Hemolymph Sugar Homeostasis and Starvation-Induced Hyperactivity Affected by Genetic Manipulations of the Adipokinetic Hormone-Encoding Gene in *Drosophila melanogaster*

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ABSTRACT

Adipokinetic hormones (AKHs) are metabolic neuropeptides, mediating mobilization of energy substrates from the fat body in many insects. In delving into the roles of the Drosophila *Akh* (*dAkh*) gene, its developmental expression patterns were examined and the physiological functions of the AKH-producing neurons were investigated using animals devoid of AKH neurons and ones with ectopically expressing *dAkh*. The *dAkh* gene is expressed exclusively in the corpora cardiaca from late embryos to adult stages. Projections emanating from the AKH neurons indicated that AKH has multiple target tissues as follows: the prothoracic gland and aorta in the larva and the crop and brain in the adult. Studies using transgenic manipulations of the *dAkh* gene demonstrated that AKH induced both hypertrehalosemia and hyperlipemia. Starved wild-type flies displayed prolonged hyperactivity prior to death; this novel behavioral pattern could be associated with food-searching activities in response to starvation. In contrast, flies devoid of AKH neurons not only lacked this type of hyperactivity, but also displayed strong resistance to starvation-induced death. From these findings, we propose another role for AKH in the regulation of starvation-induced foraging behavior.

HOMEOSTATIC regulation of blood sugar levels is a fundamental physiological process in both vertebrates and invertebrates. Failure to do so causes serious health problems such as diabetes in humans. In mammals, two important endocrine hormones, glucagon and insulin, are key physiological effectors that regulate blood glucose levels. These peptide hormones are synthesized by the endocrine glands in the pancreas and released into the bloodstream in response to internal changes in sugar levels. In target tissues, such as the liver, these pancreatic hormones activate opposing metabolic pathways (*e.g.*, glycogen breakdown by glucagon and glycogen synthesis by insulin), thereby maintaining steady-state glucose levels.

Fundamental endocrine regulations of homeostatic blood sugar levels are also conserved in insects. For instance, an insulin-related peptide, bombyxin, lowers hemolymph sugar concentrations in a dose-dependent manner in the silkworm *Bombyx mori* (Satake *et al.* 1997). Recently, genes encoding Drosophila insulin-like peptides (*dilp*) have been identified (Brogiolo *et al.* 2001), and transgenic ablation of *dilp*-producing neurons results in the elevation of total blood sugar (Rulifson *et al.* 2002).

Insects also produce peptide hormones that act as functional homologs of vertebrate glucagons (Van der Horst *et al.* 2001). Injection of the peptides into cockroaches elevates levels of hemolymph trehalose, a nonreducing disaccharide that is one of the major blood sugar molecules in insects (Bedford 1977). Thus the glucagon-like peptide in insects is referred to as *h*yper*t*rehalosemic *h*ormone (HTH). However, injection of this peptide into locusts elicits both carbohydrate and lipid mobilization from the fat body, leading to the alternative name *a*dipo*k*inetic *h*ormone (AKH). These peptide hormones form the largest neuropeptide family in arthropods, including >30 isoforms identified in >80 species encompassing all major insect phyla and several crustacean species (Gäde *et al.* 1997).

Like other neuropeptides, AKHs are multifunctional. Other known physiological effects observed for this substance include cardioacceleration in cockroaches (*e.g.*, Keeley *et al.* 1991) and migration of tegumentary and retinal distal pigments in crustaceans (Garfias *et al.* 1995; Porras *et al.* 2001). AKH also induces transcription of the cytochrome *P450* gene in the fat body of cockroaches (Bradfield *et al.* 1991) and expression of a gene encoding fatty acid binding protein in the flight muscle of locusts (Chen and Haunerland 1994). In addition, AKH peptides have excitatory effects on motor neurons in moths (Milde *et al.* 1995) and enhance amplitudes of the electroretinogram in the crayfish (Garfias *et al.* 1995).

Despite the physiological studies just described, biological functions of the AKH-encoding gene are unknown, in part due to the lack of genetic variants involv-

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ing this substance. Drosophila AKH peptide and its encoding gene (*dAkh*) sequences have been previously reported (Schaffer *et al.* 1990; Noyes *et al.* 1995). To gain insight into *in vivo* roles of AKH in Drosophila, we examined anatomical details of AKH-expressing (AKHergic) neurons in various developmental stages. We then carried out targeted ablation to obtain AKH-*c*ell-*d*eficient (AKH-CD) flies and ectopic *dAkh* expression, followed by analyses of physiological and behavioral phenotypes resulting from these transgenic manipulations. The results show that AKH functions as a metabolic stimulator causing both hypertrehalosemia and hyperlipemia. Our data also suggest that AKH is involved in the regulation of starvation-induced locomotor activities, and such roles are likely to be associated with AKH's metabolic roles to maximize the likelihood of the fly's survival when foods are scarce.

MATERIALS AND METHODS

Fly strains and genetic crosses: Flies were maintained at 25° in light:dark (12-hr:12-hr) cycles on yeast-cornmeal-agar media. Canton-S was used as the wild type and *yellow white* (*y w*) as a genetic control for some experiments. For the visualization of GAL4-expressing cells, *gal4* driver strains were crossed to a reporter transgenic strain such as UAS-*lacZ* or UAS-*NZ*, which encodes cytoplasmic or nuclear β-galactosidase, respectively (Phelps and Brand 1998), or UAS-mCD8-*gfp*, which encodes a membrane-bound derivative of green fluorescent protein (GFP; Lee and Luo 1999). A UAS-*reaper* (*rpr*) was used for cell-specific ablation and UAS-*p35* was used for the suppression of *rpr*-mediated cell death (*e.g.*, Renn *et al.* 1999). To evaluate the absence of cells resulting from *rpr*-induced apoptosis, a double transgenic line containing both UAS-*rpr* and UAS-*lacZ* (hereafter, UAS-*rpr:lacZ*) was crossed to a *dAkh-gal4* driver, and *dAkh*-expressing cells in the progeny were monitored by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) histochemistry. To rescue *rpr*-induced cell death, flies doubly homozygous for the *dAkh-gal4* and UAS-*p35* transgenes were generated by recombination and crossed to the UAS-*lacZ* or UAS-*rpr:lacZ*. Ectopic *dAkh* expression in larval fat body was accomplished by crossing an r-tetramer (r^4)-*gal4* driver (see below) to a UAS-*dAkh*. Newly generated *dAkh-gal4*, r^4-*gal4*, and UAS-*dAkh* transgenic lines are described in the following section.

Generation of transgenic fly strains: In constructing the *dAkh* promoter fused to the yeast transcription factor *gal4* gene, forward (5′-GCTCTAGAACACGCGTCGACTGAGCTT-3′) and reverse (5′-GCGGTACCTGAGTTCTATGCTGGTCCAC-3′) PCR primers were designed to encompass the 5′ upstream region extending from −1020 to +17 (+1 indicates the transcription-start site, which was previously determined by Noyes *et al.* 1995), on the basis of the nucleotide sequences flanking the *dAkh* gene (Berkeley Drosophila Genome Project; AC005814). The PCR product was subcloned into *Xba*I/*Kpn*I sites of the pPTGAL vector (Sharma *et al.* 2002). The resulting recombinant vector was injected into *y w* embryos, using standard methods for *P*-element-mediated germ-line transformation, leading to the establishment of the following three lines: *dAkh-gal4, dAkh-gal4(2),* and *dAkh-gal4(3).* A homozygous viable *dAkh-gal4* line, which has an insertion in the third chromosome, was used primarily in this study. The other two homozygous lethal strains (each of which carries an insertion in the second chromosome) were used for some experiments.

For construction of r^4-*gal4* vector, a *Spe*I/*Xba*I fragment containing r^4 was excised from a pUC/r^4 vector (An and Wensink 1995) and subcloned at an *Xba*I site of pPTGAL. The *cis*-acting "r" element has been previously known to play a role in yolk-protein gene expression in adult flies (An and Wensink 1995). Since our data showed that r^4-*gal4* (an insertion in the third chromosome) directed reporter gene expression specifically in the fat body of both sexes, this driver was employed to effect fat body-targeted ectopic *dAkh* expression. The UAS-*dAkh* vector was constructed by subcloning *dAkh* cDNA (see below) into *Not*I/*Xho*I sites of the pUAST vector (Phelps and Brand 1998). A homozygous viable UAS-*dAkh* line (an insertion in the second chromosome) was used in this study.

