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AMPK links serotonergic signaling to glutamate release to regulate feeding behavior in *C. elegans*

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Summary

Serotonergic regulation of feeding behavior has been studied intensively for understanding the basic neurocircuitry of energy balance in various organisms and as a therapeutic target for human obesity. Its underlying molecular mechanisms still remain poorly understood. Here, we show that neural serotonin signaling in *C. elegans* modulates feeding behavior through inhibition of AMP-activated kinase in interneurons expressing the *C. elegans* counterpart of human *SIM1*, a transcription factor associated with obesity. In turn, glutamatergic signaling links these interneurons to pharyngeal neurons implicated in feeding behavior. We show AMPK mediated regulation of glutamatergic release is conserved in rat hippocampal neurons. These findings reveal previously unidentified cellular and molecular mediators of serotonergic signaling.

Introduction

In *C. elegans,* as in mammals, serotonin (5-HT) signaling modulates a wide array of responses seen when food deprived animals are re-exposed to food including foraging, mating, egg-laying, metabolism, and food intake behavior (Avery and Horvitz, 1990; Loer and Kenyon, 1993; Sze et al., 2000; Waggoner et al., 1998; Srinivasan et al., 2008). Treatment of food deprived *C. elegans* with 5-HT elevates food intake behavior even in the absence of food (Horvitz et al., 1982), while mutants in tryptophan hydroxylase, *tph-1*, which lack serotonin, exhibit reduced feeding rate even in the presence food (Srinivasan et al., 2008; Sze et al., 2000). Although increased serotonin signaling in mammals is thought to initiate satiety (Tecott, 2007), the noted effects of serotonin on *C. elegans* are consistent with a role in signaling food availability.

Food intake behavior in *C. elegans* is assessed by the rate of pharyngeal pumping given that increases or decreases in pumping rate correspond to amount of ingested nutrients (Avery

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and Horvitz, 1990; Avery 1993) and that pumping rate is modulated by food availability, food quality, and the experience of food deprivation (Shtonda and Avery, 2006; You et al., 2006; Avery and Horvitz, 1990). Here, we report on delineating the cellular and molecular components of one serotonergic circuit that links external cues to the modulation of food intake behavior. The identified circuit exhibits both molecular and regulatory parallels to mammalian feeding regulatory circuits highlighting the ancient origins of known pathways while revealing new regulatory modules.

Results and Discussion

Serotonin from chemosensory ADF neurons regulates feeding through SER-5

Under well-fed normoxic conditions, *tph-1* is expressed in only a few neurons, most notably NSM, located in the pharyngeal nervous system, ADF, a head chemosensory neuron, and HSN, a hermaphrodite-specific neuron required for wild type egg laying rate (Sze et al., 2000). As previously reported (Liang et al., 2006; Figure S1A), we noted that *tph-1* levels are reduced off food and increased as animals return to food. To determine the neural source of serotonin for wild type feeding rate, we reconstituted *tph-1* in only the pair of ADF or only the pair of NSM neurons using an *srh-142* (Sagasti et al., 1999) or a *ceh-2* promoter (Aspock et al., 2003), respectively. Reconstitution in the ADF but not in the NSM neurons restored wild type pumping rate to *tph-1* mutants (Figure 1A). While animals with *tph-1* reconstituted only in ADF neurons had fast movements like *tph-1* deficient animals, NSM-specific reconstitution of *tph-1* restored wild type moving behavior (Figure S1B). Thus, distinct sources of serotonin production underlie food intake and movement behaviors.

We next implemented cell-specific RNAi to inactivate *tph-1* in ADF neurons. As a gauge of RNAi efficacy, we monitored expression of a *tph-1::gfp* reporter fusion driven by a *tph-1* promoter. Selective knockdown of *tph-1* in ADF significantly reduced pumping (Figure 1A, 1B). By contrast, a previously established line in which *tph-1* is selectively inactivated in NSM (Harris et al., 2011) showed no significant changes in pumping rate relative to wild type animals (Figure S1C), corroborating our cell-specific reconstitution studies. However, Li et al recently reported that reconstitution of *tph-1* in NSM but not ADF neurons is sufficient to confer wild type pumping rate to *tph-1* mutants (Li et al., 2012). The reason for this obvious discrepancy is not known. It is possible that depending on the environmental conditions, different sources of serotonin signaling might be engaged to modulate pharyngeal pumping.

