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Role of CYP eicosanoids in the regulation of pharyngeal pumping and food uptake in *Caenorhabditis elegans*

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Abstract Cytochrome P450 (CYP)-dependent eicosanoids comprise epoxy- and hydroxy-metabolites of long-chain PUFAs (LC-PUFAs). In mammals, CYP eicosanoids contribute to the regulation of cardiovascular and renal function. *Caenorhabditis elegans* produces a large set of CYP eicosanoids; however, their role in worm's physiology is widely unknown. Mutant strains deficient in LC-PUFA/eicosanoid biosynthesis displayed reduced pharyngeal pumping frequencies. This impairment was rescued by long-term eicosapentaenoic and/or arachidonic acid supplementation, but not with a nonmetabolizable LC-PUFA analog. Short-term treatment with 17,18-epoxyeicosatetraenoic acid (17,18-EEQ), the most abundant CYP eicosanoid in *C. elegans*, was as effective as long-term LC-PUFA supplementation in the mutant strains. In contrast, 20-HETE caused decreased pumping frequencies. The opposite effects of 17,18-EEQ and 20-HETE were mirrored by the actions of neurohormones. 17,18-EEQ mimicked the stimulating effect of serotonin when added to starved worms, whereas 20-HETE shared the inhibitory effect of octopamine in the presence of abundant food. In wild-type worms, serotonin increased free 17,18-EEQ levels, whereas octopamine selectively induced the synthesis of hydroxy-metabolites. These results suggest that CYP eicosanoids may serve as second messengers in the regulation of pharyngeal pumping and food uptake in *C. elegans*.—Y. Zhou, J. R. Falck, M. Rothe, W-H. Schunck, and R. Menzel. **Role of CYP eicosanoids in the regulation of pharyngeal pumping and food uptake in *Caenorhabditis elegans*. *J. Lipid Res.* 2015.** 56: **2110–2123.**

Supplementary key words cytochrome P450 • fatty acid • omega-3 fatty acids • lipidomics • pharynx • 17,18-epoxyeicosatetraenoic acid

Various members of the cytochrome P450 (CYP) superfamily act as epoxygenases and/or hydroxylases of long-chain PUFAs (LC-PUFAs) (1, 2). CYP enzymes differ in their individual substrate and reaction specificities and produce distinct sets of LC-PUFA-derived epoxy- and hydroxy-metabolites, collectively termed CYP eicosanoids. In mammals, these metabolites serve as second messengers of numerous hormones, growth factors, and cytokines that regulate vascular, cardiac, and renal function (3). Imbalances in CYP-eicosanoid formation are linked to the development of cardiovascular disease, inflammatory disorders, and cancer (3–5). Similar to mammals, the nematode *Caenorhabditis elegans* (Maupas, 1900) produces arachidonic acid (AA)- and EPA-derived CYP eicosanoids (6). CYP-33E2, CYP-29A3, and EMB-8, the worm's NADPH-CYP reductase (CPR), have been identified as contributing enzymes (6). CYP-33E2 prefers EPA over AA and generates 17,18-epoxyeicosatetraenoic (17,18-EEQ) as main EPA-derived metabolite (7). CYP2J2, a major epoxygenase in the heart, is the most closely related human homolog of CYP-33E2. CYP-29A3 presumably contributes to hydroxy-metabolite formation and shows sequence homology to mammalian CYP4 family members that produce 20-HETE as their main AA-derived metabolite (6).

C. elegans allows genetic dissection of the physiological functions of various PUFAs and their metabolites, with a resolution not possible in mammals (8). Mammals must obtain n-6 and n-3 PUFAs from the diet, whereas *C. elegans* endogenously produces both PUFA classes. Key genes present in *C. elegans*, but not in mammals, encode Δ-12 (*fat-2*) and n-3 fatty acyl desaturases (*fat-1*). In concert with further desaturases and elongases, *fat-2* and *fat-1* establish a biosynthetic pathway from oleic (18:1 n-9), via linoleic acid (LA; 18:2 n-6) and α-linolenic acid (ALA; 18:3 n-6), to AA (20:4 n-6) and EPA (20:5 n-3) (8, 9). Mutations in *fat-2*

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; CPR, CYP reductase; CYP, cytochrome P450; DHEQ, dihydroxyeicosaquatraenoic acid; 17,18-EEQ, 17,18-epoxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; ETYA, eicosatetraynoic acid; HEPE, hydroxyeicosapentaenoic acid; IPTG, isopropyl-β-D-thiogalactopyranosid; LA, linoleic acid; LC-PUFA, long-chain PUFA; NGM, nematode growth media; PLA_2, phospholipase A_2; RNAi, RNA interference; sEH, soluble epoxide hydrolase.

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or *fat-3* (Δ-6 fatty acid desaturase) cause C20-PUFA deficiency and lead to deficits in movement, defecation cycle, pharyngeal pumping activity, basal innate immunity, and growth (10). Almost all of these impairments are rather functional than developmental and can be rescued by feeding the mutant worms with AA and/or EPA (8, 11–13).

Searching for potential roles of CYP eicosanoids in *C. elegans*, we found that the *cyp-33E2* gene is expressed in pharyngeal marginal cells. Gene silencing or pharmacological CYP inhibition reduced pharyngeal pumping frequencies (7). This phenotype is shared by *fat-2* and *fat-3* mutants, supporting the hypothesis that AA- and/or EPA-derived CYP eicosanoids modulate pharyngeal activity. The pharynx is a rhythmically active pump that sucks, filters, and grinds nutrients (bacteria) and passes them to the intestine of *C. elegans* (14). The pharynx of nematodes appears related to the vertebrate heart: both organs move material along their lumens using binucleate cells and rely on similar electrical circuitry, and their geneses depend on related transcription factors (15). Pharyngeal pumping is controlled by endogenous and environmental cues and requires muscle-neuron interactions (16), whereby neurohormones, such as serotonin (stimulatory effect) or octopamine (inhibitory effect), play a major role (17–19).

Based on these findings, we hypothesized that CYP eicosanoids may function as second messengers of neurohormones regulating the feeding behavior of *C. elegans*. We tested the effects of 17,18-EEQ and 20-HETE on pharyngeal pumping frequencies and the uptake of fluorescent beads in both wild-type and C20-PUFA-deficient strains and analyzed the effects of serotonin and octopamine on CYP eicosanoid de novo synthesis.

MATERIALS AND METHODS

Strains and cultivation

The *C. elegans* wild-type strain used in this study was Bristol N2. Mutant strains are BX24, *fat-1(wa9)*; BX26, *fat-2(wa17)*; BX30, *fat-3(wa22)*; MJ69, *emb-8(hc69)*; and VC40814, *cyp-29A3(gk827495)*. All strains were provided by the Caenorhabditis Genetics Center. The million mutation project-derived VC40814 was outcrossed to wild-type five times before analysis. Selection of *cyp-29A3* genotype took advantage of a missing *Mst*I restriction site in the mutant allele background.

Well-fed animals were maintained on nematode growth media (NGM) plates seeded with *Escherichia coli* (Migula 1895) OP50 as food source and incubated at 20°C except *emb-8(hc69)*, which was maintained at 15°C and shifted to restrictive 25°C during the assay; for details see (6). Only hermaphrodite individuals were assayed in all experiments. Unless otherwise stated, bacteria for pumping experiments were UV-killed by 1 h exposure to 5.6 mw/cm^2 UV-light on a transilluminator (Fluo-Link FL-20-M; Bachofer, Reutlingen, Germany). UV treatment was approved as effective when no *E. coli* cells were able to grow after spreading bacterial suspension onto an lysogeny broth agar plate and incubated overnight at 37°C.

RNA interference by feeding

The RNA interference (RNAi) by feeding assay (20) was performed on NGM agar plates supplemented with additional antibiotics (50 μg/ml ampicillin, 12.5 μg/ml tetracycline) and 0.8 mM isopropyl-β-D-thiogalactopyranosid (IPTG). *E. coli* HT115 bacteria containing RNAi vectors expressing double-stranded RNA of the genes *cyp-29A3* and *cyp-33E2*, respectively, were precultured, induced with IPTG and finally used as nematode food as described previously (6).

Chemicals

IPTG and the antibiotics were purchased from Roth (Karlsruhe, Germany), octopamine, serotonin, and EPA from Sigma-Aldrich (St. Louis, MO), and AA, eicosatetraynoic acid (ETYA), and all used eicosanoids from Cayman Chemicals (Ann Arbor, MI). Both quantity and purity of prepared eicosanoid stock solutions were confirmed by LC/MS/MS measurements (data not shown). The compound used as 17,18-EEQ agonist was synthesized as described previously (21). To prevent autoxidation, all stock solutions, except for neurohormones, were prepared in an oxygen evacuated nitrogen chamber. DMSO, purchased from Sigma-Aldrich, was used as solvent; only neurohormones were dissolved in distilled water.

Preparation of assay plates and treatment

For long-term incubation, PUFA stocks were mixed with living OP50 bacteria and seeded on NGM plates at final concentration of 80 μM in the bacterial lawn, a concentration following the work of Watts et al. (10). Plates were dried in the dark. Worms from a mixed culture were chunked to assay plates to ensure that next generation was fed their whole life with dietary PUFA. Then, synchronized L1 progeny was incubated for 3 days prior use in the pumping assay. Synchronization was achieved by rinsing worms from NGM plates with M9 buffer, filtering through a 10 μm gauze membrane retaining all but first-stage juveniles, and incubating them for a further 3 days on fresh NGM/OP50 agar plates. Control experiments were included by mixing only solvent, 0.3% (v/v) DMSO, with the bacteria. All chemicals used for short-term incubation were spread onto the NGM plates together with either UV-killed OP50 or HT115 (RNAi) bacteria. Octopamine was added to a final concentration of 50 mM (17). The final concentration for eicosanoid (and EPA) treatment was 10 μM.

