, a group of 20 flies (males or females) were starved during 16 h (from 16:00 h to 08:00 h) in the presence of water. Flies were then allowed to eat for 15 min on a colored medium (7 ml H_2O, 0.15 g agar, 1.5 ml Blue dye and 0.25 g saccharose). Each group of flies was crushed in 1 ml of standard phosphate buffer saline (PBS), vortexed and centrifuged for 3 min (1500 ***g***, room temperature). An amount of the supernatant (500 μl) was collected and added to 500 μl of n-heptane. The mixture was vortexed and centrifuged as in the previous step. This step removed the lipid contaminants, which could interact with the Blue dye signal. Absorbance was read at 630 nm.

Triglyceride measurements

Samples of 10 flies (males or females separately) were weighed and crushed in 800 μl of PBT (PBS and 0.1% Triton X-100). An amount of the supernatant (100 μl) was added to 200 μl of reagent (Triglyceride LiquiColor mono-reagent, Stanbio) or to 200 μl of deionized water, which was used as a baseline level. After 15 min incubation at 37°C, absorbance was read at 500 nm and compared with a standard calibration curve. In parallel, protein levels were quantified in a second sample of 10 flies reared and treated under the same conditions. Flies were weighed and crushed in 100 μl of protein extraction buffer (deionized water, 4% SDS, 10% glycerol, 5% 2-mercaptoethanol). The mixture was vortexed for 1 min and incubated for 10 min at 70°C. An amount of PBT 0.1% (900 μl) was added, sonicated for 30 s and centrifuged (3 min, 17 000 ***g***, room temperature). An amount of each sample (50 μl) was added to 1.5 ml of Bradford reagent (Sigma). Absorbance was read at 530 nm. A calibration curve was obtained using bovine serum albumin (BSA) as a standard.

Trehalose measurements

Quantification of circulating trehalose levels was performed according to the method of Chen et al. (Chen et al., 2002), with slight modifications (Belgacem and Martin, 2006). Samples of 10 flies (males or females separately) were weighed and crushed in 250 μl of 0.25 mol l^{-1} Na_2CO_3 buffer and incubated in a 95°C water bath for 2 h to inactive all enzymes (trehalose is a non-reducing sugar resistant to temperature up to 100°C). An amount (150 μl) of 1 mol l^{-1} acetic acid and 600 μl of 0.25 mol l^{-1} sodium acetate (pH 5.2) were added and the solution was centrifuged (10 min, 12 500 ***g***, 24°C). An amount (200 μl) of supernatant was incubated overnight at 37°C with 2 μl porcine kidney trehalase (Sigma) to convert trehalose into glucose. A sample (100 μl) of this solution was added to 1 ml of a glucose oxydase solution (Thermo Electron, Melbourne, Australia) and incubated for 20 min at 37°C. The glucose concentration was quantified at 520 nm and compared with a calibration curve.

Immunohistochemistry and Hematoxylin-Eosin (HE) staining

Immunohistochemistry

Adult heads were fixed (Carnoy) for mass histology and embedded in paraffin, as previously described (Belgacem and Martin, 2006). Sections (7 μm) were blocked for 1 h in normal horse serum (PBT: 1.5% PBS+0.1% Triton X-100). A primary anti-*takeout* antibody (1:500 in PBT), kindly provided by M. Rosbash (Sarov-Blat et al., 2000), was incubated overnight at 4°C. After PBT washing, a secondary antibody (anti-rat biotinylated; Vector Laboratories, Burlingame, CA, USA) was added (1:200 in PBT) for 1.5 h. Biotin-streptavidin coupled to peroxidase (Vectastain ABC kit; Vector Laboratories) was used with either diaminobenzidine (DAB) or Vector® VIP as chromogen to visualize the primary antibody.

HE staining

Adult heads were fixed (Carnoy) for mass histology and embedded in paraffin, as previously described (Belgacem and Martin, 2006). Sections (7 μm) were deparaffinized by two successive xylene baths. After rehydration, slides were stained in Hematoxylin for 10 s and washed with water. Slides were then transferred in a solution of acid–alcohol (1 ml HCl in 200 ml ethanol) for 10 s and washed in water. Slides were then immersed in Eosin for 5 min and washed again in water. Finally, slides were gradually dehydrated and mounted in Entellan (Merck).

Electrophysiological recording of the taste neurons

For the taste cell recording technique, a fly, previously chilled on ice, was secured in a 1 ml pipette tip with the head out, and electrically grounded *via* a glass capillary filled with Ringer's solution inserted into the back of the head. To stimulate a sensillum, the tip was covered for less than 2 s with a recording electrode (Hodgson et al., 1955) containing both an electrolyte (3 mmol l^{-1} KCl) and 100 mmol l^{-1} glucose as a stimulus. All recordings were made on L-type sensilla and were stimulated only once per fly. We recorded a mean of four sensilla per fly [indistinguishable among L1, L2, L3 on both sides of the proboscis (Hiroi et al., 2002)]. The recording electrode (a glass capillary with a tip diameter of 20 μm) was connected to a Tasteprobe amplifier (Marion-Poll, 1996). The electric signals were amplified and filtered [CyberAmp 320 (Axon Instruments, Union City, CA, USA); gain: 1000; eighth-order Bessel passband filter: 1 Hz–2800 Hz]. Contacting a taste hair with the stimulus electrode triggered data acquisition [sampling rate 10 kHz, 12 bits; DT2821 (Data Translation, Marlboro, MA, USA)], and data were stored in a computer using Awave (Marion-Poll and Van der Pers, 1996). Spikes were detected and analyzed using interactive software procedures of custom-made dbWave software. When L-type sensilla are stimulated by 100 mmol l^{-1} glucose, both W and S cells respond (Hiroi et al., 2002). We sorted action potentials from W and S cells separately according to previous methods (Meunier et al., 2003b), and we evaluated the action-potential frequency by counting spikes during the first second of stimulation. A mean of four sensilla were recorded from eight different flies for each genotype and each condition (see Fig. 4 legend for details).