SER-5 is required for serotonergic regulation of feeding from ADF

How serotonin release from ADF neurons modulates pharyngeal pumping is not immediately apparent from the *C. elegans* anatomy. Serotonin produced in ADF can be taken up via the serotonin reuptake pump MOD-5 into RIH and AIM neurons, which do not normally synthesize serotonin (Jafari et al, 2011). Inhibition of MOD-5 by fluoxetine or loss of *mod-5* caused increased pumping in wild type but not in *tph-1* deficient animals (Figures 1C, 1D). Genetic or pharmacological inactivation of MOD-5 in *tph-1* mutants with *tph-1* selectively reconstituted in ADF neurons resulted in pumping rates that were indistinguishable from those of similarly treated wild type animals (Figures 1C, 1D). Thus, 5-HT produced within ADF neurons does not require *mod-5*-mediated reuptake into other neurons for feeding regulation, and 5-HT production in ADF using the *srh-142* promoter does not flood the nervous system with excessive levels of this neuromodulator.

To identify the signaling relays through which ADF-produced 5-HT regulates pumping rate, we first examined two GPCRs, encoded by *ser-1* and *ser-7*, since loss of either receptor abrogates the increased pharyngeal pumping rate caused by exogenous 5-HT (Song and Avery, 2012; Carre-Pierrat et al., 2006; Hobson et al., 2006; Srinivasan et al., 2008, Figure

1E). Loss of neither receptor altered the wild type pharyngeal pumping rate of animals in which serotonin production was specifically restored to ADF neurons (Figure 2A). We next examined *ser-5*, also encoding a putative serotonergic GPCR, with roles in serotonin-dependent behavioral processes (Hapiek et al., 2009; Harris et al., 2009; Kullyev et al., 2010), but not previously implicated in feeding behavior. As in *ser-1* and *ser-7* mutants, *ser-5* mutants displayed wild type pumping rates on well-fed conditions but failed to elevate pumping in response to exogenous 5-HT (Figure 1E). However, loss of *ser-5* abrogated the pharyngeal pumping rate of *tph-1* mutants in which *tph-1* was selectively reconstituted in ADF (Figure 2A). Thus, when 5-HT production is limited to the ADF neurons, the SER-5 receptor plays a non-redundant function in pumping rate.

SER-5 activity in SIM1/HLH-34 expressing neurons regulates feeding behavior

To begin dissecting the SER-5 feeding circuit, we generated transgenic animals expressing a P*ser-5::gfp* reporter fusion directed by 5 kb of putative *ser-5* promoter sequence. As previously reported (Carre-Pierrat et al., 2006; Hapiek et al., 2009), we noted prominent expression in numerous neurons and also in the body wall muscle, although the body wall muscle expression was quite faint in adults.

Next, we examined the roles of several *C. elegans* counterparts of human obesity and fat regulatory genes in search of additional gene inactivations that, similar to *ser-5* mutants, block the effects of 5-HT on pumping. This led to the identification of *hlh-34*, encoding a basic helix-loop-helix transcription factor, whose loss blocked the feeding increasing effects of 5-HT without altering basal pumping rate (Figure 2B). *hlh-34* is the closest *C. elegans* counterpart of *D. melanogaster* and mammalian *single minded* (*SIM1*) genes. Mammalian *SIM1* is a transcription factor expressed in various brain regions and required for development of the hypothalamic nuclei known as the PVN (Michaud et al., 2001), the site of expression of many of the signaling receptors with key roles in energy balance. To determine sites of function of *hlh-34*, we generated a transgenic reporter consisting of 2.5 kb upstream of the predicted *hlh-34* start site fused to mCherry (P*hlh-34::mCherry*). This reporter was prominently detected in a single pair of head interneurons but nowhere else (Figure 2C, S2A–C). The anatomical characteristics of neurons expressing the P*hlh-34::mCherry* reporter corresponded to either the AVJL/R or the AVHL/R pair of interneurons, which have nearly identical anatomical characteristics. To help identify *hlh-34* expressing neurons and demonstrate that they are distinct from neurons such as ASH, previously implicated in *ser-5* mediated functions (Harris et al., 2009; Harris et al., 2011) or from the pharyngeal muscle, we crossed in reporter fusions driven by the *ocr-2* (Tobin et al., 2002) and *myo-2* promoters into the P*hlh-34::mCherry* line (Figure S2G-R). To help distinguish between AVJ and AVH as the site of the P*hlh-34::mCherry* reporter, we relied on previously reported expression of *eat-4*, encoding the vesicular glutamate receptor (Lee et al., 1999), which is thought to be expressed in AVJ but not AVH (Lee et al., 1999; http://wormweb.org/neuralnet). As indicated below, glutamate signaling from the *hlh-34* expressing neurons is required for serotonergic feeding regulation, suggesting that *hlh-34* expressing neurons are AVJL/R.