To test the impact of octopamine on pumping in the presence of food, about 4 × 8 synchronized young adults were set on separated small bacterial lawns for about 10 min to let them adapt. For the corresponding control, bacteria without supplementation were applied. In the eicosanoid assay, the same procedure was carried out except that the incubation time was 40 min. In this case, DMSO containing plates served as vehicle control. The different incubation times of neurohormone and eicosanoid assays required, in the case of a combined experiment, two separate assay plates. Here, worms were set first on an eicosanoid containing plate for 30 min, then picked to a neurohormone and eicosanoid containing plate and stayed for further 10 min.

For testing the impact of serotonin in the absence of food, about 4 × 8 synchronized young adults were set on a M9-agar plate, washed two times with small M9 drops, repicked to a second M9-agar plate and incubated for 80 min. This strict procedure prevented a notable carryover of bacteria and let worms in fact starve, clearly indicated by a sharp drop of pumping frequency in the course of incubation time. Finally, worms were transferred to agar pads supplemented with 2 mM serotonin (22) for about 10 min to let them adapt. In the eicosanoid assay, the procedure was altered in the following way. After deprivation of food for 50 min, a small drop of M9 buffer mixed with 17,18-EEQ was dropped on an unseeded NGM plate. Then, the starved worms were picked into this drop and incubated for 40 min; the worms were not able to leave. To prevent evaporation, M9-infiltrated filter paper was paved inside the lid and covered up. For the

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serotonin/17,18-EEQ combined experiment, again two separate assay plates were included and carried out as mentioned above for the octopamine assay. For the corresponding control, plates without supplementation were applied.

Pharyngeal pumping assay

Because the pumping rate of young adult wild-type worms is too fast to count correctly in real time, individual videos for 1 min at ×500 magnifications were recorded using a VHX-600 digital microscope (Keyence Corporation, Osaka, Japan). At least eight animals were tested per each trial; all experiments were performed at least in triplicate. Each individual pump was very carefully counted by playing back each individual video at half to fifth speed according to the pumping frequency of different strains and conditions.

Feeding assay

We used FluoSphere® carboxylate modified microspheres (red fluorescent; 0.5 μm) from Life Technologies (Carlsbad, CA) in this assay. The original microspheres were diluted 1:50 in M9 buffer. For well-fed conditions, 25 μl of particle suspension was mixed with 175 μl of OP50 bacterial suspension [optical density $(OD)_{600} = 4.5$] containing chemicals at the concentrations as described before and pipetted to a 6 cm NGM agar plate. These exposure plates were dried in the dark. For beads accumulation in the presence of food, 50 age-synchronized young adult hermaphrodites were picked onto a 20-HETE containing or control plate for 30 min preexposure. Then, nematodes were transferred to an exposure plate to allow them to ingest of microspheres for 10 min. After that worms were anesthetized using 50 μl of sodium azide (1 M). For testing octopamine, worms were picked directly to exposure plates containing only 50 mM octopamine and incubated for 10 min. For combined exposure, 20-HETE preincubated worms were transferred to an exposure plate containing both octopamine and 20-HETE. For starved condition, worms were cultivated in the absence of food prior the test as described above: for 80 min in the case of subsequent serotonin exposure and for 50 min in the case of individual 17,18-EEQ and subsequent joint exposure. Besides that M9 buffer was used instead of bacterial suspension, all other steps were identical as in the case of the well-fed condition.

Before measuring the density of fluorescence, 35 worms were picked on an unseeded part of the used NGM plate to wash them several times with M9 buffer. Then, 5 × 7 worms were transferred to a 96-well V-bottom plate filled with 100 μl of pure ethanol and measured by using an Infinite F200 Pro (Tecan, Männedorf, Switzerland) fluorescence reader (560 nm/612 nm) for three times with a 5 min interval between each. The usage of ethanol prevented sticking of worms on the sidewall of wells and ensured the complete localization on the bottom of plates. The entire test was repeated two times. Microscopic images were acquired with an Eclipse E200 from Nikon (Chiyoda, Tokyo, Japan) coupled to a VHX-600 digital camera (Keyence Corporation).

Analysis of endogenous fatty acid and eicosanoid pattern

Synchronized 3-day-old adult worms were carefully washed off from NGM agar plates with a few milliliters of M9 buffer by slightly canting the plates back and forth. This procedure removed almost all adult worms but hardly any laid eggs from the plate. All steps were performed in the cold at 4°C. Already hatched small larvae were separated by filtering through a 10 μm gauze membrane. The resulting worm filter cake with the adult worms was rinsed from the membrane and washed two times with M9 buffer to remove adhering bacteria. Then, the worms (~50 mg fresh weight) were transferred to a 1.5 ml reaction tube and spun down at 2,000 *g* for 1 min; the pellet was frozen at −80°C. In the case of treatment with octopamine, harvested adult worms were immediately set on freshly prepared NGM plates containing 50 mM octopamine in the bacterial lawn of *E. coli* OP50. In the case of serotonin treatment, freshly prepared plates contained only 2 mM serotonin in the agar, but no bacteria. The corresponding controls without octopamine or serotonin were handled in the same way. The incubation time of this bulk culture was 15 min; afterward, the harvest was performed as described above. The CYP eicosanoid and fatty acid profiles of the harvested worms were determined using LC/MS/MS as described previously (6). To differentiate between esterified and free CYP eicosanoids, the homogenates were extracted with or without prior alkaline hydrolysis.

Statistical analyses

Pumping assay and eicosanoid pattern data sets were analyzed by *t*-test or one-way ANOVA to test for significant differences between treatments followed by the Bonferroni test to identify treatments that were significantly different from the control. All statistical tests were performed using Sigma Stat 3.5 (Systat Software Inc., San Jose, CA).

RESULTS

Effect of genetic modifications reducing pharyngeal activity on the endogenous CYP-eicosanoid profile

In a first set of experiments, we compared the endogenous CYP-eicosanoid profile of wild-type nematodes with that of mutant strains displaying impairments in pharyngeal pumping activity (**Fig. 1**). For achieving various degrees of LC-PUFA/CYP-eicosanoid depletion, we took advantage of the well-characterized pathway of LC-PUFA synthesis in *C. elegans* (8) (Fig. 1A). The *fat-1(wa9)* mutant strain was used to analyze the effect of selectively depleting n-3 PUFAs including EPA, whereas *fat-2(wa17)* and *fat-3(wa22)* served as strains deficient in both EPA and AA (see supplementary Table 1 for the detailed fatty acid profiles of the different strains). Moreover, we included the *emb-8(hc69)* strain that expresses a temperature-sensitive CPR and, thus, allows the conditional knockdown of all CYP monooxygenase activities in *C. elegans*. All strains were viable under laboratory conditions. The worms were fed with *E. coli* OP50, a bacterium that mainly contains palmitic (16:0), palmitoleic (16:1 n-7), and vaccenic (18:1 n-7) acid, but not oleic acid (18:1 n-9) or PUFAs (23, 24).

The eicosanoid pattern of N2 wild-type worms revealed that EPA, the most abundant LC-PUFA in *C. elegans*, was the preferred substrate for CYP-mediated eicosanoid production. Counting all hydroxy- and epoxy-metabolites as well as the corresponding diols, the total content of EPA-derived metabolites was more than 10-fold higher compared with their AA-derived counterparts (Fig. 1B). Among the individual metabolites, 17,18-EEQ and its hydrolysis product 17,18-DHEQ were clearly predominant (supplementary Table 2). At the restrictive temperature, the thermosensitive *emb-8(hc69)* strain showed a marked decrease in the content of all EPA- and AA-derived CYP eicosanoids (Fig. 1B and supplementary Table 2). These data are in line with the notion that shifting the worms to the restrictive temperature (25°C) resulted in cessation of

