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A glucagon-like endocrine pathway in *Drosophila* modulates both lipid and carbohydrate homeostasis

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SUMMARY

The regulation of energy homeostasis is fundamental to all organisms. The *Drosophila* fat body serves as a repository for both triglycerides and glycogen, combining the energy storage functions of mammalian adipose and hepatic tissues, respectively. Here we show that mutation of the *Drosophila* adipokinetic hormone receptor (AKHR), a functional analog of the mammalian glucagon receptor, leads to abnormal accumulation of both lipid and carbohydrate. As a consequence of their obese phenotypes, AKHR mutants are markedly starvation resistant. We show that AKHR is expressed in the fat body, and, intriguingly, in a subset of gustatory neurons that mediate sweet taste. Genetic rescue experiments establish that the metabolic phenotypes arise exclusively from the fat body AKHR expression. Behavioral experiments demonstrate that AKHR mutants are neither sedentary nor hyperphagic, suggesting the metabolic abnormalities derive from a genetic propensity to retain energy stores. Taken together, our results indicate that a single endocrine pathway contributes to both lipid and carbohydrate catabolism in the *Drosophila* fat body.

Key words: AKHR, fat body, obesity.

INTRODUCTION

The study of *Drosophila* metabolism is an emerging field that can contribute greatly to the understanding of conserved mechanisms that regulate carbohydrate and lipid homeostasis. Indeed, recent reviews highlight the remarkable parallels between metabolic pathways in *Drosophila* and mammals (Baker and Thummel, 2007; Leopold and Perrimon, 2007). Furthermore, the powerful genetic tools available in *Drosophila* research make the fly a particularly tractable model organism in which to probe metabolic pathways regulating energy balance. For example, the *Drosophila* fat body, the major depot for carbohydrate and lipid stores, is amenable to genetic manipulation specifically in either larval or adult stages (e.g. see Lazareva et al., 2007). Thus, it is becoming increasingly apparent that the powerful tools of *Drosophila* genetics can be a gateway for a better understanding of human metabolic disorders.

The molecular mechanisms by which humans and flies regulate the storage and release of fuel molecules display remarkable parallels. For example, *Drosophila* insulin-like peptides (dILPs) have a profound effect on growth and energy homeostasis, recapitulating the role of the mammalian insulin pathway (Rulifson et al., 2002). dILPs bind to a single receptor (InR) and signal through downstream effectors that are homologous to mammalian counterparts (Garofalo, 2002; Geminard et al., 2006; Goberdhan and Wilson, 2003; Lasko, 2002; Wu and Brown, 2006). The adipokinetic hormone (AKH) family of peptides is thought to play a key role in catabolism in a variety of insect species (Van der Horst, 2003). In *Drosophila*, AKH is secreted by a small group of specialized neuroendocrine cells, ablation of which results in a profound decrease in circulating carbohydrate levels (Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004). The AKH pathway has been proposed to be the functional analog of the mammalian glucagon receptor. Strikingly, the molecular mechanisms by which AKH- and dILP-secreting cells regulate carbohydrate homeostasis are similar to those employed by insulin- and glucagon-secreting pancreatic islet cells.

The fat body is the primary energy storage tissue in *Drosophila* (Canavoso et al., 2001). Glycogen and triglyceride comprise the major forms of energy storage for carbohydrate and lipids, respectively. In insects, enzymatic pathways that mediate both the synthesis and breakdown of glycogen have clear homology to those found in mammals (Orgad et al., 1987). Thus, the study of fly mutants with alterations in lipid accumulation in the fat body raises the intriguing possibility that they will provide insight into genetic determinants of human obesity and energy homeostasis (Kulkarni and Perrimon, 2005; Murphy and Bloom, 2006). In mammals, the energy storage function of the fat body is performed in separate tissues (such as liver and adipose). Because the fat body plays a major role in both carbohydrate and lipid storage, research in *Drosophila* could also illuminate the interplay between these two major arms of metabolism within a single tissue.

Although several mutants of the *Drosophila* insulin pathway have been studied, no mutants in the AKH pathway existed upon initiation of the current work. Genetic manipulation of the AKH pathway had been limited to cell ablation studies of AKH-producing corpora cardiaca cells (Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004) and ectopic expression of AKH in the fat body (Lee and Park, 2004). Biochemical studies have shown that AKH binds with high affinity to its G-protein-coupled receptor (AKHR) (Park et al., 2002; Staubli et al., 2002). Furthermore, activation of

AKHR activates many of the same second messenger pathways as the mammalian glucagon receptor (such as production of cAMP) (Gade and Auerswald, 2003; Unson, 2002). We envisioned that further analysis of the glucagon-like AKH pathway in *Drosophila* could yield general insights into the regulation of energy homeostasis by endocrine systems. To this end, we generated mutations in *Akhr* and assessed their effects on metabolism, starvation resistance, locomotor activity and feeding behavior. While this work was being prepared for publication, an independent study of *Akhr* was published (Gronke et al., 2007).