We used the 2.5 kb *hlh-34* promoter to drive expression of human SIM1 in *hlh-34* mutants. This transgene did not alter basal pumping rates but conferred responsiveness to exogenous 5-HT (Figure 2B), suggesting that *SIM1* is in fact, the functional counterpart of *hlh-34,* a remarkable observation given that *C. elegans* lack an anatomically discernable hypothalamus. We next investigated whether *hlh-34* expressing neurons may be the site of serotonin action on SER-5 since we saw overlap of P*hlh-34::mCherry* and P*ser-5::ser-5::gfp* in AVJ (Figure 2C). Selective targeting of *ser-5* to *hlh-34* expressing neurons allowed *ser-5* deficient animals to once again respond to exogenous 5-HT (Figure 2B). Targeting of the same *ser-5* construct to the pharyngeal muscle or pharyngeal neurons failed to confer

serotonin responsiveness (Figure 2B). To determine if the *hlh-34* expressing neurons contribute to pharyngeal pumping rate beyond their role in 5-HT signaling, we genetically ablated these neurons using the mammalian caspase, interleukin-1-β converting enzyme (ICE). This resulted in a ~10% reduction in basal pharyngeal pumping rate as well as unresponsiveness to exogenous 5-HT (Figure 2B). Thus, the AVJ neurons are essential for both basal as well as 5-HT mediated increased pumping rates even when all other components of serotonin signaling are intact.

AMPK activity in SIM1/HLH-34 neurons regulates feeding behavior

A second gene to emerge from our candidate screen was *aak-2*, encoding a catalytic subunit of the AMP-activated kinase (AMPK), a conserved regulator of energy balance (Hardie, 2007). As in mammals, the catalytic subunit of the *C. elegans* AMPK is encoded by one of two distinct genes, *aak-1* and *aak-2. aak-2* mutants exhibited elevated pumping even in the absence of exogenous 5-HT (Figure 3A) that did not further elevate with 5-HT (Figure 3B). This was not simply a reflection of the upper limit of pumping capacity as a similarly elevated basal pumping rate of *aak-1* mutants could be further elevated by 5-HT (Figure S3A). Moreover, treatment with aminoimidazole carboxamide ribonucleotide (AICAR), a pharmacological activator of AMPK, caused reduced pumping dependent on *aak-2* (Figure S3A), highlighting the role of this catalytic subunit in the regulation of pharyngeal pumping rate.

We next investigated the relationship between *ser-5, hlh-34*, and *aak-2* mutants. Pharyngeal pumping rates of *ser-5; aak-2* double mutants were as elevated as those of *aak-2* mutants (Figure 3A), while loss of *hlh-34* abrogated the increased rate of *aak-2* mutants (Figure 3A). *aak-2* is expressed broadly in *C. elegans* (Narbonne and Roy, 2009). To determine where *aak-2* is required to regulate pharyngeal pumping rate in response to either AICAR or 5-HT, we reconstituted *aak-2* within specific tissues *aak-2* has two predicted isoforms, *aak-2*a and *aak-2*c (wormbase release WS223). We first reconstituted both isoforms within either the pharyngeal muscle or the nervous system. Reconstitution within either of these tissues was sufficient for wild type pumping but insufficient for responsiveness to either 5-HT or AICAR. By contrast, simultaneous reconstitution within the pharyngeal muscle and the nervous system restored responsiveness to both 5-HT and AICAR (Figure S3B, C). To define the site of action of *aak-2* within the nervous system for responsiveness to 5-HT and AICAR, we reconstituted the two *aak-2* isoforms within *hlh-34* expressing cells as well as pharyngeal muscle cells of *aak-2* deficient animals. These animals had wild type pharyngeal pumping rates and were responsive to 5-HT and AICAR (Figure 3B, Figure S3B). By contrast, reconstitution within the two other pairs of interneurons, RIM and RIC, previously implicated in feeding behavior (Greer et al., 2008), or within a subset of amphid neurons did not confer responsiveness to 5-HT, highlighting the role of *aak-2* within *hlh-34* expressing neurons (Figure 3B).

Serotonin regulates AMPK via Protein Kinase A

SER-5 is predicted to be a $G_{\alpha s}$ coupled GPCR (Harris et al., 2009). To test the prediction that enhanced cAMP production in *hlh-34* neurons elevates pumping, we selectively expressed *gsa-1*(R182C), a dominant, gain-of-function mutation of *C. elegans* $G_{\alpha s}$ (Schade et al., 2005), in *hlh-34* neurons. This led to increased pumping rate (Figure 3C). In mammals, AMPK phosphorylation at Ser173 by Protein Kinase A, PKA, antagonizes activating phosphorylation at Thr172 by the LKB1 kinase (Djouder et al., 2010). To test this model, we first examined wild type animals in which adenylate cyclase was inactivated by *acy-1* RNAi and found that these animals failed to elevate pumping rates in response to 5-HT (Figure S3D). We then substituted an alanine residue at AMPK(S244), the *Ce*AAK-2 residue equivalent to mammalian Ser173 to prevent PKA-dependent phosphorylation at this

