Energy Homeostasis Control in *Drosophila* Adipokinetic Hormone Mutants

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ABSTRACT Maintenance of biological functions under negative energy balance depends on mobilization of storage lipids and carbohydrates in animals. In mammals, glucagon and glucocorticoid signaling mobilizes energy reserves, whereas adipokinetic hormones (AKHs) play a homologous role in insects. Numerous studies based on AKH injections and correlative studies in a broad range of insect species established the view that AKH acts as master regulator of energy mobilization during development, reproduction, and stress. In contrast to AKH, the second peptide, which is processed from the *Akh* encoded prohormone [termed “adipokinetic hormone precursor-related peptide” (APRP)] is functionally orphan. APRP is discussed as ecdysiotropic hormone or as scaffold peptide during AKH prohormone processing. However, as in the case of AKH, final evidence for APRP functions requires genetic mutant analysis. Here we employed CRISPR/Cas9-mediated genome engineering to create AKH and AKH plus APRP-specific mutants in the model insect *Drosophila melanogaster*. Lack of APRP did not affect any of the tested steroid-dependent processes. Similarly, *Drosophila* AKH signaling is dispensable for ontogenesis, locomotion, oogenesis, and homeostasis of lipid or carbohydrate storage until up to the end of metamorphosis. During adulthood, however, AKH regulates body fat content and the hemolymph sugar level as well as nutritional and oxidative stress responses. Finally, we provide evidence for a negative autoregulatory loop in *Akh* gene regulation.

KEYWORDS *Drosophila*; adipokinetic hormone; adipokinetic hormone precursor-related peptide; energy homeostasis; stress resistance

ENERGY homeostasis requires continuous compensation for fluctuations in the energy expenditure and availability of food resources. Organisms thus build up reserves under positive energy balance and catabolize them when the balance turns negative to retain stable levels of circulating energy fuel. Insulin signaling induces the uptake of excessive circulating sugars, thus promoting reserve accumulation (reviewed, e.g., in Saltiel and Kahn 2001; Cohen 2006), whereas energy mobilization is under the control of glucagon and glucocorticoid signaling in mammals (reviewed, e.g., in Rui 2014; Charron and Vuguin 2015) and adipokinetic hormone (AKH) signaling in insects (reviewed, e.g., in Van der Horst 2003; Lorenz and Gäde 2009; Bednářová *et al.* 2013a). Consistent with their fundamental physiological function in energy mobilization, AKHs are

found not only in insects, but are common in Protostomia, where they have been identified both in Ecdyszoa (in Arthropoda, Tardigrada, and Priapulida) and Lophotrochozoa (in Mollusca, Rotifera, and Annelida) (Gäde 2009; Hauser and Grimmelikhuijzen 2014). Nevertheless, physiological functions of AKHs have been studied mainly in Arthropoda. Similar to mammals, also insects store lipids in the form of triacylglycerides (TGs) and as carbohydrates in the form of glycogen. The main storage organ for lipid and glycogen in insects is the fat body, which can thus be considered as the functional equivalent of mammalian liver and white adipose tissue (Azeez *et al.* 2014). Under energy-demanding conditions, AKHs get released from the organ of their synthesis and storage, the corpora cardiaca (CC), into the hemolymph to reach their cognate G protein-coupled receptors (GPCRs) (called AKH receptors, AKHRs) expressed on the fat body cells. This induces TG and glycogen breakdown, which leads to the production and release of circulating carbohydrates (trehalose and glucose), lipids [diacylglycerides (DGs)], proline, or a combination of these fuel molecules, depending on the species' preference. Despite the divergence in the preferred type of circulating

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fuel, the role of AKH in the mobilization of energy stores is evolutionarily conserved among insects (reviewed in Gäde and Auerwald 2003; Lorenz and Gäde 2009). Consistently, AKH was identified as the hyperglycemic hormone in cockroaches (Steele 1961), as the hyperlipaemic hormone in *Locusta* (Beenackers 1969; Mayer and Candy 1969) and as the hyperprolinaemic hormone in the fruit beetle *Pachnoda sinuata* (Auerwald and Gäde 1999). Next to this primary, catabolic role, numerous studies have implicated AKH in a puzzling diversity of additional physiological functions ranging from behavior (Kaun *et al.* 2008), locomotion (Kodrík *et al.* 2000; Socha *et al.* 2008), and reproduction (Lorenz 2003; Attardo *et al.* 2012) to digestion (Vinokurov *et al.* 2014), heart beat control (Noyes *et al.* 1995), sleep (Metaxakis *et al.* 2014), immunity (Adamo *et al.* 2008), oxidative stress resistance (Bednářová *et al.* 2013a,b; Plavšín *et al.* 2015), aging (Katewa *et al.* 2012; Waterson *et al.* 2014), and muscle contraction (Stoffolano *et al.* 2014). It is not clear so far whether these pleiotropic effects of AKH result from changes in energy homeostasis, or rather reveal the existence of distinct AKH-regulated signaling pathways, which implement independent functions of AKH. Individual studies used typically different species of various ontogenetic stages, and thus it remains elusive, whether the described roles reflect the general functions of AKH, which might be developmentally conserved across ontogenetic stages and evolutionarily conserved across insect species. The majority of AKH studies have been done in the popular insect endocrinology models like *Locusta*, *Manduca*, *Gryllus*, or *Pyrrhocris*, where AKH roles have been addressed mostly as physiological changes induced by injections of synthetic AKH, or as correlations between the hormone titer and varying environmental or physiological conditions. A more comprehensive understanding of the AKH functions requires genetic loss-of-function analyses.

Recent advances in available technologies, like mass spectrometry (MS), and the emergence of the CRISPR/Cas9 tool for genomic engineering added to the advantages of the model insect species *Drosophila melanogaster*, strengthening its potentials in the field of insect endocrinology. *Drosophila* already proved to be an excellent model system to investigate several other conserved hormonal pathways, including, e.g., insulin/insulin-like signaling (Kannan and Fridell 2013; Nässel *et al.* 2015). However, in contrast to the numerous scientific reports dealing with AKH roles in nondrosophilid species, only a limited number of studies have focused on AKH signaling in *Drosophila* so far. In the absence of a specific mutant, tools for AKH studies have remained limited to the ablations of CC cells (Kim and Rulifson 2004; Lee and Park 2004; Isabel *et al.* 2005), stimulation of secretory activity of CC cells by their depolarization (Kim and Rulifson 2004), mutations of the receptor (Grönke *et al.* 2007; Bharucha *et al.* 2008; Waterson *et al.* 2014), overexpression of *Akh* complementary DNA (cDNA) (Kim and Rulifson 2004; Lee and Park 2004; Grönke *et al.* 2007; Katewa *et al.* 2012; Baumbach *et al.* 2014b), and *Akh* RNA interference (RNAi)

(Braco *et al.* 2012; Baumbach *et al.* 2014b). Ablation of CC cells, or their depolarization, are not AKH-specific manipulations, as these endocrine cells also produce other hormones like limostatin, which affects metabolism by regulation of insulin signaling (Alfa *et al.* 2015). In addition, it cannot be excluded that a limited amount of hormone is produced prior to the ablation and interferes with the investigation of the early developmental functions of AKH. Overexpression of AKH cDNA or RNAi are also not explicit methods to address AKH functions. Next to the typically incomplete down-regulation by RNAi, another level of complication comes from the structure of the *Akh* gene product. Processing of the 79-amino-acid-long AKH prohormone results in two peptides: AKH and adipokinetic hormone precursor-related peptide (APRP) (Figure 1A). Hence, overexpression of *Akh* RNAi decreases both AKH and APRP and overexpression of *Akh* cDNA increases both of these peptides. Differentiation between the effects of AKH and APRP loss is especially important in the light of the potential hormonal functions of APRP. Even though no role of APRP was described so far (Hatle and Spring 1999), its evolutionary conservation, common ancestry with mammalian growth hormone-releasing factors (Clynen *et al.* 2004), and release upon stimulation with melatonin (Huybrechts *et al.* 2005) all argue in favor of an endocrine function of this peptide. Thus, unequivocal study of AKH and APRP functions requires creation of specific mutants, which we describe in this study.

Biochemical studies identified a single *Drosophila* AKHR receptor (Park *et al.* 2002; Staubli *et al.* 2002), which has been functionally analyzed *in vivo* (Grönke *et al.* 2007; Bharucha *et al.* 2008). However, these studies do not exclude AKH signal transmission via other receptor pathways. Thus, throughout our study, we compared the newly created AKH mutants with mutants lacking the AKHR.

Here, we present the first functional analysis of AKH single mutants and AKH plus APRP double mutants in essential biological processes ranging from embryogenesis, metamorphosis, reproduction, lipid and carbohydrate storage, and mobilization to stress resistance. Our work shows that developmental mobilization of energy stores, oogenesis, and locomotion are under the control of AKH-independent pathways in *Drosophila*. However, *Drosophila* AKH is involved in response to stress, including nutritional and oxidative challenge. We also show that the metabolic roles of AKH in stored energy sources in *Drosophila* are developmental-stage specific; whereas AKH signaling is dispensable for accumulation and mobilization of storage lipids and glycogen during larval and pupal periods, it acquires important roles in homeostasis of storage lipids, but not storage carbohydrates during adulthood.

Materials and Methods

Fly stocks and fly husbandry

If not stated otherwise, *D. melanogaster* were reared at 25° in a 12-hr light/12-hr dark cycle, on standard *Drosophila* medium (5.43 g agar, 15.65 g yeast, 8.7 g soy flour, 69.57 g

Figure 1 Genomic organization of the *Akh* gene locus and molecular identity of the AKH single mutant *Akh^A* and the AKH plus APRP double mutants *Akh^{AP}* and *Akh^{SAP}*. (A) Genomic organization of the *Akh* gene flanked by the *Ras64B* and *CG32260* genes on the left arm of the *D. melanogaster* third chromosome (open boxes represent transcribed regions, filled boxes open reading frames). Wild-type *Akh* (*Akh⁺*) encodes a prohormone consisting of the signal peptide (yellow), the AKH octapeptide (red), a protease cleavage site (light gray), and the APRP (blue). Note that the RNAi target sequence spans most of the *Akh* open reading frame. Scale bars represent DNA sequences in base pairs (bp). Schematic drawing (A) and sequence representation (B) of the molecular lesions of *Akh* mutants compared to the prohormone (pAKH⁺) coding sequence. The AKH-specific mutant *Akh^A* lacks the sequences coding for the two amino acids (DW) at the C-terminal positions 7 and 8 of the AKH octapeptide. In AKH plus APRP double mutants *Akh^{AP}* and *Akh^{SAP}*, AKH coding sequences are deleted and APRP expression is compromised due to frame shift (*Akh^{AP}*) or due to deletion of the signal peptide coding sequence including the translation start codon (*Akh^{SAP}*). The underlined sequence in B corresponds to the *Akh* gRNA and the black triangle indicates the Cas9 cleavage site used for *Akh* mutant generation by CRISPR/Cas9-mediated *Akh* genome engineering. For details see text.

maize flour, 19.13 g beet syrup, 69.57 g malt, 5.43 ml propionic acid, and 1.3 g methyl 4-hydroxybenzoate per 1 liter of medium; supplier information available on request). Experiments were started with ~150 eggs per 68-ml vial. Freshly eclosed adults were collected and kept at density of ~50 females + 50 males per 68-ml vial and transferred to fresh media every second day. Pupae were staged according to Bainbridge and Bownes (2008).

Stocks used to create *Akh* mutant lines: The stocks used to create *Akh* mutant lines are as follows: *y¹ sc⁺ v¹*; *P{v*}*; *BFv-U6.2_Akh_gRNA}attP40* (this study); *w**; *Kr^l/CyO*; *D¹/TM3, Ser¹* (Bloomington *Drosophila* Stock Center, BDSC 7198); *P{ry⁺t7.2 = hsFLP}1, y¹ w¹¹¹⁸*; *P{y⁺t7.2 w⁺mC = UAS-Cas9.P} attP2*, *P{w⁺mC = GAL4::VP16-nos.UTR}CG6325^{MVD1}* (BDSC 54593, Port et al. 2014); and *w**; *TM3, Sb¹*, *P{2xTb1-RFP} TM3/ln(3L)D D¹* (BDSC 36338).

Mutant stocks: The mutant stocks are as follows: *w**; *Akh^A* (this study); *w**; *Akh^{AP}* (this study); *w**; *Akh^{SAP}* (this study); and *y⁺w**; *Akh^{R1}* (Grönke et al. 2007).