Whole-mount *in situ* hybridization: Since the transcription termination site of the *dAkh* gene is unknown, we performed 3′-rapid amplification of cDNA ends (RACE), using gene-specific primers in essentially the same manner as described in Park and Hall (1998). As a result, we identified a consensus polyadenylation signal (AATAAA) at an appropriate position (17-bp upstream of the 3′-end), validating our 3′-RACE experiment (not shown). On the basis of the sequence data, reverse transcription-PCR was carried out to obtain the full-length *dAkh* cDNA. The PCR product was subcloned into the pGEM-T vector (Promega, Madison, WI), from which a *Spe*I/*Eco*RI fragment was reinserted into the pBluescript (Stratagene, La Jolla, CA). Subsequently, digoxigenin (dig)-labeled antisense *dAkh* riboprobes were generated by *in vitro* transcription, using the Genius-4 RNA labeling kit (Roche, Indianapolis), and applied for whole-mount *in situ* hybridizations as described in Lee *et al.* (2000).

Histochemistry: To detect *in situ lacZ* expression, tissues were dissected, fixed, and stained with X-gal (*e.g.*, Park *et al.* 2000). After the desired staining was achieved, tissues were washed in PBS, dehydrated, and mounted on a glass slide with a mounting medium (1:6 mixture of methyl salycylate and permount). For the visualization of GFP expression, dissected tissues were fixed in 4% paraformaldehyde for 15 min on ice, washed six times with PBS, and mounted with glycerol. Signals were viewed under an Olympus BX61 microscope equipped with a CC12 digital camera and images were acquired using analySIS 3.0 software (Soft Imaging System). To detect AKH peptides in tissues, anti-AKH antibody was generated in rabbits, using keyhole limpet hemocyanin-conjugated synthetic AKH peptide as an antigen (Genemed Synthesis, San Francisco). Tissues were incubated with diluted primary antiserum (1:600) and subsequently with FITC-tagged secondary antibodies (1:200; Jackson Immunoresearch Labs, West Grove, PA), and the resulting fluorescence signals were processed as described above. To visualize lipid droplets stored in the fat cells, fat bodies were dissected from wandering third instar larvae, fixed with 4% paraformaldehyde for 10 min, rinsed in PBS, and processed for Sudan Black-mediated staining (Zinke *et al.* 1999).

Starvation-induced mortality assay: Eclosed flies were aged in a group for 7–14 days. For starvation tube preparation, glass tubes (6 × 50 mm; Fisher Scientific, Pittsburgh) were filled up to one-third with 0.5% agarose. To synchronize starvation initiation for all genotypes tested, 40–60 flies of each genotype were transferred simultaneously into a vial containing 0.5% agarose. Subsequently, each starvation tube was loaded with a single anesthetized fly, cotton plugged, and then placed in a humidified chamber in a 25° incubator supplied with 12 hr:12 hr light:dark cycles. Dead flies were counted every 6 or 12 hr and the survival rate for each genotype was plotted.

Circadian locomotor activity behavior assays: For the circadian locomotor activity assay, individual flies were loaded into a glass tube containing 4% sucrose in 2% Bactoagar medium, entrained for 3–7 days to 12-hr:12-hr light:dark (LD) cycles,

and then assessed for free-running locomotion for the next 7 days in constant darkness (DD) at 25°. For the locomotor activity assay under starvation, glass tubes containing 2% agarose only were used. The activities were monitored using a Trikinetics system interfaced with a PC computer, as described by Konopka *et al.* (1994). Numbers of activity events were recorded every half-hour (bin), and average numbers of activity events per bin per fly were calculated using Microsoft Excel software. Chi-square periodogram analysis (Sokolove and Bushell 1978) was performed using a web-derived software (http://hawk.bcm.tmc.edu/) to detect periodicity.

Sugar and lipid measurements: A group of 6–12 third instar larvae were washed twice with dH_2O and blot dried. The hemolymph was allowed to bleed out on a dimple glass plate by tearing off the cuticle; 2 μl of hemolymph was rapidly withdrawn, mixed with 38 μl of PBS, and then centrifuged for 10 min at 13,000 × *g* to precipitate blood cells and tissue debris. The supernatant was transferred to a fresh tube and an aliquot (2 μl) was applied for trehalose or glucose assays using a commercial kit (17-25; Sigma, St. Louis) as described in Rulifson *et al.* (2002).

Fat body triglyceride and hemolymph glycerol contents were assayed using a commercial kit (337-40A and 337-10B; Sigma). In principle, samples are pretreated with lipase to hydrolyze triglycerides, resulting in the production of free fatty acids and glycerol, and the latter is detected enzymatically using triglyceride reagent. Fat tissues obtained from a group of six wandering third instar larvae were suspended in 150 μl of 0.1% Tween 20 on ice, homogenized, and heated at 70° for 5 min to inactivate endogenous enzymes. After a brief vortexing, the homogenate (10 μl) was incubated with an equal volume of lipase reagent at 37° overnight. The sample was centrifuged at 13,000 × *g* for 10 min and the supernatant (10 μl) was incubated with triglyceride reagent (100 μl) at 37° for 2 hr. Once the reaction was completed, absorbance was measured at 540 nm. To assess glycerol levels, larval hemolymph was diluted 1:5 in PBS, heated at 70° for 5 min, and centrifuged at 13,000 × *g* for 10 min. The supernatant (5 μl) was assayed for the determination of glycerol as just described for triglycerides. The experiments were repeated at least twice. For statistical analysis, a Tukey-Kramer multiple comparisons test was performed using InStat software (v.2.0; GraphPad Software).

RESULTS

Corpora cardiaca-specific expression of the *dAkh* gene: The larval ring gland is an important endocrine organ in the cyclorraphous Diptera, consisting of the corpus allatum (CA), prothoracic gland, and corpora cardiaca (CC; Bodenstein 1950). Previously, the *dAkh* gene was cloned and shown to produce mRNA exclusively in the CC of third-instar larvae (Noyes *et al.* 1995); however, *dAkh*-expressing tissue types beyond this juvenile stage have not been examined.

Prior to the examination of *dAkh* expression patterns in adult flies, we performed whole-mount *in situ* hybridizations on third-instar larval tissues to validate our new antisense *dAkh* riboprobe. In agreement with previous results, the probe produced specific signals exclusively in the CC (Figure 1A, $n = 20$). Using this probe, we extended our assay to adult tissues. During metamorphosis, the ring gland migrates posteriorly and finally attaches to the esophagus, just anterior to the cardia (or proventriculus) in the adult thorax (Dai and Gilbert 1991). We detected strong and unique *in situ* hybridization signals in a tiny structure located at this position (Figure 1B, $n = 14$). The results suggest that the CC-specific *dAkh* expression pattern remains unchanged during metamorphosis.

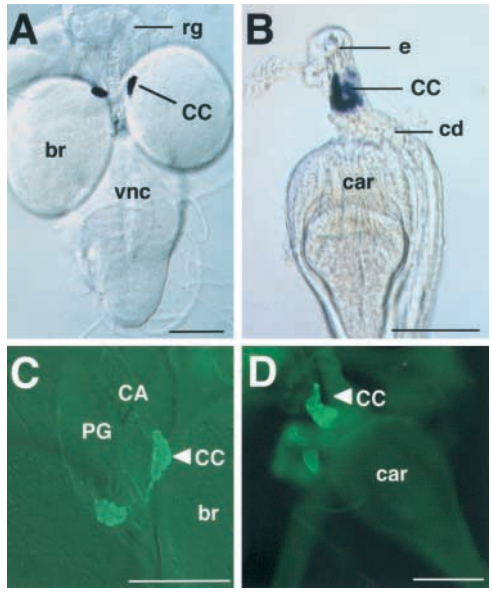

Figure 1.—Corpora cardiaca (CC)-specific expression of *dAkh* gene products. (A and B) *In situ* localizations of *dAkh* mRNA. The *dAkh* gene is transcribed exclusively in the CC of third instar larvae (A) and adults (B). (C and D) Whole-mount AKH immunohistochemistry. AKH-immunoreactive cells are indicated by arrowheads. AKH peptides are detectable only in the CC of larvae (C) and adults (D). br, brain; vnc, ventral nerve cord; e, esophagus; car, cardia; rg, ring gland; PG, prothoracic gland; CA, corpus allatum; cd, crop duct. Bar, 100 μm.