site (Djouder et al., 2010). We reconstituted wild type isoforms of *aak-2* within the pharyngeal muscle of *aak-2* deficient animals and the (S244A) versions of AAK-2 in *hlh-34* expressing neurons. These transgenic animals were unresponsive to 5-HT (Figure 3B), suggesting that PKA-dependent phosphorylation at this site is required to inactivate AAK-2. As a control, we showed that changing another PKA phosphorylation site of AAK-2, *Ce*S553 equivalent to mammalian Ser491 position, did not abrogate responsiveness to 5-HT. Combining the constitutive allele of *gsa-1* with the *aak-2*(S244A) allele in *hlh-34* neurons resulted in animals with essentially wild type pumping rates (Figure 3C), indicating that the *aak-2* and GSA-1 are likely acting in the same pathway. Together, these results suggest a model in which serotonin signaling initiated from ADF neurons acts directly on SER-5 receptors in *hlh-34* expressing neurons to inactivate AAK-2 via inhibitory phosphorylation by PKA at AAK-2(Ser244).

Glutamate signaling from SIM1/HLH-34 neurons modulates pumping rate

To investigate how loss of *aak-2* promotes pumping, we took advantage of the genes previously identified in an RNAi-based Nile Red screen, a subset of which also reduce pharyngeal pumping (Figure 4A). In most cases, treatment of *aak-2* mutants with these pharyngeal pumping reducing RNAis resulted in an intermediate rate, suggesting parallel mechanisms. One exception was *glr-7* (C43H6.9), which when inactivated lowered the pumping rate of *aak-2* mutants and wild type mutants to the same level (Figure 4A). We verified these RNAi results by examining *glr-7, aak-2* double mutants (Figure S4A).

Since *glr-7* encodes for a non-NMDA type ionotropic glutamate receptor expressed in the I1, I2, I3, M1, and NSM pairs of pharyngeal neurons (Brockie et al., 2001), we asked whether glutamate signaling could link *hlh-34* and *glr-7* expressing sets of neurons. We found that animals deficient in *eat-4*, encoding the *C. elegans* orthologue of the mammalian BNPI vesicular glutamate transporter (Lee et al., 1999) failed to elevate their pumping rates on 5-HT, and *eat-4; aak-2* double mutants exhibited the same rates of pumping as *eat-4* single mutants (Figure 4B). However, since the basal pharyngeal pumping rate of *eat-4* mutants is ~25% lower than that of wild type, we sought to distinguish between a specific requirement for *eat-4* within the serotonergic circuit from a general requirement in pharyngeal pumping. Reconstitution of *eat-4* only in the *hlh-34*-expressing cells of otherwise *eat-4* deficient animals did not alter the reduced basal rate of these animals but conferred responsiveness to 5-HT (Figure 4C), revealing a role for glutamate signaling from *hlh-34* neurons in 5-HT mediated feeding regulation.

Effect of AMPK on glutamatergic release is conserved in mammals

The mechanisms through which inhibition of AMPK leads to glutamatergic release from *hlh-34* neurons is not currently known. Nevertheless, we next asked whether a similar regulatory relationship may be conserved in mammals. The availability of experimental reagents prompted us to examine rat hippocampal neurons, which have been used extensively for studying glutamatergic synaptic vesicle recycling (Miesenbock et al., 1998; Voglmaier et al., 2006; Granseth et al., 2006). VGLUT1 is the vesicular glutamate transporter and is present in the synaptic vesicles in the majority of excitatory synapses in the hippocampus. PHluorin, a pH-sensitive form of GFP, exhibits essentially complete fluorescence quenching at the low pH inside synaptic vesicles (Miesenbock et al., 1998; Sankaranarayanan et al., 2000). The fluorescence of pHluorin increases with exocytosis and decays with endocytosis, and alkalinization of the nerve terminal with a permeant weak base (such as NH4Cl) reveals the total pool of fluorescent protein. Hippocampal neurons transfected with VGLUT1-pHluorin were subjected to electric field stimulation to elicit synaptic vesicle exocytosis. To determine the kinetics and extent of exocytosis without interference from concurrent endocytosis, bafilomycin was added during the stimulation to

prevent the re-acidfication of the newly formed synaptic vesicles. To ask if inhibition of AMPK leads to increased exocytosis, we added Compound C, an AMPK inhibitor, and measured the kinetics of exocytosis. The rate of exocytosis was significantly accelerated in the presence of Compound C (Figure 4D and 4E) without a change in the relative recycling pool size (Figure S4D). Endocytosis after synaptic vesicle exocytosis was normal, if not faster, in the presence of Compound C (Figure S4C), indicating that faster exocytosis does not necessarily result in faster depletion of the recycling pool. Taken together, these data suggest that inhibition of AMPK can increase the efficiency of glutamate vesicle release at the synapse. However, Yang and colleagues recently reported that in a state of food deprivation, AMPK is activated in the arcuate nucleus region of the hypothalamus resulting in glutamate release (Yang et al., 2011). Thus, the relationship between AMPK activity and glutamate release is likely to be dependent on specific cell types in which they are studied.