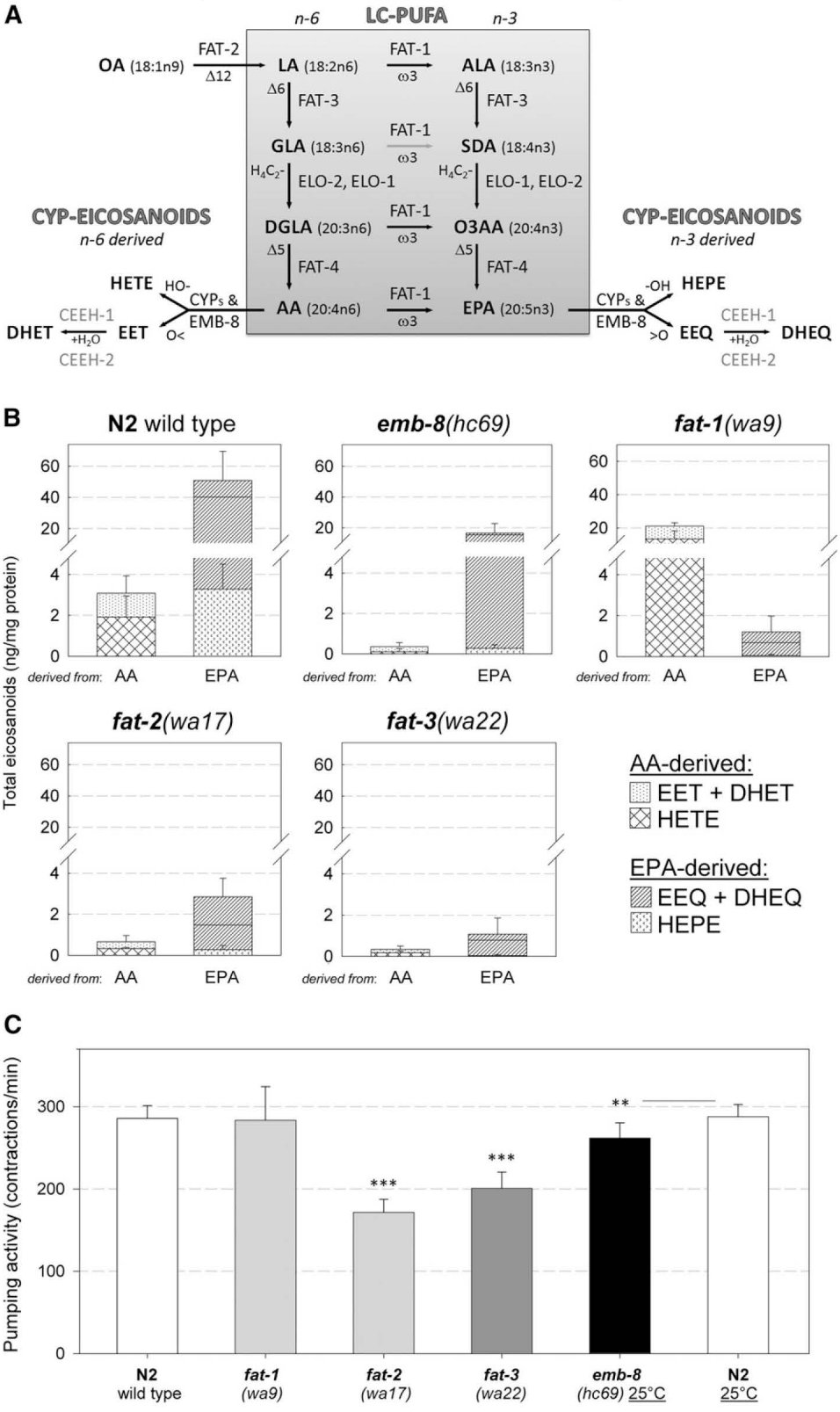

Fig. 1. Endogenous CYP-eicosanoid profiles and pumping frequencies in different *C. elegans* strains. A: Pathway of LC-PUFA and eicosanoid synthesis in *C. elegans*. AA (20:4n-6); ALA (18:3n-3); DGLA, dihomo-γ-linolenic acid (20:3n-6); EPA (20:5n-3); GLA, γ-linolenic acid; LA (18:2n-6); OA, oleic acid (C18:1n-9); O3AA, n-3 arachidonic acid; SDA, stearidonic acid (18:4n-3). Outside the gray box, CYP-dependent eicosanoid synthesis and the soluble epoxide hydrolase (sEH) (CEEH-1 and -2)-mediated hydrolysis of epoxides (40) are shown. DHEQ, dihydroxyeicosaquatraenoic acid; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HEPE, hydroxyeicosapentaenoic acid. B: Cultured wild-type and designated mutant nematodes were harvested their endogenous CYP-eicosanoid profiles were analyzed by LC/MS/MS. The

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CYP eicosanoid de novo synthesis and progressive degradation of those metabolites that were produced during the prior period at permissive temperature (15°C). Each of the desaturase mutants analyzed exhibited significant alterations in CYP-eicosanoid formation (Fig. 1B and supplementary Table 2). These alterations clearly reflected the different availabilities of LC-PUFAs in the mutant strains compared with the wild-type (for the details, see supplementary Table 1). The *fat-2(wa17)* and *fat-3(wa22)* strains almost completely lacked any of the AA- or EPA-derived CYP eicosanoids. The *fat-1(wa9)* strain was almost free of EPA-derived metabolites but produced largely increased amounts of AA-derived CYP eicosanoids compared with the wild type.

Fig. 1C compares the pharyngeal activities of the strains characterized above regarding their fatty acid and CYP-eicosanoid profiles. The pumping frequencies were determined using young adult, well-fed worms. Under these conditions, the pharynx of N2 wild-type worms regularly pumped with a frequency of 285.7 ± 15.5 (mean ± SD) contractions/min. This value is located at the upper end of the previously published range, for instance 200–300 pumps per minute for adults (25) as well as 250–300 (26) and 266.1 ± 3 (27) for young adults, in each case, measured in the presence of food. It is important to note that this work determined pumping frequency by playing back previously recorded individual videos at reduced speed and counting each individual pump carefully. Usually, pharynx pumping rates of adult worms were scored by eye under a microscope, a method that can hardly be controlled, making them more prone to errors. We added four representative videos as supplementary information. The 1-minute film of supplementary Video 1 shows an N2 wild-type worm pumping 290 times.

The *fat-2(wa17)* and *fat-3(wa22)* mutant strains displayed largely reduced pumping frequencies. Actually, these AA- and EPA-deficient worms pumped with about 110 [*fat-2(wa17)*] and 80 [*fat-3(wa22)*] contractions/min less than the N2 strain, corresponding to about 60% and 70%, respectively, of wild-type activity. Supplementary Video 2 shows a *fat-3(wa22)* worm with 207 pumps per minute. In contrast, *fat-1(wa9)* worms showed pumping frequencies almost identical to the wild type, indicating that n-3 PUFA-deficiency alone is not sufficient to impair pharyngeal activity. Inactivation of EMB-8 resulted in a moderate, but still significant decrease to ~90% of wild-type activity.

Rescue of pharyngeal activity impairments by C20-PUFAs and CYP eicosanoids

To further analyze the link between LC-PUFAs, CYP eicosanoids, and the observed phenotype, we compared EPA, AA, and ETYA, a nonmetabolizable AA analog, for their capacities to rescue the impaired pharyngeal pumping activity of the *fat-3(wa22)* and *fat-2(wa17)* mutant strains. As expected from previous studies (10, 11), long-term feeding with either EPA or AA significantly improved the impaired pharyngeal activity of both the mutant strains (**Fig. 2B**, C). However, C20-PUFA supplementation did not further increase the high pharyngeal pumping frequencies of wild-type worms that produce AA and EPA endogenously (Fig. 2A). In contrast to AA and EPA, ETYA was unable to rescue the impaired pharyngeal pumping activity of both *fat-3(wa22)* and *fat-2(wa17)* mutant strains indicating that AA- and EPA-derived metabolites, rather than the parental C20-PUFAs, were required (Fig. 2B, C). In order to distinguish between developmental or acute requirements for C20-PUFAs in unimpaired pharyngeal pumping, we provided EPA to *fat-3(wa22)* and *fat-2(wa17)* mutant strains at the last larval stage (L4), after most tissue development and differentiation has occurred. This 24 h of EPA supplementation was found sufficient to significantly rescue both *fat-3(wa22)* (Fig. 2B) and *fat-2(wa17)* (Fig. 2C) worms from deficits in pumping activity. Nevertheless, whereas in the case of *fat-3(wa22)* this rescue was in same range as in the long-term feeding, *fat-2(wa17)* worms, supplemented with EPA only 24 h post L4, pumped still significantly lower when compared with long-term feed worms; its rescue level was only half as much as when EPA was present during the complete development (Fig. 2C).

Searching for the identity of the bioactive metabolites, we next tested several of the EPA- and AA-derived CYP eicosanoids for their capacity to modulate pharyngeal activities in wild-type and mutant strains. Based on preliminary experiments with the major EPA-derived metabolite (17,18-EEQ), we selected the lowest effective concentration of 10 µM in combination with a 40 min preincubation time for comparing the effects of various compounds on pumping frequencies (supplementary Fig. 1). Please note that all concentration specifications of used compounds refer to exogenously administered media or buffers and do not reflect incorporated or internal amounts, considered as effective in lower concentrations. Under these short-term exposure conditions (**Fig. 3**), EPA had no effect, contrary to the rescue experiments described above, where we used long-term feeding for several days with 80 µM C20-PUFAs. However, short-term treatment with 17,18-EEQ accelerated pharyngeal pumping even above basal frequency in the wild type (Fig. 3A) and rescued the *fat-3(wa22)* mutant strain to the same extent as long-term EPA-feeding [compare Fig. 3B and Fig. 2B, as well as supplementary Videos 2 and 3, presenting an untreated *fat-3(wa22)* with 207 pumps/min and a 17,18-EEQ-treated

bars show the total amounts of AA- or EPA-derived metabolites and represent the sum of the corresponding n- and (n-1)-hydroxylase products as well as the sum of epoxy- and dihydroxy-metabolites. Results are means + SD from three independent experiments performed for each strain. For the detailed metabolite profiles, see supplementary Table 2. C: Eicosanoid deficiencies were associated with pumping frequency impairments. All nematodes were monitored on the first day of adulthood. Shown are the contractions per minute (three trials with n = 8–12 per trial); error bars represent SD; comparisons were made using one-way ANOVA. ** $P < 0.01$, *** $P < 0.001$.

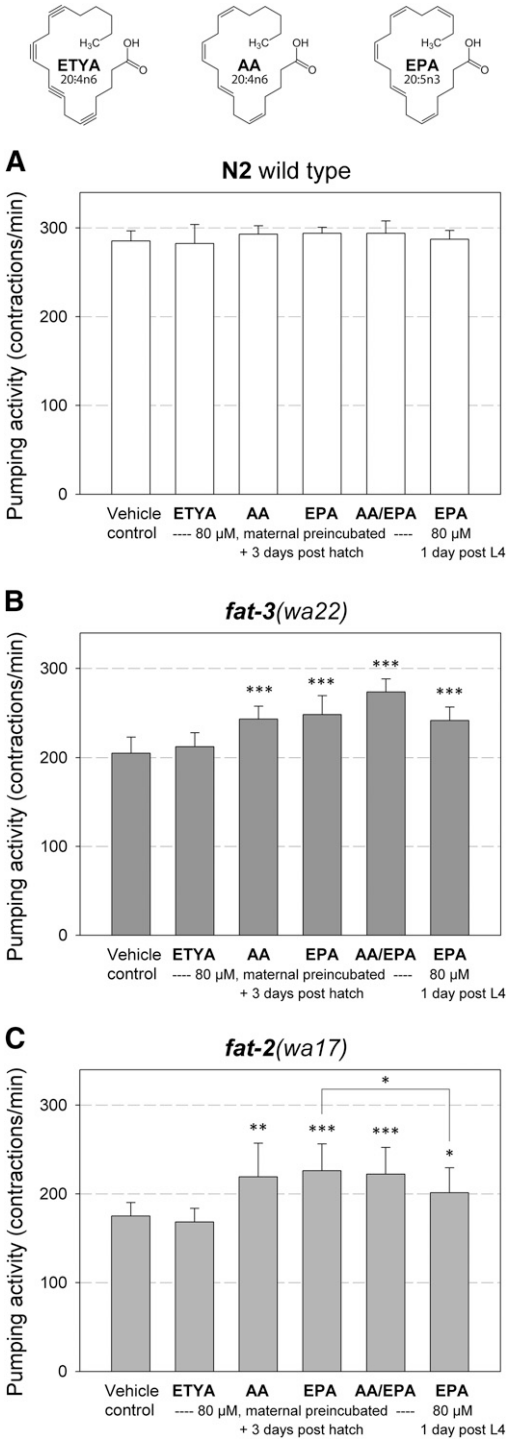

Fig. 2. Rescue of *fat-3(wa22)* mutant worms from pumping impairment. Long-term feeding with EPA and AA, but not ETYA, a nonmetabolizable AA analog, rescued the impaired pumping of the *fat-3(wa22)* (B) and *fat-2(wa17)* (C) strains but did not change pharyngeal activity in the wild type (A). Shown are the contractions per minute (three trials with n = at least 8 per trial); error bars represent SD; comparisons were made using one-way ANOVA. ** $P < 0.01$, *** $P < 0.001$.