MATERIALS AND METHODS

Fly stocks

Akhr-Gal4 transgenic *Drosophila melanogaster* Meigen were generated using standard P-element-mediated germline transformation with a 2.8 kb DNA fragment (bounded by the adjacent CG11188 gene and the *Akhr* transcription start site; forward primer: gaccgcttacgcaaagtttg and reverse primer: gtatctgaatgcaactgcatc) cloned into pCasper4-AUG-Gal4. *Akhr*–red fluorescent protein (RFP) transgenic flies were prepared by cloning the aforementioned promoter fragment into a previously reported RFP-containing plasmid (Jones et al., 2007). *Akhr* cDNA (isoform PA as annotated in FlyBase) was obtained using EST GH19447 (Bloomington) as a PCR template (forward primer: cttgtcccaaaaaatggc and reverse primer: ttacttctggcggatcgg). The resulting *Akhr* cDNA fragment was cloned into pUAST to generate the UAS-*Akhr* plasmid that was used to generate transgenic flies. A P-element excision screen was performed starting from $Akhr^{p}$ ($y^{1}w^{67c23}$;P{EPgy2}$GRHR^{EY11371}$; Bloomington stock no. 20304) and a transposase containing stock (y^{1}w*; CyO, H{w[+mC]=PDelta2-3}HoP2.1/Bc^{1}; Bloomington stock no. 2078). $Akhr^{rev}$ and $Akhr^{null}$ flies were isolated by initially screening for white-eyed flies and then further characterized by PCR using primers flanking the P-element insertion and ATG transcription start sites. The $Akhr^{rev}$ flies contain only a 17 bp remnant (catgttatttcatcatg) at the original P-element (EY11371) insertion site and an otherwise wild-type gene scaffold. The $Akhr^{null}$ flies contain an ~450 bp remnant of the excised P element fused to genomic sequence 144 bp downstream of the original *Akhr* gene locus, with removal of all intervening coding *Akhr* sequence, including the transcription start site. The P-element remnant in $Akhr^{null}$ flies is fused to the genomic region downstream of *Akhr* commencing with the following sequence: tttgaattgatatgcg. The coding sequences of the flanking genes (*Tsp* and CG1118) were intact. The flies were raised on standard medium (yeast–agar–molasses) with 12 h:12 h light:dark cycle at 25°C. The fat body r^{4}-Gal4 line (Lee and Park, 2004) was obtained from J. Park (University of Tennessee, Knoxville, TN, USA) and gustatory neuron-Gal4 lines (Wang et al., 2004) were obtained from K. Scott (University of California, Berkeley, CA, USA).

Genetic rescue experiments

Flies containing a UAS-*Akhr* insert on the third chromosome were made homozygous null for *Akhr*, which is on the second chromosome. Similarly, third chromosome inserts of fat body-Gal4 or neuronal-Gal4 stocks were established in an $Akhr^{null}$ background. The Gal4 transgenic stocks used in these experiments are described in this paper (i.e. *Akhr*-Gal4) and have been previously published (see references above); we independently confirmed the expression pattern for all published stocks. Rescue stocks were derived by crossing the just described UAS-*Akhr* and tissue-specific Gal4 stocks, allowing selective re-introduction of *Akhr* (in an otherwise null background).

RT-PCR

Total RNA of a given genotype was isolated from 50 frozen flies (1-week-old males) according to a modified solid-phase Qiagen (Valencia, CA, USA) RNeasy Mini Kit (cat. no. 74104) protocol (DGRC, CGB Technical Report 2006-10). Fat body-derived RNA was isolated using the preceding reagents from tissue pooled from approximately 20 wandering third instar larvae dissected in cold PBS. A 5 g aliquot of total RNA was subsequently treated with DNAse I and cDNA was prepared using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was performed with primers to exon 5 (forward: ggactctacaacattcgc) and exon 6 (reverse: cctcttccattcagcagc) of the *Akhr* gene.

Immunostaining

Fly brains were dissected and stained using previously described procedures (Nern et al., 2005); images were captured using a Zeiss LSM 510 confocal microscope. Mouse polyclonal AKHR antibodies were generated by the ASU CIM Antibody Core (Tempe, AZ, USA).

Nile Red staining

Fat body cells were visualized using Nile Red staining, according to a published protocol (Gronke et al., 2005).

Starvation and locomotor assay

Male flies were individually placed in *Drosophila* Activity Monitoring System (DAMS; Trikinetics, Waltham, MA, USA) testing chambers (previously capped with 2% agarose at one end). The flies were grown on a 12 h:12 h light:dark cycle at 25°C; locomotor data were collected in the dark, also at 25°C. Data were exported into Excel and average total locomotor activity (as measured by the total number of recorded midline crossings) and starvation resistance were calculated for each line (N=16 per genotype). Starvation resistance was defined as the time of death after initiation of food deprivation, estimated as the time of last recorded midline crossing for a given fly.

Body size measurements

Digital images of adult wings were obtained at constant magnification. Using the 'magic lasso' tool in PhotoShop, the area (pixel count) of each wing that was bounded by L4, L5, the anterior cross vein and the lateral wing border was measured. Higher magnification images were obtained for wing cell size measurements. A constant square area of each wing was highlighted in PhotoShop and the hairs were counted as a surrogate measure of the number of cells in that area. Mesothorax (French et al., 1998) and foreleg femur lengths were measured in arbitrary units, but the relative lengths of these measurements were preserved in the normalization.

Triglyceride and glycogen measurements

For triglyceride measurements, 1-week-old male flies (N=10 for each genotype) were homogenized for lipid extraction in 3 ml 2:1 chloroform:methanol. Total triglyceride content was determined using the Waco L-Type TG kit (Richmond, VA, USA). For glycogen measurements, 1-week-old male flies (N=40 in fed state; N=100 in starved state) were homogenized in 2 ml buffer (0.01 mol l^{-1} KH_2PO_4, 1 mmol l^{-1} EDTA, pH 7.4). The homogenate was spun for 15 min at 13,000 *g* to remove fly debris. The supernatant was aliquoted and stored at –80°C. The Sigma Starch Assay Kit (SA-20; St Louis, MO, USA) employs a two-step enzymatic approach for glycogen determination. In this assay, glycogen is first hydrolyzed by incubation with amyloglucosidase; the resulting glucose monomers are treated with hexokinase and the resulting

NADPH generated is quantified spectrophotometrically against a glucose standard curve.