To determine whether AKH peptides are actually synthesized in the CC cells, we performed whole-mount immunohistochemistry using anti-AKH antibodies (see materials and methods). Consistent with *dAkh* mRNA expression patterns, AKH-immunoreactive signals were limited to the CC of both larvae (Figure 1C, $n = 15$) and adults (Figure 1D, $n = 14$), suggesting that intrinsic neurosecretory cells in the CC actively produce AKH peptides during both juvenile and adult stages. Essentially identical expression patterns obtained by both techniques also verify the specificity of our new antibody to the AKH peptides. From the results, we conclude that the CC is the only tissue type expressing the *dAkh* gene in *Drosophila melanogaster*.

Definition of the *dAkh* promoter: To define a regulatory region responsible for CC-specific *dAkh* expression, we generated three independent fly lines bearing the *dAkh-gal4* transgene (Figure 2A). The *dAkh-gal4* flies were crossed to a UAS-*lacZ* reporter line and the progeny were processed for X-gal histochemistry. As seen in the *in situ* hybridization and immunohistochemistry results, β-galactosidase (β-gal) activity was detected only

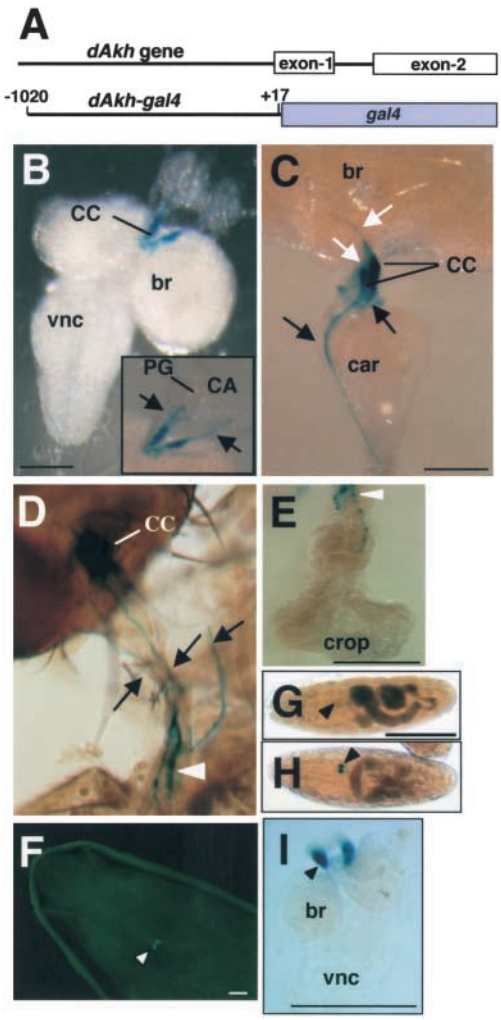

Figure 2.—Reporter gene expression mediated by *dAkh* promoter. (A) Schematics of *dAkh* gene structure and *dAkh-gal4* construct. (B–I) X-gal histochemistry or *gfp* expression of the offspring of [*dAkh-gal4* × UAS-*lacZ* or UAS-mCD8-*gfp*] cross. (B) Third instar larval central nervous system. *LacZ* expression is restricted to the CC. Processes innervating the prothoracic gland (PG) are indicated by arrows. (C–E) *LacZ* expression in adults. (C) Somata of adult AKHergic neurons are present exclusively in the CC. Projections to the brain are denoted by white arrows and ones to the crop by black arrows. (D) Projections arising from the posterior side of the CC (arrows). A white arrowhead designates a projection leading to the crop. (E) Nerve terminals at the crop duct indicated by an arrowhead. (F) Expression of *gfp* in a "live" third instar larval progeny from a [*dAkh-gal4* × UAS-mCD8-*gfp*] cross. Specific GFP-mediated fluorescence signals are clearly visible in a paired structure (arrowhead) at a position where the CC locate. (G and H) *LacZ* expression (arrowheads) during embryonic development. The expression (arrowhead) was first seen in stage-14 embryos (G) and became stronger in older embryos (stage 15; H). (I) CC-specific *lacZ* expression (arrowhead) in first instar larva. Bars, 100 μm.

in the CC of larvae (Figure 2B, $n = 25$) and adults (Figure 2C, $n = 16$). Identical expression patterns were obtained from all three *dAkh-gal4* lines. Lack of ectopic expression sites directed by this promoter was further confirmed by *dAkh-gal4*-driven *gfp* expression in "live" larvae (Figure 2F, $n = 7$). The results suggest that *cis*-acting regulatory elements necessary for CC-specific *dAkh* expression are present within the 1-kb upstream sequence.

Using the *dAkh-gal4*/UAS-*lacZ* system, we determined the earliest developmental stage of *dAkh* expression. The β-gal activity was at first faint in a paired structure in approximately stage-14 embryos (Figure 2G) and then became stronger in older embryos (Figure 2H). CC-specific expression was also observed in first-instar larvae (Figure 2I, $n = 7$); however, projections from the CC neurons were undetectable (Figure 2B *vs.* 2I), suggesting that the *dAkh* neurons in first-instar larvae have not yet been fully differentiated. Nevertheless, the overall results suggest that normal *dAkh* gene functions might be necessary from late embryonic stages onward.

Anatomy and targets of *dAkh*-expressing neurons: Little is known about neuro-anatomical details of the intrinsic neurosecretory cells in the CC of Drosophila. Since *dAkh* gene products could serve as a useful marker for such cells, we further examined characteristics of these cells in great detail, using various transgenic manipulations and histochemical assays. First, in determining the number of *dAkh*-expressing cells, *dAkh-gal4* flies were crossed to a UAS-*NZ* reporter to express β-gal in the nuclei of *dAkh* cells (Phelps and Brand 1998). As a result, we identified ~7 (±0.1, SEM) cells per each lobe of larval CC (Figure 3A, $n = 57$). For adult CC, the total number of *dAkh* cells was counted from a whole CC structure instead of per each lobe, since the boundary between lobes was not clearly recognizable, thus hampering precise counting. This yielded an average of 13 (±0.6, SEM) cells per CC ($n = 32$), ranging from 11 to 16 (Figure 3B). Since the counts in an adult CC are comparable to those observed in an entire larval CC, *dAkh* cells might be present persistently during metamorphosis.

To determine the population of *dAkh* cells in the CC, we simultaneously marked somata of *dAkh* neurons by *dAkh* promoter-driven *gfp* expression and nuclei of entire CC cells by 4′,6-diamidino-2-phenylindole (DAPI) staining. A majority of the DAPI-positive cells expressed *gfp* (Figure 3, C and D), suggesting that *dAkh* cells represent most of the CC cells.