Quantification of locomotor activity by video-tracking

We used a previously described paradigm to track fly activity (Belgacem and Martin, 2002; Martin, 2004). Briefly, a four-day-old fly was allowed to walk in a small square arena (4×4 cm, 3.5 mm high) for 5 h. A camera placed above the arena recorded the fly movements (EthoVision; Noldus, Wageningen, The Netherlands). Two parameters were monitored: total distance and number of activity and inactivity phases (start/stop). Since the level of locomotor activity could differ among groups with different genotypes, the number of start/stop phases was normalized by designating the females of each group as the reference (expressed as 100%) and comparing the males in the same group with the females. Flies and their respective controls were examined at the same time (Martin, 2004). All experiments were performed at 24°C and 60% humidity.

Methoprene treatment

To assess whether *to* could potentially be a JH carrier, the JH analog methoprene (Sigma) was applied to to^1 male flies, as previously described (Tatar et al., 2001) with slight modifications. Briefly, a wick (Q-tips) is soaked within an ethanol solution containing methoprene (20 μg ml^{-1}), and dried for 1 h at room temperature to remove the ethanol. The wick is then suspended overnight in a standard fly vial where flies are introduced and can make contact with the wick. The following day, flies are introduced into the arenas and their locomotor activity recorded for 5 h (Belgacem and Martin, 2002). Control flies were treated in parallel with ethanol (vehicle) under the same conditions.

Statistical analysis

Statistical comparisons were made with analysis of variance (ANOVA) tests, using Statistica software (StatSoft Inc., Tulsa, OK, USA).

Results

to mutant presents abnormal feeding behavior

It has previously been suggested that *to* could link circadian rhythms with feeding behavior (Sarov-Blat et al., 2000). To determine whether *to* was directly involved in feeding behavior, we quantified the food intake of to^1 compared with WT-CS by scoring the amount of Blue dye ingested along with 100 mmol l^{-1} sucrose during a 15-min period (Fig. 1A). It is well known that under regular food availability, food intake during a short time period is low (Edgecomb et al., 1994). Surprisingly, we found that the amount of food ingested by to^1 males is significantly higher (eightfold) than for WT males in similar conditions (Fig. 1A,B). Female *Drosophila* are known to eat more than males, and we found a similar result (Fig. 1A). It has been proposed that *to* may have only a minor role in females because its level of expression is rather low compared

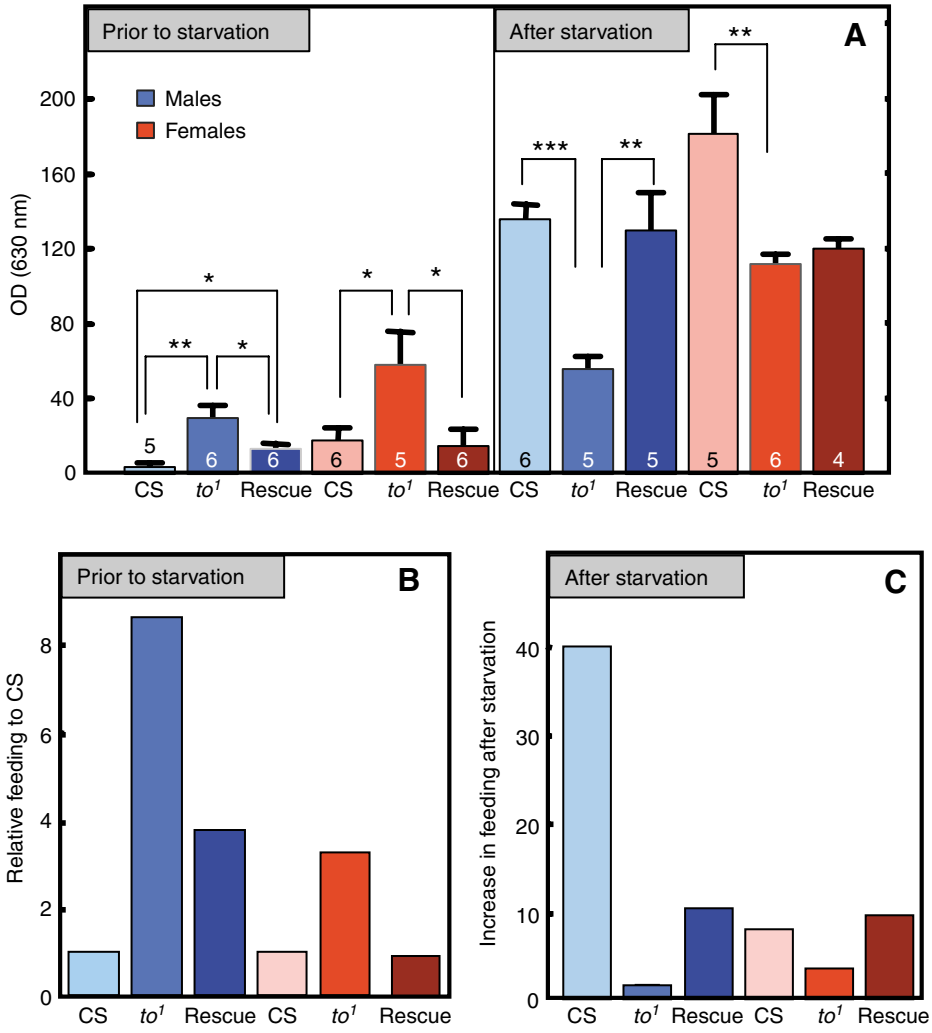

Fig. 1. Food intake under normal and starved conditions. (A) Under normal conditions, both male and female to^1 flies eat more than wild type [Canton Special (CS)]. Directed expression of the *takeout* (*to*) gene within *to*-producing cells (*tim*-GAL4/UAS-*to* in a to^1 genetic background) fully rescues the phenotype in to^1 females (rescue), but only partially in males. (B) To quantify the difference in feeding between CS and to^1 flies under normal conditions, the amount of Blue dye ingested was indexed on CS. to^1 males or females eat eight or three times more food than CS males or females, respectively. (C) After a 16-h period of starvation, the increase in food intake in CS flies (40-fold in males, eightfold in females) is significantly higher than in to^1 flies (twofold in both males and females). Targeted expression of *to* rescues food intake in males, but only partially in females. The determination of the total food ingested, measured as the total amount of Blue dye ingested (OD, optic density), was performed on groups of 10 flies. The number in each box represents the number of fly groups used for each genotype. Rescue, *tim*-GAL4/UAS-*to* in a to^1 genetic background; to^1, *to* mutant. **P*<0.05; ***P*<0.001; ****P*<0.0001. Mean ± s.e.m.