Food intake assay

Batches of 1-week-old male flies (40–60 per run) were placed in Terizaki plates (ISC Bioexpress, Kaysville, UT, USA; Cat. No. T-3017-2) in which wells were filled with 10 mmol l^{-1} sucrose in 1% agar, containing 0.5% (w/v) dye (FD&C No. 1; Spectrum Chemicals, Gardena, CA, USA). The food intake of *Akhr*^{null} and *Akhr*^{rev} flies was calculated by quantifying the absorbance of ingested dye according to a published protocol (Libert et al., 2007). Fed flies were exposed to the dye for approximately 6 h and the previously starved flies for approximately 30 min. The longer time for fed flies was needed to obtain adequate signal; previously starved flies required only 30 min exposure to ingest a measurable quantity of dye-containing food.

RESULTS

Generation of *Akhr* mutants

To assess the contribution of the AKH pathway to energy homeostasis, we generated loss-of-function mutations in *Akhr*. A P-element insertion (*Akhr*^{p}) into the non-coding exon 1 of the *Akhr* gene was found in an available collection. A null mutation (*Akhr*^{null}) and a wild-type revertant (*Akhr*^{rev}) were isolated by excision of the P element through hybrid dysgenesis (Fig. 1A) (Ryder and Russell, 2003). *Akhr*^{null} removes the entire coding sequence with minimal disruption of adjacent genes. Only ~150 bp of genomic sequence downstream of *Akhr* is removed in *Akhr*^{null} mutants, allowing phenotypes to be ascribed to the specific removal of the *Akhr* gene. The revertant retains a small remnant (<20 bp) of the ~10 kb P element, but otherwise restores wild-type gene structure (see Materials and methods section for a full description). RT-PCR analysis of whole body homogenates demonstrated *Akhr* mRNA transcript production in *Akhr*^{rev} flies but not in *Akhr*^{null} flies (data not shown). The *Akhr*^{null} mutant is homozygous viable, allowing detailed phenotypic analysis in the adult.

AKHR is expressed in the *Drosophila* fat body

To probe the expression pattern of AKHR, we generated transgenic flies that expressed the transcriptional activator Gal4 under the control of a region of DNA upstream of the *Akhr* gene likely to contain all the transcriptional elements needed to recapitulate the expression pattern of AKHR. Three independent lines were generated and were crossed to flies carrying a reporter gene with the Gal4 binding sites (i.e. UAS) fused to GFP. Robust expression of GFP was observed in the adult *Drosophila* fat body (Fig. 1C–E), the primary tissue for the storage of fuel molecules. Strong expression is observed in the body cavity as well as the pericerebral region in all three transgenic lines. Expression in the larval fat body was also observed (data not shown). RT-PCR using RNA isolated from dissected fat body tissue confirmed fat body expression. In addition, detection of *Akhr* fat body expression by *in situ* hybridization has been reported in independent work (Gronke et al., 2007). The expression of AKHR in the fat body (as reflected by GFP reporter fluorescence) is consistent with its expected role in regulating energy homeostasis.

Akhr mutants have larger lipid and carbohydrate stores

We found that 1-week-old male *Akhr*^{null} mutants had at least a twofold increase in triglyceride content when compared with