Stainings mediated by anti-AKH antibody and X-gal histochemistry were examined at higher resolution to construct a fine neural mapping involving the AKHergic neurons. In larvae, we detected two potential targets innervated by AKHergic neurons, one of which is the prothoracic gland located immediately adjacent to the CC and known to produce a molting hormone ecdysteroid (*e.g.*, Dai and Gilbert 1991). The AKHergic neurons sent two or three projections to this structure (Figures 2B and 3F). The other target is the aorta (or dorsal vessel) that is closely associated with the CC. Extensive AKH-immunoreactive varicosities observed on the aorta

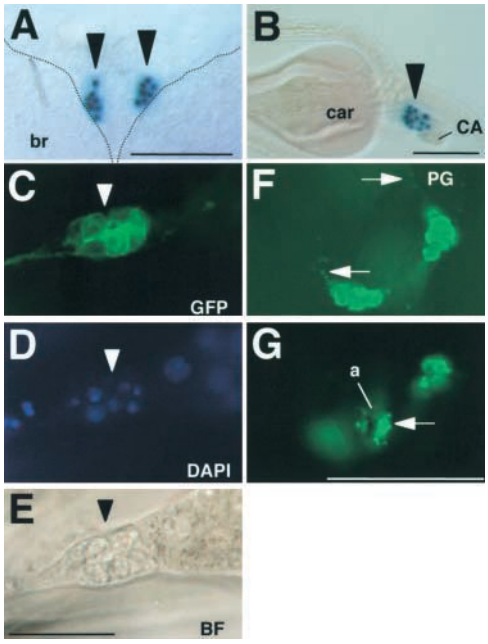

Figure 3.—Cellular characteristics of AKHergic neurons. (A and B) Dissected ring glands from the progeny of a [*dAkh-gal4* × UAS-*NZ*] cross were processed for X-gal histochemistry. Each nuclear *lacZ* expression (arrowheads) represents individual neurons in third instar larval (A) and adult CC (B). Dotted lines in A contour the brain. Bars, 100 μm. (C and D) The CC of larval offspring from a [*dAkh-gal4* × UAS-mCD8-*gfp*] cross were stained by DAPI, and the fluorescence signals were captured for either *gfp* (C) or DAPI (D). Note that most of the CC cells are AKHergic neurons. (E) A bright-field image of the same specimen shown in C and D. Bar, 25 μm. (F and G) AKH-immunoreactive projections in larval CC. Images were captured from the same specimen at different focal plans to show projections (arrows) innervating the prothoracic gland (PG) and the aorta (a). Bar, 50 μm.

indicate that AKHs are released into the circulatory system (Figure 3G).

Although it is not as clear as in larval CC, adult CC also form a bilobed structure and the *dAkh* neurons are present in both lobes (Figure 1, B and D, Figure 2C). Processes stemming from the anterior side of the lobes were traced proximate to the esophagus foramen where they are likely to enter the protocerebrum. A pair of long processes arising from the posterior side reached the crop duct at which the crop begins its expansion (Figure 2, C–E). In some insects, such as honeybees and blow flies, the crop stores liquid foods (*e.g.*, nectar or soluble nutrients), and its volume is highly variable depending on the amounts of liquid deposit (Dimitriadis and Papamanoli 1992; Lorenz *et al.* 2001b). AKH-homologous peptides have been proposed to cause regurgitation of nectars from the crop in some wasp species to increase hemolymph trehalose titers (Lorenz *et al.* 2001b). In this regard, our findings of AKH nerve terminals at the crop duct support the idea that AKH may control the crop volume in Drosophila. In addition to the brain and the crop, we occasionally observed a process whose target could not be identified (Figure 2D). Nonetheless, this implies additional physiological functions attributed to AKH in adult flies.

Targeted ablation of AKHergic neurons: To understand AKH functions in Drosophila, we obtained AKH-cell-deficient (AKH-CD) flies by expressing a pre-apoptotic gene, *reaper* (*rpr*; White *et al.* 1996), in the AKHergic neurons. Ectopic expression of *rpr* in various peptidergic neurons has been successfully employed to trigger the apoptosis of these neurons (McNabb *et al.* 1997; Renn *et al.* 1999; Park *et al.* 2003). The AKH-CD flies were generated by crossing *dAkh-gal4* flies to a UAS-*rpr* or a double transgenic UAS-*rpr:lacZ* strain, and their progeny were examined histologically to confirm the absence of the AKHergic neurons. The UAS-*rpr:lacZ* line was useful particularly for checking ablation status, since the presence or absence of target neurons could be easily judged by simple X-gal staining (Figure 4A *vs.* 4B). Indeed, we were unable to detect any β-gal activity in the CC of the *dAkh-gal4*/UAS-*rpr:lacZ* larvae and adults, which therefore indicates a complete loss of *dAkh* neurons. This was further confirmed by the lack of AKH immunoreactivities (Figure 4B). The *rpr*-mediated cell death was rescued partially by coexpression of the anti-apoptotic protein *p35* (Hay *et al.* 1994), since fewer numbers of X-gal-stained cells or less intensive AKH immunosignals were observed in the CC of *p35*-rescued animals compared with such stainings in the control animals (Figure 4C *vs.* 4A).

The AKHergic neurons apparently do not play a vital role, since AKH-CD animals developed in an ostensibly normal manner. No noticeable defects in growth, metamorphosis, eclosion, and longevity were observed. Adult AKH-CD flies also showed normal reproductive capabilities and courtship behavior (data not shown). The results suggest that AKH functions are not essential for overall development and reproduction at normal growth conditions.

Carbohydrate metabolism is affected by the ablation of AKHergic neurons and ectopic *dAkh* expression: Trehalose is a disaccharide composed of two glucose molecules and is the principal blood sugar in insects (Bedford 1977). Physiological studies in other insects have shown that AKHs elevate hemolymph trehalose titers at the expense of glycogen storage in the fat body (*e.g.*, Park and Keeley 1995). This prompted us to examine whether AKH also plays a role in the regulation of carbohydrate metabolism in Drosophila.

Hemolymph trehalose levels in AKH-CD larvae were a mere 7–26% of normal, whereas the glucose levels were unaffected (Figure 5, A and B). Moreover, the trehalose titers in *p35*-rescued larvae were intermediate between controls and AKH-CD (Figure 5B), thus revealing a positive correlation between the levels of *dAkh* expression and hemolymph trehalose concentrations. The results suggest that the AKH neurons produce a

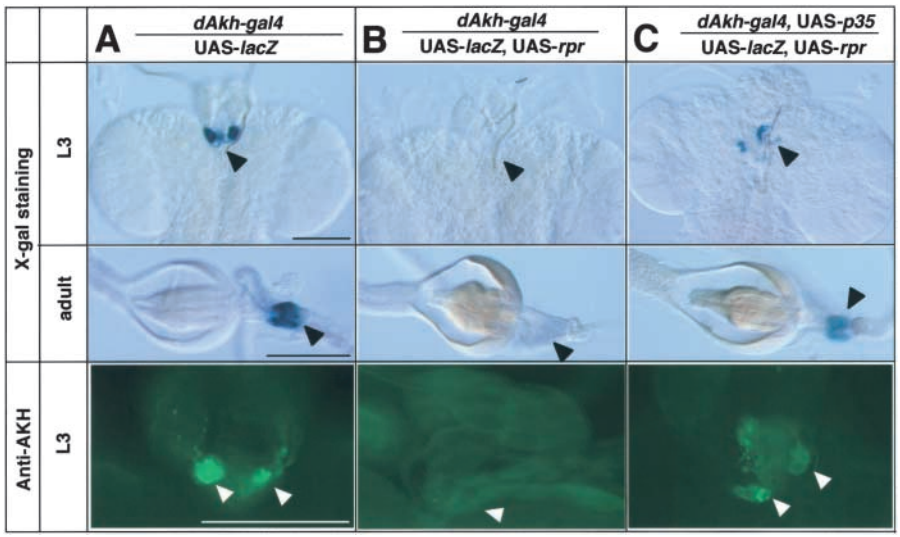

Figure 4.—Transgenic ablation of AKHergic neurons. The AKHergic neurons (arrowheads) were visualized by X-gal histochemistry and anti-AKH immunohistochemistry in the CC of third instar larvae (L3) or adults. (A) Control (*dAkh-gal4* × UAS-*lacZ*). (B) *Reaper* (*rpr*)-induced apoptosis of AKHergic neurons was confirmed by the lack of *lacZ* expression and AKH immunoreactivity (arrowheads). (C) Incomplete rescue of AKHergic neurons by coexpression of *p35*. Bar, 100 μm.

hypertrehalosemic factor essential for normal carbohydrate metabolism.