In summary, our findings reveal a circuit that links changes in environmental cues through two layers of interneurons to the pharyngeal muscle, the feeding organ of *C. elegans*. The most salient feature of our study is identification of common regulatory themes in *C. elegans* and mammalian feeding circuits. In mammals, anorexic and orexigenic signals regulate feeding behavior, in part, through modulation of AMPK in the nervous system (Minokoshi et al., 2004; Seo et al., 2008). Moreover, the PVN region of the hypothalamus has been identified as one of the sites in which peripheral signals of food availability modulate AMPK activity (Blanco Martínez de Morentin et al., 2011). Here, we found that serotonin, an indicator of food availability, regulates food intake behavior through inhibition of AMPK in neurons that are the site of action of the *C. elegans* counterpart of human *SIM1.* Thus, some of the regulatory features found in the mammalian hypothalamus predate development of this brain region. Finally, we speculate that the link between serotonin and AMPK is likely to be a common regulatory feature in a variety of different cell types in a broad range of physiological outcomes.

Experimental Procedures

Pharyngeal pumping rate assays

Gravid adults were synchronized using hypochlorite treatment and plated as L1s. Animals were grown at 20°C and feeding rate was assayed on gravid adults. Pharyngeal pumping rates were measured by counting the contraction of the pharyngeal bulb over a 10 s period using a Zeiss M2-Bio microscope. For AICAR treatment, animals were picked into liquid SM media containing OP50 bacteria and either vehicle (dH_20) or drug (2 mM AICAR) and were left at 20°C for 2 h. After treatment, animals were returned to NGM plates containing food and pharyngeal pumping rates were assayed after 15 min. For all measurements, 10 animals were counted per condition for each genotype. Wild type pumping rates were normalized to 100 and data are presented as the percentage of wild type treated with vehicle. Each experiment was repeated at least twice. Error bars represent +/− standard error of the mean (SEM) for all graphs. At least three independent lines were tested for all transgenic experiments.

Enhanced Slowing Response Locomotion Assay

This assay was performed according to Sawin et al., 2000. Briefly, well-fed, day 1 adult animals were washed twice in S-basal and placed on an unseeded NGM plate. After 5 minutes, body bends per 20 s were counted for 10 animals. The animals were left on the plate for 30 min before removal to a fresh NGM plate seeded with HB101 *E. coli.* After 5 min, body bends of 10 animals were counted. HB101 plates were incubated at 37°C overnight and cooled to room temperature prior to the start of the assay.

Hippocampal neuron cell culture and molecular biology

Hippocampal neurons isolated from day 20 rat embryos were transfected by electroporation (Amaxa) and cultured as previously described (Li et al., 2005). VGLUT1-pHluorin (Voglmaier et al., 2006) was inserted upstream of an internal ribosome entry sequence (IRES2, Clontech) driving the translation of mCherry-synaptophysin.

Hippocampal neuron imaging

Transfected neurons were imaged at 12–14 days *in vitro* as previously described (Voglmaier et al., 2006). pHluorin was imaged with 492/18 nm excitation and 535/30 nm emission filters mCherry was imaged with 580/20 nm excitation and 630/60 nm emission filters. Images were collected every 3 s. Neurons were imaged in standard Tyrode's solution (119 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 30 mM glucose and 25 mM Hepes, pH7.4). NH_4Cl buffer (69 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 50 mM NH_4Cl, 30 mM glucose, 25 mM Hepes, pH 7.4) was used to reveal total pHluorin fluorescence. Glutamate receptor antagonists 6-cyano-7 nitroquinoxaline-2,3-dione (CNQX) (10 μM) and D,L-2-amino-5-phosphonovaleric acid (APV) (50 μM) were included in the Tyrode's solution during the experiments. Bafilomycin (0.6 μM) was diluted from 0.6 mM stock solutions in DMSO, and Compound C (20 μM) was preincubated with neurons at 37°C for 2 h.

Hippocampal neuron data analysis

Regions enclosing the entire synaptic bouton were selected using mCherry-synaptophysin. Arbitrary fluorescence units were normalized to the total intracellular fluorescence (in NH_4Cl), which was determined as F_{NH4Cl}-$F_{initial.}$ To determine the kinetics of exo- and endocytosis with 10 Hz stimulation, the change in fluorescence was normalized to the maximum change in fluorescence during stimulation ($F_{poststim}$ - $F_{prestim}$). Endocytosis kinetics were fit to a single-exponential decay ($F = F_{plateau} + F_{span} \bullet e^{-kt}$). Exocytosis kinetics were fit to a single-exponential [$F = F_{max} \bullet (1 - e^{-kt})$]. Data indicate mean ± SEM, and nested Anova was used to compare groups.

