AKH-producing neuroendocrine cell ablation decreases trehalose and induces behavioral changes in *Drosophila*

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The insect fat body performs functions similar to those of the liver and the adipocytes in vertebrates. It is the major storage site for both lipid and glycogen and is also responsible for the synthesis of the bulk of hemolymph proteins. Adipokinetic hormone (AKH) was discovered as a hormone-mobilizing lipid from the fat body during flight in migratory locusts in 1969 (4, 18). Although the hormone was discovered for its adipokinetic, i.e., fat-mobilizing, activity, it also activates fat body glycogen phosphorylase, which leads to a decrease in fat body glycogen and a subsequent increase in the production of trehalose, the major circulating hemolymph carbohydrate in insects (9). Although few insect species mobilize lipids after injection of AKH or its natural homolog, virtually all stimulate fat body glycogen phosphorylase (5, 9). This may or may not lead to a measurable increase of hemolymph trehalose concentrations. For this reason, in some insect species, these hormones are also known as hyperglycemic and/or hypertrehalosemic hormones (9). Thus AKH may be functionally similar to glucagon, and the very limited sequence similarity of some of the insect AKH homologs to glucagon has been used as an argument to suggest that AKH and glucagon are derived from the same ancestral peptide (15). However, the very small peptide sequences available to assess evolutionary relationships between invertebrate and vertebrate hormones often make it difficult to ascertain that sequence similarities really reflect an evolutionary relationship and not a casual coincidence. If indeed AKH and glucagons are evolutionarily related, one would expect their receptors to be similarly related. However, the recent identification of the AKH receptors from two insect species as being closely related to the receptors of gonadotropin-releasing hormone suggests that this is probably not the case (30). Additionally, in several insect species, AKH or its homolog(s) is/are also known to increase the rate of heart beat (3), and this is the only known effect of AKH in *Drosophila*, although the concentrations needed to observe an effect on the heart are rather high (21). Thus the only demonstrated target organ in *Drosophila* is the heart, whereas fat body also appears as a likely target organ. The effects of AKH and its homologs on both the rate of heart beat and hemolymph energy substrates might suggest a physiological role for AKH-related peptides as acting as stress hormones.

In all insect species where lipids are mobilized by AKH, lipophorins, the major hemolymph lipoprotein transporting lipids in insect hemolymph, increase hemolymph lipid by associating with a small apolipoprotein, apo-III (6, 7). However, *Drosophila* lipophorin does not have such an apo-III (23); hence, there is good reason to believe that the peptide in *Drosophila* cannot mobilize lipid. If *Drosophila* AKH is not an AKH, the question arises as to whether or not AKH might mobilize trehalose. This question is of particular interest for adult *Drosophila* because flies use carbohydrates as their exclusive source for energy during flight. However, unlike other insects, which store carbohydrates as fat body glycogen, flies store carbohydrates as nectar in their crop, a blind-ended extension from the esophagus. During flight, it is these stored crop sugars that are used, probably exclusively so, since fat body glycogen stores in flies are very small. Because the release of carbohydrates from the crop in flies seems to be effected via a neural mechanism (see Ref. 34 and references therein), while AKH is released as a hormone, it remains unclear as to whether, in *Drosophila*, AKH can affect hemolymph trehalose. Indeed, in honeybees, which also use carbohydrates stored in the digestive system as fuel for flight, it has
been reported that AKH is without effect on hemolymph trehalose (16).

Given the determination of the nucleotide sequence of the *Drosophila* genome and the relative ease with which one can genetically transform this species, *Drosophila* has become a very attractive model for insect neuroendocrinology (19, 25). This stimulated us to examine trehalose levels in *Drosophila* from which the AKH-producing cells have been removed by induced apoptosis. We report here that genetic ablation of the AKH-producing cells in *Drosophila* leads to a decrease in trehalose levels in fed larvae and in starved adults. Furthermore, elimination of these cells decreased locomotor activity in the adult, whereas the survival of those flies increased during starvation.

