Energy-Dependent Modulation of Glucagon-Like Signaling in *Drosophila* via the AMP-Activated Protein Kinase

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ABSTRACT Adipokinetic hormone (AKH) is the equivalent of mammalian glucagon, as it is the primary insect hormone that causes energy mobilization. In *Drosophila*, current knowledge of the mechanisms regulating AKH signaling is limited. Here, we report that AMP-activated protein kinase (AMPK) is critical for normal AKH secretion during periods of metabolic challenges. Reduction of AMPK in AKH cells causes a suite of behavioral and physiological phenotypes resembling AKH cell ablations. Specifically, reduced AMPK function increases life span during starvation and delays starvation-induced hyperactivity. Neither AKH cell survival nor gene expression is significantly impacted by reduced AMPK function. AKH immunolabeling was significantly higher in animals with reduced AMPK function; this result is paralleled by genetic inhibition of synaptic release, suggesting that AMPK promotes AKH secretion. We observed reduced secretion in AKH cells bearing AMPK mutations employing a specific secretion reporter, confirming that AMPK functions in AKH secretion. Live-cell imaging of wild-type AKH neuroendocrine cells shows heightened excitability under reduced sugar levels, and this response was delayed and reduced in AMPK-deficient backgrounds. Furthermore, AMPK activation in AKH cells increases intracellular calcium levels in constant high sugar levels, suggesting that the underlying mechanism of AMPK action is modification of ionic currents. These results demonstrate that AMPK signaling is a critical feature that regulates AKH secretion, and, ultimately, metabolic homeostasis. The significance of these findings is that AMPK is important in the regulation of glucagon signaling, suggesting that the organization of metabolic networks is highly conserved and that AMPK plays a prominent role in these networks.

NUTRIENT availability is a dynamic variable in natural environments, and all organisms must maintain metabolic homeostasis despite food variability. Furthermore, environmental variation in food sources causes a series of physiological and behavioral responses in attempts of organisms to maintain energetic homeostasis. These responses require coordination of cellular mechanisms and hormonal signaling to produce differential effects in a wide variety of tissues (Johnson and White 2009). The connections between cell-autonomous elements and hormonal signaling to maintain metabolic homeostasis is not completely understood.

The adipokinetic hormone (AKH) is a hormone found throughout the insects and is critical for maintenance of energy homeostasis. In many insects, there is variation in the numbers and primary sequences of different forms of AKH (Gäde and Auerswald 2003). In *Drosophila*, there is a single AKH, which is an octamer, possesses a pyroglutamine residue at its N terminus, and is amidated (Schaffer *et al.* 1990). AKH expression in *Drosophila* is limited to a small group of cells that are part of the corpus cardiacum of the ring gland, and ablation of these cells leads to altered behaviors under starvation protocols and concomitant changes in carbohydrate metabolism (Lee and Park 2004; Isabel *et al.* 2005). Specifically, the loss of AKH cells leads to an increased survival when these flies are challenged with starvation conditions and an absence of starvation-induced hyperactive behaviors (Lee and Park 2004; Isabel *et al.* 2005). AKH has been hypothesized to be the functional equivalent of

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mammalian glucagon (Kim and Rulifson 2004), as it has been shown to be the primary insect hormone responsible for energy mobilization (Gäde and Auerswald 2003).

The functional relationship between insect AKH and mammalian glucagon signaling suggests that convergent mechanisms may be involved in the physiology of these cell types (AKH and pancreatic α-cells). The physiology of these cell types is similar, as expression of K^{+}_{ATP}-dependent channels is a critical element regulating pancreatic α-cell excitability (Gromada *et al.* 2004) as well as AKH neuroendocrine cells (Kim and Rulifson 2004). Thus, in both cell types, internal sensors of energy status are coupled to hormone release. Furthermore, in cultured pancreatic α-cells, the energy sensor, AMP-activated kinase (AMPK), has been reported to regulate calcium levels and, thus, glucagon secretion (Leclerc *et al.* 2011).

Given these critical roles of AMPK and the similarities between pancreatic glucagon and AKH cells, we tested the possibility that AKH signaling may be regulated by AMPK in *Drosophila*. AMPK is activated by the end product of ATP hydrolysis and thus is largely considered to function as an energy sensor (Long and Zierath 2006; Winder and Thomson 2007). Activation of AMPK leads to alterations in a series of changes in cell physiology that has the net effect of inhibiting energetically expensive processes. Functional AMPK is a heterotrimer consisting of a catalytic $\tilde{\alpha}$, a regulatory $\tilde{\gamma}$, and a scaffolding β-subunit, and, in most organisms, multiple genes are present for each of the three subunits (Riek *et al.* 2008). For example, humans possess two α-, two β-, and three γ-encoding genes; the consequence of this genetic structure is that different heterotrimeric complexes can form and genetic strategies to manipulate AMPK are problematic (Birk and Wojtaszewski 2006). In contrast, the *Drosophila* genome possesses a single gene for each of the subunits (*snf1A* encodes the α-subunit, *snf4* encodes the γ-subunit, and *alicorn* encodes the β-subunit) (Pan and Hardie 2002).

We and others recently reported the phenotypic consequences of organism-wide reduction of AMPK function in *Drosophila*. Loss of AMPK function leads to heightened starvation sensitivity, greater hyperactive responses to starvation, and reduced triglyceride levels in spite of hyperphagic behaviors (Johnson *et al.* 2010). The suspected basis for these phenotypes is a global inability of cells to reallocate energy and activities during nutritional stress (Johnson *et al.* 2010). However, in addition to the central role of energy maintenance, AMPK has a specialized function to maintain metabolic homeostasis in distinct cellular populations. For example, AMPK modulates the release of orexigenic transmitter from the mammalian hypothalamus (Claret *et al.* 2007), which would facilitate enhanced feeding under low-energy conditions. Furthermore, AMPK activation in isolated rat adipocytes inhibits lipolysis. This is also likely to be the case in *Drosophila* and other insects, as the selective reduction of AMPK function in muscle and gut tissues leads to heightened sensitivity to starvation and is thought to underlie the developmental lethality associated with a molecular null mutation in the α-subunit of AMPK (Bland *et al.* 2010; Tohyama and Yamaguchi 2010).

We report that the loss of AMPK function in AKH cells leads to a partial phenocopy of AKH cell ablations, specific knockdown of the AKH hormone, and a deletion of the AKH receptor. Notably, reduced AMPK function impacts neither AKH cell survival nor AKH expression. Furthermore, reduced levels of secretion in AKH cells bearing AMPK genetic variants were observed during a starvation paradigm. Likewise, reduced AMPK causes reduced activation of AKH cells, and activation of AMPK under constant energy levels leads to heightened calcium signals in AKH cells. Collectively, these results suggest that AMPK signaling regulates AKH secretion during heightened metabolic demand, and such results will inform future experiments in mammals with similar regulation of glucagon secretion.