Mutant and balancer lines established after backcrossing into *w¹¹¹⁸* for nine generations: The mutant and balancer

lines established after backcrossing into *w¹¹¹⁸* for nine generations are as follows: *w¹¹¹⁸*; *Akh^A/TM3, Ser¹* floating (this study); *w¹¹¹⁸*; *Akh^{AP}/TM3, Ser¹* floating (this study); *w¹¹¹⁸*; *Akh^{R1}* (this study), and *w¹¹¹⁸*; *+ /CyO*; *+ /TM3, Ser¹* (transient line).

Additional stocks: Additional stocks are as follows: *w**; *akhp-GAL4, UAS-mCD8 GFP*; *akhp-GAL4/SM5a-TM6 Tb* (Kim and Rulifson 2004); *Akh RNAi* (VDRC 11352); *w¹¹¹⁸*; *akhp-GAL4, UAS-mCD8 GFP/CyO* (this study); *w¹¹¹⁸*; *akhp-GAL4, UAS-mCD8 GFP/CyO*; *Akh^A/TM3 Ser¹* floating (this study); *w¹¹¹⁸*; *akhp-GAL4, UAS-mCD8 GFP/CyO*; *Akh^{AP}/TM3 Ser¹* (this study); *w¹¹¹⁸*; *akhp-GAL4, UAS-mCD8 GFP/CyO*; *Akh^{SAP}/TM3 Ser¹* (this study); and Canton-S; *w¹¹¹⁸* (Vienna *Drosophila* RNAi Center, VDRC 60000).

CRISPR/Cas9-mediated mutagenesis of the *Akh* gene

The *Akh^A* (AKH⁻), *Akh^{AP}*, and *Akh^{SAP}* (AKH⁻ APRP⁻) mutants were generated by CRISPR/Cas9-mediated genomic engineering according to Kondo and Ueda (2013). For details on the generation of the mutants see Supporting Information, File S1.

Creation of *Akh^A*, *Akh^{AP}*, and *Akh^{SAP}* stable stocks and backcrossing of the mutant alleles to a common genetic background: Homozygous stocks were established from the

progeny of selected males (genotype: w^* ; $Akh^*/TM3$, Sb^1 , $P\{2xTb1-RFP\}$ carrying molecular lesions in the *Akh* gene, which were molecularly characterized by genomic sequencing to reveal the following three *Akh* mutants used in this study: Akh^A , Akh^{AP} , and Akh^{SAP} . As physiological parameters are prone to confounding genetic background effects, the Akh^A , Akh^{AP} , and Akh^{SAP} alleles, together with the previously described null mutation of the AKH receptor, $Akhr^1$ (Grönke *et al.* 2007), and a CyO TM3, *Ser¹* balancer line (based on BDSC 7198) were backcrossed into standard w^{1118} genetic background (VDRC 60000) for nine generations prior to stock establishment. For primer sequences used to track the mutations during the backcrossing, see File S1.

If not stated otherwise all physiological assays were conducted on the backcrossed mutants and the genetically matched control.

Mass spectrometry

Dissection and sample preparation for mass spectrometry:

Retrocerebral complexes (RCs) of adult flies were dissected in ice-cold ammonium chloride buffer (1.404 g $Na_2HPO_3 \times 2 H_2O$, 0.262 g $NaH_2PO_4 \times H_2O$, 8.8 g NaCl in 1 liter aqua bidest; pH 7.1) using fine forceps and a stereo binocular. Single preparations were washed in an ice-cold drop of MS grade water (TraceSELECT Ultra, Fluka Analytical, St. Louis) and either transferred to a stainless steel plate for direct tissue profiling or collected in 20 μ l ice-cold extraction buffer [50% MeOH, 0.5% formic acid (FA), 49.5% H_2O (v/v)] in a 0.5-ml protein LoBind tube (Eppendorf, Hamburg). Extracts were incubated for 30 min on ice and centrifuged at 4° and 12,000 $\times g$ for 15 min. Subsequently, extracts were incubated for 1 min in an ultrasonic bath filled with ice and centrifuged for 15 min at 4° and 12,000 $\times g$. Ultrasonic bath incubation was repeated three times for better peptide extraction. Resulting supernatants were stored at –20° for further analysis. For direct tissue profiling (mass fingerprints), transferred tissues were left to dry and subsequently covered with 50–75 nl of 2,5-dihydroxybenzoic acid (Sigma-Aldrich, St. Louis; 10 mg/ml DHB, in 20% ACN, 1% FA, and 79% H_2O) matrix solution using a fine glass capillary. For on-plate disulfide reduction, samples were covered with 0.1 μ l freshly prepared 1,5-diaminonaphthalene (Sigma-Aldrich; 10 mg/ml DAN, in 50% ACN, 0.1% trifluoroacetic acid, and 49.9% H_2O) matrix solution. For control experiments and APRP identification, Canton-S (CS) wild-type flies were used.

Reduction of disulfide bonds and carbamidomethylation of cysteines:

Extracts of RCs were treated as described in Sturm and Predel (2015). Supernatants were first reduced to a volume of 5 μ l by vacuum centrifugation and mixed with 20 μ l of 50 mM ammonium bicarbonate (ABC) buffer. The pH value was adjusted to 8 with NaOH. Disulfide bonds were reduced by adding 1,4-dithiothreitol (200 mM; DTT) in ABC buffer to an end concentration of 10 mM DTT, for 1 hr at 37°. Subsequently, cysteines were carbamidomethylated by adding iodoacetamide (200 mM; IAA) in ABC buffer to an end

concentration of 40 mM, at 37° for 1 hr in darkness. Excess IAA was precipitated by adding DTT with a final concentration of 40 mM DTT. The resulting mixture was incubated for 30 min at room temperature. Samples were acidified with 0.5% acetic acid (AA) and loaded on an activated (100% MeOH) and equilibrated (0.5% AA) homemade C18 spin column (Empore 3M extraction disc; IVA Analysentechnik, Meerbusch, Germany) as described in Rappsilber *et al.* (2007). The column was rinsed with 100 μ l 0.5% AA. Peptides were eluted with different concentrations of MeOH (10, 20, 25, 30, 40, 50, 80, and 100% with 0.5% AA) onto a sample plate and subsequently mixed with DHB [sample matrix ration 1:1 (v/v)].

MALDI-TOF mass spectrometry: Mass spectra were acquired on a Bruker UltrafleXtreme TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) under manual control in reflectron positive ion mode. Instrument settings were optimized for mass ranges of m/z 600–4000, m/z 3000–10,000, and m/z 8000–15,000. The instrument was calibrated for each mass range, using a mixture of bovine insulin, glucagon, ubiquitin, and cytochrome C (Sigma-Aldrich) or the Peptide Calibration Standard II (no. 222570, Bruker Daltonik). MS² spectra were acquired in postsource decay (PSD) mode without a collision gas. All obtained data were processed with FlexAnalysis 3.4 software package (Bruker Daltonik).

Fecundity assay

Fecundity was measured as daily egg scores of individual females during the first 10 days of their lives. One female and two males of the same genotype that eclosed within a 24-hr period were placed on standard fly food with active dry yeast added on the top (~5 mg of yeast per vial). Flies were transferred daily to fresh vials and the eggs were counted under a stereomicroscope. Fecundity was expressed as mean cumulative number of eggs laid by a single female during the first 10 days of life. Females that died or escaped during the experiment were excluded from the analyses. A total of 26–30 females were tested per genotype. Data were analyzed by one-way ANOVA. After 10 days, body size of females was measured as described below.

Hatchability assay

Eggs laid by the females at days 6 and 10 of the fecundity assay were kept at 25°, and the percentage of hatched eggs (hatchability) was determined 24 hr later. Data from the 6th day are plotted in Figure 3A. Embryos from 26–30 females were tested per genotype; hence, hatchability of 2147–2695 eggs was tested per genotype. Data were arcsine square root transformed and subsequently analyzed by one-way ANOVA followed by Tukey's honest significant difference (HSD) post hoc test.

Viability (larval to adult survival) assay

Larva to adult viability was expressed as percentage of flies that eclosed from the hatched eggs. The same larvae from the hatchability assay were used. Per genotype, 1239–2473

larvae were tested. Data were arcsine square root transformed and subsequently analyzed by one-way ANOVA.

Developmental rate determination

Developmental rate was measured as the time from the egg laying to adult eclosion. Four-day-old parental flies were transferred from the standard food to standard food supplemented with sprinkled active yeast and this food was replaced daily for 3 consecutive days to prevent egg retention. Then, the parental flies were allowed to lay eggs on fresh food for 2 hr. Embryos laid within this interval were collected and their density was adjusted to ~150 embryos per 68-ml vial and kept under standard conditions afterward. The number of eclosed flies was recorded three times per day (at 8 AM, 3 PM, and 8 PM), until the last fly eclosed. The experiment was repeated twice. Between 196 and 418 eclosed flies were tested per genotype per trial. Data were log transformed before performing one-way ANOVA and Tukey's HSD post hoc test.

Body size determination

Thorax length of females used for the fecundity assay was measured using an Axiophot Zeiss microscope equipped with a digital camera AxioCam HRC and ZEN 2011 software. Thorax length was measured from the posterior tip of the scutellum to the base of the most anterior humeral bristle (French *et al.* 1998). Thorax length of 25–29 females was measured per genotype. Data were analyzed by one-way ANOVA.

Preparation of homogenates for metabolic measurements (glycogen, lipid, and protein determination)

Male flies were homogenized by Mixer Mill (MM400, Retsch), in 1.2-ml collection tubes (Qiagen) with 5-mm metal beads and 600 μ l 0.05% Tween-20. Homogenates were heat inactivated for 5 min at 70°, centrifuged for 3 min at 3500 rpm (= 2200 rcf; Heraeus Megafuge 1.0R, swing-out rotor no. 2704), and 400 μ l of the supernatant was transferred into 96 Master block well plates (Greiner Bio One) for storage.

Protein determination

Protein content was determined by Pierce BCA Protein Assay Kit according to the instructions of the manufacturer, with the following modification: samples were analyzed in a 96-well plate format, using 50 μ l of the fly homogenate per 250 μ l reaction volume. For each genotype, four to six replicates of five flies each were tested per developmental stage or starvation time point. Experiments were repeated at least three times.

Lipid determination by coupled colorimetric assay

Lipid measurements were done as described in Hildebrandt *et al.* (2011). For a more detailed description see [File S1](#).

Glycogen determination

Glycogen measurements in the 96-well plate format were based on the conversion of glycogen to glucose by amyloglucosidase (Sigma) and on its subsequent measurement by the

glucose assay (GO) kit (Sigma) as described in Tennesen *et al.* (2014). For a more detailed description see [File S1](#).

Determination of circulating sugars

Hemolymph samples (three replicates of 30 flies each per genotype) were collected by centrifugation (6 min, 9000 rcf at 4°) of decapitated flies in a holder tube (0.2-ml tube with five holes of 0.6-mm diameter) placed in a 0.5-ml collection tube. A total of 1 μ l of the collected hemolymph was diluted with 99 μ l of 0.05% Tween-20 and immediately heat inactivated at 70° for 5 min. The homogenate was further diluted 1:6 prior to the sugar measurements. Measurements of circulating sugars were performed using a modification of the Tennesen method (Tennesen *et al.* 2014). For a more detailed description see [File S1](#).

Thin layer chromatography

The thin layer chromatography (TLC) analysis was performed as described by Baumbach *et al.* 2014a), with minor modifications. For a more detailed description see [File S1](#).

Confocal laser scanning microscopy of adult fat body tissue

Adult fat body tissue of 6-day-old male flies was dissected in ice-cold 1 $\times$ PBS. Flies were mechanically fixed with a preparation pin through the thorax with the ventral side upwards. Then the fly was cut with a fine scissor in transversal plane directly after the abdominal tergite 6. Additional cuts were performed in the coronal plane along the tergital–sternal intersections. The sternal parts, the digestive and reproductive system, were removed to expose the cuticle-attached fat body. This carcass preparation was embedded in 1 $\times$ PBS containing Bodipy493/503 (38 μ M; Invitrogen, D3922) for lipid droplet staining, DAPI (3,6 μ M; Invitrogen, D1306) for nuclei staining, and CellMask Deep Red (5 μ g/mL; Invitrogen, C10046) for plasma membrane staining. Images were acquired in 16-bit mode with a Zeiss LSM-780 microscope and a C-Apochromat 40 $\times$ /1.20 W Korr FCS M27 objective adjusted in dynamic signal range for the control genotype. For fluorescence detection, the following settings were used: DAPI (Excitation (Ex): 405 nm, Emission (Em): 410–468 nm), Bodipy493/503 (Ex: 488 nm; Em: 490–534 nm), and CellMask (Ex: 561 nm; Em: 585–747 nm). Images were analyzed with ImageJ v1.49m for lipid droplet area by first applying a “Gaussian blur” filter (2.0 pixel range) to a single optical section, in to smooth the edges of the lipid droplets. Afterward, a binary image with discrete lipid droplets was generated by thresholding (removal of “below 60%”). The “watershed” tool was applied to the image to separate the area of clustered lipid droplets. Finally, the particle analyzer was applied on the picture [size (μ m²): 0.1 $\rightarrow$ ∞ ; circularity: 0.01–1.0; mark outlines] for area determination of the discretely detected particles. Lipid droplets from 29–33 cells were tested per genotype.