with males (Dauwalder et al., 2002). However, in the present study, we found that to^1 females under regular food availability ingest three times more than WT females (Fig. 1B). When flies were starved for 16 h prior to experiments, food intake significantly increased for to^1 and WT-CS (Fig. 1A). However, the relative increase on feeding after starvation was lower for to^1 (twofold for males) than for WT-CS flies [40-fold for males, in agreement with previously reported values (Edgecomb et al., 1994)] (Fig. 1C). To confirm that the lower starvation-induced food intake increases in *to* mutant flies result from the *to* mutation, we used the P[GAL4-UAS] binary expression system (Brand and Perrimon, 1993). Sarov-Blat et al. have reported that *to* expression overlaps with *tim* (Sarov-Blat et al., 2000). Consequently, the expression of *to* in *tim*-expressing cells (*tim*-GAL4/UAS-*to*, in a to^1 genetic background) could rescue circadian rhythm defects as well as the shorter survival time observed in to^1 during starvation (Sarov-Blat et al., 2000). We used the same *tim*-GAL4 driver to express *to* (*tim*-GAL4/UAS-*to*, in a to^1 genetic background) and found that this expression is sufficient to rescue food intake values to controls, for both males and females under normal feeding conditions. However, under starvation, whereas the phenotype is rescued in *tim*-GAL4/UAS-*to* males, it is only partially rescued in females (Fig. 1A). Nevertheless, taken together, these results show that *to* is involved in the regulation of feeding as well as adaptation to starvation.

Triglyceride and trehalose levels are disturbed in to mutants

The food intake quantification has revealed that to^1 flies eat more than WT flies, and thus could be qualified as hyperphagic. Consequently, we expected that to^1 flies could present alterations in their triglyceride and trehalose levels. Under normal feeding, we found that to^1 flies have an increased total triglyceride level for both sexes when compared with WT-CS flies (Fig. 2A). Normal triglyceride level is genetically rescued in both males and females in *tim*-GAL4/UAS-*to*, in to^1 genetic background flies. A second metabolic parameter related to food intake is the trehalose level, which is considered a circulating carbohydrate store in insects (Thompson, 2003). We previously reported that the trehalose level is related to insulin level, and that it slightly decreases after starvation (Belgacem and Martin, 2006; Isabel et al., 2005). We have quantified trehalose level

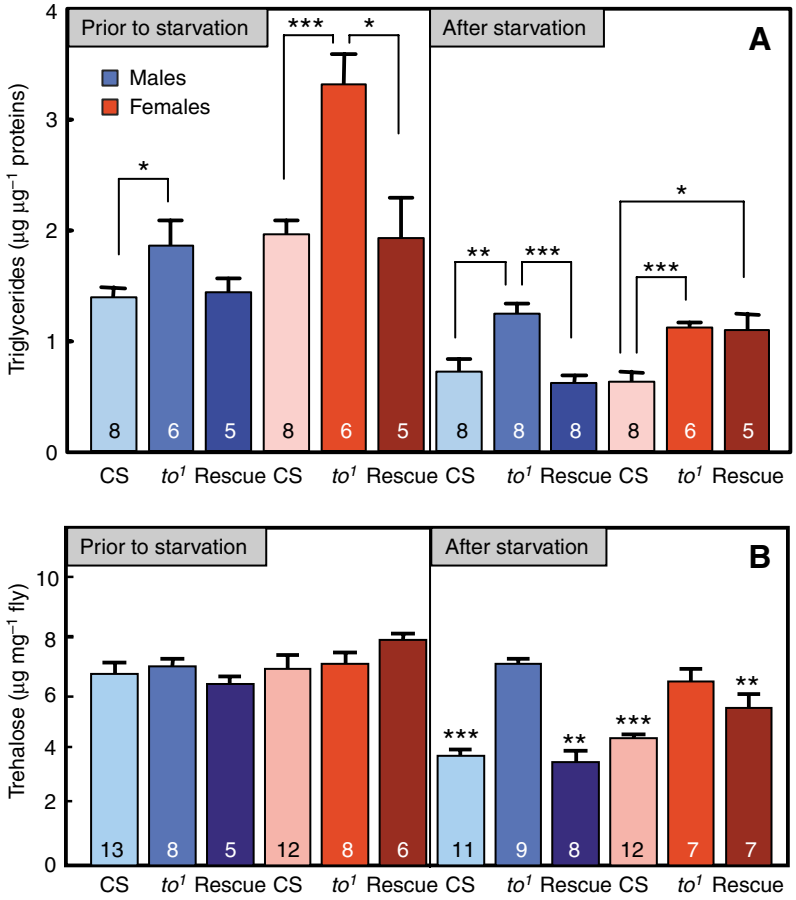

Fig. 2. Triglyceride and trehalose levels before and after starvation. (A) The total amount of triglycerides was quantified for each genotype before and after starvation. Prior to starvation, female and male *to*^{1} flies display an increase in the total amount of triglycerides. This phenotype is fully rescued by directed genetic expression of *takeout* (rescue) in both sexes. After 16 h starvation, the triglyceride level is reduced for all genotypes but the amount of triglycerides remains higher for *to*^{1}. This phenotype was also rescued in males, but not in females. (B) The total amount of trehalose was quantified for each genotype before and after starvation. Female and male wild-types displayed a decrease in their level of trehalose after starvation. However, starvation had no effect on trehalose level in *to*^{1} flies. As for the triglyceride level, this phenotype was rescued genetically. The total amount of triglycerides and trehalose was determined on groups of 10 flies. The number in each box represents the number of fly groups used for each genotype. In B, for the statistical analysis, the comparison was performed, for the same genotype, before and after starvation. CS, Canton Special; rescue, *tim*-GAL4/UAS-*to* in a *to*^{1} genetic background; *to*^{1}, *to* mutant. **P*<0.05; ***P*<0.001; ****P*<0.0001. Mean ± s.e.m.