Materials and Methods

Drosophila stock and husbandry

All flies were maintained in an incubator maintained at 25° and under a 12:12 light/dark (LD) cycle. Flies were cultured on a standard molasses–malt–cornmeal–agar–yeast medium and housed in uncrowded conditions. All transgenes were backcrossed to the w^{1118} background for five generations. The UAS-AMPK transgenes were reported previously (Johnson *et al.* 2010): UAS-snf1A ^{K57A} [Bloomington Stock (BL) # 32112], UAS-snf1A (BL# 32108), UAS-snf1A-RNAi (BL# 32371), and UAS-snf4-RNAi (BL# 34726). Other stocks used in this study were the AKH-GAL4 (BL # 25683) (Lee and Park 2004), UAS-TeTX (BL# 28839) (Sweeney *et al.* 1995), UAS-AKH-RNAi (BL#34960), UAS-rpr (BL# 5823), GAL80 (BL#7018) (McGuire *et al.* 2003), UAS-GCaMP (BL# 32236), and UAS-ANF-GFP (BL# 7001) (Rao *et al.* 2001), all procured from the Bloomington Stock Center (Table 1). The $AKHR^{01}$ and $AKHR^{rev}$ lines were kind gifts from Ronald Kuhnlein (Gronke *et al.* 2007).

Life-span measurements

We placed 30, 3- to 5-day-old mated flies (males and females housed separately) in vials with a 2% agar solution to starve the animals (Zhao *et al.* 2010). We assessed percentage survival of at least three replicate vials twice daily. For each vial, we assessed the median survival for the treatment, and data were pooled to estimate a mean median survival; we then employed a one-way ANOVA with post-hoc Tukey's comparison to determine differences between genotypes. All control lines were run simultaneously with experimental treatments.

Locomotor measurements

Locomotor activity was monitored with a TriKinetics Locomotor Monitor (Waltham, MA) on the aggregate population of 30, 3- to 5-day-old flies (Zhao *et al.* 2010). Flies were entrained to a 12:12 LD cycle for 3 days prior to the experiments. Flies were transferred to a vial containing starvation

Table 1 Genotypes of strains used in this study

Genotype	Effect	Abbreviation
UAS-rpr; AKH-GAL4	Ablation of AKH cells	AKH cell deficient (AKH-CD)
AKH-GAL4 UAS-TeTx	Inhibition of AKH cell secretion	AKH secretion deficient (AKH-SD)
AKH-GAL4 UAS-AMPKα^{K57A}	Dominant-negative AMPK in AKH cells	AMPKα-DN
AKH-GAL4 UAS-AMPKα^{WT}	Wild-type α-subunit in AKH cells	AMPKα-WT
AKH-GAL4; UAS-snf4 RNAi	RNAi knockdown of γ-subunit	AMPK$\gamma-$KD
AKH-GAL4; UAS-snf1A-RNAi	RNAi knockdown of α-subunit	AMPK$\alpha-$KD

or normal medium at Zeitgeber time 0 (ZT0). Total beam counts were monitored continuously through an automated system for 48 hr on four replicates per treatment. We determined the amount of activity during starvation relative to the activity of fed conditions for the same time period. We employed a repeated measures ANOVA to determine the time point at which starvation activity was significantly greater than activity during replete conditions. All control lines were run simultaneously with experimental treatments.

Transcript analysis

Ninety adult flies were homogenized in 1 mL of TriZol (Invitrogen), and total RNA was extracted according to the manufacturer's recommendations. RNA was then used for cDNA synthesis using a SuperScript III Reverse Transcription Kit (Invitrogen). The primers for the AKH transcript were the following: forward (5′- ATGAATCCCAAGAGCGAAGTC CTC) and reverse (5′ CTACTCGCGGTGCTTGCAGTCCAG). Specific primers were also designed to amplify the housekeeping gene *RP49* (Zhao *et al.* 2010). PCR conditions were 94° for 5 min, followed by 25 cycles of 94° for 45° sec, 55° for 45 sec, and 72° for 90 sec, followed by a single extension of 72° for 10 min. Intensity of ethidium bromide (EtBR) fluorescence was determined using a Hitachi Gene Systems equipped with a CCD camera, and PCR conditions were determined to be in the linear range of amplification. Values were normalized to RP49 across samples and expressed as percentage of baseline (replete conditions).

AKH cell imaging and immunocytochemistry

Larval and adult progeny from flies carrying the *AKH-GAL4* transgene crossed to the UAS-GFP-nls lines were dissected. Brains were fixed in a 4% paraformaldehyde, 7% picric acid fixative for 1 hr at room temperature and washed six times with phosphate buffered saline (PBS) containing Triton-X 100. A 1:1000 dilution of anti-AKH (Brown and Lea 1988) was incubated overnight at 4°. Brains were washed, and a Cy-3 conjugated anti-rabbit secondary antibody was applied overnight at 1:1000 dilution. Tissues were then mounted and viewed on a Zeiss LSM 710 confocal microscope. For calcium imaging and fused atrial naturietic factor (ANF)-GFP imaging experiments, adult ring glands were dissected and placed in adult hemolymph-like (Feng *et al.* 2004) solution containing 12 mM trehalose, which mimics replete levels (Broughton *et al.* 2005). Following a 15-min incubation period, explanted ring glands were then viewed on a Zeiss LSM 710 confocal microscope. Images were collected every 15 sec for experiments using GCaMP and once every 10 min for experiments employing the ANF-GFP in an attempt to minimize photobleaching. For sugar transition experiments, an isosmotic calcium-free solution (to reduce spontaneous activity) containing 3 mM trehalose to mimic starvation conditions (Broughton *et al.* 2005) was perfused, and images were collected. Following experiments, 3 M KCl was applied to evoke cell depolarization as a measure of cell viability; only cells that showed KCl-evoked cell increases in GCaMP fluorescence were used for analysis. Confocal settings were identical for all experiments. A region of interest was manually drawn for each ring gland, and total values for pixel intensity were assessed. Values were imported into MS Excel and normalized to baseline levels. Five replicates for each treatment and genotype were analyzed. AICAR and Compound C were purchased from Sigma Chemicals (St. Louis) and used at 2 and 0.5 mM concentrations, respectively.

Results

Reduced AMPK function in AKH cells alters starvation sensitivity

Ablation of the neuroendocrine cells that express the adipokinetic hormone, through introduction of the pro-apoptotic gene *reaper*, leads to enhanced survival during starvation challenges, the suspected mechanism of which is the concomitant loss of starvation-induced hyperactivity in animals lacking AKH cells (Lee and Park 2004; Isabel *et al.* 2005). We performed an RNAi screen to identify candidates that regulate AKH signaling using a specific AKH-GAL4 driver (Lee and Park 2004). We reasoned that the loss of elements that promote AKH release would result in a phenocopy of ablated AKH cells. From this screen, we observed that expression of RNAi elements targeting either the α-or γ-subunit of AMPK caused a significant increase in life span under starvation. Given these findings, we then selectively introduced an AMPK dominant-negative transgene (Johnson *et al.* 2010). Expression of the dominant negative α subunit also significantly increased starvation life span in females (Figure 1, A and B) and males (Figure 1B) as compared to flies expressing the wild-type α-subunit ($P < 0.001$, ANOVA). Given that overexpression of the wild-type α-subunit does not lead to overexpression of the functional heterotrimeric AMPK (Johnson *et al.* 2010), the use of the wild type is the ideal control for comparisons with the dominant-negative element, as they differ in a single amino acid (K57A). Notably, we also used