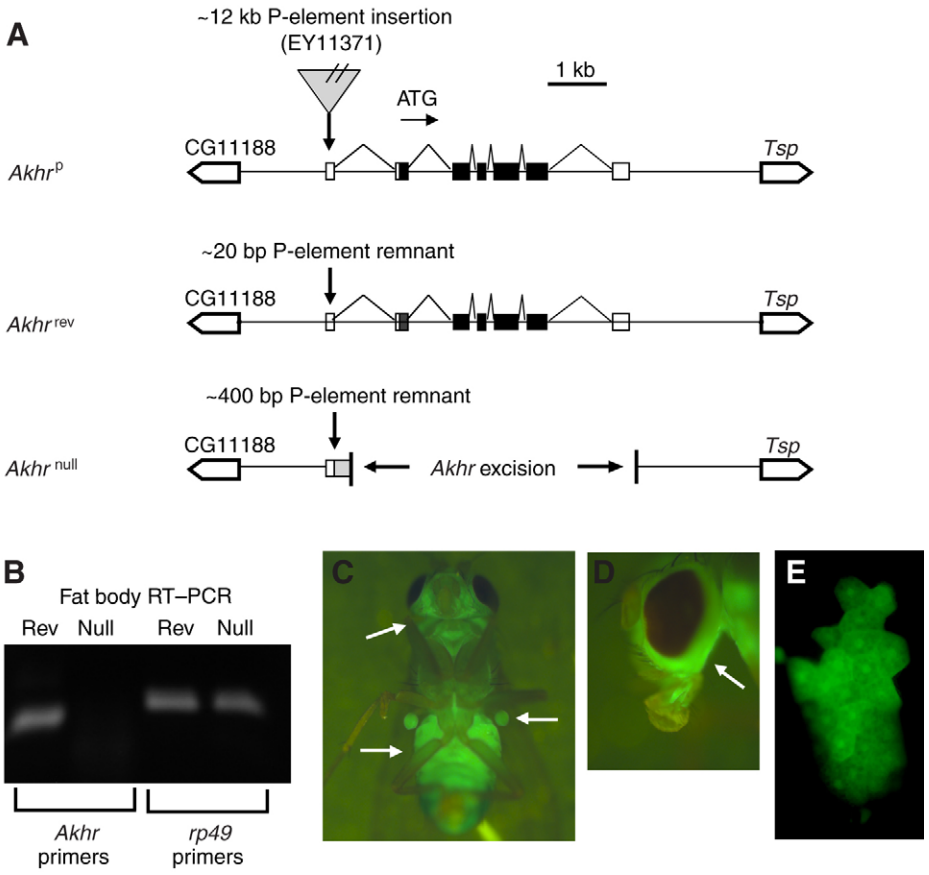

Fig. 1. Generation of *Akhr* mutants. (A) Schematic representation of *Akhr* alleles. *Akhr*^{p} (EY11371) contains a P element inserted in exon 1, upstream of the start of translation. *Akhr*^{rev} is a P-element excision allele; an ~20 bp remnant of the P element remains. *Akhr*^{null} removes the entire coding sequence of the *Akhr* gene, but does not disrupt the adjacent CG11188 and *Tsp* genes. The *Akhr*^{null} line also retains an insert of the P element (~400 bp) and removes ~150 bp of genomic sequence downstream of *Akhr*. See the Materials and methods section for further details. (B) *Akhr* mRNA is expressed in the fat body of *Akhr*^{rev} (left lane for each primer pair) but not in *Akhr*^{null} (right lane for each primer pair) as assessed using RT-PCR. *Akhr* mRNA was also detected in $y^{1}w^{67c23}$ and *Akhr*^{p} (EY11371) lines (data not shown). All bands are of the expected molecular mass; see the Materials and methods section for a description of the PCR protocol. (C) Transgenic flies that expressed the yeast transcriptional activator Gal4 under the control of the *Akhr* promoter (*Akhr*-Gal4) were crossed to reporter flies (UAS-GFP). Expression in the fat body (white arrows) was observed throughout the adult body, including head (D) and dissected fat body tissue (E); expression in the larval fat body was also observed (data not shown).

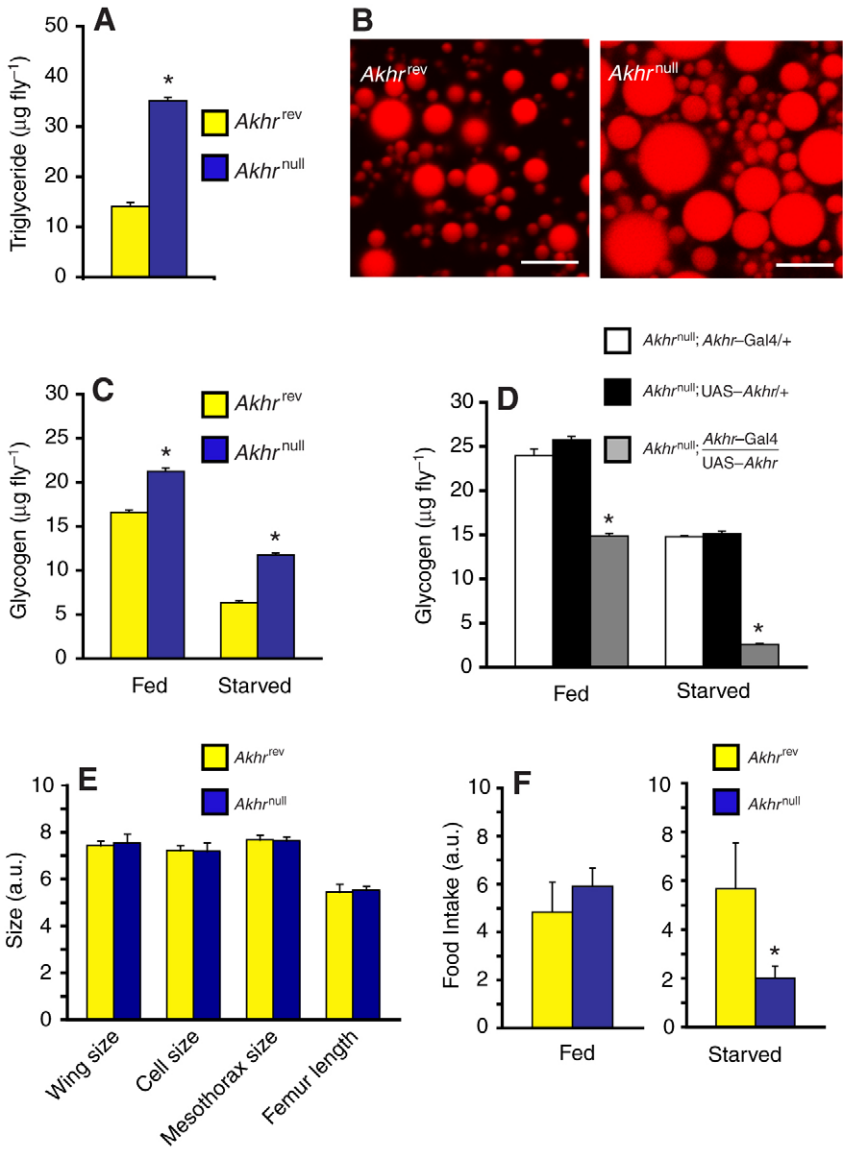

Fig. 2. *Akhr* mutants have abnormal lipid and carbohydrate homeostasis. All experiments were performed with 1-week-old male flies unless otherwise noted. In the bar graphs data for $Akhr^{null}$ flies are shown in blue and those for $Akhr^{rev}$ flies in yellow. (A) $Akhr^{null}$ flies have higher total body triglyceride content than $Akhr^{rev}$ (*ad libitum* fed). Triglyceride values are averages of triplicate measurements, with corresponding standard deviations. (B) $Akhr^{null}$ flies had larger lipid cells than control flies, consistent with the total body triglyceride measurements. Representative images are shown for Nile Red staining of fat body tissue from ~1-week-old male $Akhr^{null}$ (right panel) and $Akhr^{rev}$ (left panel) flies. Scale bars, 10 μm. (C) $Akhr^{null}$ flies have higher glycogen content (measurements obtained from whole body homogenates). Differences between $Akhr^{null}$ and $Akhr^{rev}$ flies are accentuated after 24 h of starvation (Student's *t*-test, $^*P<0.05$). (D) Genetic rescue experiments demonstrate that glycogen levels decrease when *Akhr* is re-introduced in an otherwise $Akhr^{null}$ background. Data from Gal4 flies are shown as white bars and those for UAS control flies as black bars; data from flies containing both Gal4 and UAS transgenes are shown in grey. Gal4 and UAS control flies have a higher glycogen content than flies that contain both transgenes (which can now express *Akhr*; Student's *t*-test, $^*P<0.05$). (E) Tissue and cell size in $Akhr^{null}$ and $Akhr^{rev}$ were indistinguishable. Measurements for wing size, wing cell size, mesothorax size and foreleg femur length are shown. (F) $Akhr^{null}$ flies are not hyperphagic. The ingestion of FD&C No. 1 blue was quantified for both fed and starved (18–24 h) 1-week-old male flies. Food intake is shown in arbitrary units which are proportional to the measured absorbance of ingested dye as per a published protocol (Libert et al., 2006). With prior starvation, $Akhr^{null}$ flies had lower food ingestion during the first 30 min (Student's *t*-test, $^*P<0.05$).