**MATERIAL AND METHODS**

**Transposon construction.** The *Drosophila* AKH gene was identified by blasting the peptide sequence from the Berkeley *Drosophila* Genome Project website. The putative promoter of the gene was amplified by PCR with the following primers: 5'-GGGATATCC-CATTCAAGAACGCAATGGA-3' (*Eco*RI site underlined) and 5'-CCGGGATCCCGCGTCAGAATGCTT-3' (*Bam*HI site underlined). The 944-bp fragment was cloned in pCR2.1-TOPO vector (Invitrogen) from which it was recovered by digestion with *Bam*HI and *Eco*RI (because of the presence of an *Eco*RI site inside the promoter, the latter digest was only partial). pGATB vector (5') was cut with *Bam*HI and *Spe*I to obtain the GAL4 coding sequence, and the rest of the necessary elements for a functional P-element were obtained by digesting pCaSpeR with *Eco*RI and *Xho*I. The three fragments (AKH promoter, GAL4 coding sequence and the P-element backbone from pCaSpeR) were ligated together to produce pAKH-GAL4. The structure of pAKH-GAL4 was confirmed by restriction digests and DNA sequence analysis of crucial parts of the plasmid.

**Drosophila transformation.** The pAKH-GAL4 transposon and the helper plasmid p*pr*($\Delta 2–3$) were dissolved together at concentrations of 300 and 100 $\mu$g/ml, respectively, and were injected in water microbiolizes. Two independent transformants were obtained that had the P-element inserted on the third chromosome (pAKH02) and on the X chromosome (pAKH03). Thereafter, those two homozygous-viable lines were outcrossed in Canton S background (CS), used as a wild-type strain.

**Drosophila stocks.** Flies were maintained on standard *Drosophila* medium and kept at 25°C under 60% humidity conditions (2). The UAS-head-involutio deffective (UAS-hid), UAS-creaper (UAS-rpr), UAS-Bax, and UAS-CD8-green fluorescent protein (GFP) strains were obtained from Bloomington stock center. We used either a line carrying the two transgenes, UAS-hid and UAS-rpr, both located on the X chromosome or UAS-Bax located on the second chromosome to generate AKH-ablated *Drosophila*.

**Antibody production.** pGlu-Leu-The-Phe-Pro-Asp-Trp-Gly-Lys (2 mg), custom synthesized by Research Genetics (Huntsville, AL), was coupled via its Lys residue to 5 mg BSA with difluoro-dinitrobenzene, as described by Tager (31). The peptide-BSA conjugate was separated from unreacted peptide and reaction products by dialysis against PBS. The antigen was injected subcutaneously in two New Zealand White rabbits, using Freund complete adjuvant for the first injections and incomplete Freund’s adjuvant for the booster injections. Injections were ~6 wk apart. Blood was collected before the first injection (preimmune serum) and 10 days after each booster injection; serum was collected and stored frozen. Antiserum from rabbit 2 of December 2000 had the highest titer and was used throughout this study.

**Immunohistochemistry.** The nervous system of third instar larvae with the retrocerebral complex attached were dissected in 9% NaCl and fixed for 2 h at room temperature or overnight at 4°C in 4% paraformaldehyde following procedures described elsewhere (14, 35). The unlabeled antiserum was used at a dilution of 1:4,000 in combination with a rhodamine-labeled secondary antibody. We also purified the IgG of the antiserum and labeled them with rhodamine using methods described previously (36); this directly labeled antibody was used at a dilution of 1:500 overnight. For comparison of GFP expression in the progeny of a cross between a pAKH-GAL4 strain and a UAS-CD8-GFP strain with that of *Drosophila* AKH, tissues were processed as described above, and the directly labeled AKH antibody was used to visualize the AKH endocrine cells. The retrocerebral complex can be accidentally removed during dissection. For flies in which we intended to eliminate the AKH cells by genetic ablation, we added, therefore, a fluorescently labeled antibody to the *Drosophila* precursor of the diuretic peptides capa-1 and capa-2 (14). The latter antibody labels neuroendocrine axons in the retrocerebral complex, which allows its unambiguous identification in insects from which the AKH-producing cells have been ablated. To visualize corpora cardiaca in adults, paraffin sections of pAKH/UAS-CD8GFP flies were placed in mass histology “collars,” fixed for 4 h in Carnoy’s solution, dehydrated 2 h, immersed overnight in methyl benzate, and imbedded in different baths of paraffin at the 70°C for 4 h (10). Heads and thoraxes were cut in 7-μm serial frontal sections that were deposited on slides and dehydrated according to standard procedures. Staining with GFP antibody is as described (11). Stained sections were inspected with a Leica microscope.