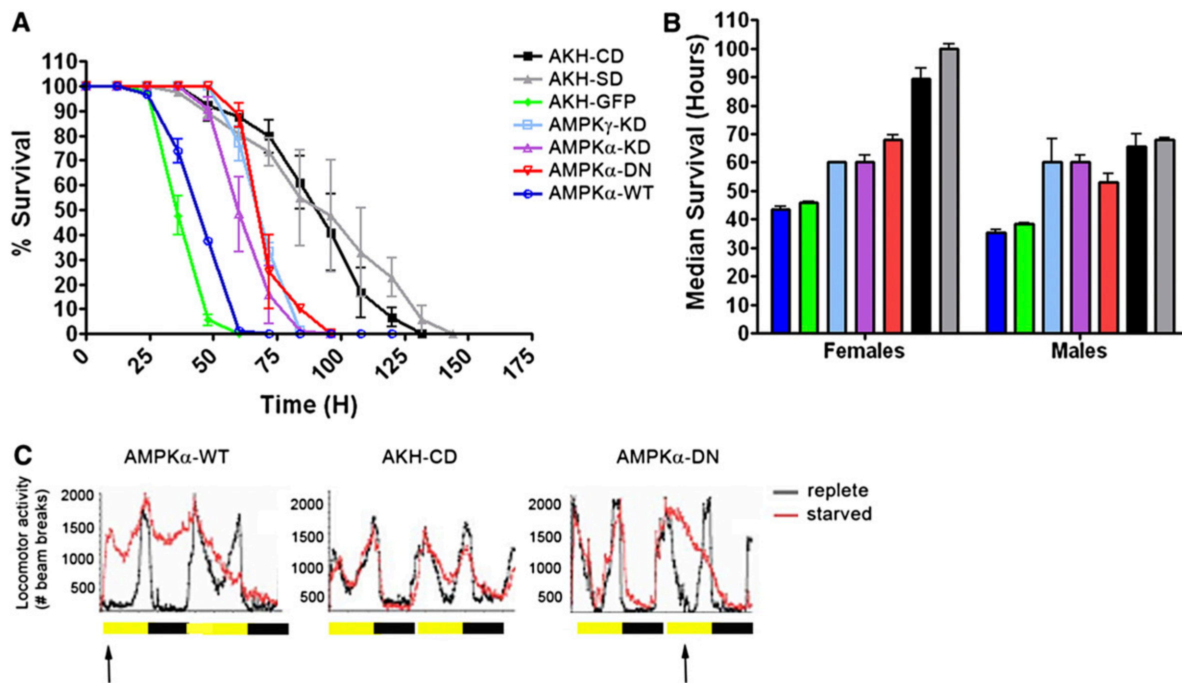

Figure 1 Reduced AMPK function in AKH cells causes similar phenotypes as loss of AKH genetic variants. (A) Life-span measurements during starvation of adult female animals with wild-type AKH cells (AKH-GFP and AKH-α WT, green and dark-blue lines, respectively); no AKH cells (AKH-CD, black line), or defective AKH secretion (AKH-SD, gray line). Compare to results from animals expressing a dominant-negative AMPK α-transgene (dark-gray lines) or RNAi elements targeting the AMPK γ-subunit (light-blue line) or the α-subunit (purple line). (B) Mean median survival ±SEM for different AKH genetic manipulations (same legend as in A); females (left) and males (right). (C) Locomotor activity under a 12:12 LD cycle (yellow and black bars indicate the duration of lights-on and lights-off, respectively) in animals with wild-type AKH neuroendocrine cell function (left), ablated AKH cells (AKD-CD) (center), and in animals expressing the AMPK αDN transgene (right) under fed (black lines) and starvation (red lines). Arrows indicate the time point that starvation activity is significantly greater than activity during replete conditions as evaluated by a repeated measures ANOVA.

the GAL80 element (McGuire *et al.* 2003), which inhibits GAL4-mediated transcription and found that survival phenotypes were dependent upon GAL4 transcription (supporting information, Figure S1A). However, while reduced AMPK in AKH cells function causes increased starvation life span, the extent of this lengthening is considerably less than in animals lacking AKH cells or to animals expressing tetanus toxin, which prevents secretion of this hormone (Sweeney *et al.* 1995).

Likewise, stress-induced hyperactivity was also impacted in flies with altered AMPK function in AKH cells. While hyperactive responses were observed, the onset of this behavioral response was significantly delayed compared to female flies expressing wild-type AMPK (Figure 1C). Specifically, we observed significant increases in activity in wild-type animals during the first hour under starvation conditions, whereas increased activity was observed following 24 hr of starvation in animals expressing the AMPK-DN transgene. We observed no significant increase in activity in animals lacking AKH cells as previously reported (Lee and Park 2004; Isabel *et al.* 2005). While all these genetic manipulations are restricted to the AKH cell population, we wanted to ensure that these behavioral phenotypes were attributable to a loss of AKH function. To test the notion that it was an AKH deficit rather than another unknown co-expressed hormone, we employed an RNAi directed against the AKH hormone and a genetic strain bearing a deletion in the AKH receptor (Gronke *et al.* 2007). Both of these lines significantly differed from wild type in starvation survival (Figure S1A). The AKH-RNAi line eliminated AKH immunosignals (Figure S1B), and the AKHR mutant line showed no increase in locomotor activity under starvation conditions (Figure S1C). Collectively, these results suggest that the consequences of reduced AMPK function reflect a specific defect in AKH signaling.

Reduced AMPK function affects neither AKH cell survival nor AKH expression

Given that reduced AMPK function in AKH cells leads to phenotypes similar to those of AKH cell ablations, we reasoned that AMPK, following verification of expression of AMPK in AKH cells (Figure S2), may be critical for AKH cell survival or development. To determine if AMPK impacted AKH cell survival, we introduced a GFP reporter that possessed a nuclear localization signal to facilitate AKH cell counts. We observed no differences ($P = 0.72$, ANOVA) in the number of GFP-labeled nuclei in wild-type larval (16 ± 0) as compared to animals expressing the γRNAi transgene (16 ± 0). In adults, we observed 11.6 ± 0.4 GFP-labeled cells in wild-type animals and 11.25 ± 0.7 GFP-labeled cells expressing the γRNAi element (Figure 2, A and B). Given that GAL4-driven expression of multiple UAS elements effectively reduces the dosage from a single UAS element, we tested behavioral phenotypes of animals expressing the AMPK