Statistical significance of differences between the lipid droplet area sizes of controls and *Akh^A* mutants were tested using the Mann–Whitney test with OriginPro 9.1.0.

Ex vivo confocal laser scanning microscopy and quantification of corpora cardiaca cells

For analysis of GFP expression, samples were handled as described by Pitman *et al.* (2011). Adult male and female flies were collected 6 days after eclosion and RCs were dissected in ice-cold phosphate-buffered saline (1.86 mM NaH₂PO₄, 8.41 mM Na₂HPO₄, 175 mM NaCl) containing 4% paraformaldehyde and fixed for 120 min under vacuum at room temperature. Samples were washed three times for 10 min in PBS containing 0.1% Triton X-100 and twice in PBS before mounting in glycerol with 20% PBS. Imaging was performed on a Zeiss LSM Meta 510 microscope and images were processed in Amira 5.4 (FEI, Hillsboro, OR). Resulting image stacks were contrast adjusted and the cell numbers were independently counted by two experimenters.

Starvation resistance assay, lipid and glycogen mobilization upon starvation

Seven-day-old flies (three to five replicates per genotype, 25–30 flies per replicate) were transferred to 0.6% agarose (in water) and the dead flies were scored every 7–10 hr. The statistical significance of differences between starvation survival curves was analyzed by log-rank test. The mobilization of energy stores upon starvation was analyzed by lipid and glycogen measurements on five to six replicates of five flies each per genotype and starvation time point as described above. Experiments were repeated in at least two independent trials. Effect of the genotype was analyzed by one-way ANOVA followed by Tukey's HSD post hoc test. To test for the effects of genotype, duration of starvation exposure, and their interactions, two-way ANOVA was used.

Food intake assay by food labeling

Seven-day-old mated female flies were transferred to fly food medium containing 0.04% bromophenol blue and supplied with or without 20 mM paraquat for 4 hr. At this point in time, the number of flies with blue dye in the abdomen was scored by visual inspection. The assay was done as a blinded experiment, meaning that the genotypes were anonymized to the experimenter during scoring. The experiment was repeated at least twice, with 30–80 flies per genotype and tested time point. Data were analyzed for statistical significance by two-tailed Fischer exact test.

Paraquat resistance assay: application of paraquat on the nerve cord

The assay was done according to Cassar *et al.* (2015) with minor modifications. For a more detailed description see [File S1](#).

Locomotor activity assay

Spontaneous activity of *ad libitum*-fed flies and starvation-induced hyperactivity of starved flies was tested using the *Drosophila* Activity Monitor 2 system (TriKinetics). Flies were briefly anesthetized with CO₂ and loaded individually into the monitoring tubes containing standard medium or 0.6% agarose (in water). Spontaneous locomotion was recorded

over the first week of life. Test of the starvation-induced hyperactivity was started on 7-day-old flies; in parallel, siblings of these flies were monitored under *ad libitum* feeding conditions. For each genotype and feeding condition, 32 male flies were analyzed. Experiments were repeated at least twice. Spontaneous locomotor activity was measured as total number of midline crossings over the first week of life. Starvation-induced hyperactivity was analyzed both by visual inspection of the locomotion patterns of individual flies and by counting the total number of midline crossings over the complete starvation period and during the last 12 hr *pre mortem*. Quantitative data were analyzed by one-way ANOVA followed by Tukey's HSD post hoc test.

Startle-induced vertical climbing

The climbing assay is based on the “countercurrent distribution” method described by Benzer (1967) with modifications. For a more detailed description see [File S1](#).

Flight performance assay

Flight performance analysis was based on the assay developed by Benzer (1973) and modified by Babcock and Ganetzky (2014). For a more detailed description see [File S1](#).

Data availability

All fly strains generated in this work (see [Supporting Information](#) for details) are available on request.

Results

Generation of AKH single mutant and AKH plus APRP double mutant flies by CRISPR/Cas9-mediated genome engineering

The *Akh* gene encodes a prohormone that gives rise to the mature AKH and APRP peptides after signal peptide removal and proteolytical processing (Figure 1A). To study the developmental and metabolic functions of AKH and APRP, we employed CRISPR/Cas9-assisted mutagenesis to create an AKH-specific mutant (*Akh^A*) and AKH plus APRP double mutant flies (*Akh^{AP}* and *Akh^{SAP}*) (Figure 1, A and B). Mutations were obtained according to Kondo and Ueda (2013) by mutagenesis in the male germline expressing *Akh*-targeting gRNA, *UAS-Cas9*, and *nanos-GAL4* (Port *et al.* 2014). The *Akh^A* allele represents an AKH-specific in-frame deletion of the two C-terminal amino acids of the AKH octapeptide, which leaves the APRP sequence unaffected (Figure 1, A and B). MS analysis on RC peptides confirmed the presence of the predicted AKH hexapeptide and its processing intermediates in *Akh^A* mutant flies (Figure 2). Moreover, *Akh^A* mutants express APRP peptide dimers indistinguishable from *Akh⁺* control flies (Figure 2, [Figure S1](#), and [Figure S2](#)). In contrast, MS analysis detected no peptides encoded by the *Akh* gene in the *Akh^{AP}* and *Akh^{SAP}* mutants or in flies subject to an *Akh* RNAi knockdown, while the profile of other RC peptides was unchanged (Figure 2). This is consistent with the molecular identity of the *Akh^{AP}* and *Akh^{SAP}* mutants. The

Akh^{AP} allele carries a 19-bp deletion in the AKH region, which causes a frameshift upstream of the APRP coding sequence (Figure 1, A and B). Similarly, the 206-bp deletion in the *Akh^{SAP}* allele also removes the AKH coding sequence along with the signal peptide sequence and the translation initiation side of the prohormone (Figure 1, A and B). Collectively, the sequencing and peptide MS data identify *Akh^{AP}* and *Akh^{SAP}* as specific AKH plus APRP double loss-of-function mutants. We also propose *Akh^A* to be a AKH-specific null allele of *Akh* as the AKH^A hexapeptide lacks the tryptophan at position 8, which was shown to be essential for *Drosophila* AKH receptor activation by structure–activity studies (Caers *et al.* 2012).

Thus, the new *Akh* mutants allow addressing AKH-specific functions (revealed by the *Akh^A*) and APRP-specific functions (revealed by the comparison of *Akh^{AP}* and *Akh^A* phenotypes). Moreover, comparison of *Akh^A* mutant phenotypes with phenotypes of the AKH receptor deletion mutant *Akhr¹* (Grönke *et al.* 2007) can support or challenge the view of a single ligand/receptor pair in *Drosophila* AKH signaling.

AKH signaling and APRP are dispensable for developmental and fitness-related functions in *Drosophila*

AKH signaling is regarded as the central regulator in systemic energy mobilization control acting antagonistically to insulin signaling. Accordingly, we first tested the *Akh* dependency of nonfeeding ontogenetic stages (*i.e.*, embryogenesis and metamorphosis) and of biosynthetically demanding processes such as oogenesis. As developmental and physiological traits are sensitive to confounding genetic background effects, all AKH pathway and APRP mutants were crossed into a common *w¹¹¹⁸* background for nine generations prior to the phenotypic analyses.

Comparative analysis of *Akh^A*, *Akh^{AP}*, *Akhr¹*, and the genetically matched control flies revealed no gross abnormalities in hatchability (*i.e.*, egg to L1 larval survival; Figure 3A), viability (*i.e.*, L1 larval to adult survival; Figure 3B), developmental time (egg to adult; Figure 3C), or female fecundity (Figure 3D) in flies lacking AKH signaling. Moreover, the body size of AKH signaling mutants was unaffected (Figure 3E, Figure S3). We noticed slightly, but significantly reduced hatchability in *Akh^A*. However, this effect was not caused by the deficiency in the AKH signaling itself, as the *Akhr* mutant and AKH plus APRP double mutants had normal hatchability and larval to adult survival (Figure 3, A and B).

Taken together, in contrast to the dramatic effects of insulin signaling deficiency (Grönke *et al.* 2010), absence of AKH does not cause gross abnormalities in development or reproduction.

AKH signaling and APRP are dispensable for mobilization of lipids and glycogen during *Drosophila* development

Development encompasses nonfeeding periods like embryogenesis, moltings, and metamorphosis, when the organism

relies exclusively on stored energy reserves. To address the fuel utilization during *Drosophila* metamorphosis, and to test a possible involvement of AKH in this process, we followed the glycogen and lipid changes in *Drosophila* from the wandering stage of the third instar larvae (3L, end of the feeding) through pupation (P0), termination of the pupal development (P13–P15), until the posteclosion values in the immature adults (within 10 hr after eclosion) (Figure 4, A and B). As expected, the developmental stage had a highly significant effect on the amount of lipid reserves (see statistical analysis for Figure 4A). Lipid stores increased shortly before initiation of metamorphosis, reaching their highest values at early pupation and gradually decreased afterward (Figure 4A). In contrast to lipids, the glycogen content already reached its maximum at the 3L larval stage, decreased toward the P0 stage, fell to very low levels at P13–P15, and increased within the first day after eclosion (Figure 4B).

In analogy to AKH functions in nondrosophilid insects, *Drosophila* AKH can be anticipated to regulate synthesis of fat stores before metamorphosis, and their utilization during this process. However, *Akh^A*, *Akh^{AP}*, or *Akhr¹* accumulated comparable amount of lipids at both 3L larval stage and P0 pupal stage. Similarly, lipid mobilization proceeded normally in all tested genotypes, and lipid stores were comparable between all mutants and control at the end of metamorphosis (stage P13–P15) (Figure 4A). There was a nonsignificant trend toward increased lipid levels in freshly eclosed flies (Figure 4A); however, this phenomenon is likely not connected with the metamorphosis itself, but rather foretells the adult-specific role of AKH signaling, as described in *Deficiency in AKH-signaling results in adult-onset obesity and hypoglycemia*. Altogether, deficiencies of AKH signaling or APRP had no effect on the lipid content at any tested developmental stage or on the storage lipid mobilization during metamorphosis (see statistical analyzes in Figure 4A).

Adipokinetic hormone was described to act hypertrehalosaemic in several insect species including *Drosophila* larvae (Kim and Rulifson 2004; Lee and Park 2004). Thus, we hypothesized that the loss of AKH function might result in impaired glycogen mobilization, and consequently in higher body glycogen levels. However, similarly to lipid reserves, glycogen profiles of *Akh^A*, *Akh^{AP}*, and *Akhr¹* did not differ from the profiles of controls at any of the tested developmental stages (Figure 4B). We observed a nonsignificant trend toward lower glycogen storage in *Akh^A*, *Akh^{AP}*, and *Akhr¹* mutants at the 3L and P0 stages (Figure 4B). Lowered glycogen storage reached statistical significance if data from different developmental time points were analyzed together, with the genotype and developmental stage as fixed effects (see statistical analyzes in Figure 4B). However, despite the trend toward lower glycogen storage at the onset of metamorphosis, the following mobilization of glycogen was not affected, and there was no interaction between the effect of genotype and developmental stage (see statistical analyzes in Figure 4B).

Thus, the above-presented experiments show that in *Drosophila*, AKH pathway and APRP are not required for the

nals are labeled with monoisotopic masses. Signal intensities were scaled (100%) to control fly pQLTFSPDWGK-OH [M+H]⁺ signal (1161.6 *m/z*) in A and to the dimer [M+H]⁺ signal (10292.0 *m/z*) in B. Note that peak labels annotated in A and B are bold in C.