fat-3(wa22) worm pumping with 237 pumps/min, respectively]. Moreover, 17,18-EEQ significantly increased the pumping frequencies in those strains that were designed to have limited capacities for CYP-eicosanoid formation due to CPR inactivation (Fig. 3D) or RNAi-mediated inhibition of *cyp-29A3/cyp-33E2* expression (Fig. 3F). In the case of *fat-2(wa17)* (Fig. 3C), only the more effective concentration of 20 µM (compare supplementary Fig. 1) was sufficient to significantly alter the pharyngeal pumping activity, indicating again developmental deficits in this strain not fully compensable by dietary supplementation. Notably, a synthetic 17,18-EEQ analog was as effective as the natural 17,18-EEQ in elevating the pumping frequencies (Fig. 3F, G). Further confirming the high specificity of the 17,18-EEQ effect, neither its hydrolysis product, the corresponding diol (17,18-DHEQ), nor the AA-derived metabolite 14,15- EET did increase the pumping frequencies (Fig. 3F, G). Only the AA-metabolite 11,12-EET was able to mirror the 17,18-EEQ effect (Fig. 3F, G).

The AA-derived 20-HETE showed opposite properties compared with 17,18-EEQ. This hydroxy-metabolite significantly decreased pharyngeal pumping frequencies in the wild as well as in all genetically modified strains tested (Fig. 3A–E). The 1-minute film of supplementary Video 4 shows a 20-HETE treated N2 wild-type worm pumping 257 times/min. In wild-type worms, both its regioisomer 19-HETE and the EPA-derived analog 20-HEPE were not able to reduce pumping frequency (Fig. 3A). Moreover, the joint treatment of worms with both 20-HETE and 19-HETE only marginally reduced the 20-HETE activity (Fig. 3A).

Comparison of CYP-eicosanoid and neurohormone effects on pumping frequency

Whereas effects of CYP eicosanoids on pharyngeal activity of *C. elegans* were never described before, it has been well established that the pumping frequency is regulated by neurohormones, such as the biogenic amines serotonin and octopamine (17–19). Accordingly, the following experiments were designed to gain insight into potential links between neurohormone and CYP-eicosanoid actions. A first series of experiments was performed using worms completely deprived of food for 90 min, taking into account that serotonin exerts its stimulatory effect most strongly after starvation and mimics the worm's response to refeeding. During the starvation period, the pharyngeal pumping frequency of the wild-type strain declined from about 285.7 ± 15.5 to 82.2 ± 11.0 contractions/min and became highly responsive to exogenously added serotonin. In contrast, we used well-fed worms for studying the effect of octopamine because this neurohormone is thought to reduce pharyngeal pumping under conditions of satiation.

As shown in **Fig. 4A**, starved wild-type worms responded to exogenously administered serotonin with a very strong acceleration of pharyngeal pumping. 17,18-EEQ mimicked the effect of serotonin and was actually as effective as the neuromodulator. The remarkable capacity of 17,18-EEQ to stimulate the pumping frequency of starved worms was detectable not only in the wild type, but also in the *emb-8(hc69)*, *fat-3(wa22)*, and *fat-2(wa17)* mutant strains, respectively. An even higher stimulating effect was achieved by combined administration of serotonin and 17,18-EEQ, significant in the case of *emb-8(hc69)* and *fat-2(wa17)*.

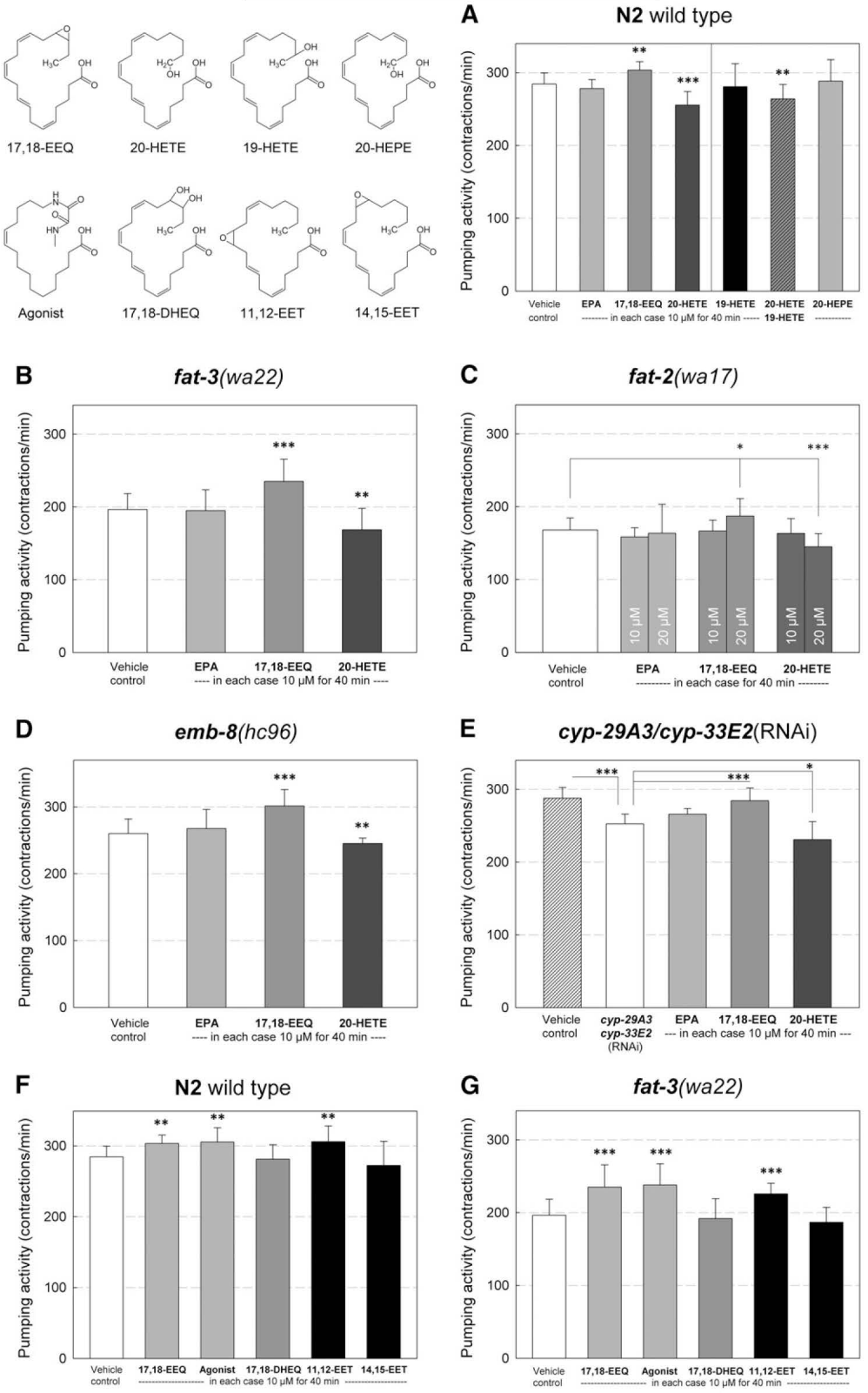

Fig. 3. Contrasting effects of different CYP eicosanoids on pumping activity. A–E: 17,18-EEQ increased whereas 20-HETE decreased the pumping frequency in the wild type and in the genetically modified strains *fat-3(wa22)*, *fat-2(wa17)*, *emb-8(hc69)*, and *cyp-29A3/cyp-33E2*(RNAi). F, G: The 17,18-EEQ effect was mimicked by a synthetic agonist and 11,12-EET but not by 17,18-DHEQ or 14,15-EET. Shown are in each case the contractions per minute (three trials with n = at least 8 per trial); error bars represent SD; comparisons were made using one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; vehicle: 0.3% DMSO.