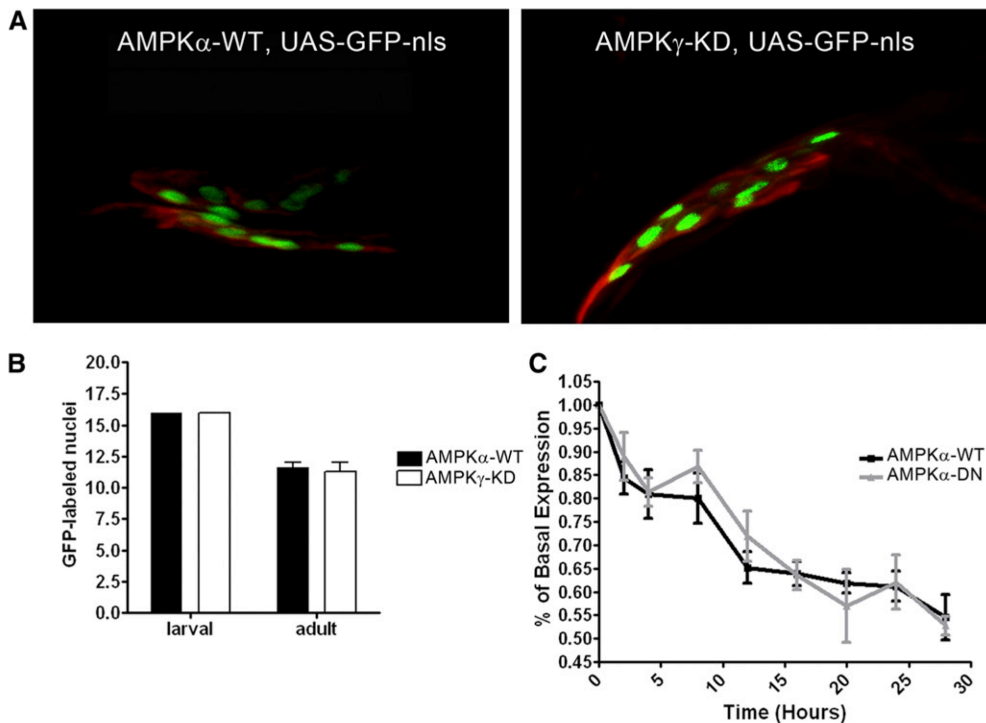

Figure 2 AKH cell survival or expression is not altered by reduced AMPK. (A) Representative images of adult AKH cells expressing nuclear GFP and counterstained with an anti-AKH antibody in wild-type animals (left) and γRNAi-expressing animals (right). (B) Quantification of larval and adult cell counts of AKH cells from wild-type AMPK (solid bars) and RNAi targeting the γ-subunit (open bars). Mean counts ±SEM from 10 different animals and no significant differences between genotypes were observed (two-way ANOVA genotype: $P = 0.715$) (C) Relative AKH transcript levels were first normalized to RP49 and then to baseline (prior to starvation—time 0) for each genotype. There was a significant reduction in AKH transcript levels as a function of starvation, but there was no significant difference between wild-type and AMPK-deficient AKH cells (ANOVA, $P = 0.4919$).

transgenes in combination with the nuclear GFP. We observed that neither RNAi element in combination with nuclear GFP differed phenotypically from the RNAi element expressed alone (Figure S1A). In contrast, behavioral phenotypes of nuclear GFP in combination with the dominant-negative α-construct were wild type (Figure 1B and Figure S1A), which is consistent with the notion of different dosage requirements of the dominant negative, which competes with endogenous wild-type α-subunits during heterotrimer formation (Riek *et al.* 2008), as compared to the effective dosages of RNAi elements. Based on this finding, for all following experiments where multiple UAS elements were employed, we limited our experiments to the RNAi elements.

Given that there was no significant effect of AMPK on AKH cell survival, we reasoned that there may be a role for AMPK regulation of AKH gene expression. We measured AKH transcript abundance under replete and starvation conditions in animals differing in AMPK function in AKH neuroendocrine cells. We found that the AKH expression profile was similarly independent of genotype as it pertained to AMPK function and expression (Figure 2C). We did find a significant downregulation of AKH expression due to starvation, which is supported by previous experiments showing downregulation of aspects of the AKH-signaling pathway, specifically the AKH receptor (Fujikawa *et al.* 2009) and other downstream effectors of AKH, including the TAG lipase and other metabolic enzymes (Grönke *et al.* 2005).

AMPK regulates AKH secretion

We then tested the hypothesis that AKH secretion was reliant on AMPK function. Using an antibody against the AKH hormone (Brown and Lea 1988), we quantified the relative amounts of AKH immunolabeling in animals with wild-type or altered AMPK in AKH neuroendocrine cells. Our reasoning was that AKH cell immunolabeling is inversely correlated with AKH secretion. Under replete or starved conditions, larval AKH immunolabeling was constant in animals with wild-type AMPK function; however, animals expressing either the dominant-negative αAMPK transgene or a tetanus toxin construct (to block synaptic transmission) had consistently elevated AKH immunolabeling (Figure 3). While these results are consistent with the notion that AMPK may impact AKH cell secretion, we were surprised that there were no observed differences in AKH immunolabels as a function of starvation since previous reports implicated heightened AKH secretion during starvation (Gronke *et al.* 2007; Bharucha *et al.* 2008).

We attributed this to low sensitivity of the assay and suspect that the amount of AKH secreted is low compared to the cellular stores of the hormone. This possibility is supported by measurements of the high affinity (subnanomolar EC_{50}) that the AKH receptor has for the AKH hormone (Park *et al.* 2002; Staubli *et al.* 2002). Therefore, we employed a GFP-fused ANF-GFP to further evaluate the roles of AMPK on AKH secretion. The ANF-GFP reporter has been used extensively to record secretion events in different peptidergic cells in *Drosophila* (Rao *et al.* 2001; Husain and Ewer 2004). We isolated adult AKH neuroendocrine cells from wild-type animals, and, upon transition from high to low trehalose (the major sugar used in insects) concentrations to mimic starvation, observed a significant loss of ANF-GFP signals. In contrast, the difference in ANF-GFP signals as a function of starvation in AKH cells expressing the AMPK αRNAi element was reduced in comparison to wild type