Figure 2 Characterization of AKH single and AKH plus APRP double mutants by mass spectrometry. Comparison of recorded MALDI-TOF mass spectra of single retrocerebral complex preparations from *Akh*⁺ (black) control, *Akh* mutants (*Akh*^A in blue, *Akh*^{AP} in red, *Akh*^{SAP} in green) and *Akh* RNAi knockdown (gray) flies in the mass ranges *m/z* 700–1340 (A) and *m/z* 10,220–10,320 (B). In the control flies, the full set of *Akh* gene products (black labels) was detected (see panel C and Figure 1). Deletion of the codons for the two C-terminal AKH amino acids DW in *Akh*^A mutants resulted in truncated AKH products (blue labels; pQLTFSPa, 696.3 [M+Na]⁺; pQLTFSPGK-OH, 860.4 [M+H]⁺, 882.3 [M+Na]⁺; pQLTFSPGKR-OH, 1016.4 [M+H]⁺), leaving the APRP sequence unaffected. *Akh*^{AP} flies, which carry a deletion causing a frame shift mutation, and *Akh*^{SAP} flies, which miss the signal peptide coding for the sequence including the *Akh* translation start codon, showed no ion signals for putative *Akh* translation products in all analyzed ranges. Also *Akh* RNAi flies lack the AKH and the peptides. Ion signals from neuropeptides of other genes (pink peak labels) were unaffected in all genotypes analyzed and served as marker (*Dm*-sNPF-14–11/sNPF-2^{12–19}, 974.6 [M+H]⁺; *Dm*-sNPF-3, 982.6 [M+H]⁺; *Dm*-sNPF-4, 985.6 [M+H]⁺; *Dm*-MS 1247.7, [M+H]⁺; *Dm*-sNPF-1, 1329.8 [M+H]⁺). All spectra were recorded in reflectron positive mode and all ion sig-

dynamic changes in storage lipids and carbohydrates, which are characteristic for metamorphosis.

Deficiency in AKH signaling results in adult-onset obesity and hypoglycemia

After the analyses of the roles of AKH signaling and APRP during metamorphosis, we focused on their potential metabolic functions at the adult stage of *Drosophila*. Interestingly, *Akh*^A, *Akh*^{AP}, and *AkhR*¹ mutants already developed adult-specific obesity within the first week after eclosion (Figure 5A). Thin layer chromatography confirmed that among the neutral lipids, storage TGs were particularly increased in the obese AKH signaling mutants (Figure 5B). When examining the age-dependent changes in the fat content during the first week of adult life (the first day after eclosion vs. 1 week later), we noticed that the lipid content of controls dramatically decreased (Figure 4A and Figure 5A). This drop in storage lipids corresponds to the histolysis of the larval/pupal fat body cells during and shortly after the metamorphosis and their functional replacement by the adult fat body cells (Nelliot *et al.* 2006). In contrast to the control flies, lipid content of mature *Akh*^A, *Akh*^{AP}, and *AkhR*¹ mutants remained at the posteclosion levels (Figure 4A and Figure 5A). This observation raised the question of whether the obesity of the AKH signaling mutants is based on defective clearance of larval/pupal fat body cells or on excessive lipid loading of adult fat body cells. These types of fat body are morphologically distinguishable, and thus we examined the fat body composition of 1-week-old mutants. However, dissection, staining, and confocal imaging of fat body suggests that the obesity resulted from increased cellular lipid loading of adult

fat body cells (Figure 5, C and D). Altogether our data showed that AKH deficiency results in adult-onset obesity coupled with adult fat body cell hypertrophy.

Next, we tested whether the stored carbohydrates, *i.e.*, glycogen, also increased in response to the loss of AKH signaling. However, this was not the case. On the contrary, we observed a nonsignificant trend toward decreased glycogen values in all *Akh*^A, *Akh*^{AP}, and *AkhR*¹ mutants (Figure 5D). Next to the stored carbohydrates, we tested also the free circulating sugars (trehalose and glucose). When analyzing whole body samples, we were not able to detect any significant difference between the mutants and controls (data not shown), whereas hemolymph samples revealed a significant hypoglycemia in all *Akh*^A, *Akh*^{AP}, and *AkhR*¹ mutants (Figure 5E).

Altogether, these data show that AKH signaling fulfills important functions in the homeostasis of stored lipids and of circulating, but not stored carbohydrates in mature adult *Drosophila* flies.

AKH signaling and APRP are dispensable for spontaneous locomotor activity, startle-induced climbing, and flight performance

Mobilization of energy reserves to sustain locomotion is one of the main general functions of AKHs. However, when testing spontaneous locomotion in the absence of AKH, APRP, and AKHR over a 1-week period of time, we did not find any significant reduction of the locomotion in the obese and hypoglycemic *Akh*^A, *Akh*^{AP}, and *AkhR*¹ mutants (Figure 6A). Next, we addressed a potential role of AKH signaling or APRP in forced locomotion using a startle-induced climbing

Figure 3 AKH, APRP, and AKHR are dispensable for preadult development. Plotted are means $\pm$ SEM. (A) AKH, APRP, and AKHR are not required for embryogenesis. Slightly, but significantly decreased hatchability of *Akh^A* as compared to controls; *Akh^{AP}* and *AkhR¹* had normal hatchability (one-way ANOVA, $F_{3,105} = 8.65$, $P < 0.001$; Tukey's HSD: $P < 0.05$). (B) AKH, APRP, and AKHR are not necessary for larval to adult survival; no significant difference between any of the mutants when compared to control (one-way ANOVA, $F_{3,101} = 3.86$, $P < 0.05$; Tukey's HSD: $P < 0.05$). (C) Developmental rate (measured as time span from egg laying to adult eclosion) was not extended in AKH single mutants nor AKH plus APRP double mutants nor *AkhR* mutants. On the opposite, deficiency in the AKH signaling slightly increased the speed of development; in the case of *Akh^{AP}* and *AkhR¹*, this effect reached statistical significance (one-way ANOVA, $F_{3,585} = 71.44$, $P < 0.001$; Tukey's HSD: $P < 0.05$). (D) AKH, APRP, and AKHR are all dispensable for female fecundity. No difference in egg laying of the mutants compared to the genetic control (one-way ANOVA, $F_{3,107} = 1.8$, $P = 0.15$). (E) AKH, APRP, and AKHR are all dispensable for regulation of body size. No difference in the thorax length between mutants and genetic control is seen (one-way ANOVA, $F_{3,103} = 1.94$, $P = 0.13$). Plotted data were obtained on female flies.

paradigm. Climbing of *Akh^{AP}* and *AkhR¹* mutants was indistinguishable from controls, while the *Akh^A* flies showed reduced climbing performance (Figure 6B). However, this effect was unlikely caused by the absence of AKH signaling, as the receptor mutant and AKH plus APRP double mutants climbed normally. Loss of AKH signaling and APRP also did not affect flight performance (Figure 6C).

AKH signaling contributes to the mobilization of lipids under starvation

Mobilization of energy reserves in periods of negative energy balance is the main function of AKH hormones. We used extended food deprivation to address the energy mobilization capacity of flies lacking AKH, APRP, or AKHR. Consistent with their higher body fat content, *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants were all more starvation resistant than controls (Figure 7A). Monitoring of lipid and carbohydrate stores during starvation revealed that all three mutants mobilized both storage energy sources (Figure 7, B and C). However, analysis of the interactions between the genotype and the starvation-dependent lipid changes revealed a significant interaction between these two factors (Figure 7B), suggesting that the lack of AKH function modulates the lipid mobilization profile.

In contrast to the controls, *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants were not able to mobilize their lipid reserves completely, and thus died with higher residual lipid content (Figure 7B).

In contrast to the storage lipids, the glycogen reserves of *ad libitum* fed *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutant flies were not increased. All mutants were able to mobilize glycogen stores at a rate comparable with controls (Figure 7C). This suggests that increased starvation survival of *Akh^A*, *Akh^{AP}*, and *AkhR¹* was predominantly driven by the increased lipid reserves. Next, we tested whether potential changes in locomotion under starvation might contribute to the differential survival of the *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants.

AKH signaling promotes starvation-induced hyperactivity

Locomotion adds to the negative energy balance during starvation and therefore reduces the survival time under food deprivation. Hence, we aimed to test whether the lack of AKH function contributes to starvation resistance of *Akh^A*, *Akh^{AP}*, and *AkhR¹* via inducing hypoactivity under nutritional shortage. Indeed, total starvation lifetime locomotion of long-lived *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutant flies was unchanged compared to the short-lived controls (Figure 7D). Accordingly,

(one-way ANOVA, $F_{3,16} = 2.57$, $P = 0.09$). Note the statistically nonsignificant trend toward lower glycogen levels in all AKH signaling mutants compared to the controls. Strong glycogen mobilization during metamorphosis is observed; no interaction between the genotype and tested developmental stages is observed (two-way ANOVA, genotype and developmental stage as fixed effects, genotype: $F_{3,66} = 2.94$, $P = 0.04$, developmental stage: $F_{3,66} = 119.9$, $P < 0.001$, genotype $\times$ developmental stage: $F_{9,66} = 1.86$, $P = 0.07$). (B) Glycogen content. No significant difference is observed in the glycogen content in the mutants at the stage of wandering third instar larvae (3L) (one-way ANOVA, $F_{3,20} = 3.67$, $P = 0.03$), or at the beginning of metamorphosis at P0 (one-way ANOVA, $F_{3,20} = 2.94$, $P = 0.06$), or at the end of pupal development at P13–P15 (one-way ANOVA, $F_{3,16} = 2.56$, $P = 0.43$). Note the nonsignificant trend toward increased lipid content in the *Akh* and *Akh^R* mutants at the first day after adult eclosion (one-way ANOVA, $F_{3,16} = 3.28$, $P = 0.05$). Strong lipid mobilization is observed in all genotypes during metamorphosis; no significant interaction is observed between the genotype and the effect of the developmental stages (two-way ANOVA, genotype and developmental stage as fixed effects, genotype: $F_{3,66} = 2.94$, $P = 0.04$, developmental stage: $F_{3,66} = 119.9$, $P < 0.001$, genotype $\times$ developmental stage: $F_{9,66} = 1.86$, $P = 0.07$).

the average locomotion per hour of starvation lifetime was reduced in the absence of AKH signaling.

As described previously, locomotion of starved control flies shortly before death typically exceeded the activity of *ad libitum*-fed siblings, reflecting a behavioral strategy of flies during extended food deprivation, which is interpreted as food-seeking behavior. This starvation-induced hyperactivity was abolished in CC-ablated flies (Lee and Park 2004), suggesting that AKH, APRP, or another factor produced in these cells is required for the process. By visual inspection of the locomotory patterns of individual flies, we noted that the starvation-induced hyperactivity was suppressed in all *Akh^A*, *Akh^{AP}*, and *Akh^{R1}* mutants (Figure 7E). Quantification of the mean activity of individual flies during their last 12 hr of starvation survival confirmed the dramatic decrease of locomotion of *Akh^A*, *Akh^{AP}*, and *Akh^{R1}* compared to controls (Figure 7F). We did not observe any significant differences between the AKH- and AKHR-deficient mutants (Figure 7F).

Taken together, these data show that AKH is necessary for the starvation-induced hyperactivity and that the AKH signal is transduced via the canonical AKHR receptor. Since hypoactivity is an energy-saving strategy, it might contribute to the starvation resistance of the *Akh^A*, *Akh^{AP}*, and *Akh^{R1}* mutants.

AKH signaling confers oxidative stress resistance

Food deprivation is one form of metabolic stress commonly experienced by *Drosophila* in natural environments. AKH has also been implicated in coping with other forms of stress conditions such as oxidative stress (e.g., Kodrík *et al.* 2007;

Večeřa *et al.* 2007). Exposure to foodborne paraquat showed apparently increased oxidative stress resistance of *Akh^A*, *Akh^{AP}*, and *Akh^{R1}* mutants (Figure S4). However, a food-intake assay revealed increased paraquat-induced food aversion of *Akh^A*, *Akh^{AP}*, and *Akh^{R1}* mutants (Figure 8A). As reduced paraquat intake could be causative for the observed apparent oxidative stress resistance of *Akh* signaling mutants, we switched to application of paraquat directly on the nerve cord. This food intake independent drug application revealed increased paraquat sensitivity of AKH-deficient flies (Figure 8B), suggesting a protective role of AKH in coping with oxidative stress.

Taken together, our data on nutritional and oxidative stress resistance suggest that AKH orchestrates metabolic changes in flies challenged with environmental stress factors.