(in whole flies) in both conditions, pre- and post-starvation. Wild-type males and females exhibit a decrease in trehalose level after 16 h of starvation, as previously reported (Isabel et al., 2005) (Fig. 2B). Surprisingly, in *to*^{1} flies trehalose level is not reduced after starvation, in both males and females, suggesting that *to* product could play a role in the fine regulation of trehalose level when flies are starved.

Head fat body of both male and female to^{1} *is hypertrophic*

Northern blot analysis has shown that *to* is expressed in the head (Sarov-Blat et al., 2000) and, by *in situ* hybridization, in male brain-associated fat body (Dauwalder et al., 2002). The structure of the head fat body was examined using HE staining. This stain labels the cell membrane and thus can be used to reveal putative changes in cell volume and shape. In male and female *to*^{1}, we observed that the cells of head fat body are larger than in WT and contain larger lipid vacuoles (Fig. 3B). Since triglycerides are stored in this tissue, the increased triglyceride levels observed in *to*^{1} may be correlated to the hypertrophy of its head fat body.

Excess of triglyceride storage in to^{1} *is not based on mobilization failure*

As lipids and carbohydrates are consumed first during starvation (Marron et al., 2003), we questioned whether this hypertrophy of the fat body could be because of either a defect in lipid stock mobilization or an excess of lipid storage. We examined the evolution of total triglyceride level (Fig. 2A) and the structure of the head fat body after 16 h starvation (Fig. 3B). A 50% reduction of the total triglyceride level was observed after starvation in control and mutant flies. This result suggests that every fly genotype tested is able to mobilize their fat storage. Head fat body cells exhibit a more condensed morphology after starvation. Lipid vacuoles are almost absent in these starved cells. By contrast, in *to*^{1} starved head fat body cells, lipid vacuoles are still evident, although are smaller than non-starved *to*^{1} cells (Fig. 3B). This difference is consistent with the fact that *to*^{1} have more reserves at the beginning of starvation compared with WT flies, and thus they still have some lipid reserve after 16 h starvation, whereas WT flies have almost completely mobilized their energy storage. This result confirmed that all genotype flies were able to use their lipid reserves. Therefore, the excessive lipid storage in *to* mutants is not because of a defect in energy store mobilization, but rather a direct consequence of the hyperphagia.

Physiological adaptation to starvation of taste neurons is absent in to mutants

In situ hybridization has previously demonstrated that in addition to the expression in the head fat body, *to* is also expressed in the antennae, the main organ of the olfactory

system (Dauwalder et al., 2002). Using the *to*-specific antibody generated by Sarov-Blat and colleagues (Sarov-Blat et al., 2000), we found that TO is indeed present in olfactory sensilla, confirming previous data using *in situ* hybridization (Dauwalder et al., 2002) (Fig. 3A). We also confirmed the presence of TO in the head fat body of males and females (Fig. 3A). Dauwalder and colleagues did not find any *to* expression in the head fat body of females (Dauwalder et al., 2002), and thus the detected TO protein in the female head fat body may come from another organ, as it is supposed to be a circulating protein (Sarov-Blat et al., 2000). It may well be that the *in situ* hybridization technique used by Dauwalder and colleagues failed to detect it, possibly because of a weak relative expression in the female. Interestingly, we observed that TO was present in the gustatory sensilla of the labellum, the main taste organ of flies (Fig. 3A), in both males and