**Measurement of the rate of heart beat.** Experiments were performed as described (21). Transparencies of prepupaee illuminated from below allowed visualization of the heart. The number of contractions was counted for 20 s, and pAKH/UAS-Bax animals were counted in alternation with appropriate controls.

**Trehalose quantification.** Flies or larvae were individually homogenized with a plastic pestle in 0.5 ml of 70% ethanol in an Eppendorf tube, and the pestle was washed two times with 0.5 ml of 70% ethanol.

The homogenate was centrifuged at room temperature for 5 min at 13,000 rpm. Because preliminary experiments using up to five larvae or flies per tube had shown that no additional trehalose could be recovered from the pellets, these were discarded. The supernatant was collected in a glass tube and dried in a Speed Vac Concentrator. The lophophilized material was dissolved in 200 μl of 2% NaOH and incubated at 100°C for 10 min to destroy reducing sugars, after which the tubes were rapidly cooled in ice-cold water. The nonreducing trehalose was subsequently quantified by adding 1.5 ml cold freshly prepared anthranile reagent. Trehalose standards were dissolved in 70% ethanol and subjected to the same protocol as the *Drosophila* samples.

It is well known from other insect species that trehalose metabolism varies largely from one larval stage to another and within a given larval stage. We therefore only used the third larval instar (the first and second larval stages contain too little trehalose to be measured individually), and we weighed the larva immediately before homogenizing it to the nearest microgram to allow a correlation with the physiological stage of the insect. For the adult 5- to 7-day-old flies, we used the same procedure as for the larvae.

**Quantification of locomotor activity by video-tracking.** Intact single *Drosophila* flies (male and female) walked freely in a square chamber of 40 × 40 mm and 3.5 mm high (to limit the vertical movement), made of two glass plates and trans-illuminated from below (Martin J-R, unpublished observation). All experiments were performed at 24°C and 60% relative humidity, starting at 1800, with 7-day-old males and mated females. Twenty-four flies (12 males and 12 females) were simultaneously videotaped at a capture rate of 5 images/s for 12 h. Traces were generated using EthoVision software (Noldus). Paths joining contiguous traces were graphically represented as dots (center of gravity of the fly) connected by lines (path traveled between frames). From these paths, two main parameters were extracted. First, we measured the total distance moved (mm). Second, to reveal its temporal distribution, the distance moved was determined for each successive 10-min period. For statistical comparisons, ANOVA tests were applied, using Statistica (StatSoft).
**Resistance to starvation.** Adults were transferred to fresh medium each day. After 5 to 7 days, female and male flies were handled with CO₂ anesthesia, collected, and separated in distinct vials. On regularly hydrated Whatman (3MM) filter paper, adults of the same sex were kept at a density of 25–30/vial without medium. Deaths were scored two times per day. Starvation survival was calculated using the product limit or Kaplan-Meier method with Prism software (Graph-PAD Software for Science, San Diego, CA). For calculating the logrank test, Prism uses the method called the Mantel-Haenszel logrank test (1).

**RESULTS**

Identification of AKH-producing cells. Various antibodies have been raised against AKH or its analogs, but none of these recognize AKH-producing cells in *Drosophila* or other flies (25, 26, 27). To localize AKH expression and verify its absence in flies from which the AKH-producing cells were ablated, we produced a new antibody specific to *Drosophila* AKH. The cells recognized by this antiserum are the same as those previously identified as containing AKH mRNA by in situ hybridization (21, 25). However, the localization of the peptide rather than the mRNA allows a more accurate visualization of cell bodies than is possible with the localization of mRNA. As in other insect species (e.g., see Ref. 8), the AKH-producing endocrine cells in the retrocerebral complex of third instar larvae have axon-like structures protruding from their cell bodies (Fig. 1A), which are also present in the adult.

GAL4 expressed by the pAKH-GAL4 mimics expression of AKH. Expression of GAL4 in the pAKH-GAL4 lines was studied in the progeny of crosses between this line and a UAS-CD8-GFP line. The observed expression of a membrane-associated GFP appeared similar to that observed using immunohistology with the AKH antibody (Fig. 1, B and C). Immunohistology performed on these insects confirmed that the two methods indeed identify the same cells. These results show that the region identified as the putative promoter of the AKH gene is sufficient to induce spatially specific expression of the AKH gene.