The *Akh* gene is controlled by negative autoregulation

Homeostatic modulation of metabolism in response to changing environments requires prompt feedback regulation. Endocrine systems in general involve negative feedback loops controlling the hormone production. However, nothing is known about autoregulation of AKH levels. Glucagon, the mammalian homolog of AKH, negatively regulates its own production as revealed by compensatory overproliferation of glucagon-positive pancreatic alpha cells (Gelling *et al.* 2003). To test whether AKH acts in an analogous manner, we visualized the CC cells of *Akh⁺*, *Akh^A*, and *Akh^{AP}* flies by expressing GFP under indirect control of the AKH promoter (*akhp*-Gal4 > UAS-mCD8 GFP). The number of CC cells did not differ in any

Figure 5 Adult-onset obesity and hypoglycemia in AKH signaling mutants. Shown are carbohydrate and lipid levels in 7-day-old AKH single, AKH plus APRP double, and AKHR mutants. Plotted are means $\pm$ SEM. (A) Deficiency in AKH signaling resulted in adult-onset obesity; magnitude of the phenotype was the same for *Akh^A*, *Akh^{AP}*, and *AkhR¹* (one-way ANOVA, $F_{3,20} = 23.84$, $P < 0.001$; Tukey's HSD: $P < 0.05$). (B) TLC analysis illustrated that the *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants predominately accumulate triglycerides (TGs) (FA, fatty acids; DG, diacylglyceride; MG, monoacylglyceride; S, standard. Note: As indicated by the dashed line between the control and *AkhR¹* lanes, an unrelated sample has been removed from the TLC plate image). (C) Fat body cell hypertrophy in AKH signaling mutants as illustrated by confocal imaging of increased cellular lipid loading. Lipid droplets are shown in green (Bodipy493/503), cell membranes in red (CellMask Deep Red), and nuclei in blue (DAPI). Bar, 25 μm. (D) Quantification of the lipid droplet (LD) area per fat body cell. Mann-Whitney test, $P < 0.001$, $n = 33$ cells (*Akh^A*) and $n = 29$ cells (control). (E) Non-significant decrease in glycogen levels in *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants compared to controls (one-way ANOVA, $F_{3,20} = 2.49$, $P = 0.091$). (F) Hypoglycemia of *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants as revealed by circulating sugar quantification (one-way ANOVA, $F_{3,8} = 9.97$; $P < 0.01$; Tukey's HSD: $P < 0.05$).

of the tested mutants (Figure 9A and Figure S5), suggesting that in contrast to its mammalian functional homolog, the insect hormone does not feed back to the cells of AKH origin via proliferation control. Interestingly, we observed a significant increase of GFP signal intensity in a subset of CC cells in AKH-deficient mutants (Figure 9, B and C), suggesting an increased *Akh* promoter activity in response to the lack of AKH. Consistently, the *Akh* messenger RNA (mRNA) abundance was increased in the *Akh^A* mutants (Figure 9D). These data provide first evidence that *Akh* is subjected to negative autoregulation at the mRNA level. Future studies will address how the AKH levels are sensed and which factors contribute to the proposed regulatory loop.

Discussion

AKH signaling has been initially described as a master regulator of insect energy mobilization in a broad range of biological contexts. This view has been shaped by numerous elegant studies on the effects of ectopic application of AKH and on correlations between the hormone titer and insect physiology (metabolism, developmental stage, behavior) in diverse insect species (reviewed in Vroemen *et al.* 1998; Van der Horst *et al.* 2001; Gäde and Auerswald 2003; Van der Horst 2003; Lorenz and Gäde 2009). However, data on the conse-

quences of selective impairment of AKH function are scarce. Here, we conducted the first comparative loss-of-function analysis of *Drosophila* AKH mutants and AKH plus APRP double mutants. These specific mutants, together with the AKH receptor mutant in the same genetic background, allowed testing of developmental, reproduction-, stress-, and metabolism-related functions of AKH signaling, its dependency on the *AkhR*, as well as addressing the putative hormonal roles of the functionally enigmatic APRP. We discuss below our main results in the context of general predictions of AKH roles in insects, which have so far been based mainly on AKH studies in nondrosophilid species.

Developmental roles of AKH signaling

Insect embryogenesis, as a nonfeeding stage, is dependent on mobilization of maternally supplied lipid reserves (Beenakkers *et al.* 1985; Arrese and Soulages 2010). Intriguingly, *Drosophila Akh* and *AkhR* are already expressed at embryogenesis (Kim and Rulifson 2004; Lee and Park 2004; Grönke *et al.* 2007), suggesting that AKH might have biological functions in energy mobilization from early developmental stages onwards. However, our data show that AKH, APRP, and AKHR are dispensable for embryonic development.

Next to embryogenesis, nonfeeding periods of insect life cycle also include moltings and metamorphosis. Metamorphosis

Figure 6 No requirement of AKH signaling and APRP for spontaneous locomotor activity, or for startle-induced vertical climbing, or for flight performance. (A) No spontaneous locomotion defects of *Akh^A*, *Akh^{AP}*, or *Akh^{R1}* compared to controls as revealed by cumulative activity monitoring for 1 week using the DAM2 system (one-way ANOVA, $F_{3,29} = 10.11$, $P = 0.04$; Tukey's HSD: $P < 0.05$). (B) Reduced startle-induced climbing ability of *Akh^A* but not *Akh^{AP}* or *Akh^{R1}* compared to controls (one-way ANOVA, $F_{3,29} = 10.11$, $P < 0.001$; Tukey's HSD: $P < 0.05$). (C) No defects in flight performance of *Akh^A*, *Akh^{AP}*, or *Akh^{R1}* (one-way ANOVA, $F_{3,123} = 1.28$, $P = 0.29$).

involves rebuilding of larval structures into adult body, and this process completely depends on oxidizing energy stores accumulated during the larval period (Agrell and Lundquist 1973). A general role of AKH in this process has been predicted based on, e.g., experimental evidence that AKH injections cause differential glycogen or lipid mobilization in a developmental stage-specific manner in *Manduca sexta* (Gäde and Beenackers 1977) or *Zophobas atratus* (Slocinska *et al.* 2013; Gołębowski *et al.* 2014). Preference for lipids or glycogen as energy sources and dynamics of their usage during metamorphosis vary considerably among species (Nestel *et al.* 2003; Dutra *et al.* 2007). Similarly, as recently described by Matsuda *et al.* (2015), we also noticed that metamorphosis of *Drosophila* is fueled to a considerable extent by glycogen, but lipids are mobilized as well. We monitored the effect of AKH signaling deficiency upon glycogen and lipid levels at several time points before and during *Drosophila* metamorphosis to detect potential differences in the dynamics of lipid and glycogen mobilization in the absence of AKH signaling or APRP. Consistent with the previously described absence of any effects of CC ablation on larval lipids (Lee and Park 2004), we did not detect significant changes in the larval lipid content in any of the *Akh^A*, *Akh^{AP}*, and *Akh^{R1}* mutants. Larval glycogen values also did not statistically differ from controls, meaning that AKH deficiency did not affect starting levels of energy sources at the onset of metamorphosis. Measurements of glycogen and lipids throughout metamorphosis excluded any differences in the energy mobilization of the tested mutants. Thus, *Drosophila* AKH signaling is dispensable for proper accumula-

tion of energy stores at larval stage, as well as for their mobilization during metamorphosis. Consistent with the lack of metabolic phenotypes during metamorphosis, larval to adult survival of flies deficient in AKH signaling did not differ from the genetically matched controls. Thus, similarly to its mammalian functional homolog glucagon (Gelling *et al.* 2003), AKH is dispensable for developmental functions. Nevertheless, it is possible that other pathways compensate for absence of AKH, thus obscuring the detection of AKH-related developmental functions in the mutants. A prime candidate for such an alternative lipid mobilization pathway involves the Brummer lipase, as *bmm* mutants are embryonic lethal (Grönke *et al.* 2005), and the gene is known to work in parallel with *AkhR* in starvation-induced storage fat mobilization in *Drosophila* adults (Grönke *et al.* 2007). Alternatively, AKH signaling might instead have fine-tuning functions that would become obvious only under particular suboptimal conditions. Our study was conducted under a protected laboratory environment, with controlled diet, temperature, animal density, etc. However, development is highly plastic, and life history traits like developmental time, body size, fecundity, and viability are sensitive to environmental changes. AKH signaling was repeatedly connected with stress responses (Bednářová *et al.* 2013a), and thus, it might also play some context-dependent roles in adjusting metabolism and speed of development during metamorphosis. This hypothesis argues favorably for the recent finding that the developmental time is extended in the *AkhR* mutants, when raised on a low-yeast diet (Kim and Neufeld 2015).

Figure 7 AKH signaling regulates the starvation response. (A) Increased starvation resistance of *Akh^A*, *Akh^{AP}*, and *AkhR¹* compared to control (log-rank test, *Akh^A* vs. control: $\chi^2 = 137.63$, $P < 0.001$; *Akh^{AP}* vs. control: $\chi^2 = 131.66$, $P < 0.001$; *AkhR¹* vs. control: $\chi^2 = 135.53$, $P < 0.001$; $n[Akh^A] = 82$, $n[Akh^{AP}] = 95$, $n[AkhR^1] = 105$, $n[control] = 110$). (B) Functional but impaired storage lipid mobilization of obese *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants as revealed by strong interaction between the genotype and starvation duration (two-way ANOVA, genotype and starvation time as fixed effects, genotype: $F_{3,100} = 237.7$, $P < 0.001$, starvation time: $F_{4,100} = 389.6$, $P < 0.001$, genotype $\times$ starvation time: $F_{12,100} = 14.31$, $P < 0.001$). Note that *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants did not mobilize lipid reserves completely in contrast to controls (total starvation, TS, one-way ANOVA, genotype as fixed effect, $F_{3,20} = 7.39$, $P < 0.05$; Tukey's HSD: $P < 0.05$). (C) Functional but impaired glycogen storage mobilization of *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants. Note that glycogen content did not differ from each other nor from the controls at any individual time point tested (one-way ANOVA, genotype as fixed effect, 0 hr: $F_{3,20} = 2.49$, $P = 0.09$; 8 hr: $F_{3,18} = 2.61$, $P = 0.08$; 16 hr: $F_{3,20} = 2.45$, $P = 0.09$; 24 hr: $F_{3,18} = 2.54$, $P = 0.09$), but AKH signaling deficiency had a significant effect on the glycogen starvation response (two-way ANOVA, genotype and starvation time as fixed effects, genotype: $F_{3,76} = 3.09$, $P = 0.03$, starvation time: $F_{3,76} = 152.8$, $P < 0.001$, genotype $\times$ starvation time point: $F_{9,76} = 2.44$, $P < 0.05$). (D) No difference in the starvation lifetime locomotor activity between all genotypes (one-way ANOVA, genotype as fixed effect, $F_{3,123} = 1.5$, $P = 0.22$). Note that since all mutants are starvation resistant (A), average locomotor activity per day is reduced compared to controls. (E) Representative figure showing the locomotor activity distribution in AKH-deficient and control flies during starvation (TS) compared to *ad libitum*-fed siblings (AL). Note the starvation-induced hyperactivity shortly before death of control flies when compared to *ad libitum* fed siblings. Starvation-induced hyperactivity is absent in *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants (last 12 hr of life are highlighted in black rectangle). *Akh^A* AL = mean locomotor activity of *Akh^A* siblings ($n = 32$) fed on standard medium. *Akh^A* TS = locomotor activity of representative individual *Akh^A* male on total starvation. Control AL = mean locomotor activity of control siblings ($n = 31$) fed *ad libitum* on standard medium. Control TS = locomotor activity of representative individual control male on total starvation. Bar below the graphs illustrates the 12-hr light (yellow)/12-hr dark (blue) cycle. (F) Quantitative analysis of the *pre mortem* locomotor activity supports the absence of starvation-induced hyperactivity in *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants (one-way ANOVA, genotype as fixed effect, $F_{3,122} = 47.78$, $P < 0.001$; Tukey's HSD: $P < 0.05$).