Cell-specific RNAi

Neuron-specific RNAi transgenes were constructed by fusing neuron-selective promoters to the 5′ and 3′ genomic regions of target genes. Promoter regions were fused to both the 5′ and 3′ ends of the genomic region, with the 3′ promoter in the 3′-5′ orientation to create a 5′-promoter-3′-5′-genomic region-3′-3′-promoter-5′ fused PCR product. PCR products were pooled and injected into the gonads of animals with a co-injection marker.

Statistical Analysis

Student's t test was used for pairwise comparisons. For multiple comparisons, ANOVA with Bonferroni correction was used. Error bars represent +/− standard error of the mean (SEM) for all graphs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *C. elegans* counterparts of obesity genes mediate serotonergic feeding behavior
- Serotonin signaling causes inhibition of AMP-activated kinase
- Inhibition of AMP-activated kinase promotes glutamatergic release

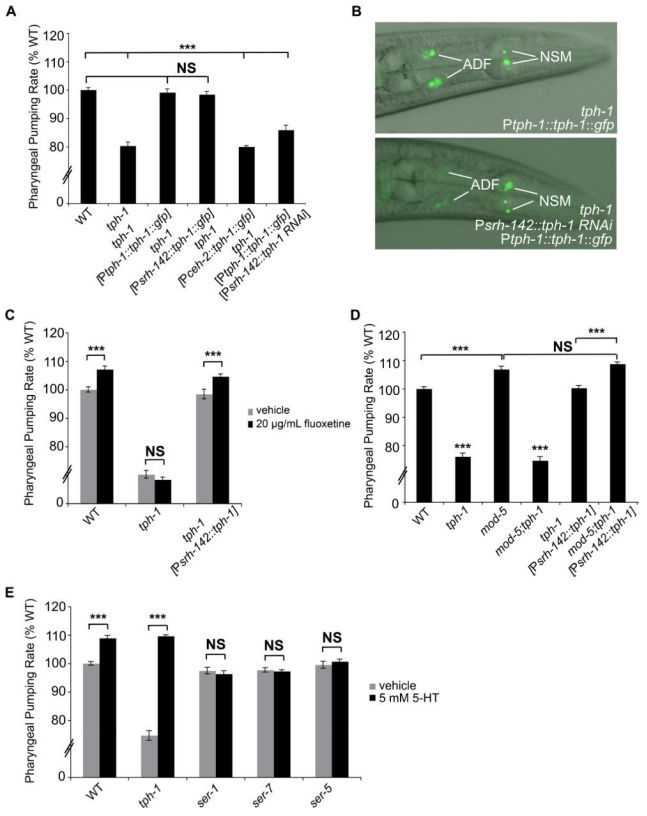

Figure 1. ADF serotonin is necessary and sufficient for wild type feeding

A. Reconstitution of *tph-1* in NSM and ADF by *tph-1*[P*tph-1::tph-1*] or only in ADF by *tph-1*[P*srh-142::tph-1*] but not in NSM *tph-1*[P*ceh-2::tph-1*] confers WT pumping rates to *tph-1* mutants. **B.** Cell-specific RNAi of *tph-1* in ADF results in reduced pharyngeal pumping rates. Efficacy of ADF-specific *tph-1* RNAi as monitored by TPH-1::GFP levels. **C.** Fluoxetine treatment (20 μg/mL) and **D.** *mod-5(n3314)* increase pharyngeal pumping rate in WT animals and when *tph-1* is reconstituted in ADF of *tph-1* deficient animals. **E.** Animals lacking *ser-1*, *ser-7*, or *ser-5* fail to elevate pumping rate in the presence of 5-HT. Data are normalized mean +/− SEM. For **A** and **D**, ANOVA with Bonferroni correction for multiple comparisons, for **C** and **E**, Student's t-test. *** $p < 0.001$ versus well-fed animals, unless otherwise indicated. See also Figure S1.