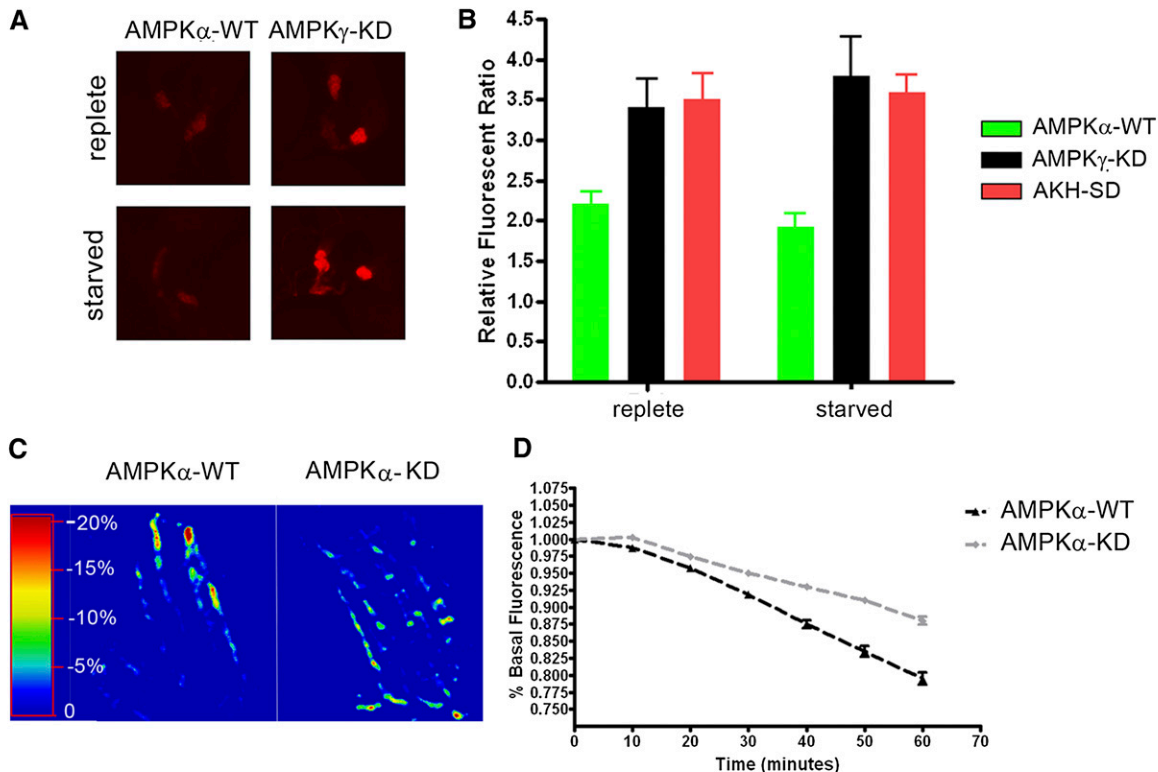

Figure 3 AMPK mediates AKH hormone secretion. (A) Representative images of larval AKH cells stained with an antibody specific for AKH in wild-type ring glands (left) and *snf4*-RNAi-expressing glands (right) under replete (top) and starvation (bottom) conditions. (B) Quantification of immunofluorescence from flies with wild-type AKH cells, expressing the *snf4*-RNAi, and the TeTX (tetanus toxin) construct. Mean fluorescent values ±SEM from five animals. (C) Representative heat maps of AKH cell terminals expressing the ANF-GFP secretion reporter with wild-type AMPK (top) and γRNAi during transition from high to low sugar conditions. Note heat map reports percentage difference not percentage increase. (D) Quantification of ANF-GFP levels in wild-type (black line) and AKH cells expressing the RNAi targeting the α-subunit (gray line) during high-to-low sugar transition. Mean ± SEM fluorescence values were normalized to initial fluorescent levels and were derived from five different ring glands.

(Figure 3) (P = 0.04, ANOVA). Notably, there were no differences in GFP fluorescent levels observed in either genotype when held at constant high trehalose levels (P = 0.86, ANOVA) (data not shown).

AMPK impacts AKH cell excitability

Since our results with the ANF-GFP reporter show deficits in AKH cell secretion caused by reduced AMPK function, we next performed experiments to discern potential mechanisms, *i.e.*, whether AMPK modulates AKH secretion machinery and/or AKH cell excitability. We used the fluorescent calcium reporter GCaMP to observe changes in AKH neuroendocrine cell excitability. A previous study established that AKH cell excitability increases in response to lowered trehalose levels (Kim and Rulifson 2004). Consistent with this report, we observed a rapid increase in calcium fluorescence upon transitioning from high to low trehalose levels in adult AKH neuroendocrine cells. In contrast, either pharmacological inhibition of AMPK function by Compound C [an AMPK antagonist (Gao *et al.* 2008)] or genetic reduction of AMPK function by the γRNAi attenuates the response to starvation (Figure 4). Specifically, wild-type AKH cells respond within 10 min to the transition from high to low trehalose (replete to starved), whereas in AKH cells with reduced AMPK function, both the onset and magnitude of the response are altered. Notably, addition of Compound C in animals expressing the RNAi element targeting the γ-subunit produces a response indistinguishable from either alone, suggesting that the effects of Compound C are specific to AMPK (Figure S3).

We next reasoned that this altered response in AKH cell excitability could be caused either by a blunted response to the change in trehalose concentration or, alternatively and/or additionally, by modulation of specific ion channels regulating AKH cell excitability. To test these hypotheses, we applied an AMP mimetic and specific activator of AMPK, AICAR (Merrill *et al.* 1997), to AKH cells under constant high trehalose concentrations. We observed that, in lieu of any change in sugar levels, activation of AMPK significantly increased GCaMP fluorescence (Figure 5). Notably, the increase in GCaMP fluorescence with AICAR addition to γRNAi-expressing AKH cells was significantly reduced compared to wild type (P = 0.0001, repeated measures ANOVA). While we cannot rule out the possibility that AMPK activation may alter sugar sensitivity of AKH cells, these results clearly show that AMPK activation alters AKH cell excitability by increasing intracellular calcium. Because AMPK has been shown to regulate trafficking of ion channels in different cell types (Alzamora *et al.* 2010; Alesutan *et al.* 2011), we tested whether, in