AKH cell ablation. To assess the biological role of AKH, either cell death genes *rpr*, *hid* (19), or *Bax* were activated in the AKH cells using the UAS-GAL4 system with the aim of pushing the AKH-producing cells into apoptosis. We analyzed at least 20 neuroendocrine systems for each combination and observed a consistent and complete elimination of AKH-producing cells in third instar wandering larvae when the larvae were the progeny of crosses between females carrying the two UAS-*rpr* + UAS-*hid* transgenes or UAS-*Bax* and male pAKH-GAL4 lines (Fig. 2). However, when we used UAS-*hid* or UAS-*rpr* alone, to induce apoptosis, in some of the larvae a few of the AKH cells survived. Because AKH is generally present in very large quantities in the corpus cardiacum, it is conceivable that a single AKH-producing endocrine cell has sufficient AKH-secreting capacity for normal homeostasis. We therefore used pAKH-GAL4/UAS-*rpr* + UAS-*hid* or pAKH-GAL4/UAS-*Bax* genotype for producing AKH-less *Drosophila*. All experiments were made with appropriate genetic controls. Ideally, one would have liked to measure AKH individually in adult flies. However, the quantities present in a single fly are in the low femtomolar range; hence, if after incomplete ablation of the AKH cells some flies would still produce around a tenth of their normal quantity of AKH, this would be undetectable, even by a very sensitive RIA or enzyme-linked immunosorbent assay (ELISA; the best detection limit published for an AKH-RIA is 8 fmol; see Ref. 20). It thus seems likely that the immunohistological approach to check the presence of AKH is much more sensitive than measuring AKH by RIA or ELISA.

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![Fig. 1. Localization of adipokinetic hormone (AKH) in the neuroendocrine system of a late third instar larva carrying the transgenes pAKH02-GAL4 and UAS-CD8-green fluorescent protein (GFP). A-C: late third instar larva; D: adult. Scale bars, 50 μm. In A, cells are localized by immunohistology using antiserum against *Drosophila* AKH. In B the GFP expression is visualized, and, in C, the images are superimposed. Notice that these endocrine cells have axon-like protrusions, some of which contain significant amounts of AKH immunoreactivity. In D, GFP expression is also visualized in the corpora cardiaca; the dorsal part of the fly at top left.](http://ajpregu.physiology.org/doi/10.1152/ajpregu.00281.2004)
The rate of heart beat. AKH family peptides have been observed to generate cardioacceleration in several insects (9), including Drosophila prepupae (21). To assess if the absence of AKH leads to a decrease in the spontaneous rate of heart beat, we compared the rate of heart beat in pAKH/UAS-Bax with the rate in appropriate genetic controls. No significant differences were found for either females ($P = 0.29$; $n = 7$ for pAKH/UAS-Bax vs. $n = 16$ for pAKH/CS) or males ($P = 0.72$; $n = 11$ vs. $n = 9$). No difference between sexes was found ($P = 0.77$, $n = 16$ for females vs. $n = 9$ for males).

Trehalose quantification. AKH and its analogs are known to mobilize energy substrates in insects (9). Because it appeared unlikely that AKH could mobilize lipids (see INTRODUCTION), we measured trehalose levels. In our first set of experiments, we eliminated AKH-producing cells by crossing males from two independent pAKH-GAL4 lines with females of a UAS-rpr + UAS-hid line, and we measured total trehalose in third instar larvae. As controls, we used a wild-type CS strain, as well as the parent lines used to produce larvae without AKH cells. From work on caterpillars, it is well known that carbohydrate metabolism changes dramatically during the last larval instar (e.g., see Ref. 28); therefore, we anticipated a similar phenomenon in Drosophila. The last larval instar in Drosophila lasts only 24 h, which makes it very difficult to stage the insects accurately. We therefore weighed the last larval instars to use weight as an approximation for physiological stage within the third instar. As can be seen from the results (Fig. 3), there is a clear correlation between weight and trehalose levels in CS larvae, whereas in the other control strains trehalose is present in quantities that appear either the same or slightly higher than in the wild-type strain. However, in flies from which the AKH-producing cells were ablated, the quantities of trehalose for the same weights are significantly lower, especially in heavier, i.e., older, larvae. Our results thus suggest that, particularly during the latter part of the third instar, AKH is required to maintain normal trehalose levels.