Despite the results, it was still uncertain whether subnormal trehalose levels observed in AKH-CD are due to the lack of AKH or other coexisting hypertrehalosemic factor(s). Thus, we examined the effects of overexpression and misexpression of the *dAkh* gene on trehalose titers. If AKH is the principal effector for hypertrehalosemia, then increasing AKH production in such transgenically modified animals should elevate trehalose concentrations in the hemolymph.

Overexpression of *dAkh* in the native neurons was accomplished by crossing *dAkh-gal4* flies to a UAS-*dAkh*; however, the overexpression did not alter the trehalose levels (Figure 5C). This is perhaps because *dAkh* expression levels are not proportional to the amounts of AKH peptide released; thus, circulating AKH levels in UAS-*dAkh*/+; *dAkh-gal4*/+ animals may approximate those in wild type. In support of this, Harthoorn *et al.* (2001) showed the absence of coupling between release and biosynthesis of AKH peptides in locust CC. The lack of phenotypic effect by overexpression of a given neuropeptide gene in its usual location is not unprecedented. For instance, overexpression of the *Pdf* gene—which encodes a principal circadian clock output factor (Park 2002)—in pacemaker neurons that normally contain PDF peptides did not affect circadian rhythmicity (Helfrich-Förster *et al.* 2000).

As an alternative tactic, we misexpressed *dAkh* in the fat body, using a fat body-specific GAL4 driver (r^4-*gal4*) that directed strong and constitutive expression of a reporter gene in the fat body in a sex-nonspecific manner from late embryo to adult stages (Figure 5E). We reasoned that expression of AKH in its target tissue could be the most effective way of activating AKH-dependent metabolism. Since adipose tissues are an important endocrine organ, producing several bioactive peptides in mammals and growth factors in flies (Britton and Edgar 1998; Bradley *et al.* 2001), we speculate that AKH precursors encoded by *dAkh* in the fat body undergo appropriate processing, thereby producing functional AKH peptides.

Ectopic expression of *dAkh* (AKH-EE) in the fat body was accomplished by crossing the r^4-*gal4* to a UAS-*dAkh*. Overall developmental processes were not interfered with by the misexpression of *dAkh*. Production of AKH peptides in the fat body was verified by AKH immunofluorescence. Although wild-type fat bodies do not produce AKH, the peptides bound to fat body receptors could misguide our interpretation of the origin of AKH immunosignals. To avoid this, the fat body from AKH-CD larvae was employed as control tissue ($n = 9$). As shown in Figure 5F, AKH-CD fat bodies gave rise to background signals originating from endogenous autofluorogenic materials in this tissue (Rizki 1961). By comparison, immunofluorescent signals detected in AKH-EE fat bodies ($n = 10$) were considerably greater (~1.5-fold) than those in AKH-CD (Figure 5G), thus verifying that AKH is indeed overproduced by this type of transgenic modification.

Next, attempts were made to determine whether hemolymph trehalose levels are altered in AKH-EE. Consistent with AKH's suggested role as a hypertrehalosemic effector, significant elevation of trehalose levels (~34% above normal) was observed in AKH-EE larvae (Figure 5D). Such hypertrehalosemic response to AKH-EE is unlikely due to an ectopic overexpression artifact, since the trehalose titers were unchanged by ectopic expression of another neuropeptide *Pdf* gene in the fat body (data not shown).

As summarized in Figure 5H, hemolymph trehalose titers are nicely correlated with the levels of *dAkh* expression affected by various transgenic modifications. The data thus strongly suggest that AKH plays a major role in the regulation of carbohydrate metabolism in Drosophila. However, there must be AKH-independent path-

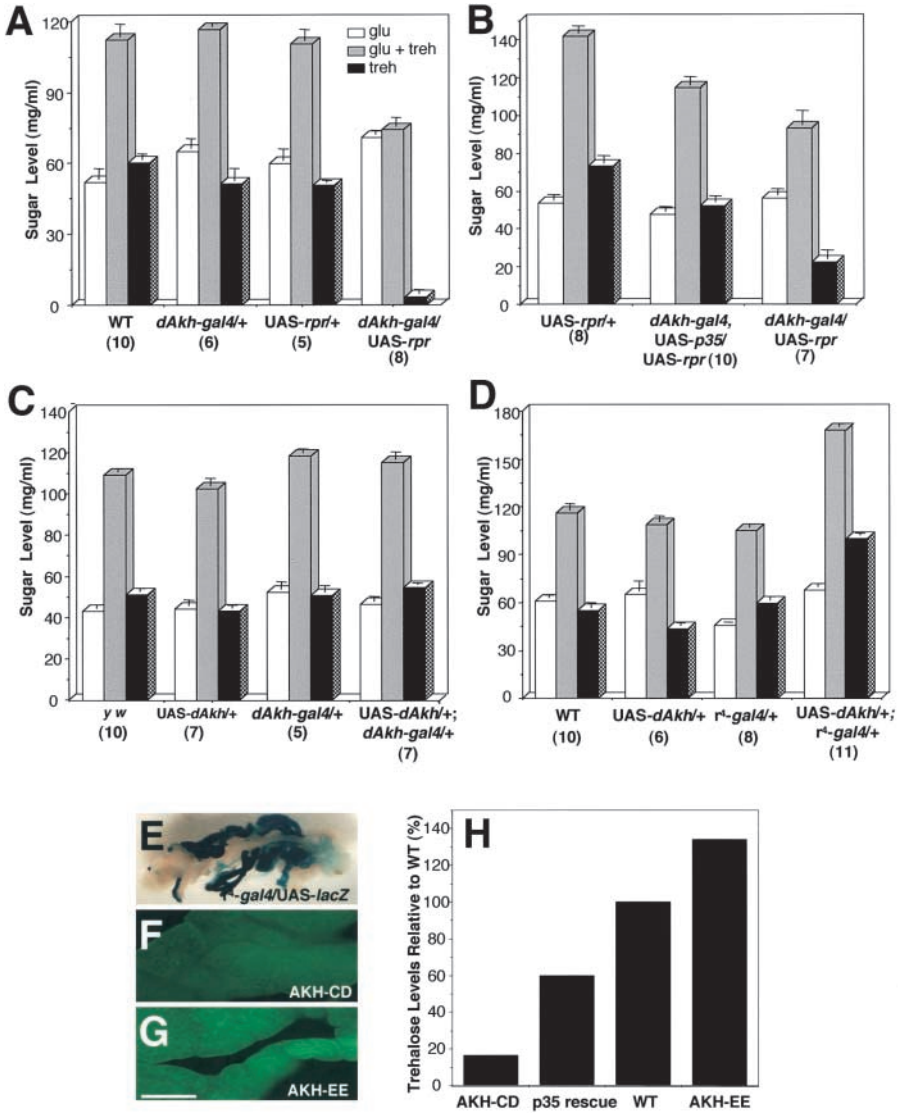

FIGURE 5.—Effect of altered *dAkh* expression on larval hemolymph sugar levels. Numbers in parentheses indicate the number of samples tested for each genotype. (A) Ablation of AKHergic neurons (*dAkh-gal4*/UAS-*rpr*) caused significant reduction in trehalose levels. (B) Partial rescue of *rpr*-mediated ablation by *p35* (*dAkh-gal4*, UAS-*p35*/UAS-*rpr*) incompletely restored trehalose titers. (C) Hemolymph trehalose concentrations were unchanged by overexpression of *dAkh* in AKHergic neurons (UAS-*dAkh*/+; *dAkh-gal4*/+), whereas (D) ~34% increase was triggered by ectopic *dAkh* expression in the fat body (UAS-*dAkh*/+; r^4-*gal4*/+). (E) Fat body-specific *lacZ* expression driven by r^4-*gal4* in larvae. (F and G) AKH immunohistochemistry of the fat body. (F) Control, nonspecific autofluorescence in AKH-CD fat body. (G) AKH immunofluorescence in AKH-EE fat body. Note that the signals shown here are much stronger than those in AKH-CD. (H) A summary for the trehalose phenotypes affected by various transgenic manipulations of the *dAkh* gene.

ways for this type of physiological reaction, since detectable amounts of trehalose are still present in animals devoid of AKHergic neurons (Figure 5, A and B).