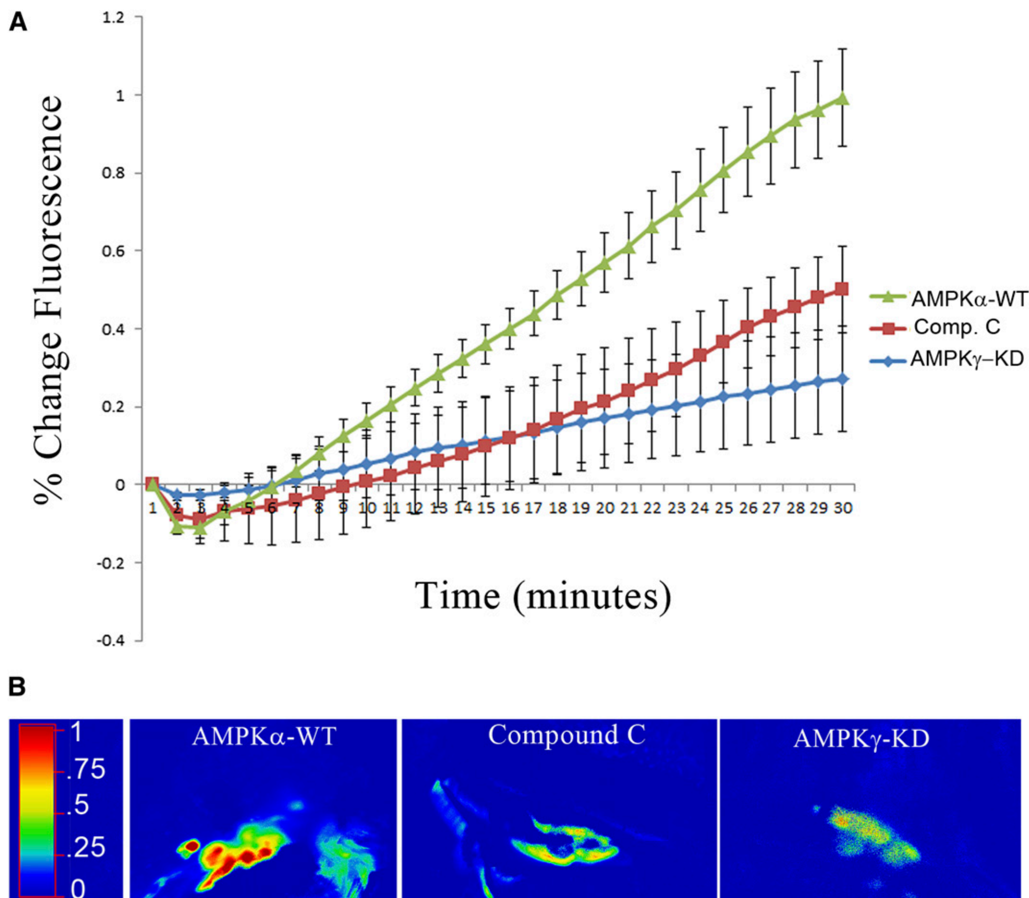

Figure 4 AMPK is required for AKH cell excitability changes during starvation. (A) Quantification of starvation responses (high-to-low sugar transition) from explanted AKH cells expressing the calcium reporter GCaMP. Mean percentage change ±SEM from baseline fluorescence from wild-type AKH cells (green line), wild-type cells treated with Compound C (red line), and AKH cells expressing the RNAi targeting the γ-subunit (blue line) (*n* = 5 replicates per treatment). (B) Representative heat maps of AKH cells expressing GCaMP with wild-type AMPK (left), Compound C treated wild-type AKH cells (center), and AKH cells co-expressing the γRNAi element (right) during transition from high to low trehalose-containing solutions.

AMPK-deficient AKH cells, evoked depolarizations were equivalent to wild-type AKH cells. Application of KCl to explanted wild-type AKH cells caused an expected increase in GCaMP fluorescent signals that was equivalent to that in AMPK-deficient AKH cells (Figure S4).

Discussion

We report that the selective reduction of AMPK function in AKH neuroendocrine cells results in a series of behavioral and physiological phenotypes consistent with a loss of function of AKH itself. Specifically, animals have increased life span during starvation as do animals lacking the AKH hormone. Furthermore, animals deficient in AKH exhibit a loss of starvation-induced hyperactivity, and the selective loss of AMPK function in these cells leads to a delay in this behavioral response. We conclude that AMPK is a critical component that regulates AKH secretion via modulation of cell excitability based on our observations that AMPK is not necessary for cell survival or AKH expression and the results demonstrating reduced secretion and GCaMP fluorescence during starvation challenges. We propose a model in which AMPK acts as an energy sensor in the AKH cell population to control secretion and ultimately coordinate physiological and behavioral responses to maintain metabolic homeostasis.

Processing of the AKH peptide relies on cleavage of the prohormone and subsequent amidation of the N terminus, and these events are required for AKH bioactivity (Rhea *et al.* 2010). While we cannot rule out the possibility that AMPK may be impacting AKH hormone processing, we consider this insufficient to explain the ensemble of phenotypes associated with reduced AMPK function in AKH cells. First, we observed partial phenocopies of AKH cell ablation, whereas in contrast, the loss of processing causes complete loss of function phenotypes (Rhea *et al.* 2010). Second, our observation of delayed locomotor responses present in animals with compromised AMPK function suggests at least minimal levels of bioactive AKH as the complete loss of the hormone eliminates this behavioral response. Third, reduced levels of bioactive AKH, which may be caused by reduced AMPK function, are insufficient to explain the changes in AKH cell excitability.

While we cannot completely rule out the possibility that another hormone co-expressed in the AKH cell population is responsible for some of the behavioral phenotypes that we observed, we consider this unlikely. First, there is an extensive literature establishing the roles of adipokinetic hormone in mediating metabolic homeostasis (for review, see Gäde and Auerswald 2003). Second, our observations targeting the AKH hormone with a specific RNAi leads to phenotypes consistent with the AKH cell ablations. Finally,

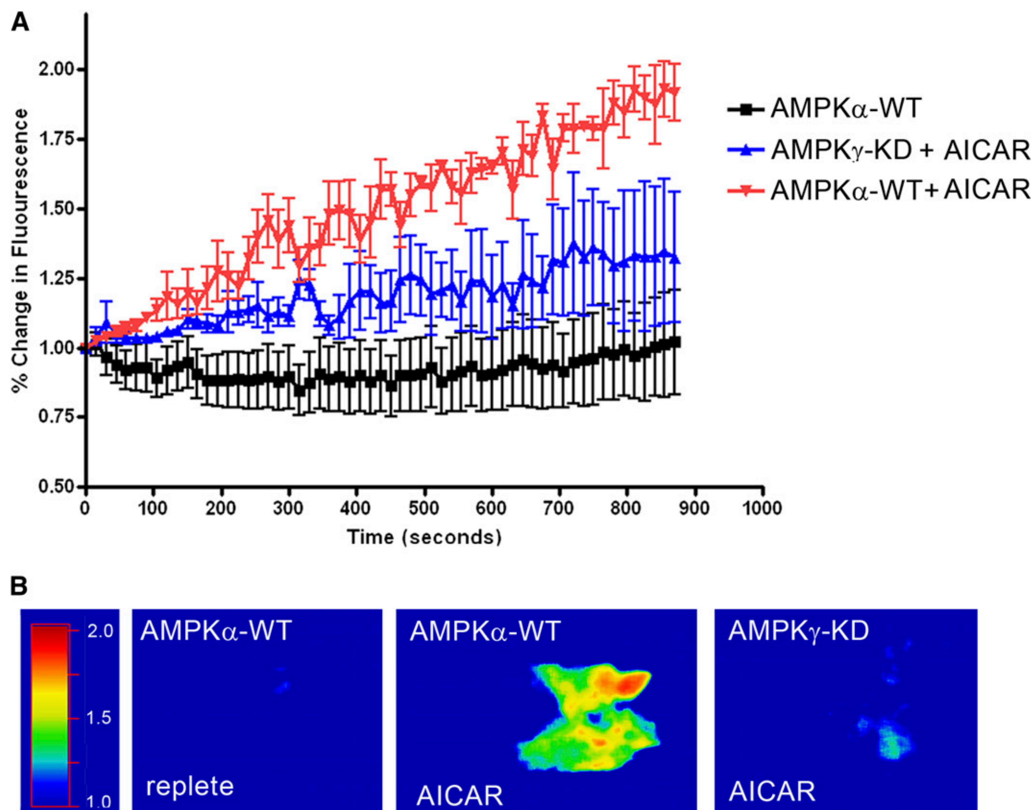

Figure 5 Activation of AMPK in AKH cells leads to elevated calcium levels during replete conditions. (A) Time course of GCaMP fluorescence responses. Data are mean ±SEM from three to five replicates from wild-type AKH cells held in constant high-trehalose solution (black line) and treated with AICAR (red line) and RNAi knockdown of the γ-subunit-expressing cells treated with AICAR (blue line). (B) Representative images of AKH cells expressing the GCaMP reporter during constant high (replete) sugar conditions in wild-type animals (left), in wild-type animals treated with AICAR (center), and in animals expressing the γRNAi treated with AICAR (right).

results with a deletion of the receptor highly specific for the AKH peptide are also consistent with the behavioral phenotypes from AKH cell ablations (Grönke *et al.* 2007; Bharucha *et al.* 2008). Collectively, these results make a compelling case that it is AKH as opposed to another hormone that is relevant in mediating metabolic homeostasis. Nonetheless, we note that, even if there were other hormones coexpressed with AKH that are relevant, the actions of AMPK strongly cement this kinase as a critical regulator of AKH cell excitability and, by extension, hormonal regulation.