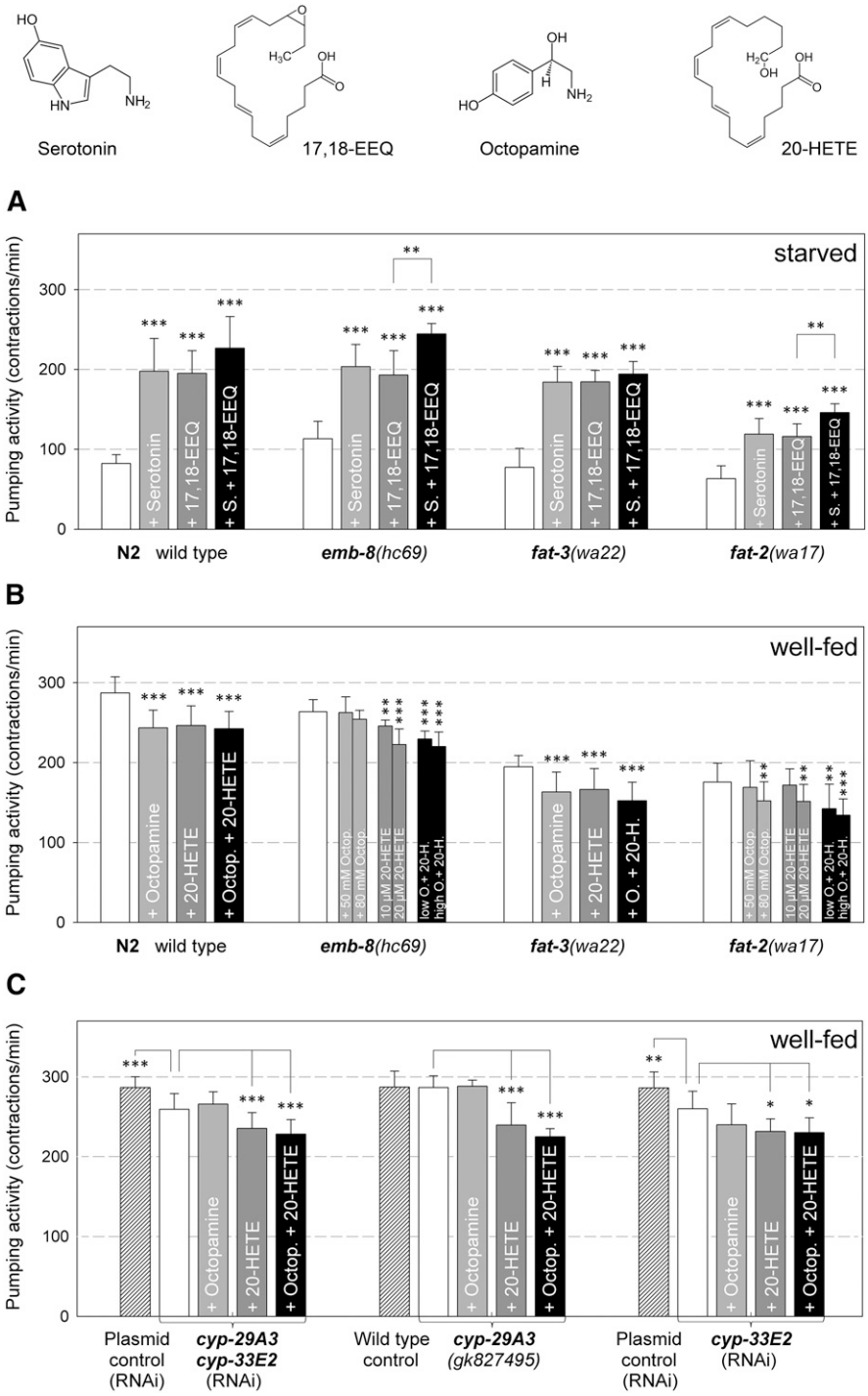

Fig. 4. Comparison of CYP-eicosanoid- and neurohormone-induced effects on pharyngeal pumping frequencies. A: 17,18-EEQ mimicked the strong stimulating effect of serotonin when applied to the starved wild type or *fat-3(wa22)*, *fat-2(wa17)*, and *emb-8(hc69)* mutant strains. B: Exogenous administration of octopamine as well as 20-HETE downregulated the pumping frequency in well-fed wild-type worms. The *fat-2(wa17)* and *emb-8(hc69)* mutant worms were partially octopamine resistant but responded to 20-HETE with decreased pumping activity. C: CYP-29A3-deficient strains did not respond to 50 mM octopamine but still to 20-HETE. Shown are the contractions per minute (three trials with n = at least 8 per trial); error bars represent SD; comparisons were made using one-way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; plasmid control: empty RNAi feeding control. O./Octop., octopamine; S., serotonin; 20-H., 20-HETE. Unless otherwise specified, the nominal concentration of serotonin was 2 mM; octopamine, 50 mM; and 10 µM, in the case of 17,18-EEQ and 20-HETE.

As shown in Fig. 4B, octopamine exerted its expected effects by moderately reducing the pumping frequency of well-fed wild-type and *fat-3(wa22)* worms. The *emb-8(hc69)* strain, cultivated at restrictive temperature, as well as the PUFA-deficient *fat-2* strain, failed to respond to octopamine (50 mM). Raising the octopamine concentration to 80 mM overcame this ineffectiveness to some extent, significantly in the case of *fat-2(wa17)*. 20-HETE was clearly effective when added on top of octopamine, suggesting that lack of endogenous 20-HETE formation might be responsible for the inability of the *emb-8(hc69)* and *fat-2(wa17)* strains to response to 50 mM octopamine (Fig. 4B). Based on this notion, we next tested the potential involvement of CYP-29A3 and/or CYP-33E2 in providing 20-HETE for the octopamine response (Fig. 4C). RNAi-mediated silencing of both genes indeed abolished the response of wild-type worms to octopamine (50 mM). Similarly, the *cyp-29A3(gk827495)* strain that carries a loss-of-function mutation in the *cyp-29A3* gene was resistant against octopamine. However, the *cyp-29A3(gk827495)* strain still clearly responded to exogenous 20-HETE (Fig. 4C), resembling the results obtained with *emb-8(hc69)* and *fat-2(wa17)*, the two other octopamine-resistant strains (compare Fig. 4B). Selective downregulation of CYP-33E2 produced a different phenotype. RNAi-mediated silencing of *cyp-33E2* alone caused already a significant deceleration of pumping frequency in well-fed worms. Treatment with octopamine trended to result in a further decrease of pumping activity; however, this effect was only significant compared with the RNAi plasmid control (Fig. 4C).

Effect of neurohormones on endogenous CYP-eicosanoid formation

Next, we analyzed acute effects of serotonin and octopamine on the formation of CYP eicosanoids in *C. elegans* (**Fig. 5**). Again, we used starved and well-fed worms for testing the responses to serotonin and octopamine, respectively. Without any neurohormone treatment, starved and well-fed wild-type worms showed clear differences in the endogenous levels of free CYP eicosanoids (compare the left panels in Fig. 5A, C). In particular, the 17,18-EEQ levels were significantly (almost 3-fold) lower in starved than well-fed worms.

Serotonin treatment for 15 min resulted in a significant, almost 2-fold, increase of free 17,18-EEQ levels in starved wild-type worms (Fig. 5A). Simultaneously, serotonin

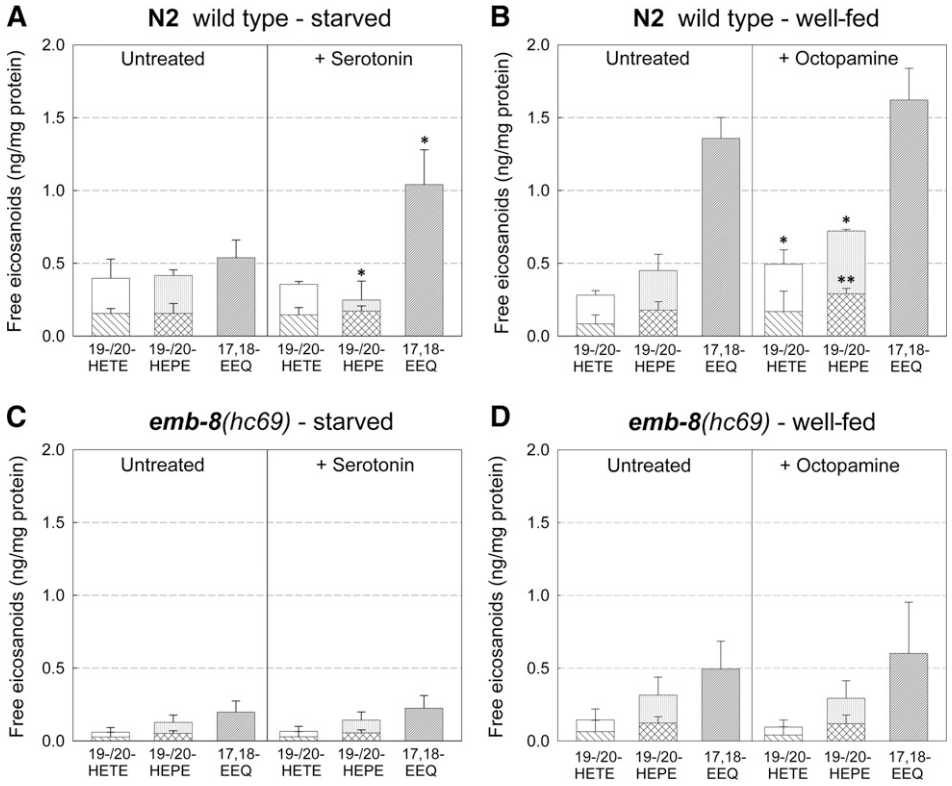

Fig. 5. Effect of neurohormones on CYP-eicosanoid formation. A: The addition of serotonin to starved wild-type worms caused increased endogenous levels of free 17,18-EEQ but not of hydroxy-metabolites. B: Octopamine treatment of well-fed wild-type worms selectively induced the formation of free AA- and EPA-derived hydroxy metabolites including 19-HETE, 20-HETE and 19-HEPE, and 20-HEPE. C, D: Starved as well as well-fed *emb-8(hc69)* worms incubated at the restrictive temperature featured strongly reduced CYP-eicosanoid levels compared with the corresponding wild-type controls. Moreover, these CPR-deficient worms neither responded to serotonin nor to octopamine with changes in CYP-eicosanoid formation. Results are means + SD from three independent experiments performed for each strain. Note that in the case of divided boxes the upper part presents the 19-hydroxy metabolite and the lower part the 20-hydroxy metabolite. Comparisons were made using *t*-test. * $P < 0.05$, ** $P < 0.01$.

induced a decline of free hydroxy-metabolites that was significant for 19-HEPE but not for 19-/20-HETE (Fig. 5A). Compared with wild type, the CPR-deficient *emb-8(hc69)* strain displayed largely reduced levels of free CYP eicosanoids already under basal conditions and was unable to increase 17,18-EEQ formation in response to serotonin (Fig. 5C).