AKH signaling controls lipid homeostasis in adult *Drosophila*

Metabolic pathways governing the energy balance during preadult and adult development might differ from those maintaining this balance during adulthood. Several genes such as *inositol 1,4,5-tris-phosphate receptor Itp-r83A* (Subramanian *et al.* 2013) or *perilipin1* (Beller *et al.* 2010) act as antiobesity genes specifically at the adult stage of *Drosophila*. Here we show that this is also the case for AKH signaling. Obesity of *Akh^A* and *Akh^{AP}* mutants is in line with the previous reports on the effect of mutations in the AKH receptor (Grönke *et al.* 2007; Bharucha *et al.* 2008). Earlier data on the AKH roles in glycogen storage were rather contradictory, reporting both

no changes (Grönke *et al.* 2007), as well as significant increase in the body glycogen content (Bharucha *et al.* 2008) of AKH receptor mutants. In contrast, the role of AKH in circulating sugars in adults was not addressed previously. In larva, however, CC cell ablation was shown to cause hypoglycemia (Kim and Rulifson 2004; Lee and Park 2004; Isabel *et al.* 2005). We were not able to detect any significant differences in the free sugars when analyzing whole body samples; however, when hemolymph samples were analyzed, we observed significant hypoglycemia in *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants. This reduction of circulating sugars was not coupled with an increase in the stored carbohydrates. On the contrary, we observed the opposite trend toward lowered glycogen levels in *Akh^A*, *Akh^{AP}*,

Figure 8 AKH signaling regulates the oxidative stress response. (A) Significantly reduced intake of paraquat-supplemented food in *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants compared to controls challenges foodborne paraquat as a suitable measure to test oxidative stress resistance. Food intake was assayed using blue dye labeling of food supplemented with (+) or without (–) 20 mM paraquat for 4 hr prior to visual inspection of abdominal coloring. Fischer exact test, *Akh^A* vs. control: $P < 0.001$; *Akh^{AP}* vs. control: $P < 0.001$; *AkhR¹* vs. control: $P < 0.001$; no significant difference among *Akh^A*, *Akh^{AP}*, and *AkhR¹* ($n[Akh^A] = 66$, $n[Akh^{AP}] = 64$, $n[AkhR^1] = 70$, $n[control] = 62$). Note that differential food intake resulted from differential aversion to paraquat, as

there was no difference among the genotypes when fed on regular food (Fischer exact test, for all comparisons, $P > 0.05$, ($n[Akh^A] = 80$, $n[Akh^{AP}] = 81$, $n[AkhR^1] = 73$, $n[control] = 81$). (B) Direct application of paraquat to the nerve cord revealed oxidative stress sensitivity of *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants compared to controls. Fischer exact test, *Akh^A* vs. control: $P < 0.001$; *Akh^{AP}* vs. control: $P < 0.001$; *AkhR¹* vs. control: $P < 0.05$; *Akh^A* vs. *Akh^{AP}*: $P = 0.74$; *Akh^A* vs. *AkhR¹*: $P = 0.04$; *Akh^{AP}* vs. *AkhR¹*: $P = 0.12$ ($n[Akh^A] = 89$, $n[Akh^{AP}] = 93$, $n[AkhR^1] = 91$, $n[control] = 90$).

and *AkhR¹* mutants. Therefore, increased uptake of circulating sugars and their subsequent use for lipogenesis is one hypothesis on the etiology of *Akh^A*, *Akh^{AP}*, and *AkhR¹*-dependent obesity to be tested in the future.

AKH signaling is not required for *Drosophila* reproduction

Insect reproduction is an energetically demanding process, as females deposit a considerable amount of energy reserves into the developing oocytes. Mobilization of energy reserves for oogenesis was predicted to be AKH regulated (Lorenz and Gäde 2009). Consistently, AKH is required for reproduction of tsetse fly *Glossina morsitans* (Attardo *et al.* 2012), but on the contrary, AKH prevents vitellogenesis and egg production in the locust *Locusta migratoria* (Glinka *et al.* 1995) and the cricket *Gryllus bimaculatus* (Lorenz 2003). Accordingly, relevance and mode of action of AKH signaling in insect oogenesis appears to vary considerably among species. This diversity of AKH functions is also reflected by the differential expression of the AKH receptor in ovaries. For example, AKHR is expressed in the ovaries of the mosquito *Aedes aegypti* (Kaufmann *et al.* 2009), but not in those of *Drosophila* (Grönke *et al.* 2007; Bharucha *et al.* 2008). In the current study, we did not detect any changes in fecundity in the absence of AKH signaling, suggesting that in *Drosophila*, oogenesis-dependent fat mobilization is under the control of an alternative lipolytic pathway. However, we cannot exclude that AKH plays a role in reproduction and vitellogenesis under natural conditions, which likely requires more metabolic adaptability to environmental changes.

Roles of AKH in locomotion

It is widely accepted that AKH has an important regulatory function in insect locomotion (Lorenz 2003; Van der Horst 2003; Lorenz and Gäde 2009). This view is supported by many studies describing correlations between the release of AKH and activities like flight and walking (Lorenz 2003), and on experimental increase of locomotion, such as walking of the firebug *Pyrrhocoris apterus*, by ectopic applications of

AKH (Kodrík *et al.* 2000, 2002). Ablation of CC cells in *Drosophila* decreased spontaneous locomotion (Isabel *et al.* 2005). However, this effect was likely caused by other factors produced in CC cells, as the *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants tested in this study had normal spontaneous locomotion. Thus, neither the AKH signaling deficiency nor the resulting obesity affected spontaneous movement. This suggests that either the locomotion-related roles of AKH signaling are not evolutionarily conserved or that the regulatory potential of AKH as demonstrated by gain-of-function studies is not exploited by *Drosophila* under laboratory environmental conditions.

AKH signaling, together with octopamine, was also hypothesized to act analogously to vertebrate adrenaline during the “flight or fight” reaction (Lorenz and Gäde 2009). However, we did not detect any defects in the startle-induced climbing of AKH signaling mutants. Thus, other pathways exist to ensure the energy supply for this kind of movement in *Drosophila*.

Roles of AKH in the starvation response

Periods of starvation are coupled with rapid mobilization of energy reserves (Arrese and Soulages 2010). Starvation-induced mobilization of lipids in *Rhodnius prolixus* has been recently shown to be dependent on the AKH receptor (Alves-Bezerra *et al.* 2015). Given the hyperglycemic and hyperlipaemic effects of AKH in adult insects subjected to negative energy balance (Gäde and Auerswald 2003; Van Der Horst 2003; Lorenz and Gäde 2009), we studied *Drosophila* AKH functions during starvation. *Akh^A*, *Akh^{AP}*, *AkhR¹* mutants were considerably more resistant to starvation than their genetically matched controls. Lipids were mobilized in all *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants, however, to a lower extent than in controls, resulting in higher residual lipids in flies starved to death. These data are consistent with the earlier finding that AKHR cooperates with a second lipolytic pathway involving the Brummer lipase to orchestrate starvation-induced storage lipid mobilization (Grönke *et al.* 2007). Nevertheless, in the context of starvation, this alternative lipolytic pathway

Figure 9 AKH deficiency does not affect corpora cardiaca cell number but reveals a negative autoregulatory loop on *Akh* transcription. Fluorescence tagging of CC cells under indirect control of the *Akh* promoter (*akhp-Gal4* > *UAS-mCD8 GFP*) detected no change in CC cell number between *Akh⁺* controls and in *Akh^A* mutants (A; two-tailed Student's *t*-test: $P = 0.34$; *Akh⁺* $n = 10$ flies, *Akh^A* $n = 9$ flies) but increased somatic GFP signal in a subset of CC cells (arrowheads in the maximum intensity projection in B, quantification in C; two-tailed Student's *t*-test: $P = 0.004$ *Akh⁺* $n = 160$ cells, *Akh^A* $n = 140$ cells). (D) Increased *Akh* mRNA abundance in *Akh^A* mutants compared to *Akh⁺* controls as revealed by qPCR (two-tailed Student's *t*-test: $P < 0.001$; $n = 3$ biological replicates per genotype).

compensates for AKH absence only to a limited extent, as lipid mobilization is not completed at the time of death.

In contrast to lipids, the glycogen-mobilization response of all *Akh^A*, *Akh^{AP}*, and *AkhR¹* mutants was similar to controls during the first 24 hr of starvation, when glycogen reserves became almost completely depleted. Accordingly, AKH signaling mutants survive a considerably long period without glycogen reserves, fueling their metabolism by stored lipids.

Prolonged starvation increases foraging behavior, which can be observed as increased locomotion that exceeds the activity of *ad libitum*-fed flies and overwrites the circadian activity pattern. In *Drosophila*, CC cell ablation suppresses this hyperactivity (Lee and Park 2004), consistent with the view that this behavior requires *Akh* gene products or other CC cell-produced factors. On the contrary, locomotion of *AkhR* mutants under starvation was described as identical to controls (Bharucha *et al.* 2008). In our study, both visual inspections of locomotory patterns of individual flies under starvation, as well as quantification of their total activity shortly before death revealed that in contrast to control flies, starvation failed to increase locomotion in *Akh^A* and *Akh^{AP}* but also *AkhR¹* mutants. Thus, starvation triggers increase in locomotion via AKH, and its signal is transduced via the canonical AKHR.

Altogether, starvation resistance of flies that are deficient in AKH signaling results from increased lipid reserves, but reduced energy expenditure due to absence of starvation-induced hyperactivity might contribute to the resistance as well. While the starvation resistance of obese AKH-deficient flies might be advantageous to survive periods of paucity even under natural environmental conditions, the failure to induce foraging behavior would likely have fatal consequences. Thus, under natural conditions, AKH signaling is likely required to

orchestrate adaptive responses to nutritional shortage, resulting in increased foraging.

Roles of AKH signaling in oxidative stress resistance

As documented by numerous studies on nondrosophilid insects, AKH plays a complex role in adaptation to oxidative stress. Ectopic applications of AKH to the linden bug *P. apterus* increased sensitivity to insecticide-triggered oxidative stress, such as to endosulfan, to malathion (Velki *et al.* 2011), and to permethrin (Kodrík *et al.* 2010). However, the AKH titer positively correlated with oxidative stress induced by bacterial toxin in *Leptinotarsa decemlineata* (Kodrík *et al.* 2007), and by paraquat in *P. apterus* (Večeřa *et al.* 2007) and *L. decemlineata* (Kodrík *et al.* 2007). Moreover, co-injection of AKH alleviated the effect of paraquat by enhancing the antioxidative capacity in the firebug (Večeřa *et al.* 2007), suggesting a protective role of AKH during oxidative stress response.

In *Drosophila*, foodborne paraquat exposure is the standard assay for testing oxidative stress resistance (Rzezniczak *et al.* 2011; Sun *et al.* 2013) and many fly studies successfully used this single agent (e.g., Alic *et al.* 2011; Barnes *et al.* 2014; Lin *et al.* 2014). Following this protocol, we observed increased paraquat resistance in all tested mutants deficient in AKH signaling. However, when analyzing the food intake during the experiment, we observed that AKH signaling mutants were hypophagic, when compared to controls fed on the same concentration of paraquat. Accordingly, reduced paraquat intake, not the oxidative stress resistance itself, might be causative for the extended survival time of the mutants exposed to foodborne paraquat. Indeed, AKH deficiency reduced paraquat resistance when differences in paraquat intake were bypassed by direct application of the drug

on the nerve cord. Consistent with the protective role of AKH in the context of paraquat resistance in firebug (Večeřa *et al.* 2007), and with positive regulation of antioxidant enzymes by AKH as recently described in *Drosophila* (Bednářová *et al.* 2015), our experiment argues for the conserved role of AKH signaling in antioxidant defense. The exact mode of action whereby AKH facilitates the adaptive response to oxidative challenge awaits further detailed studies.

Our experiment on paraquat feeding vs. paraquat application also showed that the very same stressor could have differential effects on the very same genotypes, depending on the mode of its application. This outlines the importance of reevaluation of oxidative stress resistance tests dependent on spontaneous feeding, in particular when animals with unequal energy reserves are tested.

The functionally orphan adipokinetic hormone precursor-related peptide

APRP is the second peptide being processed from the *Akh*-encoded prohormone with currently unknown biological function (Figure 1, A and B). Our data confirmed the presence of APRP dimers in the CC cells of *Drosophila*; however, flies lacking APRP revealed no physiological defects in any of the tested processes. In particular, *Akh^{AP}* were fully viable, had normal developmental timing, body size, and oogenesis, which argues against the discussed ecdysiotropic role of APRP (De Loof *et al.* 2009) in *Drosophila*. Moreover, APRP plus AKH double mutants were indistinguishable from AKH single and *Akhr* mutants in all of the tested metabolic phenotypes.

The lack of systemic functions of *Drosophila* APRP is in line with the missing metabolic effects in response to injections of APRPs from the lubber grasshopper *Romalea microptera* (Hatle and Spring 1999). Together with the recent finding of high sequence variability at certain positions of the APRP peptide in cockroaches (Sturm and Predel 2015) this supports the view that APRP might mainly or exclusively have scaffold peptide function as proposed by the model of AKH processing in *L. migratoria* (Baggerman *et al.* 2002). However, a comprehensive analysis of potential systemic APRP functions awaits the availability of an APRP-specific single mutant.