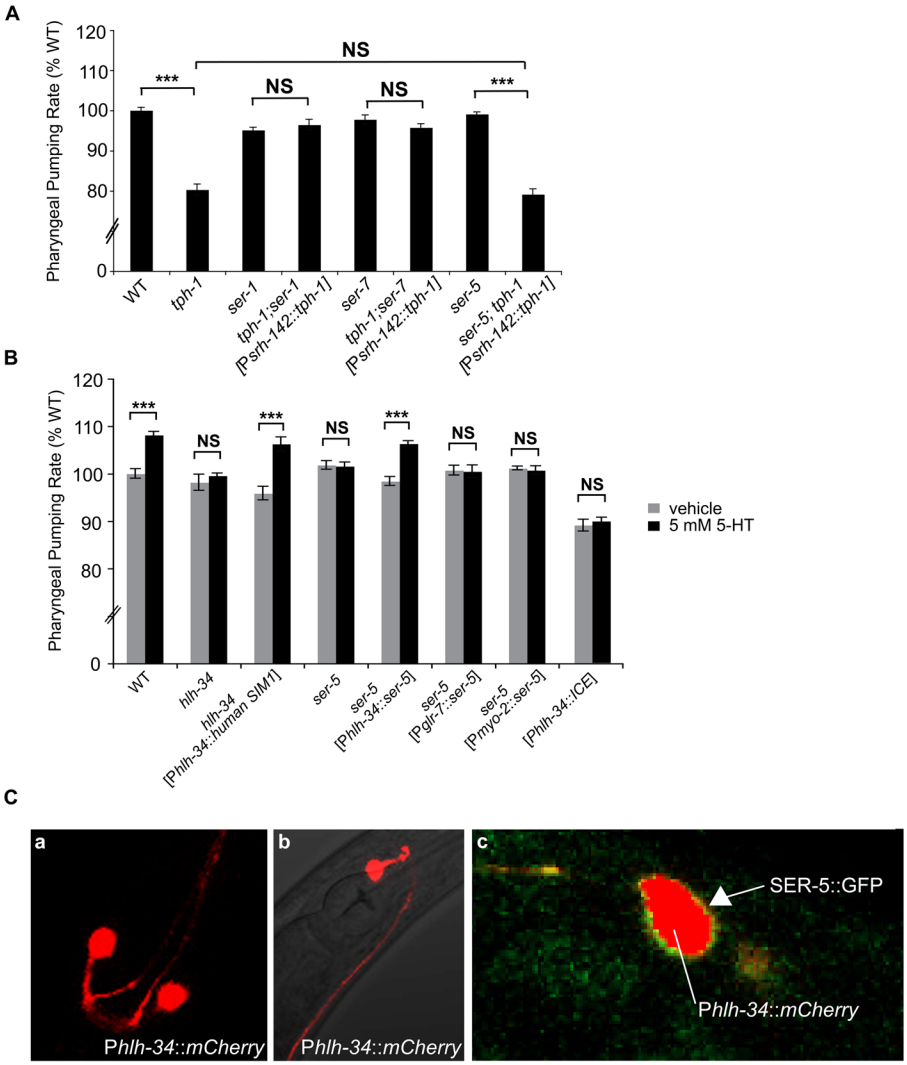

Figure 2. Serotonin from ADF requires *ser-5* in AVJ to elevate pumping

A. WT pumping rate of *tph-1* mutants in which *tph-1* is reconstituted in ADF alone, is dependent on *ser-5 (ser-5; tph-1*[P*srh-142::tph-1*]) but not *ser-1* (*tph-1;ser-1*[P*srh-142::tph-1*]) or *ser-7* (*tph-1;ser-7*[P*srh-142::tph-1*]). **B.** *ser-5* and *hlh-34* are required for elevation of pumping rate in response to 5 mM 5-HT. Reconstitution of *ser-5* within *hlh-34*-expressing neurons, but not pharyngeal neurons (P*glr-7::ser-5*) or pharyngeal muscle (P*myo-2::ser-5*) confers responsiveness to 5-HT. Genetic ablation of *hlh-34* neurons (P*hlh-34::ICE*) abrogates responsiveness to 5-HT. Data are normalized mean +/− SEM. For **A**, ANOVA with Bonferroni correction for multiple comparisons. For **B**, Student's t-test. *** $p < 0.001$ versus well-fed animals, unless otherwise indicated**. C.** Panels **a.** and **b.** 2.5 kilobases upstream of the *hlh-34* start site was used to drive expression of *mCherry*. Panel **c.** Expression of P*hlh-34::mcherry* and P*ser-5::ser-5::gfp* overlap in *hlh-34* neurons. Images were taken using a spectral confocal microscope using a Z-stack and compressed into a single file. See also Figure S2.