In contrast to serotonin, octopamine primarily induced the formation of hydroxy-metabolites (Fig. 5B). Actually, the free levels of 19-/20-HETE as well as of 19-/20-HEPE were almost doubled after treating well-fed wild-type worms for 15 min with octopamine. However, octopamine had no significant effect on 17,18-EEQ that only slightly increased its high level as characteristic for well-fed worms. Compared with the wild type, the *emb-8(hc69)* strain showed significantly lower basal levels of 19-/20-HETE as well as of 19-/20-HEPE and 17,18-EEQ (Fig. 5D). Importantly, the well-fed CPR-deficient worms also lost the ability to increase hydroxy-metabolite formation in response to octopamine, indicating that de novo CYP-eicosanoid synthesis was required for the octopamine-induced changes in the free hydroxy-metabolite levels observed in the wild-type strain (compare Fig. 5B, D).

Effects of CYP eicosanoids and neurohormones on food uptake

Finally, we analyzed the efficiency of food uptake by quantifying the incorporation of fluorescent beads (**Fig. 6**). This assay provides information not only on pumping, typically counted as cycle of contraction and relaxation of the terminal bulb, but also on peristalsis as an essential second feeding motion generating a moving wave of contraction of the muscles of the posterior isthmus that carries food from the corpus to the terminal bulb (25). Several studies indicated that the uptake of particles was indeed related to pharyngeal pumping rate and responded to changes in nutritional status (22, 28–30). Nevertheless, terminal bulb contractions, which are not timed properly with those in the corpus, can prevent transport of food (or beads) into the intestine. We used this assay also to demonstrate concentration- and time-dependent effects of 17,18-EEQ treatment on pharyngeal activity (see supplementary Fig. 1).

The feeding assay was performed both with well-fed worms in the presence of food and starved worms, exclusively incubated with beads. Starved worms incorporated significantly more beads when treated with serotonin;

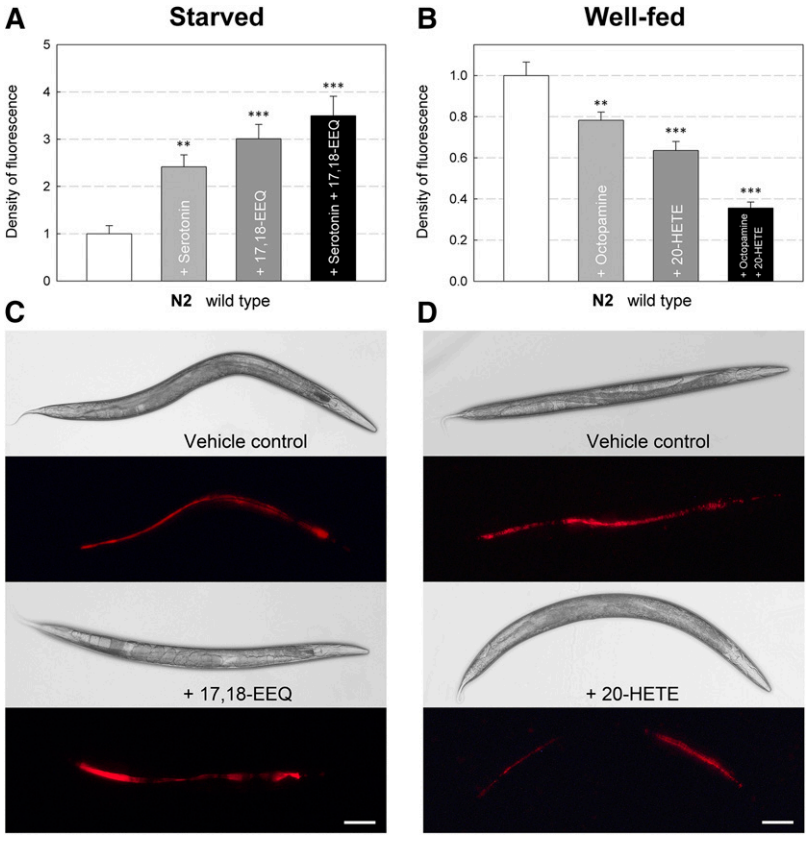

Fig. 6. Contrasting effects of 17,18-EEQ and 20-HETE on the food uptake. Shown is the relative effect of neurohormones and CYP eicosanoids on the accumulation of 0.5 µm fluorescent beads. The accumulation was assayed with starved worms (A), incubated in the absence of *E. coli* cells, and well-fed worms (B), incubated in the presence of *E. coli* cells. Representative bright field and fluorescence images, arranged as pairs one below the other, show starved (C) and well-fed (D) adult worms allowed to uptake the red fluorescent beads for 10 min at 20°C. The photographs were taken at ×100 magnification, using an exposure time of 1/30 s for the fluorescence images in C and of 1/5 s in D; white bars correspond to 100 µm. Data are means + SEM; n = 15. *P* values were obtained from one way ANOVA. ** $P < 0.01$, *** $P < 0.001$; vehicle: 0.3% DMSO.

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17,18-EEQ successfully mimicked this effect and the combined exposure of both agents was slightly more effective than serotonin or 17,18-EEQ alone (Fig. 6A). Well-fed worms reduced the uptake of beads when treated with octopamine, an effect that was also elicited by 20-HETE; a combined treatment resulted in an additive effect (Fig. 6B). Representative microscopic images are shown in Fig. 6C, D. The labeling is visible in the terminal bulb of the pharynx and the whole intestinal lumen; the overall intensity of fluorescence mirrored the contrasting effects of 17,18-EEQ (excitatory) and 20-HETE (inhibitory), respectively.

DISCUSSION

The present study provides direct experimental evidence of a role for CYP eicosanoids in the regulation of pharyngeal pumping and food uptake in the nematode *C. elegans*. In particular, we found that 17,18-EEQ contributed to restoration of high pumping frequencies in LC-PUFA deficient strains, whereas 20-HETE reduced pharyngeal activities and food uptake. Moreover, we show that the formation and action of 17,18-EEQ did closely mimic the stimulatory effect of the neurohormone serotonin. In contrast, 20-HETE and related hydroxy-metabolites were possibly linked to the decelerating effect of octopamine.

Confirming and extending previous results (8, 10, 11), we detected impaired pharyngeal activities in all mutant strains that were deficient in the endogenous production of both EPA and AA. Consequently, these mutant strains also almost completely lacked EPA- and AA-derived metabolites, raising the question whether the parental fatty acids or more directly the corresponding CYP eicosanoids were required for maintaining pharyngeal activities. The same question also applies to the other behavioral phenotypes associated with C20-PUFA-deficiency. As shown recently, the touch sensation defect of *fat-3(wa22)* can be rescued supplementing the worms during complete development with EPA and AA, but also with ETYA, an AA-analog (31). ETYA harbors triple instead of double bonds (32) and has been used as a nonmetabolizable analog of AA and inhibitor of AA-derived eicosanoid formation (33–35). These findings suggested that C20-PUFAs modulate *C. elegans*' touch sensation while being incorporated into membrane phospholipids and that CYP-eicosanoid formation is not required to maintain this phenotype (31). In clear contrast, we found that pharyngeal pumping frequencies can be only rescued by EPA and AA, but not by ETYA. This outcome of the ETYA experiment, combined with our observation of reduced pumping activities in the CPR- and CYP-33E2-deficient strains, clearly indicated that AA- and EPA-derived metabolites rather than the parental C20-PUFAs are required for maintaining high pharyngeal activities. The function of C20-PUFA in pharyngeal activity appears to be in fact independent of developmental roles of these PUFAs, as providing EPA for 24 h to *fat-3(wa22)* post L4 stage was sufficient for restoration of high pumping frequency in young adults. It seems likely that in the *fat-3(wa22)* mutant the enrichment of C18 LA and ALA (compare supplementary Table 1) as well as derived metabolites can partially compensate the absence of C20-PUFA. In contrast, this kind of rescue was incomplete in *fat-2(wa17)* worms, producing no PUFA at all. These data indicate that there are at least two separable requirements for EPA (and probably other PUFAs) in normal adult pumping behavior including both acute and developmental requirements.

Searching for the identity of the bioactive metabolites, we first tested 17,18-EEQ, the major CYP eicosanoid endogenously produced in wild-type worms. Indeed, 17,18-EEQ was alone sufficient to rescue impaired pharyngeal pumping in the LC-PUFA- and in the CPR- or CYP-33E2-deficient strains. Moreover, we found that the formation and action of 17,18-EEQ was associated with the typical behavioral response of *C. elegans* to refeeding. Deprivation of food (bacteria) resulted in a strong deceleration of pharyngeal pumping frequencies and simultaneously in a reduction of free 17,18-EEQ levels. After starvation, the worms responded to exogenously supplied 17,18-EEQ with a marked acceleration of pharyngeal pumping and concomitantly with increased food uptake capacity as visualized by enhanced incorporation of fluorescent-labeled beads. However, 17,18-EEQ-treatment exerted a slight stimulating effect already in well-fed worms, suggesting that the endogenous 17,18-EEQ levels did not fully saturate the stimulating mechanisms even in the presence of abundant food. It might be argued that in isolated mammalian cells an even lower concentration of, e.g., 17-18-EEQ was found effective, as 1 μM, with respect to Ristocetin-induced thrombocyte aggregation (36), or even 30 nM, which caused antiarrhythmic effects in isolated neonatal rat cardiomyocytes (37). However, *C. elegans* as living organism cannot be considered as comparably accessible as vulnerable segregated cells. This applies even more in the light of an encapsulated pharynx, isolated from the rest of the worm by a specialized basal lamina. Moreover, recent investigations revealed that the more economic approach of spotting compounds on the agar surface achieved lower absorption efficiency in worms when compared with other drug delivering methods, as pouring compounds together with agar (38). In the case of the spot method, the solution will immerse into the NGM agar, which reduces the availability.