Lipid metabolism induced by ectopic expression of *dAkh*: Another well-documented physiological AKH function is to mobilize lipid storage from the fat body via lipase activation; the resulting metabolites serve as energy substrates in locusts for long-term flight (STONE *et al.* 1976; VAN DER HORST *et al.* 1979; VAN HEUSDEN *et al.* 1984; CHINO *et al.* 1989) and in tobacco hornworms during periods of starvation (ZIEGLER *et al.* 1990; ZIEGLER 1991; ARRESE and WELLS 1997).

The fat body of Drosophila also stores large amounts of lipids, which are consumed rapidly upon starvation (ZINKE *et al.* 1999; BRITTON *et al.* 2002). However, it is unknown whether lipid metabolism is regulated by AKH in Drosophila. To address this question, lipid droplets stored in the fat cells were visually examined in well-fed AKH-EE and control larvae by using the Sudan Black staining method (for example, ZINKE *et al.* 1999). The lipid droplets were substantially smaller and fewer in the AKH-EE than in the controls (Figure 6, A–C, *vs.* 6D). Consistent with the results, a quantitative assay also revealed a significant decrease of triglyceride content—the main storage form of lipids in the fat body—in AKH-EE (Figure 6E).

Reduction of endogenous triglyceride levels in AKH-EE fat bodies could be a consequence of either subnormal synthesis or supernormal degradation (hydrolysis) of the triglycerides. If the latter is the case, one can expect an increase of metabolites derived from the hydrolysis of triglycerides (*i.e.*, free fatty acids and glycerol) in the serum of AKH-EE. In accordance with the prediction, hemolymph glycerol concentrations were significantly higher in AKH-EE than in controls (Figure 6F), thus supporting that reduction of triglyceride contents in the AKH-EE fat body is due to an enhanced lipolytic response to AKH.

If AKH is the sole effector for the hydrolysis of triglycerides, then complete suppression of lipolysis in AKH-

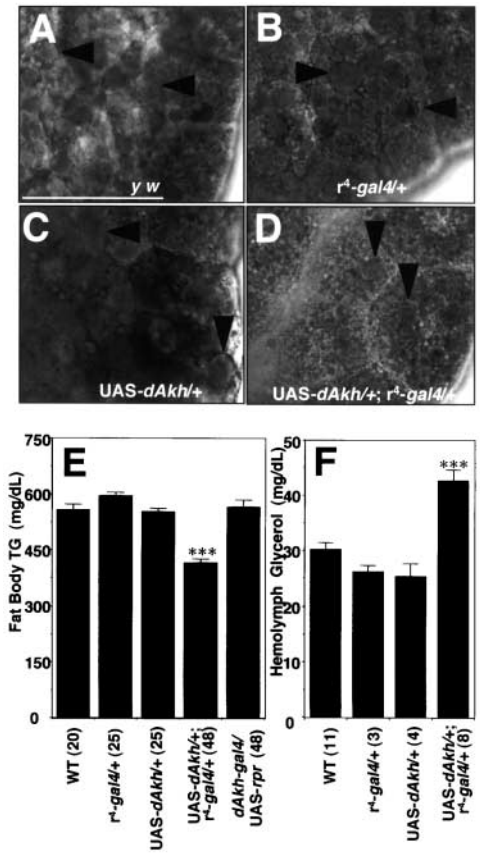

FIGURE 6.—Effect of AKH-EE on lipid contents within larval fat cells. Lipid droplets were stained by Sudan Black. The sizes and amounts of the droplets (arrowheads) are significantly reduced in AKH-EE (D) compared to those of genetic controls (A–C). Bar, 50 μm. (E and F) Quantitative measurements of triglyceride (TG) content in the fat body (E) or glycerol in the hemolymph (F). Numbers in parentheses designate the numbers of samples tested. (E) Significant reduction of TG or (F) elevation of glycerol is observed in AKH-EE (UAS-*dAkh*/+; r^4-*gal4*/+). Asterisks indicate statistical significance ($P < 0.001$).

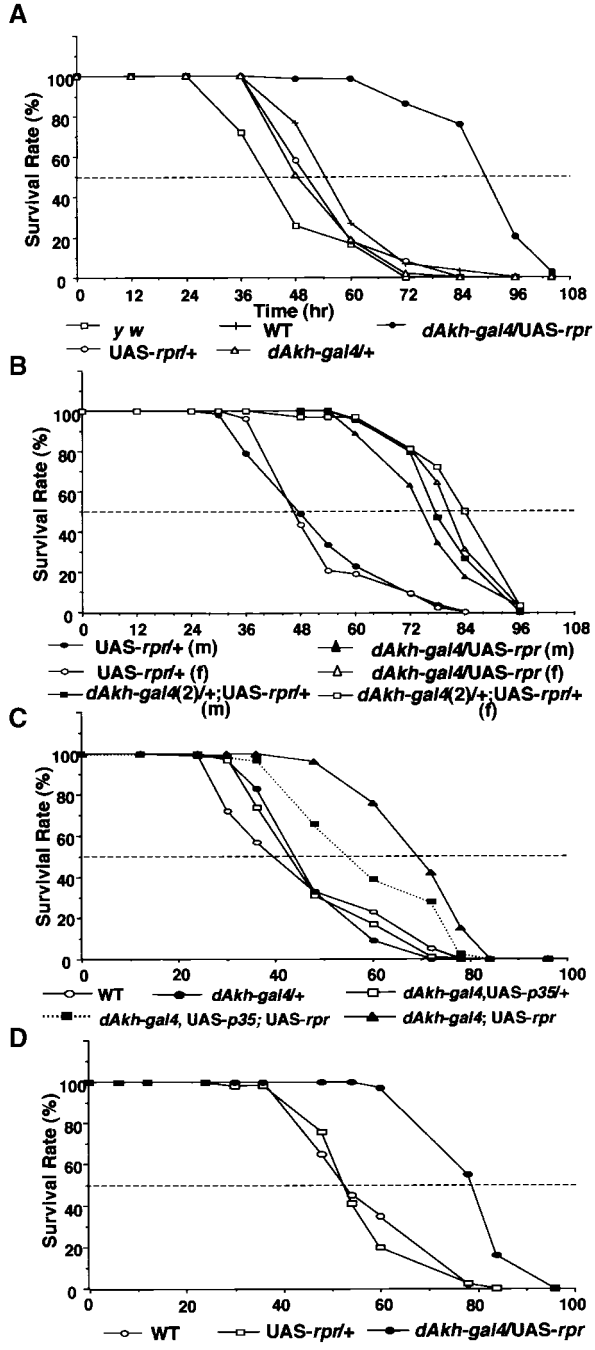

FIGURE 7.—Survival curves in response to starvation. (A) At 48 hr after starvation initiation, ~50% of control flies died, whereas most of the AKH-CD flies (*dAkh-gal4*/UAS-*rpr*) were still alive. (B) Both male (m) and female (f) AKH-CD flies display similar levels of tolerance to starvation. (C) Rescue of AKH-CD by coexpression of *p35* (*dAkh-gal4,* UAS-*p35*/UAS-*rpr*) shows intermediate levels of resistance to starvation. (D) Degrees of resistance to starvation-induced death are indistinguishable between young (<30 hr after eclosion) and old AKH-CD flies. At least 40 flies were tested for each genotype or sex.

CD would increase triglyceride storage in AKH-CD fat body. Our data, however, showed that fat body triglyceride contents in AKH-CD were comparable to those of controls (Figure 6E). The results indicate that lipid metabolism occurs normally in the absence of AKH, thus foretelling the existence of alternative lipolytic pathways that are independent of AKH.

AKH-CD flies are resistant to starvation-induced death: Since animals have to survive on nutrients stored in the body when foods are not available, slower catabolism of such limited resources would help them to survive longer. In this context, AKH-CD flies are expected to live longer than wild type, as the foregoing results indicate that catabolic activities are appreciably attenuated in AKH-CD. To test the hypothesis, mortalities of AKH-CD and control flies, when supplied only with water, were monitored.