We repeated these experiments in adults. In both male and female fed flies, the total amounts of trehalose are only slightly lower in flies from which the AKH cells have been ablated than in controls, and the difference is not statistically significant (Fig. 4). However, although fed and starved fly masses are not significantly different (data not shown), when flies are starved, the amounts of trehalose decrease, and the differences between flies with and without AKH-producing cells become statistically significant in both males and females (Fig. 4).

Locomotor behavior. Our results show that the elimination of the AKH-producing cells lowers trehalose levels, but the question remains as to whether the observed decreases in total trehalose are physiologically relevant in that they reduce trehalose utilization and thus may affect the general physiology of Drosophila. If indeed trehalose cannot be used to the same extent as in normal flies, one might expect a difference in general physiological activity. This led us to quantify locomotor activity in normal and AKH cell-ablated flies. Figure 5A shows the total distance moved by the flies during the 12-h recording, while Fig. 5B shows the time course of the distance moved, the parameter used to quantify locomotion. In normal flies, locomotor activity gradually increases with time, whereas in AKH-ablated flies the level of locomotor activity gradually decreases. Although their locomotion behavior is being quan-

Fig. 2. Ablation of AKH-producing cells in third instar larva. A: AKH-positive cells are localized by immunohistology using antisera against Drosophila AKH in wild-type strain. B: same larva in A visualized in bright field. C: same staining in pAKH-GAL4–02/UAS-reaper (rpr) + UAS-head-invagination defective (hid) larvae. Staining is completely absent in ring gland and other parts of larva. Localization of corpora cardiaca is indicated by arrows; the dorsal part of the fly is up, and larval brain is silhouetted in white. Scale bar, 100 μm.
tified, the flies are gradually starved. In wild-type flies, locomotor activity increases after ~2 h without feeding, an effect that we interpret as an intensification of the search for food. However, in AKH-less flies, this increase does not occur, but rather these flies gradually decrease their locomotor activity.

Longevity during starvation is increased in AKH-ablated flies. Because flies without AKH cells strongly reduce their locomotor activity (Fig. 5), we questioned whether these AKH cell-ablated flies might be exhausted and prone to die more quickly than the wild type. We therefore determined the longevity of both control and AKH-ablated flies during starvation. Normal flies start to die after ~12 h of starvation, and 50% of flies are dead ~30 h after the start of the experiment. AKH cell-ablated flies survive much longer, with a median survival time of 60 h for both sexes (Fig. 6, AKH-ablated vs. control male flies, logrank test $P < 0.0001$, hazard ratio 2.37; AKH-ablated vs. control female flies, logrank $P < 0.0001$, hazard ratio 2.64). Moreover, the difference in survival rate is significant between sexes for AKH-ablated flies (males vs. females, logrank test $P = 0.0372$, hazard ratio 0.86).

Fig. 3. Ablation of AKH-producing cells reduces trehalose levels in larvae. A: trehalose levels in Canton S background (CS; wild-type) larvae. Diamonds represent individual values. Data for CS fit a linear regression $y = 11.3x - 6.6$ with $R^2 = 0.69$; $n = 36$; $F = 136$; $P < 0.001$; the same line is used in B-F for comparison. B: trehalose level for larvae of parental line $y, w, hid$, and rpr (UAS-rpr + UAS-hid). C and D: parental pAKH-GAL4–02 (C) and pAKH-GAL4–03 (D) lines. E and F: larvae without AKH cells [pAKH-GAL4–02/UAS-rpr + UAS-hid (E) and pAKH-GAL4–03/UAS-rpr + UAS-hid (F)].

Fig. 4. Histograms of trehalose levels per fly as a function of sex [males (m) and females (f)], feeding regime, and presence of AKH cells. Number of flies is $n > 32$ per group. Test of significance: 2-tailed $t$-test, **$P < 0.01$ and ***$P < 0.001$. 
AKH are unusual insect neuropeptides for different reasons. First, they are generally present in large quantities in the corpora cardiaca (9). Second, the structures of the peptides identified from the various insect species are very variable, clearly much more so than other similarly sized insect neuropeptides or the closely related crustacean red pigment concentrating hormone (38). Third, some, but not all, insect species have two or three different AKH whose functions are likely to be different, since they have slightly different physiological effects (e.g., in Manduca sexta: controls fat body glycogen phosphorylase in starving larvae, whereas, in adults, it controls lipid mobilization during flight; see Ref. 22). These peculiarities have largely contributed to the fact that the AKH are the best-studied insect neuropeptides, yet several aspects of their functions remain poorly understood. It is plausible that the powerful genetic tools available in Drosophila will be helpful to resolve some of these questions.