$Akhr^{rev}$ flies (Fig. 2A); $Akhr^{p}$ flies had intermediate triglyceride levels under normal feeding conditions (data not shown). Furthermore, Nile Red staining of dissected fat body tissue (Gronke et al., 2005) demonstrated markedly larger cells in $Akhr^{null}$ flies than in control $Akhr^{rev}$ flies (Fig. 2B), consistent with the total body lipid measurements. $Akhr^{null}$ mutants showed a modest increase in glycogen content compared to $Akhr^{rev}$ control flies, but the difference became more apparent after 24 h of starvation (Fig. 2C). Genetic rescue experiments provide further confirmation of the carbohydrate phenotype. $Akhr^{null}$ mutants containing either the *akhr*-Gal4 or UAS-*Akhr* transgene had higher glycogen content than rescue flies with both transgenes (both in the fed and starved states; Fig. 2D).

Akhr mutants do not display any growth phenotypes, in contrast to mutants in the *Drosophila* insulin signaling cascade (which have decreased body and cell size; Fig. 2E) (Wu and Brown, 2006). Quantification of food intake in the fed state did not reveal any gross differences in ingestion between $Akhr^{null}$ and $Akhr^{rev}$ flies (Fig. 2F). In fact, $Akhr^{null}$ flies consumed less of a dye-containing sucrose meal after 18–24 h of starvation. Thus, the higher total body triglyceride content of $Akhr^{null}$ flies indicates an 'obese' phenotype rather than arising from an increase in overall body size. In humans, the term obesity is used as a measurement of mass corrected for size. Because $Akhr^{null}$ flies do not have any growth phenotypes, we describe their higher total triglyceride content as reflecting an obese phenotype rather than merely arising from an increase in total body size. Intriguingly, based on our feeding experiments, the etiology of the obese phenotypes is unlikely to arise from hyperphagic behavior of fed $Akhr^{null}$ mutants.

Akhr mutants are starvation resistant

Previous work in *Drosophila* has shown that starvation resistance correlates strongly with lipid content (Djawdan et al., 1998). We therefore asked whether the higher triglyceride content of $Akhr^{null}$ flies confers a survival benefit during starvation. *A priori*, if the AKH axis were the only pathway mediating lipolysis, one would expect that mutant flies would *not* be starvation resistant. However, we found $Akhr^{null}$ flies were markedly starvation resistant when compared with control flies (Fig. 3A,B). $Akhr^{null}$ flies survived for about 3–4 days under starvation conditions,