Autoregulation of the *Akh* gene

Glucagon, the functional homolog of AKH in mammals, autoregulates its own production via proliferation control of the pancreatic alpha cells (Gelling *et al.* 2003) and via regulation of their secretory activity (Ma *et al.* 2005; Cabrera *et al.* 2008). Our data show that unlike its mammalian homolog, AKH does not affect the number of CC cells. Nevertheless, CC cell numbers appear to be subject to environmental or genetic variation, as our study detected more adult CC cells (16 ± 0.15 SEM) than was described previously using the same technical approach (13 ± 0.6 SEM) (Lee and Park 2004).

Regulation of the circulating AKH titer at the level of secretory activity of CC cells under energy-demanding con-

ditions has been linked to the AMPK pathway (Braco *et al.* 2012). However, a role of AKH in AMPK activation, which would establish an autoregulatory loop, has not been addressed. Similarly, up-regulation of *Akh* on the transcriptional level in response to loss of insulin signaling has been described in *Drosophila* (Buch *et al.* 2008). But again, it is unclear whether this antagonistic regulation involves an autoregulatory mechanism. Here, we have shown that lack of AKH function increases *Akh* mRNA levels. Consistently, a GFP reporter under the indirect control of the *Akh* promoter showed the same regulatory response as did the endogenous *Akh* gene. These data provide the first evidence for a negative autoregulation of AKH, which is mediated by the *Akh* promoter. Future work will address the regulatory factor(s) acting on the *Akh* promoter, and the biological relevance of the predicted negative autoregulation of the *Akh* gene.

Conclusions

AKH signaling is considered to be a master regulator of energy mobilization in insects in various biological contexts, including development, reproduction, locomotion, and stress response. Whereas many of these functions were derived from AKH gain-of-function and correlation studies, here we addressed for the first time AKH *in vivo* functions using *Drosophila* AKH and AKH plus APRP double mutants. We showed that AKH signaling is dispensable for energy mobilization during the preadult stages of ontogenesis, but this pathway is of importance for storage lipid homeostasis in *Drosophila* adults. *Ad libitum*-fed AKH-deficient flies are obese and hypoglycemic, suggesting that lipid accumulation might result from increased cellular uptake of circulating sugars and enhanced lipogenesis in the fat body. Under food deprivation, AKH signaling contributes to lipid mobilization and induces starvation-induced hyperactivity, which likely reflects foraging behavior. We also provide evidence that AKH signaling confers oxidative stress resistance. Our study did not find any phenotype that could be attributed to the lack of APRP, arguing against its endocrine role in all of the tested processes. Comparison between the effects of *Akh^A* vs. *Akhr¹* showed that the metabolic phenotypes of AKH are transduced via the canonical AKHR receptor. Surprisingly, several vital energy-demanding processes, such as locomotion, reproduction, and lipid and glycogen mobilization during preadult development are independent of AKH signaling. These results could be explained by evolutionary divergence of energy mobilization pathways in insects, reflecting the variability in insect life histories connected with differential preference for fueling energy stores as lipids, glycogens, or proteins. The rapid advance of genome engineering technologies, such as the CRISPR/Cas9 system used in this study, will hopefully result in AKH-specific mutants in a wider range of insect species, thus contributing to better understanding of the physiological functions of this ancient hormone system and their diversification during insect evolution.

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Note added in proof: While this manuscript was in preparation, Sajwan *et al.* (2015) published an independent *Drosophila* *Akh* mutant (called *Akh*¹), which lacks the leucine at position 2 of the mature AKH octapeptide. Consistent with our findings *Akh*¹ mutants are viable and *Akh*¹ virgin female flies are starvation resistant. Moreover, the authors report a reduction of free carbohydrates in *Akh*¹ mutant larvae and a reduction in the CO₂ production in adult *Akh*¹ mutants. These parameters have not been addressed in the present study. In contrast to our finding that the body size of *Akh*^A or *Akh*^{AP} mutants is identical to the size of genetically matched controls, Sajwan *et al.* (2015) report that *Akh*¹ mutant flies have increased body mass.

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Energy Homeostasis Control in *Drosophila* Adipokinetic Hormone Mutants

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SUPPLEMENTARY MATERIAL AND METHODS

Creation of *Akh* gRNA transgenic flies: A gRNA target site in the mature AKH coding sequence was identified using the target prediction tool available on www.shigen.nig.ac.jp/fly/nigfly/cas9. For construction of the *Akh*-targeting transgene construct (pBFv.U6.2-*Akh*; MG457), oligonucleotides 5` CTTCGTTGACCTTCTCGCCGGATT 3` (RKO895) and 5` AAACAATCCGGCGAGAAGGTCAAC 3` (RKO896) were annealed and the resulting gRNA-coding DNA was cloned via *Bbs*I into the pBFv.U6.2 vector (KONDO and UEDA 2013). The pBFv.U6.2-*Akh* plasmid was injected into the attP40 strain ($y^1 w^{67c23}; P\{CaryP\}attP40$) by BestGene, the F1 generation was crossed to $y^1 sc^* v^1$; *In(2LR)Gla*, *wg^{Gla-1} Bc¹* / CyO (BDSC35781), and the following transgenic line with ubiquitous expression of *Akh* gRNA was generated by BestGene: $y^1 sc^* v^1; P\{v^*; BFv-U6.2-Akh.gRNA\}attP40$.

Generation of the *Akh* mutant flies: For the induction of *Akh* mutations, $y^1 sc^* v^1; P\{v^*; BFv-U6.2-Akh.gRNA\}attP40$ was crossed to $P\{ry^{+7.2}=hsFLP\}1, y^1 w^{1118}; P\{y^{+7.7} w^{+mC}=UAS-Cas9.P\}attP2, P\{w^{+mC}=GAL4::VP16-nos.UTR\}CG6325^{MVD1}$ to generate founder males that expressed both CAS9 and *Akh* gRNA in the germline. Founder males were crossed to $w^*; Kr^{f1} / CyO; D^1 / TM3, Ser^1$ females. Male *Akh* candidate mutants were subsequently selected for the presence of both balancers and against the presence of w^{+mC} (genotype: $w^*; + / CyO; Akh^{*2} / TM3, Ser^1$). Selection for the absence of w^{+mC} guaranteed absence of *nos*-GAL4 and *UAS*-CAS9 constructs, whereas selection for the presence of CyO ensured absence of the *Akh* gRNA in the male genome. These males were mated to the $w^*; TM3, Sb^1, P\{2xTb1-RFP\}TM3/In(3L) D^1$ females in a single male crosses, and after mating, candidate mutant males were individually genotyped for the presence of the mutations within the *Akh* region by T7 endonuclease assay.

T7 endonuclease assay: Primers 5`TGTACATGTCCCCAGTCGGA3` (MGO921) and 5` CTATCTACTCGCGGTGCTT3` (MGO922) were used to PCR-amplify *Akh* coding region of heterozygous (over TM3 balancer) *Akh* candidate mutant males. PCR amplification of *Akh* mutations resulted in heteroduplex formation, which was detected by T7 endonuclease assay (New England Biolabs). The assays were done with 2 µl of the PCR reaction in a 10 µl reaction volume (NEBuffer2), with incubation time 15 min at 37°C. Cleavage products were subsequently analyzed by agarose gel electrophoresis.

Creation of *Akh^A*, *Akh^{AP}* and *Akh^{SAP}* stable stocks and backcrossing of the mutant alleles to a common genetic background: The following primer pairs were used to track the mutations during the backcrossing:

5`ACCTTCTCGCCGGGCAAG3` (MGO944) and 5`ATTGGCACGATCGGTTGGGT3` (MGO945) for *Akh^A*,

5'ATTACCAATCGTGGCTCGCA3' (MGO946) and 5'CACCGAACGCTTGTCAGCT3' (MGO947) for *Akh^{AP}*,
5'TGTACATGTCCCCAGTCGGA3' (MGO921) and 5'GCTATCTACTCGCGGTGCTT3' (MGO922) for *Akh^{SAP}* and
5'ACGCATTGAGGTGTATAGTCC3' (RKO377) and 5'TCAATCCCGAAACATGCTTAC 3' (RKO438) for *Akh^{R1}*.

Wing area measurement

Wing area of female flies used for the fecundity assay was measured using a Zeiss Axiophot microscope equipped with a digital camera AxioCam HRc and ZEN 2011 software. The left wing of each female was removed and fixed between two microscope slides. Wing outlines were traced as described by Klepsatel and colleagues (KLEPSATEL *et al.* 2013) and wing area was calculated using ZEN 2011 software. Wing area of 25 to 26 females was measured per genotype. Data were analyzed by one-way ANOVA.

Lipid determination by coupled colorimetric assay

Lipid measurements were done as described in Hildebrandt *et al.* (HILDEBRANDT *et al.* 2011). Lipid data were normalized to protein content. For each genotype, 4-6 replicas of 5 flies each were tested per developmental stage or starvation time point. Experiments were repeated at least three times. Lipid data were normalized to protein levels. Data were analyzed by one-way ANOVA to test the effect of genotype, or by two-way ANOVA to test the effects of genotype and starvation exposure or developmental time point, and their interactions.

Glycogen determination

Glycogen measurements in the 96 well plate format were based on the conversion of glycogen to glucose by amyloglucosidase (Sigma) and on its subsequent measurement by the glucose assay (GO) kit (Sigma) as described in Tennessen *et al.* (TENNESSEN *et al.* 2014). Fly homogenates were diluted with 0.05% Tween-20, in order to retain the sugar amounts within the linear range of the assay (0 to 0.16 µg of glycogen or glucose per µl of the analyzed homogenate). Each reaction was run with 30 µl of the fly homogenate. For each sample blank absorbance measurements at 540 nm were performed on two 30µl aliquots of diluted homogenates to which 100 µl of the GO reagent supplemented with 1 µl of amyloglucosidase (for total glucose determination) or no amyloglucosidase (for free glucose determination) was added. Samples were incubated for 30 min at 37°C with mild shaking before the reaction was stopped by adding 100 µl of 12N H₂SO₄. Final absorbance was measured at 540 nm. Initial absorbance of the homogenates was subtracted from the final absorbance, and glycogen and glucose levels were determined based on the absorbance of glucose and bovine liver glycogen standards (both from Sigma). The glycogen content was calculated from the difference between the total and the free glucose values. Obtained glycogen data were normalized to the protein content. For each genotype, 4-6 replicas of 5 flies

each were tested per developmental stage or starvation time point. Experiments were repeated at least three times. Data were analyzed by one-way ANOVA followed by Tukey's HSD post-hoc test to detect the effect of genotype, or by two-way ANOVA to test the effects of genotype and starvation exposure or developmental time point, and their interactions.

Determination of circulating sugars

Hemolymph samples (three replicates of 30 flies each per genotype) were collected by centrifugation (6 min, 9.000 rcf at 4°C) of decapitated flies in a holder tube (0.2 ml tube with 5 holes of 0.6 mm diameter) placed in a 0.5 ml collection tube. One µl of the collected hemolymph was diluted with 99 µl of 0.05% Tween-20 and immediately heat inactivated at 70°C for 5 min. The homogenate was further diluted 1:6 prior to the sugar measurements. Measurements of circulating sugars were performed using a modification of the Tennesen method (TENNESSEN *et al.* 2014). Briefly, the assay was based on conversion of trehalose to glucose by porcine trehalase (Sigma), and subsequent measurement of glucose by the glucose assay (GO) kit (Sigma). Measurements were done in 96 well plate format. Per sample, 30 µl of the diluted homogenate was used. First, absorbance of the samples and standards was measured at 540 nm, then 100 µl of the GO reagent (pH adjusted to 6.8 by 1M phosphoric acid) + 0.3 µl of porcine trehalase (Sigma, T8778-1U) were added. Samples were incubated overnight at 37°C with mild shaking. The reaction was stopped by adding 100 µl of 12N H₂SO₄. Samples were mixed and final OD was measured at 540 nm. Initial absorbance of the homogenates was subtracted from the final absorbance, and sugar levels were determined based on the standard curves. The experiment was repeated three times. Data were expressed as circulating sugars per 1 µl of hemolymph and analyzed by one-way ANOVA followed by Tukey's HSD post-hoc test.