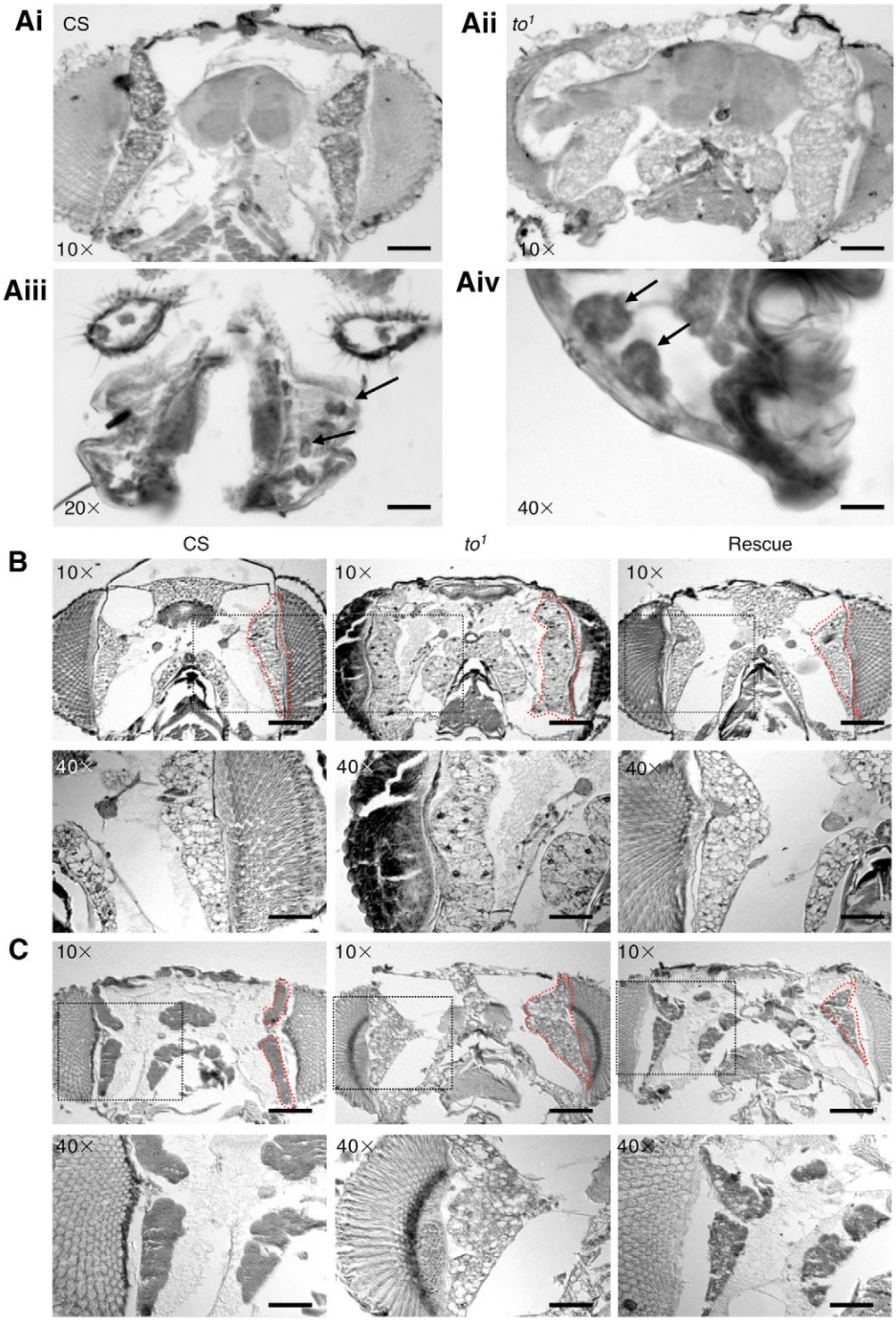

Fig. 3. Presence of Takeout (TO) in the head fat body, which is hypertrophic in to^1. (A) Immunohistological staining using an anti-TO antibody. (Ai,Aii) TO is detected in the head fat body (arrows) of a wild-type Canton Special (CS) male, whereas in a to^1 mutant it is not detected (10×; scale bar, 100 μm). (Aiii,Aiv) In wild-type CS, TO is also detected in the gustatory sensilla (arrows) (Aiii: 20×; scale bar, 50 μm. Aiv: 40×; scale bar, 25 μm). (B) Hematoxylin-Eosin (HE) staining reveals that under normal feeding conditions, *to* flies exhibit a hypertrophic head fat body (outlined in red). The 40× field corresponds to the 10× outlined area. The head fat body cells in to^1 exhibit large lipid vacuoles (40× image). This phenotype is rescued by directed genetic expression of *to* within the *to*-producing cells. (40×: scale bar, 25 μm; 10×: scale bar, 100 μm). (C) After a 16-h period of starvation, the head fat body is reduced in wild-type flies, because they have mobilized their energy resource. The HE staining is more intense in the head fat body of wild-type flies as cells have shrunk and cell membranes are tightly regrouped. In to^1 flies, the fat body is also reduced, but less so than in wild type. This phenotype is also rescued by directed genetic expression of *to* within TO-producing cells. Rescue, *tim*-GAL4/UAS-*to* in a to^1 genetic background; to^1, *to* mutant.

females. This expression pattern, associated with the fact that to^1 flies failed to adapt their food intake to food availability, raises the question as to whether the peripheral sensitivity to food in to^1 was normal.

In many insects, four or more taste neurons are generally clustered in a hair-like structure called basiconic sensillum. In *Drosophila*, each one of these four neurons responds preferentially to sugars, bitter compounds, salts and water and is named accordingly (Meunier et al., 2003a). The electrical activity of the sugar cell responding to compounds known as sweet for humans is a good and relevant estimator of fly appetence for food (Dethier, 1976). The sensitivity of this taste neuron can be estimated by recording extracellularly the response of one sensillum (Hodgson et al., 1955). It has previously been shown that the sensitivity of neurons responsive to sugars increases after starvation in many insects, including the fly *Phormia regina* (Amakawa, 2001). We recorded the activity of taste neurons sensitive to sugar and water under normal feeding conditions or after 16 h starvation (Meunier et al., 2000). We observed that in WT-CS flies, there is a significant increase in the activity of neurons sensitive to sugar after starvation compared with flies kept on normal food medium (Fig. 4). The sensitivity of neurons responsive to water does not change (data not shown). The increase in the activity of neurons sensitive to sugar is absent in to^1 and is rescued by expressing *to* under the *timeless* promoter (*tim*-GAL4/UAS-*to*, in a to^1 genetic background). This result suggests that the defective food intake in to^1 flies could be caused, at least in part, by an inability of to^1 flies to adapt their taste sensitivity in relation to their metabolic and physiological status.

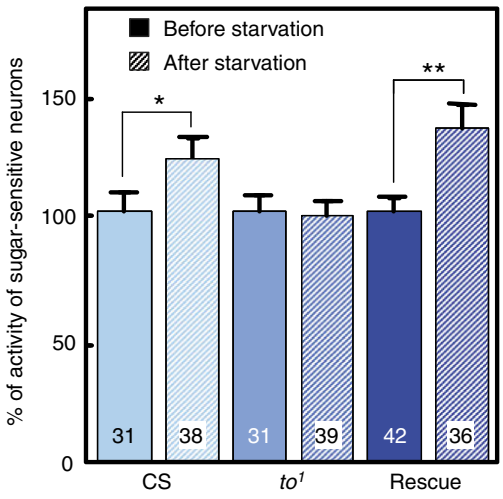

Fig. 4. Modulation of taste sensitivity of gustatory neurons to sugar after starvation. The response of the gustatory neurons sensitive to sugar is estimated by the number of spikes during the first second after stimulation with 100 mmol l^{-1} glucose. Results are normalized on response prior to starvation for each genotype. After starvation, the activity of neurons sensitive to sugars is increased by approximately 20% in wild type, whereas it remains unchanged for to^1 (*takeout* mutant), suggesting that to^1 gustatory neurons do not modulate their activity after a starvation period. This phenotype is rescued by directed genetic expression of *to* within the *to*-producing cells. The number in each box represents the number of sensilla recorded for each genotype. CS, Canton Special; rescue, *tim*-GAL4/UAS-*to* in a to^1 genetic background. Data presented are recorded from males. *P<0.05; **P<0.001. Mean ± s.e.m.