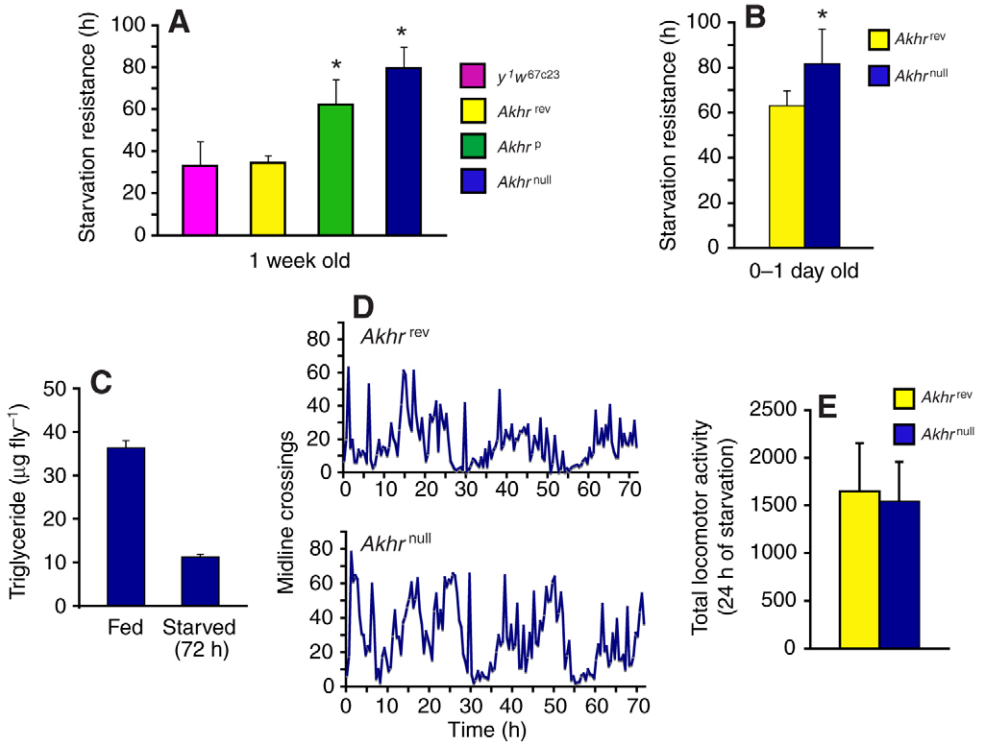

Fig. 3. *Akhr* mutants are starvation resistant. All experiments were performed on 1-week-old male flies, except when specifically noted otherwise. Throughout the figure, data for $Akhr^{null}$ flies and $Akhr^{rev}$ flies are represented in blue and yellow, respectively. (A) Starvation resistance of *Akhr* mutant flies. Starvation resistance profiles were quantified on the Trikinetics *Drosophila* Activity Monitoring System (DAMS), with individual monitoring tubes containing 2% agarose, but no other food source. The time of death of an individual male fly was defined to be the time of last recorded locomotor activity, which correlated well with starvation profiles obtained from direct observation. Average starvation resistances are given for each genotype (N=16) with their corresponding standard deviations. $Akhr^{rev}$ flies have starvation resistance that is comparable to y^1w^{67c23} flies, the genetic background from which the $Akhr^{p}$ line was generated. $Akhr^{null}$ flies were markedly starvation resistant when compared to $Akhr^{rev}$ control flies, and $Akhr^{p}$ flies showed an intermediate phenotype (Student's *t*-test, *P<0.05 for both comparisons). Starvation resistance comparisons between genotypes were repeated at least three times. (B) Both young and older (1 week) $Akhr^{null}$ flies have enhanced starvation resistance when compared to age-matched $Akhr^{rev}$ control flies (Student's *t*-test, *P<0.05). (C) $Akhr^{null}$ flies are able to mobilize triglyceride stores, as reflected by the decreased lipid levels of flies starved for 72 h. (D) *Akhr* mutants do not have defective locomotor activity or circadian rhythm. Average number of midline crossings (N=16 for each genotype) were recorded every 30 min for fed $Akhr^{rev}$ and $Akhr^{null}$ using the DAMS. No gross defects in locomotor activity or circadian rhythm were observed in $Akhr^{null}$ flies. (E) Total locomotor activity (counted as number of midline crossings) for the first 24 h of starvation for each genotype, with corresponding standard deviations (N=16 for each genotype). The differences in locomotor activity between all starved lines were not statistically significant.

whereas control flies lived for 1–2 days. Young $Akhr^{rev}$ flies were more starvation resistant than older flies, but young $Akhr^{null}$ flies were as starvation resistant as their older counterparts (Fig. 3B). It has been shown that larval fat body cells can persist in the newly emerged adult (Aguila et al., 2007). We have observed that the older mutant and revertant flies lack the characteristically dissociable residual larval fat body cells of younger flies. Taken together, these results suggest that $Akhr^{null}$ flies have a higher triglyceride content (and are thus more starvation resistant) than $Akhr^{rev}$ flies because of continued accumulation of lipid stores during the initial days of adulthood.

We observed that after 48 h of starvation, nearly all revertant flies do not survive, in contrast to null mutant flies (see Fig. 3A for a quantification of starvation resistance profiles). In addition, we found that $Akhr^{null}$ flies were fertile even after 48 h of starvation (data not shown), a time point at which nearly all wild-type flies are dead. Thus, the starvation resistant $Akhr^{null}$ mutant flies can be maintained and propagated, despite being subjected to a potentially lethal environmental stressor. $Akhr^{null}$ flies retain the ability to mobilize triglyceride but have significant stores even after 72 h of starvation, around the average time of death (Fig. 3C).

Decreased locomotor activity does not contribute to the starvation resistance of *Akhr* mutants

AKH is thought to play a role in modulating locomotor activity, as ablation of AKH cells decreased locomotor activity under starvation conditions (Isabel et al., 2005; Lee and Park, 2004). The starvation resistance of *Akhr* mutant flies could thus also reflect a decrease in energy expenditure resulting from reduced locomotion. To ascertain whether the starvation resistance of $Akhr^{null}$ flies was affected by decreased energy expenditure in locomotion, we monitored the activity of individual revertant and mutant flies. One-week-old male $Akhr^{null}$ mutants in the fed state had no obvious defects in locomotor behavior or circadian rhythm (Fig. 3D). Importantly, no statistically significant differences were seen between $Akhr^{null}$ and $Akhr^{rev}$ flies during the first 24 h of starvation (Fig. 3E), a time point at which the vast majority of $Akhr^{rev}$ flies were still alive. As previous studies, in which AKH-secreting cells were ablated, suggested a role for AKH in regulating locomotion under starvation conditions, we were surprised that loss of AKHR did not affect locomotion. Although the underlying reason for this discrepancy is not known, locomotor activity may be regulated by other hormones released from AKH-secreting endocrine cells or another AKH receptor. In summary,

these data indicate that there is no contribution of abnormal locomotor activity to the starvation resistant phenotypes of AKHR mutant flies.

AKHR is expressed in attractive gustatory neurons

We asked whether AKHR is expressed in the nervous system, as many peptides hormones (and their receptors) have been shown to be critical for regulating energy homeostasis and food intake in both insects and mammals (Schwartz and Porte, 2005; Wu et al., 2005). We observed an intriguing expression pattern of AKHR in a subset of neurons in the adult subesophageal ganglion (Fig. 4A–F), a site of projection for the majority of peripheral gustatory neurons in *Drosophila* (Amrein and Thorne, 2005; Scott, 2005). A similar expression pattern was observed in three independent transgenic lines. However, verification of gustatory neuronal expression by *in situ* hybridization was unsuccessful, as is the case for the majority of published gustatory neuron-Gal4 transgenic lines. Unfortunately, antibody staining also failed to give independent evidence for gustatory neuron expression. As additional evidence for *Akhr* expression in the gustatory system, *Akhr* was independently identified in a genome-wide microarray screen for genes specifically expressed in the *Drosophila* gustatory system; the *Akhr* gene mRNA transcript was found to be significantly downregulated (by 2.942-fold; Student's *t*-test, *P* value <0.01) in *poxn* mutants (which lack gustatory neurons) when compared to heterozygous controls (P. Cameron and K. Scott, personal communication).