Remarkably, AKH-CD flies survived for at least 24 hr longer than wild type or any other genetic controls (Figure 7). Resistance to starvation-induced death was consistently observed for all *dAkh-gal4* transgenic lines regardless of gender (Figure 7B). Of importance, the

survival rate of *p35*-rescued flies was intermediate between those of controls and AKH-CD (Figure 7C). This nicely correlates with *dAkh* expression levels in the *p35*-rescued flies that are also intermediate between normal and fully ablated (Figure 4C). The data suggest that degrees of resistance to the starvation-induced death are most likely AKH-dose dependent.

Extended longevity of AKH-CD flies under starvation may be due to their abnormal feeding habits (for instance, more frequent feeding or a larger amount of food intake per meal) in response to the reduction of blood sugar levels, resulting in a larger amount of nutrients taken in by AKH-CD flies than by wild types. If so, then young flies have less time to feed than the older flies do, thereby storing relatively low energy reserves. As a consequence, young AKH-CD flies could be more sensitive to starvation than older AKH-CD flies. We tested this hypothesis by assessing the phenotype of very young flies (the majority of whom were younger than 30 hr after eclosion). Survival rates displayed by young AKH-CD flies (Figure 7D) were similar to those of older flies, suggesting that the feeding anomaly (proposed above) may not be an influential factor for the phenotype exhibited by Drosophila ablated of their AKH cells.

Clock-independent locomotor activity patterns upon starvation: Recent studies show that locomotor activities of honeybees and wasps are unable to be sustained in the absence of available energy substrates (Lorenz *et al.* 2001a,b). The studies led us to propose that subnormal levels of energy substrates observed in AKH-CD may affect motility of these animals. To test this hypothesis, we monitored the flies' circadian locomotor activities, using an infrared emitter-detector system as described previously (Konopka *et al.* 1994).

First we measured daily locomotor activities of wild-type and AKH-CD flies fed on 4% sucrose-agar medium. Under 12-hr:12-hr LD conditions, wild type showed typical bimodal activity peaks, one at dawn and the other at dusk; in subsequent DD conditions, robust circadian rhythmicity was sustained (Figure 8). Quite similar rhythmic activity patterns were observed in AKH-CD flies, suggesting that normal functions of AKH are not involved in clock-controlled locomotor activity rhythms.

We extended our studies to detect any differences in locomotor activities between starved and fed wild-type flies or between wild-type and AKH-CD in the absence of food. In doing so, flies were provided with water only in a form of agarose block. Under this assay condition, the nonfeeding wild-type flies were persistently active at Zeitgeber times (in LD cycles) while feeding flies were normally quiescent (Figure 9A). Most of the starved flies died after the onset of accentuated locomotion. Although the durations and amplitudes of such hyperactivities varied individually, this type of behavioral pattern was observed in >90% of hungry wild-type flies ($n = 80$) and other genetic controls [*y w* ($n = 40$), UAS-*rpr*/+ ($n = 36$), and *dAkh-gal4*/+ ($n = 30$)]. The hunger-driven prolonged hyperactivity may reflect avid (even desperate) search for food.

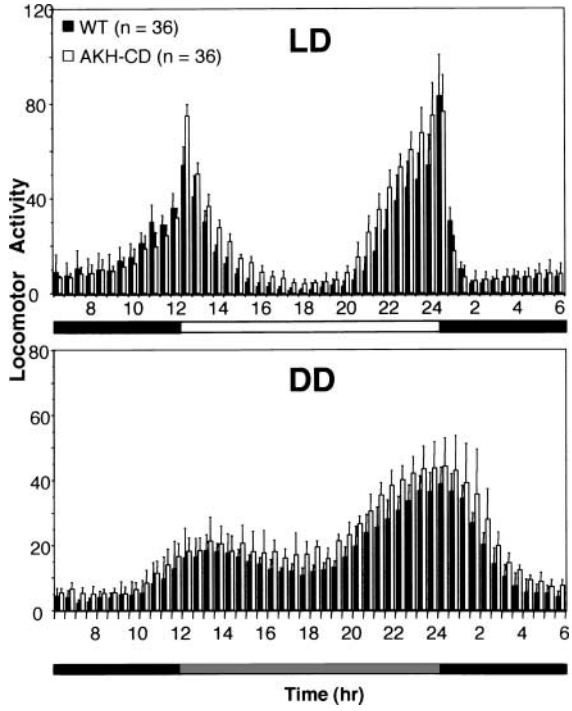

Figure 8.—Circadian locomotor activity rhythms of fed flies. Flies were entrained to four cycles of 12 hr:12 hr light:dark (LD) and then proceeded to 7 days of constant darkness (DD). Bars represent average numbers of activity events per half-hour bin for the number of flies tested. Horizontal open and solid boxes indicate the light-on and light-off phases, respectively, and the shaded box denotes subjective day under DD condition. Numbers on the *x*-axis are Zeitgeber (ZT) times (light is on at ZT-0 and off at ZT-12). Chi-square periodogram analysis revealed free-running rhythms with the following periodicities: 24.0 ± 0.5 (mean ± SEM) for wild-type and 24.2 ± 0.5 for AKH-CD flies. Most of flies tested in each group were rhythmic.

Intriguingly, the majority of AKH-CD flies ($n = 45$) did not show pronounced starvation-induced hyperactivity (Figure 9B), suggesting a role for AKH in the regulation of this novel phenotype. Lack of hyperactivity in starved AKH-CD flies is unlikely due to their general weakness, since they are as robust as wild type when food is ample (Figure 8). Instead, this could be a consequence of lower levels of energy substrates in the hemolymph of AKH-CD. If this is true, then higher levels of energy substrates in the hemolymph of AKH-EE may cause them to be excessively hyperactive. However, starvation-dependent activity patterns of AKH-EE were not much different from those of the control (data not shown), indicating that the fat body's metabolic activity may not be a causative factor for the accentuated locomotive behavior. Perhaps neural inputs from the AKH neurons (Figure 2C) play a role in the starvation-induced behavioral change.

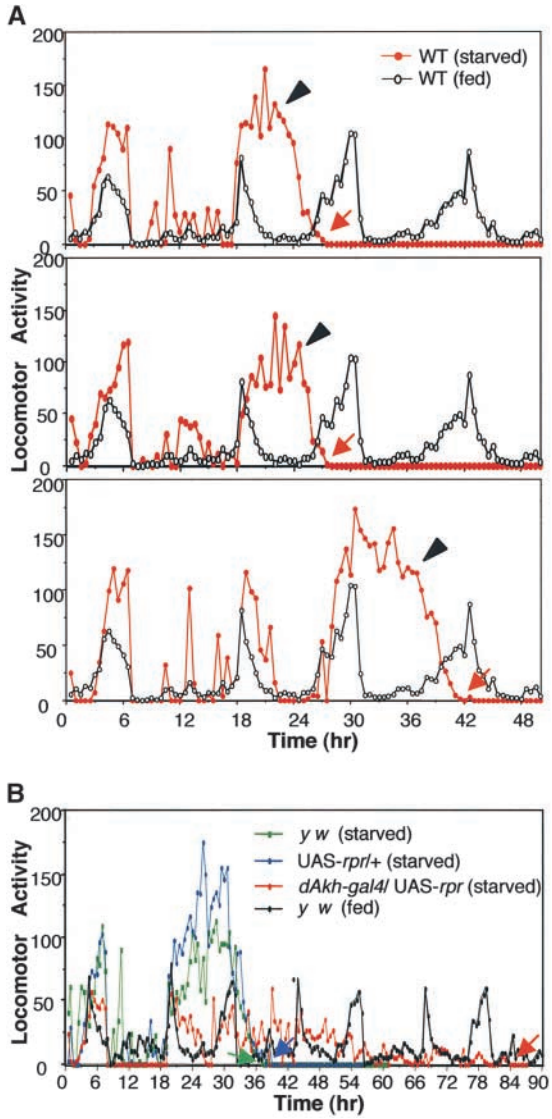

FIGURE 9.—(A) Three representative locomotor activity patterns of starved wild-type (WT) flies. In contrast to rhythmic activities displayed by fed flies, clock-independent prolonged hyperactivities (arrowheads) are evident in hungry flies prior to starvation-mediated death (arrows). (B) Similar hyperactive behaviors are observed in other genetic controls (*y w* or UAS-*rpr*/+), whereas this type of behavior is suppressed in the starved AKH-CD flies. Arrows indicate time points of immobilization as in A.