How might AMPK be altering AKH cell excitability? Our results implicate an acute modulation of channel activity. AMPK has been shown to modulate the biophysical properties of the twin pore K^+ (TWIK) channels, and while it is currently unknown if AKH cells express similar channels, we speculate that AMPK is similarly modulating an unknown channel conductance in AKH cells. There is evidence that AKH cells express the K^+_{ATP} channels based on *in situ* analysis and that dietary introduction of a specific K^+_{ATP} channel antagonist, tolbutamide (Henquin 1980), leads to behavioral phenotypes consistent with blocking AKH release (Kim and Rulifson 2004). AMPK has been shown to regulate the activation of this channel subtype (Yoshida *et al.* 2012). Given the energy-sensing roles of K^+_{ATP} channel conductance, we are currently testing the contribution of this conductance in the regulation of AKH signaling and whether this intersects with AMPK signaling.

We note that AMPK-deficient AKH cells still respond to sugar changes as directly observed with GCaMP, albeit those responses are diminished and delayed. This may reflect residual wild-type AMPK function or, more likely, redundant mechanisms present in AKH cells to regulate AKH secretion. Therefore, we suspect that AKH release in an AMPK-deficient background may result from other signaling processes. In support of that notion, autophagy, which also facilitates increased cellular energy availability, has been shown to occur independently of AMPK activation (Williams *et al.* 2009). Another candidate that may be involved in AMPK-independent regulation of AKH is the activity-regulated cytoskeletal-associated gene, which is specifically expressed in AKH cells; mutants in this gene fail to exhibit normal starvation-induced hyperactivity (Mattaliano *et al.* 2007).

While the distinct changes in the responses to sugar transitions in explanted AKH cells implicate other AKH cell-autonomous elements, we also note the delay in hyperactive behaviors in animals with reduced AMPK function. Many different hormones in a variety of insects have been implicated as AKH release factors, including but not limited to, tachykinin-like peptides, octopamine, and proctolin (Nassel *et al.* 1999; Clark *et al.* 2006). While it is currently unknown if these hormones are operating in a similar fashion in *Drosophila*, we speculate that these or other hormonal factors may also be responsible for AKH release in animals with reduced AMPK function in AKH cells. We also suspect that some of these or other regulatory hormones may operate through AMPK. For example, AMPK has been shown to be a critical component of leptin signaling and a target of follicle-stimulating hormone modulation in mammals (Andreelli *et al*. 2006; Chen and

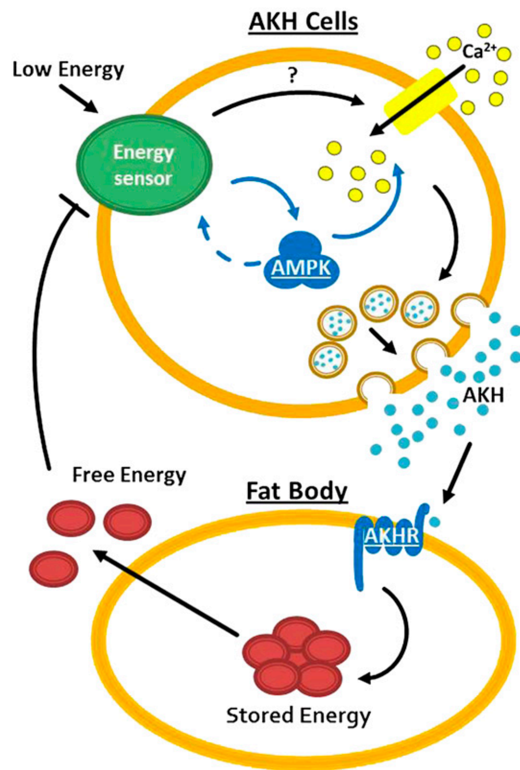

Figure 6 Model of AMPK function in AKH neuroendocrine cells. AMPK activation by low trehalose levels are detected through a currently unknown mechanism. Upon activation, AMPK leads to enhanced calcium levels in AKH cells, which subsequently leads to elevated levels of AKH release. The AKH hormone stimulates the fat body to release stored energy, which leads to the inhibition of AMPK and AKH hormone secretion.

Downs 2007). We are currently evaluating which hormones regulate AKH secretion and assessing whether hormonal signaling pathways modulate AMPK activity.

We note that the regulation of AKH via AMPK is similar to the regulation of glucagon signaling via AMPK. Specifically, AMPK activity in pancreatic α-cells is required for elevated calcium levels upon lowered glucose levels, akin to our demonstration of AKH calcium levels requiring AMPK. These similarities suggest that the signaling networks dedicated to maintaining metabolic homeostasis are highly conserved across broad phylogenetic distances. Our results suggest that the mechanism underlying AMPK regulation of glucagon signaling in mammals may be caused by changes in pancreatic α-cell excitability.

We propose a model in which AMPK acts to integrate energetic balance from internal cues and subsequently facilitate AKH release (Figure 6). Low energy levels are specifically detected by AMPK, which leads to AMPK activation and modulation of components that regulate AKH cell excitability. This change in excitability leads to enhanced AKH release. Secreted AKH subsequently binds to its receptors in the fat body to facilitate the mobilization of energy, thereby increasing hemolymph concentrations of sugars that consequently cause the inactivation of AMPK. Future studies will be needed to identify the direct targets of AMPK activation in *Drosophila*, how AKH cells sense sugar changes, and what are the AMPK-independent components regulating AKH signaling.

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Energy-Dependent Modulation of Glucagon-Like Signaling in *Drosophila* via the AMP-Activated Protein Kinase