The locomotor activity of to mutants reveals that to^1 fails to adapt its foraging activity

Previous reports show that to^1 flies exhibit aberrant locomotor activity (Sarov-Blat et al., 2000). Interestingly, in the broad array of adaptive behavior related to food intake, the foraging behavior, used to find a new food source, is one of the most important (Barton-Browne, 1975; Bell et al., 1985; Osborne et al., 1997; Ye et al., 1994). Locomotor activity is an essential part of foraging behavior (Isabel et al., 2005; Knoppien et al., 2000; Lee and Park, 2004; Overton and Williams, 2004). To assess whether *to* is involved in the regulation of foraging activity, we quantified the locomotor activity of to^1 by measuring the total distance traveled during 5 h of recording (Belgacem and Martin, 2002; Martin, 2004). In this paradigm, the flies have no access to food, and are gradually starved. We found that female and male to^1 moved significantly less than WT flies (Fig. 5). Furthermore, this lower locomotor activity could be rescued completely for males and partially for females by expressing *to* under the *tim*-GAL4 driver (Fig. 5). These results suggest that to^1 flies lack physiological adaptation to nutrient availability.

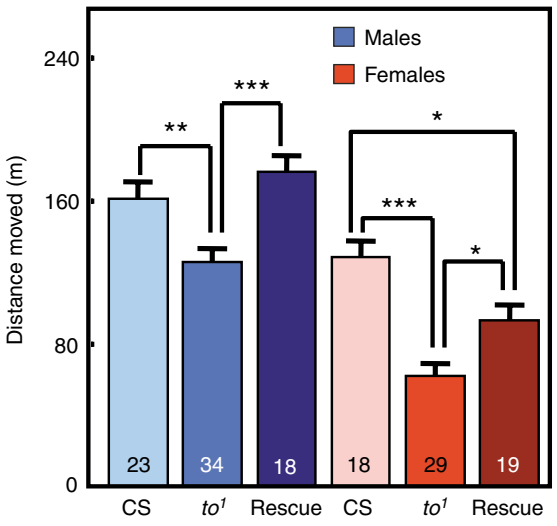

Fig. 5. *takeout* mutants (to^1) displayed disturbed locomotor activity. Total distance traveled by flies during a 5-h period of recording of locomotor activity, as measured by video-tracking. Male and female to^1 flies displayed lower total distance traveled, which can be correlated to lower foraging activity. This phenotype is fully rescued in males, whereas it is only partially rescued in females. The number in each box represents the number of flies recorded for each genotype. CS, Canton Special; rescue, *tim*-GAL4/UAS-*to* in a to^1 genetic background. *P<0.05; **P<0.001. Mean ± s.e.m.

Sexual dimorphism in locomotor activity is abolished in to[1] mutants

The molecular mechanisms underlying *to* activity remain elusive. It has been proposed that TO could be a JH carrier according to its amino acid sequence homology to JHBP (Bohbot and Vogt, 2005; Du et al., 2003; Sarov-Blat et al., 2000). To investigate this hypothesis further, we studied the sexual dimorphism of locomotor activity, which we have previously reported to be linked to JH (Belgacem and Martin, 2002). The sexual dimorphism in locomotor activity displayed by WT flies has been described using different recording devices (Gatti et al., 2000; Belgacem and Martin, 2002; Martin, 2004). We used the video-tracking paradigm during which the number of start/stop phases is recorded for a given time period. The number of start/stop phases is higher for WT females than males (Belgacem and Martin, 2002). By contrast, no significant difference in the number of start/stop phases is found between to^1 males and females (Fig. 6A), suggesting that to^1 males display a female-like activity pattern. Expressing *to* under the *tim* promoter rescues the sexual dimorphism, confirming that the defect in male locomotor activity depends on *to* gene product. We have formerly shown that disrupting JH synthesis pathways causes males to walk like females, and that this effect can be rescued by application of methoprene, a JH analog (Belgacem and Martin, 2002). If the absence of sexual dimorphism of locomotor activity observed in to^1 is based on an insufficient level of circulating JH, the phenotype should be rescued by methoprene. Methoprene application decreases the number of start/stop phases in males, rescuing pharmacologically the to^1 phenotype (Fig. 6B). Such treatment had no significant effect on to^1 females or on WT flies (data not shown).