It is important to keep in mind that the physiological effects observed after the elimination of the AKH-producing cells can only be attributed to the elimination of these cells, not necessarily the hormone that these cells produce and secrete, i.e., AKH. However, in our opinion, it is likely that the physiological effects of the genetic ablation of the AKH-producing cells in Drosophila are exclusively the result of the absence of the secretion of AKH, just like the physiological effects of alloxan, which kills the pancreatic cells and induces diabetes in dogs, are the result of the absence of insulin secretion (24). Indeed, if the AKH-producing cells were to produce a second hormone, the physiological effects observed after ablating the AKH cells could be because of this second hormone. Fortunately, the very extensive analysis of AKH in a large number of insect species has only revealed the presence of AKH-related peptides in those cells (9) for which the Drosophila genome codes only a single peptide.

The classical function of AKH and its homologs is to stimulate the liberation of energy substrates from the fat body into the hemolymph, whether it is lipid, trehalose, or even proline in some insects (37). The liberation of AKH is thus most easily demonstrated when demand on energy substrates is high, such as during flight. Many studies have therefore focused on the role of AKH during flight. However, it has previously been pointed out that AKH is not only present during the larval stages (29) but also physiologically important at that stage, notably during starvation of caterpillars, when AKH is needed to maintain hemolymph trehalose to a minimum concentration (39). It is thus known that the requirement for AKH is important when demands on energy substrates are higher than absorption from the digestive tract can supply, such as during flight and starvation. However, we show here that ablation of the AKH-producing cells significantly reduces trehalose levels, even in fed insects, both in third instar larvae and in adult males. Although this reduction is much more pronounced in males than in females (indeed in females it does not attain statistical significance), it suggests that the role of AKH is not merely restricted to increasing hemolymph trehalose or the closely related crustacean red pigment concentrating hormone (38). Third, some, but not all, insect species have two or three different AKH whose functions are likely to be different, since they have slightly different physiological effects (e.g., in Manduca sexta: controls fat body glycogen phosphorylase in starving larvae, whereas, in adults, it controls lipid mobilization during flight; see Ref. 22). These peculiarities have largely contributed to the fact that the AKH are the best-studied insect neuropeptides, yet several aspects of their functions remain poorly understood. It is plausible that the powerful genetic tools available in Drosophila will be helpful to resolve some of these questions.

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Fig. 5. Locomotor activity. A: histograms of the total distance moved (in mm) during the 12 h of recording. Values shown are means ± SE. Control males and females traveled about the same distance (~320,000 mm), suggesting a similar level in locomotor activity, whereas AKH-ablated flies, both males and females, present a lower level of locomotor activity (~225,000 mm) compared with control flies. Number of recorded flies are indicated in bars. B: time course of the distance moved for the 4 groups of flies presented in A. Curves represent means ± SE of the distance moved for each successive 10-min period for the 12-h recording. M, males; F, females; Cont, control flies; AKH−, heterozygous AKH-ablated flies.

Fig. 6. Survival during starvation of control and AKH-ablated flies. The survivorship curve (expressed in percentage) of the control flies [M-cont: AKH/CS (n = 143) and F-cont: AKH/CS (n = 143)] and AKH cell-ablated male or female flies [M-AKH− (n = 134) and F-AKH− (n = 84)] under starvation. Flies are kept under starvation in a room at 24°C and 60% humidity, and with access to water, to avoid dehydration. AKH-ablated fly survival was analyzed by the Kaplan-Meier method. Starvation survival was significantly increased in both male and female AKH-ablated flies when compared with the control group.
lose when energy demands are high but also to maintaining hemolymph trehalose to a minimum concentration, even when energy demands are low.