Recent work has demonstrated that *Drosophila* gustatory neurons are functionally segregated into neurons that mediate attractive taste and aversive taste modalities. Interestingly, immunohistochemical double labeling with markers for different subclasses of gustatory neurons indicated that AKHR is expressed in neurons associated with attractive taste (e.g. *Gr5a*-expressing neurons; Fig. 4A–C), but strictly excluded from neurons mediating aversive taste (e.g. *Gr66a*-expressing neurons; Fig. 4D–F). We postulate that additional AKHR-expressing neurons probably represent other gustatory neurons mediating attractive taste. Taken together, the labeling studies indicate that AKHR is expressed in a highly selective fashion in only a subset of gustatory neurons, strictly excluded from all neurons known to be associated with aversive taste. Given the co-expression in a major subset of attractive-gustatory neurons, an intriguing possibility is that AKHR may be expressed in all neurons mediating attractive taste.

Fat body AKHR expression mediates metabolic phenotypes

To determine whether AKHR expression is required in the fat body or the nervous system to mediate starvation resistance, we performed genetic rescue experiments. In these experiments, *Akhr* expression was induced in either the fat body or *Gr5a* gustatory neurons, in an

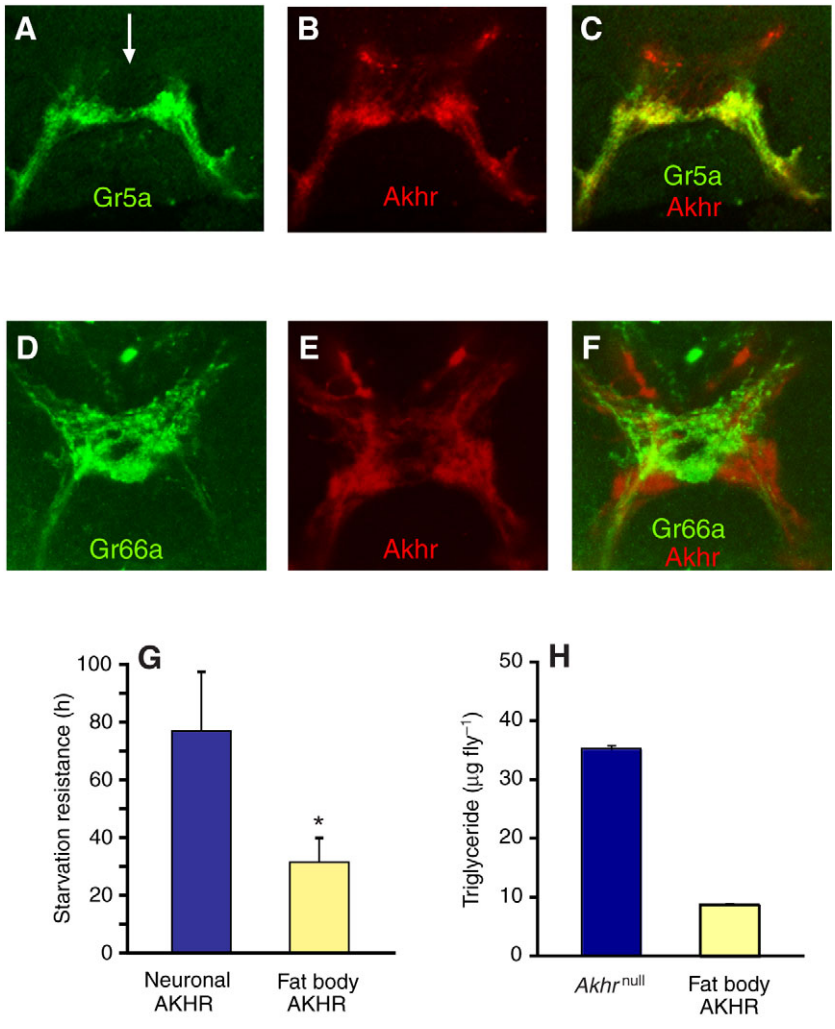

Fig. 4. Fat body (but not neuronal) AKHR expression substantiates observed metabolic phenotypes. (A–C) The axon projection pattern of *akhr*-Gal4-expressing gustatory neurons in the adult subesophageal ganglion. The dark region at the top of the picture (white arrow) is the esophagus. Shown from left to right, *Gr5a*-Gal4/UAS-GFP, *Akhr*-RFP, and the merged images, demonstrating co-expression of Gr5a and AKHR. (D–F) Double-labeling experiments with Gr66a-expressing neurons demonstrated exclusion of AKHR. Additional *Akhr* fibers likely represent axon projections of other attractive taste neurons. Single sections are displayed (1 μm); similar results were obtained through all projection layers in the subesophageal ganglion. (G) Genetic rescue experiments demonstrate that AKHR expression in the fat body of $Akhr^{null}$ flies restores wild-type starvation resistance (Student's *t*-test, $*P<0.05$). All genetic rescue experiments were done in an $Akhr^{null}$ background in 1-week-old male flies. R^4-Gal4 was used as a fat body driver. *Gr5a*-Gal4 (which drives Gal4 expression in the majority of attractive-gustatory neurons) was used as the gustatory neuron driver. The results are average starvation resistances from separate experiments ($N=4$ for fat body rescue, with 16 flies for each experiment; $N=3$ for *Gr5a* rescue, with 16 flies for each experiment), and standard deviations are shown. The starvation resistance of neuronal rescue flies is indistinguishable from $Akhr^{null}$ flies. (H) Expression of AKHR in the fat body (in an otherwise $Akhr^{null}$ background) dramatically reduces total body triglyceride content; the triglyceride content of $Akhr^{null}$ flies is shown for comparison.

otherwise *Akhr* homozygous null mutant background (see the Materials and methods section for a more detailed description). A dramatic decrease in starvation resistance in $Akhr^{null}$ mutants was observed after expressing AKHR under the control of a fat body-Gal4 driver (r^4-Gal4; Fig. 4G) (Lee, 2004). Similar rescue experiments using *Gr5a*-Gal4, which drives Gal4 expression in a majority of attractive-gustatory neurons, did not revert starvation resistance (Wang, 2004). Fat body AKHR expression also greatly reduced triglyceride content (Fig. 4H). Our data suggest that the regulation of total body triglyceride content is probably independent of gustatory AKHR expression. In summary, genetic rescue experiments demonstrate that manipulation of expression of AKHR in the fat body is sufficient to modulate triglyceride content and starvation resistance.