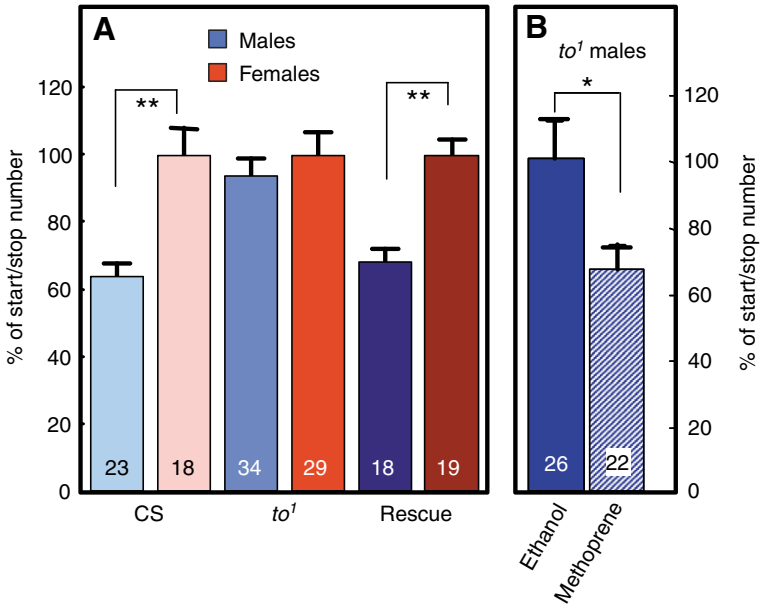

Fig. 6. to^1 (*takeout* mutant) defects in sexual dimorphism of locomotor activity are related to juvenile hormone (JH) level. (A) Wild-type flies present a sexual dimorphism in locomotor activity, as the males do not have the same number of start/stop phases compared with females. This sexual dimorphism is absent in to^1. This phenotype is rescued by directed genetic expression of *to* within the *to*-producing cells. (B) The normal number of start/stop phases is rescued by local application of methoprene (a JH analog) compared with the vehicle alone (ethanol), suggesting that *to* serves as a JH carrier. The number in each box represents the number of flies recorded for each genotype. CS, Canton Special; rescue, *tim*-GAL4/UAS-*to* in a to^1 genetic background. $*P<0.05$; $**P<0.001$. Mean ± s.e.m.

Discussion

Using the *Drosophila* genetic model system, we report that to^1 flies are deficient in their food intake adaptation, and appear hyperphagic. In parallel, their triglyceride stores are increased, a phenotype that resembles in part obesity. Furthermore, their taste sensitivity to sugars and foraging activity do not change under starvation conditions.

to is an important component of food intake regulation

It has been previously shown that *to* is expressed in structures related to feeding, such as cardia, crop and antennae, and that this expression is induced by starvation. This adaptive mechanism might help to increase starvation resistance (Sarov-Blat et al., 2000). We report here that *to* is also expressed in the labellum, a structure directly related to food intake. Furthermore, when reared on normal laboratory food medium, to^1 flies consume more food than WT flies. This hyperphagy leads to an increase in fat energy storage, as indicated by an increase in the size of the head fat body. Moreover, after starvation, in to^1 flies, the increase in food intake is significantly lower than for WT flies. These phenotypes depend on *to* product, because they could mostly be rescued by directed expression of the *to* gene in *to*-producing cells. The discrepancy that we observed for some phenotypes between WT and genetically rescued flies suggests that the expression pattern between the endogenous *to* expression does not match exactly the expression pattern of the driver *tim*-GAL4. Another possibility could be that, in some cases, the level of expression of *to* (UAS-*to* driven by *tim*-GAL4) is not sufficient to totally rescue the to^1 phenotype. Altogether, our results show that *to* plays an important role in food intake regulation, at different levels of integration.

to modulates taste sensitivity in Drosophila

It is well known that the nutritional status of the organism plays an important role in food intake regulation. Most studies have focused on the role of the CNS (Hillebrand et al., 2002; Wu et al., 2005a). However, some experiments have shown that part of this adaptation could also occur at the peripheral nervous system. Indeed, early studies in the blowfly have established that the taste threshold for sugars is modulated by

starvation state (Omand, 1971). Similar modulation of the sensitivity of taste neurons occurs in the locust depending on the quality of diet (Abisgold and Simpson, 1988; Simpson et al., 1991). We observed that in *Drosophila*, the sensitivity of taste neurons to glucose increases by approximately 20% in response to starvation. A similar modulation of the sensitivity of taste neurons to carbohydrates has also been observed in a bigger fly, *Phormia regina*, after injection of sugars directly into the animal (Amakawa, 2001). We found that TO is expressed in taste organs and that taste neurons in to^1 showed no change in their sensitivity to glucose after a period of starvation. This result suggests that *to* product could serve as a signal of the general nutritional status of the fly for taste neurons. A similar adaptation has been shown to occur in mice and has been related to leptin level. Indeed, Kawai and coworkers show that injection of leptin reduces taste sensitivity specifically to sugars (Kawai et al., 2000). The capacity to modulate taste sensitivity is not the only property that *to* shares with leptin. Both leptin and TO are synthesized by cells specific to fat storage. Long-term energy storage is based on lipids in animals. Given the lack of homology between TO and leptin (Sarov-Blat et al., 2000), it may be an evolutionary convergence of mammals and insects to send a signal from fat stores to regulate food intake. Leptin is mainly known for its effects on the hypothalamus (Stanley et al., 2005), and one could wonder about the role of *to* in the CNS, which still needs to be clarified.

to is involved in foraging activity

Food intake *per se* is only part of the complex and fully integrated feeding behavior. Foraging, the search for a food source, is another important component related to food intake that is regulated. Such regulation has been reported in mammals (Overton and Williams, 2004) as well as in insects (Lee and Park, 2004; Osborne et al., 1997). Indeed, WT flies increase their level of locomotor activity when gradual starvation occurs (Martin, 2003; Martin, 2004). Interestingly, in such conditions, to^1 do not display a significant increase in locomotor activity, revealing a lack of proper adaptation of foraging activity to starvation. Moreover, the sensitivity of gustatory neurons to sugar does not change upon starvation, suggesting a general lack of adaptation to food availability in to^1.

Why do to^1 flies die faster than WT during starvation?

to^1 flies are known to have a low survival rate during starvation (Sarov-Blat et al., 2000). In the present study we found that there is a larger fat storage in to^1 when compared with WT flies. One would expect that with increased energy storage, flies would survive for a longer time period, especially for to^1, which displayed a lower locomotor activity than WT flies during starvation. However, we also found that the trehalose level remains high during starvation in to^1, whereas it decreases in WT flies. The carbohydrate pool (mainly glycogen) may be depleted faster in to^1 even with a higher triglyceride level, as gluconeogenesis cannot occur from triglycerides in insects (Thompson, 2003). A faster depletion of carbohydrate level during starvation may be a possible explanation for the lower resistance of to^1 to food deprivation, because carbohydrates are essential for neuronal survival.