A reaction potentially limiting 17,18-EEQ levels consists in the hydrolysis of this epoxy-metabolite to the corresponding vicinal diol (17,18-DHEQ). Supporting this notion, our LC/MS/MS data showed, besides 17,18-EEQ, also high 17,18-DHEQ levels in the wild-type worms. Furthermore, we found that 17,18-DHEQ did not share the capacity of 17,18-EEQ to stimulate pharyngeal activity. In mammals, hydrolysis of LC-PUFA-derived epoxy-metabolites is catalyzed by sEH and frequently also results in a loss of their biological activities (39). Candidates for mediating 17,18-EEQ inactivation in *C. elegans* are *ceeh-1* and *ceeh-2*, two genes encoding enzymes orthologous to mammalian sEH (40). Our study also indicates that 17,18-EEQ, if not available, can be replaced by other metabolites, as 11,12-EET. Strong evidence for this notion also comes from the

finding that the EPA-deficient *fat-1(wa9)* strain did not show any impairment in pharyngeal pumping activity. This strain produced largely increased amounts of AA-derived metabolites.

Contrary to the stimulating effect of 17,18-EEQ, 20-HETE decreased pharyngeal activities when added to well-fed worms. 20-HETE thus elicited a response otherwise indicating satiation. This effect was detectable not only in the wild type but also in all of the genetically modified strains tested. The requirement of a doubled concentration of eicosanoids for being active also in *fat-2(wa17)* gave further evidence that the complete absence of PUFAs in worm's development resulted in impairments that interfere with the restoration of high pumping frequencies. We selected 20-HETE as a representative of the various hydroxy-metabolites present in *C. elegans*. An additional testing of 19-HETE and 20-HEPE did not reduce the pumping speed of well-fed young wild-type adults, confirming the high specificity of the 20-HETE effect. However, we cannot exclude and will have to test in future experiments that the inhibitory effect of 20-HETE is shared by further AA- and EPA-derived hydroxy-metabolites such as specific stereoisomers of HETE and HEPE.

We speculated that CYP eicosanoids are integrated as second messengers of neurohormones into the complex regulation of pharyngeal activity and food uptake in *C. elegans*. As reviewed by Avery and You (25), it has been well-established that pharyngeal muscle activities are coordinated by the pharyngeal nervous system for allowing efficient contractions and stimulation of fast pumping in response to food and slow pumping in response to starvation or satiation. In the absence of the pharyngeal nervous system, a muscle-intrinsic pathway promotes only very slow pumping (26 pumps/min in the presence of food) (41). In the unaffected wild type, both serotonin and acetylcholine stimulate fast pumping, whereas glutamate acts as an inhibitory neurotransmitter (17, 42, 43). Also octopamine reduces pharyngeal pumping when added to intact worms (17) or isolated pharynx preparation (18). The cellular origins of this bioamine neurotransmitter are ring interneurons C of the head region and gonadal sheath cells (44), known octopamine receptors, SER-3 and OCTR-1, are expressed in head neurons, too (45, 46). 20-HETE not only mimicked the inhibitory effect of octopamine but was also increasingly produced in response to octopamine. Moreover, we found that the CPR- or CYP-29A3-deficient strains failed to respond to the inhibitory neurotransmitter, but still responded to 20-HETE. On the other hand, C20-PUFA-deficient strains *fat-3(wa22)* and *fat-2(wa17)* were able to respond to octopamine treatment with a drop in pumping frequency, but the latter only after increasing octopamine's nominal concentration to 80 mM. Also the data of the fluorescent beads uptake experiments with the wild type in the presence of octopamine and 20-HETE tend to argue for a rather independent (additive) action of both substances. Further work will be necessary to close this knowledge gap, e.g., the open question why a notably high 20-HETE concentration in the *fat-1(wa9)* mutant, producing only n-6 PUFA and derived CYP eicosanoids, is not sufficient to lower its pumping frequency.

The interpretation of our results with 17,18-EEQ is similarly complex. Our data show that 17,18-EEQ is involved in maintaining high pumping and food uptake rates in the presence of bacteria. Moreover, 17,18-EEQ was increasingly produced in response to serotonin and elicited, like this neuromodulator, the behavioral response of refeeding. However, 17,18-EEQ was obviously not essential for mediating the serotonin effect. Supporting this notion, we found that CPR-deficient worms responded to serotonin with increased pumping frequencies, despite their inability for de novo 17,18-EEQ synthesis. Furthermore, pharyngeal pumping of *fat-2* mutant worms was serotonin responsive, although this strain was completely devoid of any PUFAs and CYP eicosanoids. These results indicate that serotonin can exert its stimulatory effect both via 17,18-EEQ-dependent and independent pathways. Moreover, it appears possible that 17,18-EEQ is used as a second messenger primarily by other transmitters that act downstream (e.g., acetylcholine) or independent of serotonin. Mutants defective in the serotonin receptor SER-7 do not respond to serotonin, but still respond to bacterial food, suggesting that serotonin is indeed not the only transmitter relaying the physiological response of refeeding (47).

Various aspects of the hypothetical pathway remain to be clarified. A first question concerns the identity and cellular localization of the CYP isoforms involved. CYP-29A3 appears responsible for the formation of 20-HETE and other hydroxy-metabolites as indicated by the lack of clear octopamine response in the CYP-29A3 knockout strain. However, the expression site of CYP-29A3 remains to be defined considering that the pharyngeal machinery is constituted by various neuronal, marginal, and muscle cells. CYP-33E2 is the leading candidate for producing 17,18-EEQ, based on the activity of the recombinant enzyme, its localization in pharyngeal marginal cells, and the reduced pharyngeal activities observed after downregulating CYP-33E2 expression (7). However, pharyngeal marginal cells also express CYP-13A12, a CYP enzyme that is involved in *C. elegans'* response to hypoxia/reoxygenation and presumably shares with CYP-33E2 the capacity of producing 17,18-EEQ (48). Another important open question concerns the selective induction of 17,18-EEQ versus 20-HETE formation, respectively. CYP enzymes require free fatty acids as substrates. This feature ensures coupling of CYP-eicosanoid de novo synthesis to the action of extracellular signals that activate phospholipase A_2 (PLA_2), which in turn release free C20-PUFAs from membrane phospholipids (1, 49). Currently, it is unclear whether one of the serotonin or octopamine receptors may trigger PLA_2 activation and which of the diverse PLA_2 enzymes expressed in *C. elegans* are involved. Finally, it may be presumed, but has to be directly shown, that pharyngeal muscle cells contain receptors selectively recognizing 17,18-EEQ or 20-HETE and in turn trigger signaling pathways accelerating or decelerating pharyngeal pumping.

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From a more general perspective, the questions raised above similarly apply to the formation and action of CYP eicosanoids in mammalian systems. Resembling our findings on the opposite roles of 17,18-EEQ and 20-HETE in *C. elegans*, EETs act as second messengers of vasodilatory hormones, whereas 20-HETE mediates vasoconstriction in the mammalian vasculature (50, 51). EETs were first characterized as endothelium-dependent hyperpolarizing factors when analyzing the components mediating the vasorelaxing effects of acetylcholine and bradykinin (52). EET-generating CYP enzymes are primarily localized in endothelial cells and 20-HETE formation occurs predominantly in vascular smooth muscle cells. Partially explaining the opposite effects, EETs activate whereas 20-HETE inhibits large conductance Ca^{2+}-activated potassium (BK) channels in vascular smooth muscle cells (50, 53). In *C. elegans*, BK channels are involved in regulating muscle Ca^{2+}-transients (54, 55) as well as neurotransmitter release at neuromuscular junction (56). 17,18-EEQ shares and, in several vascular beds, even largely exceeds the vasodilatory (57) and BK channel activating effects of EETs (58). Moreover, 17,18-EEQ relaxes airway smooth muscle cells in the human lung (59) and potently modulates the contractility of cardiomyocytes (37). Interestingly, a synthetic compound developed to mimic the effect of 17,18-EEQ on cardiomyocytes (21) was also effective when assayed in our *C. elegans* model. In mammalian systems, CYP eicosanoids probably act via both intracellular targets such as peroxisome-proliferator activated receptors and membrane receptors that remain to be identified (49, 60, 61). Considering the parallels between nematodes and mammalian systems revealed in the present study, we believe that *C. elegans* provides a suitable model for elucidating evolutionary conserved mechanisms and key components of CYP-eicosanoid signaling.