DISCUSSION

AKHR and the hungry fly

The *Drosophila* fat body serves as a major depot for storage of carbohydrates and lipids. We have shown that the AKH pathways serves as a critical determinant of both glycogen and triglyceride homeostasis. Interestingly, *Akhr* mutants are starvation resistant, retaining the ability to mobilize their lipids stores. Thus, it appears that the AKH pathways acts as a generalized catabolic signal, mobilizing both lipid and carbohydrate energy stores. Interestingly, our work suggests that the obese phenotypes of *Akhr* mutants do not result from increased food intake. In fact, *Akhr* mutants appear to ingest less when previously challenged with starvation. We therefore propose that the obese phenotypes result from a genetic propensity to retain energy stores rather than by increased food ingestion. *Akhr* mutants do not have any gross defects in locomotor activity (as measured by DAMS), suggesting that the greater energy reserves of mutant flies do not result from decreased energy expenditure in locomotor behavior.

Other lipolytic mechanisms (independent of the AKH pathway) must exist in *Drosophila* that enable *Akhr* mutants to utilize their triglyceride stores and affect their starvation resistance. Recently, the AKH and *brummer* lipase pathways were shown to be two major pathways regulating lipolysis in *Drosophila* (Gronke et al., 2007), but they concluded that AKHR does not affect carbohydrate homeostasis. Here, in striking contrast, we demonstrate that AKHR affects *both* total body carbohydrate and lipid content. In the fed state, the percentage differences in glycogen content between $Akhr^{null}$ and $Akhr^{rev}$ flies were not as pronounced as the differences in lipid content, perhaps accounting for this discrepancy. However, we show that differences in glycogen content between $Akhr^{null}$ and $Akhr^{rev}$ flies are more readily apparent after 24 h of starvation. Our genetic rescue experiments provide further support for the effect of *Akhr* expression on carbohydrate homeostasis. Because *Akhr* mutants (and *brummer* mutants) retain their ability to access their glycogen stores, we predict that additional pathways exist that regulate carbohydrate homeostasis.

The selective expression of *Akhr* in gustatory neurons that mediate attractive taste raises the interesting possibility that the AKH pathway coordinates a fly's response to hunger in two ways: (1) by mobilizing internal energy stores by its action on the fat body, and (2) increasing food intake by its action on attractive-gustatory neurons. Starved *Akhr* mutants display decreased food intake when re-introduced to food. However, genetic rescue experiments (using flies of the same genotype as those used for rescue of metabolic phenotypes) did not allow us to definitively attribute this altered behavior to loss of AKHR function. Therefore, we cannot rigorously exclude the possibility that the observed feeding behavior results from a background effect. Nonetheless, it is intriguing to speculate that activation of AKHR in the gustatory system promotes food intake in the hungry fly. Further work will be needed to delineate the role of gustatory *Akhr* expression in the context of an emerging picture of the *Drosophila* neuronal feeding circuit (Melcher and Pankratz, 2005).

Modulation of energy homeostasis by the AKH pathway

Genes that modulate the retention of fuel molecules can provide an adaptive survival benefit during periods of decreased food availability (Neel, 1962; Speakman, 2004). Our results are consistent with the idea that specific genetic mutations in *Drosophila* can serve to prolong long-term survival when flies are challenged with food deprivation. There is evidence that selective pressures can be used to increase the triglyceride content of flies both in nature and in the laboratory. For example, naturally occurring mutants of the *adipose* gene have higher triglyceride stores and are starvation resistant (Hader et al., 2003). In addition, flies with higher triglyceride stores can be generated by selecting for starvation-resistant phenotypes over several generations (Baldal et al., 2006; Djawdan et al., 1998; Hoffmann and Harshman, 1999). Overall, more work is needed to understand better how specific genetic mechanisms contribute to the adaptation of *Drosophila* to specific ecological niches differing in food availability (Hoffmann and Weeks, 2007; Montooth et al., 2003; Reaume and Sokolowski, 2006).

Over the approximately 600 million years of evolution that separate humans from flies from common urbilaterial ancestors (De Robertis and Sasai, 1996), mammals have evolved discrete liver and adipose tissues that have energy storage functions performed jointly by the *Drosophila* fat body. Thus, AKHR expression in the fat body is uniquely poised to control mobilization of both carbohydrates and lipids. Mammals may require a more elaborate array of endocrine signals that coordinate carbohydrate and lipid homeostasis during periods of food deprivation. For example, specific genetic manipulation of the mammalian glucagon pathway is rendered difficult by the complex structure of the preproglucagon gene (Drucker, 2001). Although murine glucagon receptor knockouts have abnormal carbohydrate metabolism, no obese phenotypes have been observed (Conarello et al., 2007; Gelling et al., 2003; Parker et al., 2002). Significantly, these results are confounded by upregulation of other hormone pathways. Thus, *Drosophila* offers a genetically tractable model organism to dissect pathways involved with energy mobilization.

We anticipate that further study of the AKHR pathway will provide a better understanding of the downstream signaling components regulating glycogenolysis and lipolysis that are conserved between flies and mammals. In addition, the power of forward genetic screens in the *Drosophila* may uncover other determinants of energy homeostasis that have relevance to the study of human disorders of lipid and carbohydrate metabolism, such as obesity and diabetes (Gronke et al., 2005; Ruden et al., 2005).

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