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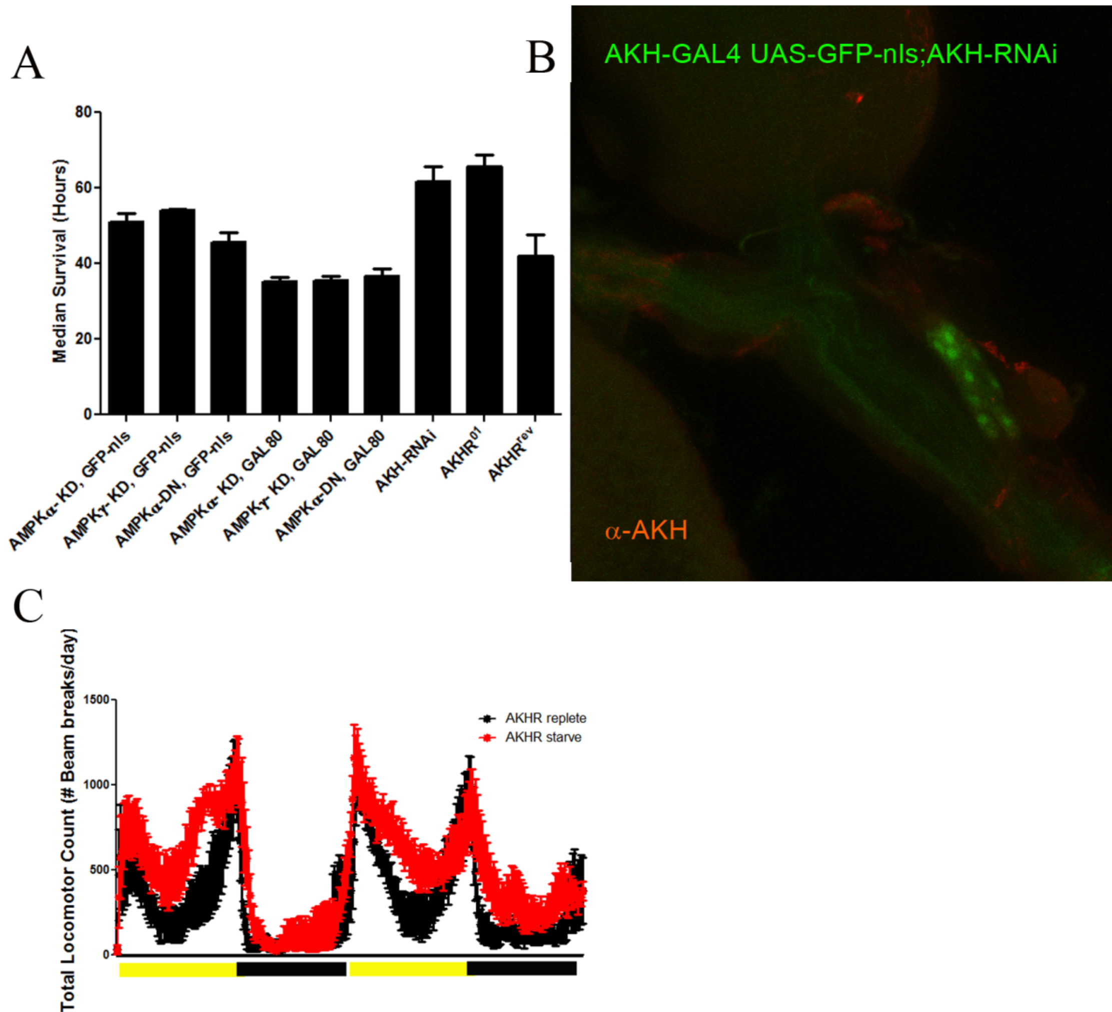

Figure S1 Median survival rates of different AKH genetic manipulations. A. Mean (+/- SEM) median survival rates for three replicates of male animals bearing different AKH genetic manipulations. Combination of the UAS-GFP-nls transgenes with either the a or g RNAi elements produced significant differences in lifespan as compared to controls (compare to Figure 1B) and also were significantly different than each transgene when tested in combination with the GAL80 element (which inhibits GAL4-mediated transcription). Also shown are values for an RNAi element targeting the AKH transgene directly and an AKHR deletion (*AKHR*^{01}) and a revertant line (*AKHR*^{rev}). B.The AKH-RNAi element showed no evidence of AKH immunosignals indicating that this line produced the desired effect of reduced AKH expression. C. The AKHR deletion line exhibits no significant difference in locomotor activity under replete or starved conditions.

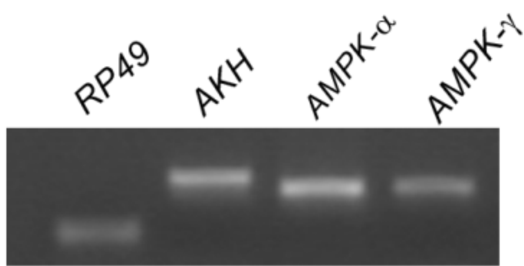

Figure S2 Single cell RT-PCR confirms AMPK expression in AKH cells. Individual AKH cells were isolated from animals expressing GFP under the control of the AKH-GAL4 driver. AMPK primers were designed to flank intronic sequences in order to determine whether amplicons were derived from cDNA via reverse transcription or contaminant genomic DNA sequences. A Qiagen single-cell RT PCR kit was employed to on a total of five individual AKH expressing cells. Specific AMPK primers used were previously reported (Johnson et al., 2010). All amplicons were of the correct expected length and sequence verified.

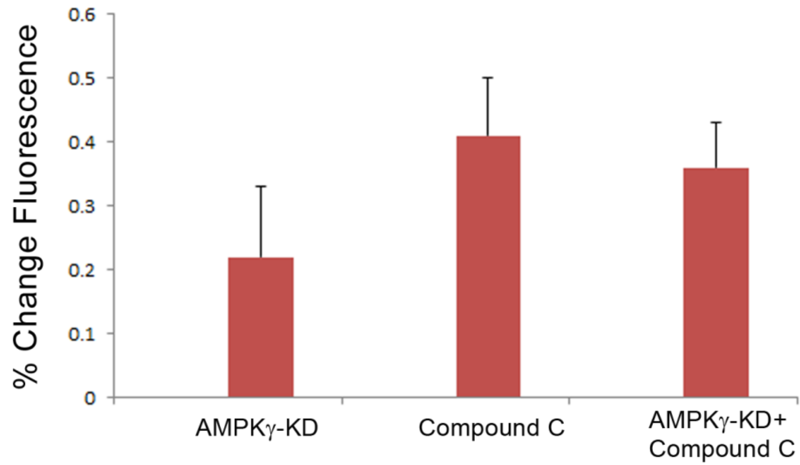

Figure S3 Compound C effects are largely mediated through inhibition of AMPK. We measured GCaMP fluorescence as described in the text under transitioning explanted AKH neuroendocrine cells from high to low trehalose containing solutions. We measured initial fluorescence and final fluorescence (30 minutes in low trehalose solution) and determined the percent change. There were no significant differences between wild-type AKH cells treated with Compound C and AMPKγ-KD expressing AKH cells treated with Compound C (ANOVA, $P= 0.83$).

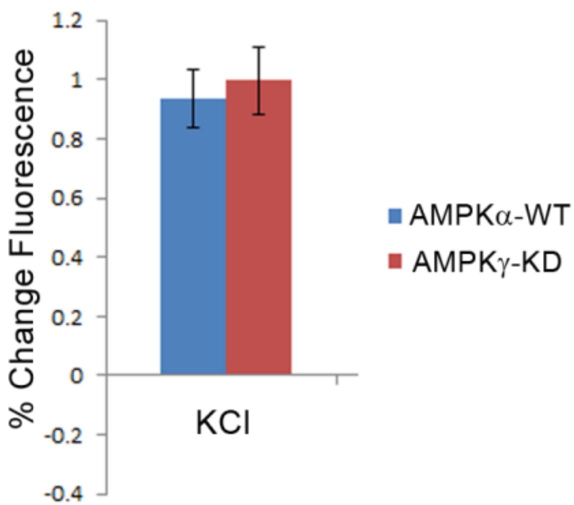

Figure S4 KCl evoked depolarizations are similar in different AMPK genetic backgrounds. GCaMP images were collected as described in the methods section. To test the possibility that AMPK may be regulating ion channel abundance in AKH cells and globally impact AKH cell excitability 3M KCl was added to confirm cell viability. To test the hypothesis that AMPK generally impacted AKH cell excitability, we measured fluorescence prior to addition of 3M KCl and again within ten seconds of application. Fluorescence levels were normalized to baseline levels and we observed no significant differences in the responses to KCl between wild-type and AMPKγ-KD. (Two tailed T-test, $P = 0.78$).