The physiological role of to is related to JH effect

JH is the most versatile hormone in animals. It is mainly known for its role in metamorphosis, reproduction and more recently longevity (Flatt et al., 2005). Interestingly, JH has been shown to modulate olfactory interneuron sensitivity to pheromones in locust (Anton and Gadenne, 1999). In honeybees, high JH levels are also known to increase foraging activity (Robinson, 1985), whereas it lowers the sensitivity threshold to sugar (Pankiw and Page, Jr, 2003). Our results indicate that TO could mimic these effects because the absence of TO leads to a decrease in starvation-induced locomotor activity and to an absence of adaptation of the sensitivity of taste neurons to sugars. The molecular identification of the *to* gene has revealed that it shares structural homology with JHBPs (Sarov-Blat et al., 2000). JH, synthesized in the *corpus allatum*, circulates in haemolymph bound to JHBPs, which serve as carriers for this hydrophobic hormone (Park et al., 1993). Further investigation needs to be done to demonstrate that TO could be a carrier for JH.

We have previously shown that JH is involved in sexual dimorphism of locomotor activity. Indeed, pharmacologically provoked JH deficiency in males produces a female-like activity profile (Belgacem and Martin, 2002). However, the mechanisms underlying JH-induced effects remain unclear, particularly because the JH receptor has not been characterized (Flatt et al., 2005). Here, we show that, like the JH-deficient male flies, to^1 males exhibit a female-like activity profile. Moreover, this phenotype is rescued by methoprene treatment, a JH analog. These results suggest that TO could act as a JH carrier. We hypothesize that TO could modulate JH availability in the organism (Fig. 7). Further experiments will be required to demonstrate the mode of action of TO.

TO is a key regulator of feeding behavior in Drosophila

It is well known that the nutritional status of the organism plays an important role in the regulation of food intake. In mammals, the hypothalamus has been identified as a potential center of feeding regulation (Hetherington, 1941). However, apart from the role of insulin (Woods, 1979), the fine hormonal regulation and the related neuronal circuitry involved in this process remained unclear for a long time. During the past decade, a complex pattern of food intake regulation in mammals has emerged (Jobst et al., 2004). In insects, even though former studies have reported the dependence of feeding regulation on their nutritional status (Dethier, 1976; Edgecomb et al., 1994), the neuronal and hormonal bases involved remain elusive. Recent advances in molecular genetics have allowed the identification of several genes involved in this regulation (Zinke et al., 1999; Wu et al., 2003; Melcher and Pankratz, 2005; Wu et al., 2005b), but no clear link has been made between those genes and the adaptation of feeding to food availability.

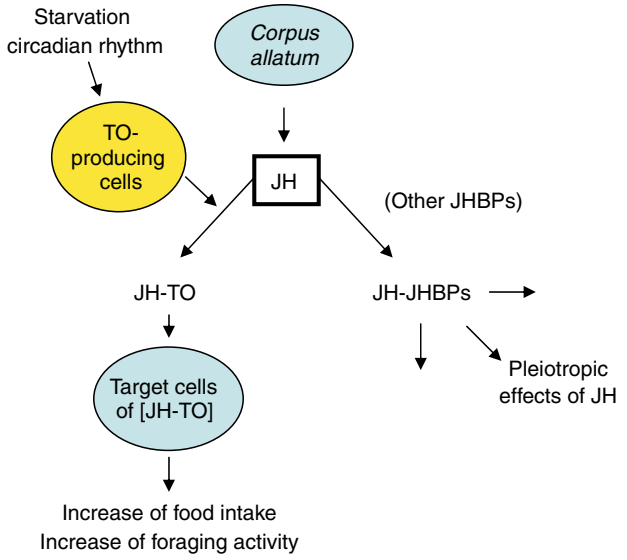

Fig. 7. Proposed model of Takeout (TO) action. Juvenile hormone (JH) produced by *corpus allatum* could have pleiotropic effects according to the circulating juvenile hormone binding proteins (JHBPs). During starvation, TO level is increased and the binding of JH-TO could act specifically on target cells to regulate feeding behavior.

Several studies have underlined the role of biogenic amines in food intake and foraging regulation in honeybees (Pankiw and Page, Jr, 2003; Scheiner et al., 2002), as well as in the blowfly (Brookhart et al., 1987; Long et al., 1986). Octopamine and more recently tyramine have been shown to enhance the sensitivity to sugars and to promote feeding in the blowfly (Nisimura et al., 2005). Like other amino acid-derived hormones, these biogenic amines act within a time course of minutes, enabling it to rapidly adapt the animal feeding behavior according to external stimuli, such as immediate stress or odors in the environment. Interestingly, some of these studies have also pointed out that a high JH level leads to an increase in the foraging activity and a decrease in the threshold of sugar detection in honeybees (Pankiw and Page, Jr, 2003). By modifying the availability of JH levels, TO could act on food intake as a circadian regulator as well as during long-term stress (Fig. 7). Further experiments will be required to precisely associate *to* with other genes related to food intake. For example, it would be worth measuring TO level over time in genetic background mutants of well-characterized genes, such as *hugin*, *klumpfuss* and *pumpless*.

The ry^{506}, to^{1} were kindly provided by B. Dauwalder. The *tim*-GAL4/CyO; ry^{506}, to^{1} and the UAS-*to* (141)/CyO fly lines, as well as the anti-*takeout* antibody, were kindly provided by M. Rosbash. We are grateful to F. Marion-Poll for allowing us to use his electrophysiological set-up. We are also grateful to E. Gouadon, A. Klarsfeld, J. M. Jallon, P. Newland and L. Borodinsky for critical comments on the manuscript.

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