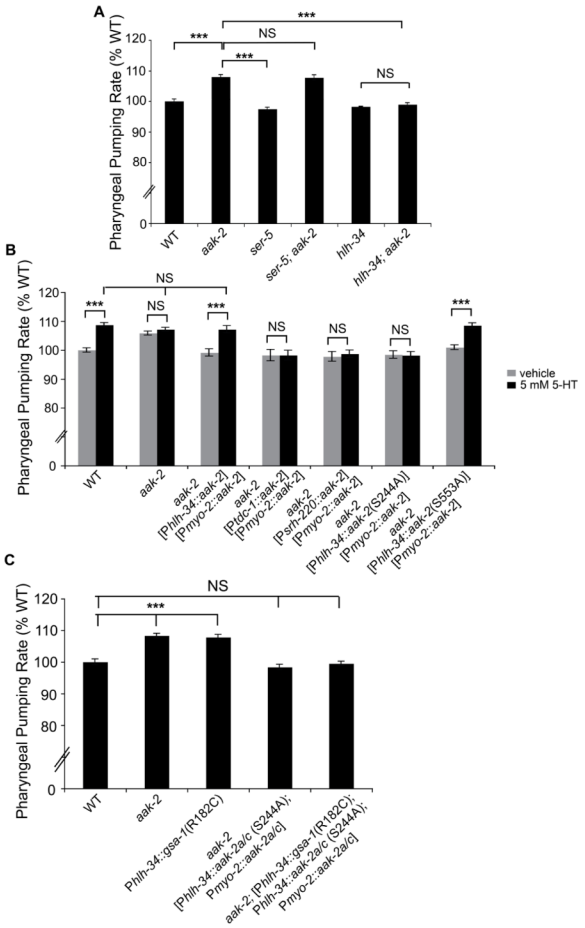

Figure 3. Serotonin regulates AMPK via Protein Kinase A

A. Pharyngeal pumping rates of indicated strains. **B.** Reconstitution of *aak-2* in *hlh-34*-expressing neurons (P*hlh-34::aak-2*) is sufficient for responsiveness to 5-HT. Reconstitution of *aak-2* within RIM/RIC interneurons (P*tdc-1::aak-2*) or ADL amphid neurons (P*srh-220::aak-2*) (McCarroll et al., 2005) is not sufficient. Alanine substitution at AMPK Ser244 but not Ser553 blocks the pumping effect of 5-HT. In all *aak-2* reconstitution studies, *aak-2* is expressed in the pharyngeal muscle, using the *myo-2* promoter. **C.** Pharyngeal pumping rates of animals expressing the constitutively active *gsa-1*(R182C) in *hlh-34*-expressing neurons. For **A–C**, data are normalized mean +/− SEM. ANOVA with Bonferroni correction for multiple comparisons was used for statistical analysis. *** $p < 0.001$ versus well-fed animals, unless otherwise indicated. See also Figure S3.

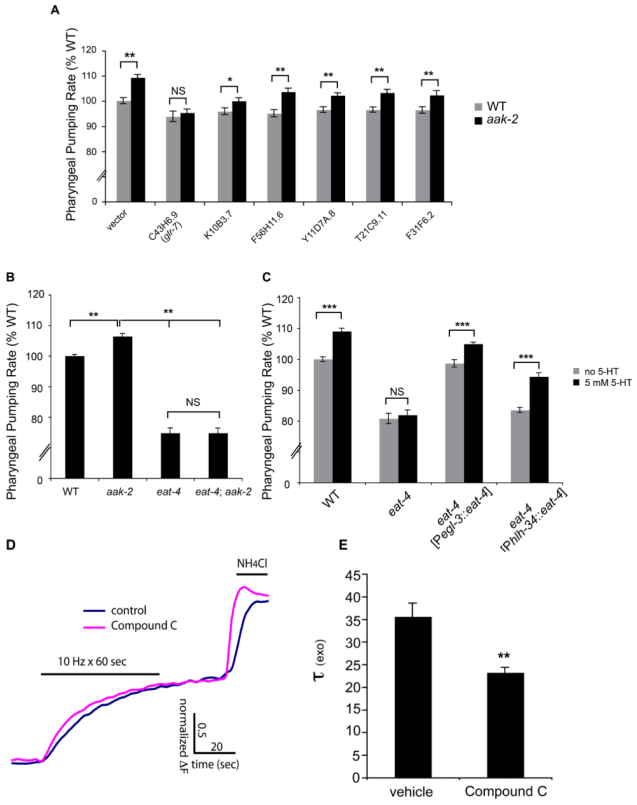

Figure 4. AMPK links serotonin to glutamatergic signaling in *hlh-34* neurons

A. Pumping rates of RNAi treated wild type (WT) and *aak-2* mutants. Data are normalized mean +/− SEM. Student's t-test was used for statistical analysis. * $p < 0.05$, ** $p < 0.01$ versus vector treated animals. **B.** Pumping rates of *eat-4; aak-2* relative to *eat-4* mutants. Data are normalized mean +/− SEM. ANOVA with Bonferroni correction for multiple comparisons was used for statistical analysis. ** $p < 0.01$ **C.** Reconstitution of *eat-4* only in *hlh-34*-expressing neurons confers 5-HT responsiveness. Data are normalized mean +/− SEM. Student's t-test was used for statistical analysis. *** $p < 0.001$. **D.** Synaptic vesicle exocytosis is accelerated in the presence of Compound C. **E.** The time constant for exocytosis (τ_{exo}) is significantly faster in the presence of Compound C. $p = 0.00154$; $n = 8$. 5 coverslips containing a total of 300 boutons for each group. See also Figure S4.