Even though we have demonstrated both in larvae and in adults a decrease in trehalose, it remained to be seen whether this decrease has any physiological consequences. Trehalose is synthesized by the fat body from glucose, which has either been absorbed from the gut or is produced from fat body glycogen; after stimulation of glycogen phosphorylase by AKH, it subsequently diffuses passively in the hemolymph. Trehalose is thus present in both the hemolymph and the intracellular fat body space. In flies, the total compartment containing trehalose is ~30% volume/body wt (34). Thus we can estimate from the observed total quantities of trehalose an approximate hemolymph trehalose concentration of 2.5 mM for flies from which the AKH cells have been ablated vs. 5 mM for control flies. The $K_m$ for insect trehalase, the key enzyme permitting trehalose utilization, is ~1–2 mM (e.g., see Ref. 32). This suggests that, even when hemolymph trehalose is maintained at 2.5 mM instead of 5 mM, Drosophila (both larvae and adults) should still be able to utilize trehalose. Indeed, during flight in the migratory locust, hemolymph trehalose falls to ~2.5 mM, or one-third its preflight concentration, but at the same time the trehalose turnover rate is increased fivefold (12). Although one would expect that trehalose turnover rates in AKH-ablated flies are probably decreased, this example does show that low hemolymph concentrations of trehalose do not mean that trehalose cannot be used as an energy substrate. Because determination of trehalose turnover rates in insects as small as Drosophila is not practical, we looked for other ways to demonstrate that the absence of the secretion of AKH interfered with its basic physiology.

It was reported that AKH peptide causes cardioacceleration in a number of insects (9) and in Drosophila prepupa (21). However, in AKH-less Drosophila prepupa, a normal rate of heart beat was found. This is probably not surprising, since the concentrations of AKH needed to do so are much higher than those normally needed to activate glycogen phosphorylase; hence, the effects are likely to be pharmacological rather than physiological.

The locomotor activity of the AKH-ablated flies presents two main differences compared with control flies: first, for the first 4 h, both male and female AKH-ablated flies present a higher level of locomotor activity. Second, after ~4 h, the AKH-ablated flies decrease their activity rather than increase it, as control flies do. Locomotor activity is a complex behavioral trait that may reflect the motivational state of the fly for walking to find a sexual partner, food, and/or a place to oviposit. Differences between the motivation of the sexes, males looking for females, females looking for protein food sources to feed on and to oviposit in, are likely responsible for the observed differences between the sexes in locomotion observed in both control and AKH-ablated flies, whereas differences between normal and AKH-ablated flies should be because of the absence of AKH. For about the first 2 h of the experiment, males decrease their locomotor activity from an initially high level. In females, locomotor activity starts at a lower level than in males but then gradually increases. This first phase corresponds to a reactivity phase (Ref. 17 and Martin J-R, unpublished observation) that could be interpreted as a stress response, since the fly has been manipulated when put in the apparatus, which is a novel environment. It is tempting to speculate that the increase in locomotor activity seen after ~1 h for female and 2 h for male control flies reflects an increase in food-searching behavior, since they are emptying their guts and get hungry. If so, it would appear that the flies with AKH cells ablated are already “hungry” before being analyzed for locomotor behavior. Flies have no access to the food while their locomotor activity is analyzed, and, consequently, they gradually starve as time goes by. In both control and AKH cell-ablated flies, trehalose decreases during the experiment (Fig. 3), but the decrease in control flies is limited while the decrease in AKH-ablated flies is much larger. In AKH-ablated flies, AKH cannot activate trehalose synthesis from fat body glycogen; trehalose that is still being synthesized by these flies must be generated by other mechanisms, either because other hormones, such as e.g., octopamine (33, 22), activate fat body glycogen phosphorylase or because the key enzymes in the synthesis of trehalose are stimulated directly, e.g., by low trehalose concentrations. Once starvation starts, less and less glucose will be absorbed from the gut; consequently, less glucose will be available for fat body trehalose synthetase, and trehalose will gradually decrease to a level that precludes normal locomotor activity. What is not clear is whether this decrease in locomotor activity is a direct physiological effect of low hemolymph trehalose, i.e., the flies literally don’t have the energy to do so, or whether this is a behavioral adaptation to the low hemolymph trehalose concentrations. As discussed above, trehalose hemolymph concentrations in AKH-ablated flies, although significantly lower than in control flies, should be sufficient to allow a normal trehalose utilization. This seems to suggest that the lower activity observed in AKH-ablated flies may well be an indirect behavioral adaptation. Such a behavioral adaptation might well explain why the AKH-ablated flies survived ~24 h longer than the control wild-type flies under starvation conditions.

NOTE ADDED IN PROOF

During the review process, another group published closely related data on AKH using similar techniques and arriving at the same major conclusions (G. Lee and J. H. Park. Genetics 167: 311–323, 2004).

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