DISCUSSION

Active food acquisition and storage and utilization of energy substrates are critical for an animal's survival. Recent studies in mammals suggest that neuro-peptidergic signals from the hypothalamic-pituitary system controlled by the brain play pivotal roles in the regulation of energy metabolism and behavioral aspects of feeding (RAYNER and TRAYHURN 2001; MATTSON 2002). The pars intercerebralis (PI)-CC complex in insects has been considered as the functional counterpart of the vertebrate hypothalamic-pituitary system (VEELAERT *et al.* 1998). To date, AKH is the only known neuropeptide expressed in the intrinsic neurosecretory cells of the CC in many insect species including *D. melanogaster* (NOYES *et al.* 1995; DIEDEREN *et al.* 2002). Currently our studies have explored developmental regulation of Drosophila *Akh* gene expression, its essential roles in energy metabolism, and function associated with starvation-induced feeding behavior.

AKHergic neurons: Unlike in other insect species, larval CC of Drosophila and other cyclorraphous dipterans are fused to other endocrine glands, forming a ring-like structure called the ring gland (BODENSTEIN 1950). Using *dAkh* as a marker gene for the CC, we summarize detailed neuro-anatomical aspects of the CC in Drosophila. First, most (if not all) of the CC cells are AKH-positive; therefore, characteristics of AKHergic neurons represent overall morphology of the CC at least in larvae. There are ~7 AKHergic cells in each larval CC lobe and 13 such cells in the entire adult CC. The latter count (of adult AKHergic cells) agrees with the electron microscopic observation, which estimated ~12 intrinsic cells in the CC of Drosophila adults (AGGARWAL and KING 1971). Second, the adult CC also form bilobed structure (analogous to the larval version of this organ). The lobes are closely associated with each other, so that they often appear to be a single mass of tissue. Third, larval AKHergic neurons send projections into the aorta, where AKH is likely to be released into the circulatory system to reach distantly located target tissues (*e.g.*, fat body). In addition, we found projections invading the prothoracic gland, which is the source of a molting hormone ecdysteroid (DAI and GILBERT 1991). Thus, it is tempting to speculate that AKH has a role in metamorphosing processes. However, since AKH-CD larvae and pupae molted in a normal fashion, we currently do not know the neurological roles of the projections just described. Fourth, adult AKHergic neurons project to the brain and the crop. These potential targets are likely to be associated with metabolism/feeding-related roles of AKH, as discussed below.

Roles of Drosophila AKH in energy metabolism: It has been well documented that members of the AKH family play a pivotal role in the stimulation of intermediary metabolism in the fat body of various insects (VAN DER HORST *et al.* 2001). For instance, in locusts, AKH-mediated lipid and carbohydrate mobilizations from the fat body provide energy substrates for the flight muscles. In the horse fly (*Tabanus atratus*), injection of AKH causes hyperlipemia but not hypertrehalosemia (JAFFE *et al.* 1989), and in the blow fly (*Phormia terraenovae*), it causes hypertrehalosemia, but not the other (GÄDE *et al.* 1990). By comparison, our genetic data show that AKH induces both hyperlipemia and hypertrehalosemia in the fruit fly (*D. melanogaster*). Perhaps the fruit flies may need (as do locusts) a combination of carbohydrates and lipids as energy sources for a variety of energy-requiring conditions such as starvation, flight, and other locomotor activities.

Insect AKH is apparently a functional homolog of verte-

brate glucagon. Recently, Drosophila insulin-like peptide (*dilp*) has been shown to produce a physiological activity opposite to AKH with respect to carbohydrate metabolism (Rulifson *et al.* 2002). These studies combined with our results suggest that hormonal regulatory mechanisms for homeostatic carbohydrate metabolism are conserved between Drosophila and vertebrates. Of interest, nerve fibers from the *dilp* neurons project to the AKHergic neurons, implicating intercellular interactions between these cell types. If in fact this is true, it will be interesting to determine whether these peptidergic neurons regulate each other, so that only one type of peptide is dominantly produced under a certain physiological circumstance. Exploiting cellular and molecular mechanisms involved in sensing hemolymph sugar titers is another avenue of inquiry prompted by the results we now present.

Although AKH-mediated carbohydrate metabolism in the fat body is the principal cause of hyperglycemia in some insects, studies done in hymenopteran insects have proposed another mechanism of hyperglycemia caused by this peptide. Lorenz *et al.* (2001a) reported that workers of bumblebees, honeybees, and *Vespula vulgaris* store most carbohydrates in the crop and essentially lack fat body storage of carbohydrates. Despite this, injection of AKH into well-fed animals (whose crops were presumably full) still elicited significant hyperglycemia, whereas no such effect was found in the animals with empty crops (Lorenz *et al.* 2001b). The results suggest that the crop is a principal carbohydrate storage organ in certain insects and that AKH induces hyperglycemia perhaps by stimulating crop-emptying activity. In line with this, potential innervation of the crop by AKHergic neurons indicates that the crop could be another source of AKH-dependent hyperglycemia in Drosophila. AKH may modulate crop muscle contractions, squeezing out sugar-containing fluid into the midgut from which sugar molecules are transported into the hemocoel through the gut epithelium.

Implications of AKH function for coordinating adult feeding behavior: When foods are abundant, wild-type flies show robust daily activity-rest rhythms that are governed by a circadian pacemaker system (Hall 2003). However, we are the first to demonstrate that the clock system fails to control normal rhythmicity when animals are stressed by adverse environmental conditions. Prolonged hyperactivities displayed by starved wild-type flies prior to death could be a desperate attempt to acquire food that would be the key to their survival. Food is not always available in nature; thus, this kind of accentuated locomotion, regardless of the time of day, might be an important behavioral component for the survival of hungry animals. This theory is supported by evidence that food availability is an important environmental factor that controls animals' circadian behavior (reviewed in Stephan 2002).

Intuitively, persistent hyperactive behavior may augment the likelihood of starvation-induced death, since this would facilitate rapid consumption of energy resources. Conversely, suppression of such behavior may help animals to survive longer during periods of starvation. This is what we observed in AKH-CD flies, which not only lacked hyperactive locomotion, but also survived ~24 hr longer than wild type under starvation condition. Assuming that average life spans for humans and flies, under normal living conditions, are 70 years and 45 days, respectively (Ashburner 1989), 24 hr of fly life is equivalent to ~570 days of that in humans. By comparison, timings of starvation-induced death of AKH-EE flies did not deviate from those of wild type, perhaps because AKH-EE flies displayed wild-type-like hyperactivity patterns (data not shown). From these data, we speculate that prolonged hyperactive locomotion is causally associated with starvation-induced lethality.

On the basis of our findings, we propose that AKH acts in two ways to regulate separate phenotypes in Drosophila; in one way, AKH stimulates intermediary metabolism in the fat body, leading to hypertrehalosemia and hyperlipemia. In the other way, AKH may carry out a central function involving hyperactive behavior in response to starvation. Apparently the central brain controls the fly's locomotor activities, because lack of pacemaker neurons or "behavioral output factor" (PDF peptide) normally possessed by such cells disrupts circadian activity rhythms (Renn *et al.* 1999; Park 2002). The fact that no motor neurons in the brain are responsible for locomotion implies the presence of a complex neural network that controls the fly's general locomotion. AKHergic neuronal projections entering the brain may be a part of the network. Evidence from studies in other insects supports the central role of AKH for locomotion; for instance, injection of AKH into the mesothoracic neuropile elicits marked motor response in a moth (Milde *et al.* 1995). Nevertheless, central functions of AKH seem to be complementary to its hormonal roles, since AKH-mediated prolonged hyperactivities (central role) are likely to be supported by AKH-dependent fat body metabolism (hormonal role). Therefore, such multidirectional AKH functions maximize the fly's best chances for survival particularly when the food source is limited.

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