Thin layer chromatography (TLC)

The TLC analysis was performed as described by Baumbach et al (BAUMBACH *et al.* 2014a), with minor modifications. In detail, lipids were extracted according to Bligh and Dyer (BLIGH and DYER 1959). Two biological replicates of five flies each were homogenized in 150 µl methanol, 75 µl chloroform and 60 µl water by ten 1.4 mm ceramic cylinders (Pqclab) using a Pqclab Precellys 24 instrument (10 sec, 5000 rpm). Samples were incubated for one hour in a water bath at 37°C. Afterwards, first 75 µl chloroform and then 75 µl 1M KCl were added and vigorously mixed. Separation of phases was achieved by centrifugation (1000 x g; 2 min) and the chloroform phase was transferred into a new 1.5 ml tube. Solvent was evaporated in a SpeedVac concentrator. Lipid pellets were resuspended in 100 µl chloroform / methanol (1:1) for the control genotype. The re-suspension volume for the *Akh^A*, *Akh^{AP}* and *AkhR¹* was adjusted to the equivalent protein amount of the control.

Fly protein content of all genotypes was determined by the protein assay (see section 2.10) using independent sibling flies. Finally, 20 µl of the mix was separated on high performance thin layer chromatography (HPTLC) plates (Merck, 105633) using n-hexane / diethylether / acetic acid (70:30:1, v/v/v; Merck) for unpolar lipids (TG, DG, MG and FA). As lipid standard, we used 40 µg of glyceryltriolate, 40 µg of 1,3-diolein, 40 µg of 1,2-dioleoyl-*rac*-glycerol, 40 µg of monoolein (all as mix in SUPELCO Mono-, Di-, Triglyceride Mix, SIGMA 1787-1AMP) supplemented with 4 µg oleic acid (FA; CALBIOCHEM #4954). Plates were air-dried, immersed in 8% (w/v) H₃PO₄ containing 10% (w/v) copper (II) sulfate pentahydrate, charred for 5 min at 180 °C and imaged on a Canon LiDE220 scanner.

Paraquat resistance assay - application of paraquat on the nerve cord

The assay was done according to Cassar and colleagues (CASSAR *et al.* 2015) with minor modifications. The assay is based on survival of flies after direct exposure to paraquat applied on the nerve cord of decapitated flies. In accordance to the literature our pilot experiment showed that decapitation and application of the vehicle on the nerve cord does not affect viability of flies for at least 24 h after decapitation, given that flies are housed in humid chambers (data not shown). Paraquat resistance was assayed by dipping the neck of cold anesthetized decapitated flies into PBS with or without 40mM paraquat, housing the decapitated flies in humid chambers at 25°C, and recording the survival rate 5 h after the paraquat application. Flies that did not recover from cold anesthesia were excluded from the analysis. Statistical significance of differences in the survival among the genotypes was tested by two-tailed Fischer exact test.

Startle-induced vertical climbing

The climbing assay is based on the 'countercurrent distribution' method described by Benzer (BENZER 1967) with modifications. Flies were CO₂-anesthetised and sorted not later than 24 h prior to the assay. The climbing assay device consisted of two detachable tubes (upper and lower tube; Polystyrene, 17 x 95 mm) connected by a funnel in vertical head-to-head position (50 x 40 mm part was attached to bottom tube, stem part 10 x 12 mm, was connected to the upper tube). Flies were transferred to the lower tube and the device assembled as described. After 30 sec of adaption, flies were tapped down to induce climbing. Flies that climbed into the upper tube within 60 sec (with every 15 sec gentle tapping for startle induction) were flipped into a new lower tube for another round of climbing as described. After six rounds of the assay, flies from the upper tube were transferred to a new tube, which was considered as 7th lower tube for the calculation of the climbing index (CI). Then, the number of flies in the lower tubes (number 1 to 7) was counted and the climbing index was calculated according to (SHCHERBATA *et al.* 2007):

$$CI = \frac{\sum_{n=1}^7 \text{number of flies in each lower tube} \times n}{\text{total number of flies in replica} \times 7}$$

Assay was done on ≥ 8 replicates per genotype per trial. Each replica consisted of 13 to 25 male 7-day-old flies. The climbing index of these replicas was used to calculate the averaged climbing index. The experiment was repeated at least twice. Statistical significance of the genotype effect on climbing ability was analyzed by one-way ANOVA followed by Tukey's HSD post-hoc test.

Flight performance assay

Flight performance analysis was based on the assay developed by Benzer (BENZER 1973) and modified by Babcock and Ganetzky (BABCOCK and GANETZKY 2014). Briefly, flight performance was measurement as landing position of flies ejected into the flight tester. Flight tester consisted of 45 cm long plastic cylinder with inner diameter of 9 cm, a short adapter connecting the cylinder to a plastic funnel, and of a 21 cm long drop tube (diameter 4.5 cm) attached to the funnel. The walls of the plastic cylinder were covered with paper sheet with sticky substance (beet syrup), that ensured capturing flies at their first attempt to land. Bottom of the cylinder was covered with a layer of water, in order to capture also the fraction of flies that did not start flying in the tester. An unplugged vial (diameter 3.5 cm) with at least 25 flies was dropped into the drop tube, and as the vial fell down and hit the funnel, flies were ejected into the flight tester. Measurement was based on the assumption that the attempt to land corresponds to the moment when the fly starts the flight. Flies were captured at their first attempt to land, as they stuck to the tester sheet of paper. After the trial, the paper sheet with flies was removed and photographed. Landing high was calculated as the distance from the bottom of the tester. Measurements were done in ImageJ. Assay was done on male flies of 4-5 days of age. The experiment was repeated twice. Quantitative data were analyzed by one-way ANOVA followed by Tukey's HSD post-hoc test.

Statistical analyses

Measurement variables were analyzed by two-tailed Student's t-test, by one-way analysis of variance (ANOVA) with genotype as fixed effect, followed by Tukey's HSD (honest significant difference) post-hoc tests, or by two-way ANOVA if the effect of genotype and other nominal category and their interactions were tested. The only exception was lipid droplet area measurement, where Mann-Whitney-test was used. Nominal variables were analyzed by two-tailed Fischer exact test, starvation survival data by log-rank test.

Hatchability and larva-to-adult survival data were arcsine square root transformed before performing one-way ANOVA. Developmental rate data were log transformed before performing one-way ANOVA.

Samples that did not differ significantly from each other ($P > 0.05$) were labeled with identical letter codes in the

figures; samples that differ significantly ($P < 0.05$) from each other were labeled with different letter codes. In case where only two genotypes were compared, P values were indicated by star symbols (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). In panels aiming to illustrate effects of two nominal variables (effect of genotype and effect of the developmental stage or starvation exposure), letter code was not used, and the reader is directed to consult the corresponding figure legend for F and P values.

Unless stated otherwise, data on measurement variables and survival (log rank tests) were analyzed using PAST (HAMMER *et al.* 2001): http://palaeo-electronica.org/2001_1/past/issue1_01.htm. Data on nominal variables were analyzed by Graphpad QuickCalcs.

Fly stocks

Genotype	Reference / stock number	Internal stock number
$y^1 sc^* v^1$; $P\{v^*; BFv-U6.2_Akh_gRNA\}attP40$	this study	MGF1492
w^* ; Kr^{lf-1}/CyO ; $D^1/TM3, Ser^1$	BDSC7198	RKF1365
$P\{ry^{+t7.2}=hsFLP\}1, y^1 w^{1118}; P\{y^{+t7.7} w^{+mC}=UAS-Cas9.P\}attP2, P\{w^{+mC}=GAL4::VP16-nos.UTR\}CG6325^{MVD1}$	(PORT <i>et al.</i> 2014), BDSC54593	MGF1476
w^* ; $TM3, Sb^1, P\{2xTb1-RFP\}TM3/ln(3L)D D^1$	BDSC36338	
w^* ; Akh^A	this study	MGF1594
w^* ; Akh^{AP}	this study	MGF1595
w^* ; Akh^{SAP}	this study	MGF1596
$y^* w^*$; $AkhR^1$	(GRÖNKE <i>et al.</i> 2007)	RKF639
$w^{1118}; Akh^A/TM3, Ser^1$ floating	this study	MGF1629
$w^{1118}; Akh^{AP}/TM3, Ser^1$ floating	this study	MGF1630
$w^{1118}; AkhR^1$	this study	MGF1634
w^* ; $akhp-GAL4, UAS-mCD8 GFP; akhp-GAL4/SM5a-TM6 Tb$	(KIM and RULIFSON 2004)	RKF694
$w^{1118}; Akh RNAi$	VDRC11352	RKF1054
$w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO$	this study	MGF1632
$w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO; Akh^A / TM3 Ser^1$	this study	MGF1639
$w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO; Akh^{AP}/TM3 Ser^1$	this study	MGF1640
$w^{1118}; akhp-GAL4, UAS-mCD8 GFP/CyO; Akh^{SAP}/TM3 Ser^1$	this study	MGF1641
Canton S		
w^{1118}	VDRC60000	RKF1084

SUPPLEMENTARY FIGURES

Figure S1 Mass spectrometry characterization of APRP dimers and monomers. MALDI-TOF mass spectra in positive reflectron mode from single *Drosophila* RC preparations before (A) and after (B) reduction of disulfide bonds. (C) Carbamidomethylated APRP monomer in a pooled extract of 20 RCs. (A) Direct tissue profiling of a single RC showed mass matches for all known AKH products in the mass range m/z 900-1300 (inset, 1 pQLTFSPDWa, 997.5 [M+Na]⁺; 2 pQLTFSPDWGK-OH, 1161.6 [M+H]⁺, 3 1183.6 [M+Na]⁺; 4 pQLTFSPDWGKR-OH, 1317.7 [M+H]⁺). The ion signal recorded at m/z 10292.1 matched a putative APRP dimer. (B) Reduction of cysteine bonds by direct profiling of a single RC with DAN matrix revealed a mass match for a possible APRP monomer (m/z 5148.5) and confirmed a dimeric structure. The ion signal was not recorded prior to reduction of disulfide bonds. (C) Carbamidomethylation of cysteines resulted in a mass shift of m/z +114 and confirmed the presence of two cysteines. Subsequent fragmentation of the precursor ion m/z 5262.5 confirmed the predicted APRP monomer sequence (see Fig. S2). All ion signals are labeled with monoisotopic masses. Cys-CAM, carbamidomethylation of cysteines.

Figure S2 Tandem mass spectrometry characterization of the APRP monomer. Detail of MALDI-TOF MS² fragment ion spectrum of the precursor ion at m/z 5262.5 $[M+H]^+$ (see Fig. S1). Ion signals of b-type fragments are labeled in green, y-type fragments in red. Identified fragments confirmed the predicted APRP sequence with sequence coverage of 64.4%. C* carbamidomethylated cysteine.

Figure S3 AKH signaling and APRP are dispensable for regulation of body size. Wing area measurement showed slightly increased wing size in *Akh^A*, however, the effect was most likely independent of AKH signaling, as *Akh^{AP}* and *Akh^{R1}* had normal size (one-way ANOVA, $F_{3,94} = 7.47$, $P < 0.001$).

Figure S4 Standard paraquat resistance assay based on drug feeding revealed increased oxidative stress resistance of *Akh^A*, *Akh^{AP}* and *Akh^{R1}* mutants when compared to controls. **(A)** Assay conducted on males. Fischer exact test: *Akh^A* vs. control: $P < 0.001$; *Akh^{AP}* vs. control: $P < 0.001$; *Akh^{R1}* vs. control: $P < 0.001$; *Akh^A* vs. *Akh^{AP}*: $P > 0.05$; *Akh^A* vs. *Akh^{R1}*: $P < 0.001$; *Akh^{AP}* vs. *Akh^{R1}*: $P < 0.001$ ($n[Akh^A] = 97$, $n[Akh^{AP}] = 110$, $n[Akh^{R1}] = 81$, $n[control] = 98$). **(B)** Assay conducted on females. Fischer exact test: *Akh^A* vs. control: $P < 0.001$; *Akh^{AP}* vs. control: $P < 0.001$; *Akh^{R1}* vs. control: $P < 0.001$; *Akh^A* vs. *Akh^{AP}*: $P > 0.05$; *Akh^A* vs. *Akh^{R1}*: $P < 0.001$; *Akh^{AP}* vs. *Akh^{R1}*: $P < 0.001$ ($n[Akh^A] = 109$, $n[Akh^{AP}] = 81$, $n[Akh^{R1}] = 92$, $n[control] = 116$).

Figure S5 Corpora cardiaca cell number is independent of APRP. Represented are cell counts of CC cells tagged by fluorescence under indirect control of the *Akh* promoter (*akhp*-Gal4 > *UAS*-mCD8 GFP) in *Akh^{AP}* and *Akh^{SAP}* mutants compared to *Akh⁺* controls (one-way ANOVA, $F_{2,27} = 0.3$, $P = 0.74$). Note that the *Akh⁺* control is identical to the one used in Figure 9A.

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