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REFERENCES

1. Capdevila, J. H., and J. R. Falck. 2002. Biochemical and molecular properties of the cytochrome P450 arachidonic acid monooxygenases. *Prostaglandins Other Lipid Mediat.* **68–69:** 325–344.
2. Konkel, A., and W. H. Schunck. 2011. Role of cytochrome P450 enzymes in the bioactivation of polyunsaturated fatty acids. *Biochim. Biophys. Acta.* **1814:** 210–222.
3. Roman, R. J. 2002. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol. Rev.* **82:** 131–185.
4. Wu, C. C., T. Gupta, V. Garcia, Y. Ding, and M. L. Schwartzman. 2014. 20-HETE and blood pressure regulation: clinical implications. *Cardiol. Rev.* **22:** 1–12.
5. Panigrahy, D., M. L. Edin, C. R. Lee, S. Huang, D. R. Bielenberg, C. E. Butterfield, C. M. Barnes, A. Mammoto, T. Mammoto, A. Luria, et al. 2012. Epoxyeicosanoids stimulate multiorgan metastasis and tumor dormancy escape in mice. *J. Clin. Invest.* **122:** 178–191.
6. Kulas, J., C. Schmidt, M. Rothe, W. H. Schunck, and R. Menzel. 2008. Cytochrome P450-dependent metabolism of eicosapentaenoic acid in the nematode *Caenorhabditis elegans*. *Arch. Biochem. Biophys.* **472:** 65–75.
7. Kosel, M., W. Wild, A. Bell, M. Rothe, C. Lindschau, C. E. W. Steinberg, W. H. Schunck, and R. Menzel. 2011. Eicosanoid formation by a cytochrome P450 isoform expressed in the pharynx of *Caenorhabditis elegans*. *Biochem. J.* **435:** 689–700.
8. Watts, J. L., and J. Browse. 2002. Genetic dissection of polyunsaturated fatty acid synthesis in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA.* **99:** 5854–5859.
9. Hutzell, P. A., and L. R. Krusberg. 1982. Fatty acid compositions of *Caenorhabditis elegans* and *C. briggsae*. Comp. Biochem. Physiol. B Comp. *Biochem.* **73:** 517–520.
10. Watts, J. L., E. Phillips, K. R. Griffing, and J. Browse. 2003. Deficiencies in C20 polyunsaturated fatty acids cause behavioral and developmental defects in *Caenorhabditis elegans fat-3* mutants. *Genetics.* **163:** 581–589.
11. Lesa, G. M., M. Palfreyman, D. H. Hall, M. T. Clandinin, C. Rudolph, E. M. Jorgensen, and G. Schiavo. 2003. Long chain polyunsaturated fatty acids are required for efficient neurotransmission in *C. elegans*. *J. Cell Sci.* **116:** 4965–4975.
12. Nandakumar, M., and M. W. Tan. 2008. Gamma-linolenic and stearidonic acids are required for basal immunity in *Caenorhabditis elegans* through their effects on p38 MAP kinase activity. *PLoS Genet.* **4:** e1000273.
13. Raabe, R. C., L. D. Mathies, A. G. Davies, and J. C. Bettinger. 2014. The omega-3 fatty acid eicosapentaenoic acid is required for normal alcohol response behaviors in *C. elegans*. *PLoS One.* **9:** e105999.
14. Albertson, D. G., and J. N. Thomson. 1976. The pharynx of *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **275:** 299–325.
15. Mango, S. E. 2007. The *C. elegans* pharynx: a model for organogenesis. *In* WormBook. The *C. elegans* Research Community, editors. WormBook, doi:10.1895/wormbook.1.129.1. Accessed September 29, 2015, at http://www.wormbook.org.
16. Song, B. M., and L. Avery. 2013. The pharynx of the nematode *C. elegans*: a model system for the study of motor control. *Worm.* **2:** e21833.
17. Horvitz, H. R., M. Chalfie, C. Trent, J. E. Sulston, and P. D. Evans. 1982. Serotonin and octopamine in the nematode *Caenorhabditis elegans*. *Science.* **216:** 1012–1014.
18. Rogers, C. M., C. J. Franks, R. J. Walker, J. F. Burke, and L. Holden-Dye. 2001. Regulation of the pharynx of *Caenorhabditis elegans* by 5-HT, octopamine, and FMRFamide-like neuropeptides. *J. Neurobiol.* **49:** 235–244.
19. Luedtke, S., V. O'Connor, L. Holden-Dye, and R. J. Walker. 2010. The regulation of feeding and metabolism in response to food deprivation in *Caenorhabditis elegans*. *Invert. Neurosci.* **10:** 63–76.
20. Hull, D., and L. Timmons. 2004. Methods for delivery of double-stranded RNA into *Caenorhabditis elegans*. *Methods Mol. Biol.* **265:** 23–58.
21. Falck, J. R., G. Wallukat, N. Puli, M. Goli, C. Arnold, A. Konkel, M. Rothe, R. Fischer, D. N. Muller, and W. H. Schunck. 2011. 17(R),18(S)-epoxyeicosatetraenoic acid, a potent eicosapentaenoic acid (EPA) derived regulator of cardiomyocyte contraction: structure-activity relationships and stable analogues. *J. Med. Chem.* **54:** 4109–4118.
22. Avery, L., and H. R. Horvitz. 1990. Effects of starvation and neuroactive drugs on feeding in *Caenorhabditis elegans*. *J. Exp. Zool.* **253:** 263–270.
23. Satouchi, K., K. Hirano, M. Sakaguchi, H. Takehara, and F. Matsuura. 1993. Phospholipids from the free-living nematode *Caenorhabditis elegans*. *Lipids.* **28:** 837–840.
24. Tanaka, T., K. Ikita, T. Ashida, Y. Motoyama, Y. Yamaguchi, and K. Satouchi. 1996. Effects of growth temperature on the fatty acid composition of the free-living nematode *Caenorhabditis elegans*. *Lipids.* **31:** 1173–1178.
25. Avery, L., and Y. J. You. 2012. *C. elegans* feeding. *In* WormBook. The *C. elegans* Research Community, editors. WormBook, doi:10.1895/wormbook.1.150.1. Accessed September 29, 2015, at http://www.wormbook.org.
26. Wilson, M. A., B. Shukitt-Hale, W. Kalt, D. K. Ingram, J. A. Joseph, and C. A. Wolkow. 2006. Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. *Aging Cell.* **5:** 59–68.
27. Song, B. M., and L. Avery. 2012. Serotonin activates overall feeding by activating two separate neural pathways in *Caenorhabditis elegans*. *J. Neurosci.* **32:** 1920–1931.
28. Klass, M. R. 1983. A method for the isolation of longevity mutants in the nematode *Caenorhabditis elegans* and initial results. *Mech. Ageing Dev.* **22:** 279–286.

29. Boyd, W. A., S. J. McBride, and J. H. Freedman. 2007. Effects of genetic mutations and chemical exposures on *Caenorhabditis elegans* feeding: evaluation of a novel, high-throughput screening assay. *PLoS One.* **2:** e1259.
30. Kiyama, Y., K. Miyahara, and Y. Ohshima. 2012. Active uptake of artificial particles in the nematode *Caenorhabditis elegans*. *J. Exp. Biol.* **215:** 1178–1183.
31. Vásquez, V., M. Krieg, D. Lockhead, and M. B. Goodman. 2014. Phospholipids that contain polyunsaturated fatty acids enhance neuronal cell mechanics and touch sensation. *Cell Reports.* **6:** 70–80.
32. Tobias, L. D., and J. G. Hamilton. 1979. The effect of 5,8,11,14-eicosatetraynoic acid on lipid metabolism. *Lipids.* **14:** 181–193.
33. Morisaki, N., J. A. Lindsey, J. M. Stitts, H. Zhang, and D. G. Cornwell. 1984. Fatty acid metabolism and cell proliferation. V. Evaluation of pathways for the generation of lipid peroxides. *Lipids.* **19:** 381–394.
34. Harris, R. C., K. A. Munger, K. F. Badr, and K. Takahashi. 1990. Mediation of renal vascular effects of epidermal growth factor by arachidonate metabolites. *FASEB J.* **4:** 1654–1660.
35. McGiff, J. C. 1991. Cytochrome P-450 metabolism of arachidonic acid. *Annu. Rev. Pharmacol. Toxicol.* **31:** 339–369.
36. Jung, F., C. Schulz, F. Blaschke, D. N. Muller, C. Mrowietz, R. P. Franke, A. Lendlein, and W. H. Schunck. 2012. Effect of cytochrome P450-dependent epoxyeicosanoids on Ristocetin-induced thrombocyte aggregation. *Clin. Hemorheol. Microcirc.* **52:** 403–416.
37. Arnold, C., M. Markovic, K. Blossey, G. Wallukat, R. Fischer, R. Dechend, A. Konkel, C. von Schacky, F. C. Luft, D. N. Muller, et al. 2010. Arachidonic acid-metabolizing cytochrome P450 enzymes are targets of omega-3 fatty acids. *J. Biol. Chem.* **285:** 32720–32733.
38. Zheng, S. Q., A. J. Ding, G. P. Li, G. S. Wu, and H. R. Luo. 2013. Drug absorption efficiency in *Caenorhbditis elegans* delivered by different methods. *PLoS One.* **8:** e56877.
39. Harris, T. R., and B. D. Hammock. 2013. Soluble epoxide hydrolase: gene structure, expression and deletion. *Gene.* **526:** 61–74.
40. Harris, T. R., P. A. Aronov, P. D. Jones, H. Tanaka, M. Arand, and B. D. Hammock. 2008. Identification of two epoxide hydrolases in *Caenorhabditis elegans* that metabolize mammalian lipid signaling molecules. *Arch. Biochem. Biophys.* **472:** 139–149.
41. Avery, L., and R. Horvitz. 1989. Pharyngeal pumping continues after laser killing of the pharyngeal nervous-system of *C. elegans*. *Neuron.* **3:** 473–485.
42. Dent, J. A., M. W. Davis, and L. Avery. 1997. *avr-15* encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. *EMBO J.* **16:** 5867–5879.
43. McKay, J. P., D. M. Raizen, A. Gottschalk, W. R. Schafer, and L. Avery. 2004. *eat-2* and *eat-18* are required for nicotinic neurotransmission in the *Caenorhabditis elegans* pharynx. *Genetics.* **166:** 161–169.
44. Alkema, M. J., M. Hunter-Ensor, N. Ringstad, and H. R. Horvitz. 2005. Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. *Neuron.* **46:** 247–260.
45. Suo, S., Y. Kimura, and H. H. M. Van Tol. 2006. Starvation induces cAMP response element-binding protein-dependent gene expression through octopamine-G(q) signaling in *Caenorhabditis elegans*. *J. Neurosci.* **26:** 10082–10090.
46. Sun, J., V. Singh, R. Kajino-Sakamoto, and A. Aballay. 2011. Neuronal GPCR controls innate immunity by regulating noncanonical unfolded protein response genes. *Science.* **332:** 729–732.
47. Hobson, R. J., V. M. Hapiak, H. Xiao, K. L. Buehrer, P. R. Komuniecki, and R. W. Komuniecki. 2006. SER-7, a *Caenorhabditis elegans* 5–HT7-like receptor, is essential for the 5-HT stimulation of pharyngeal pumping and egg laying. *Genetics.* **172:** 159–169.
48. Ma, D. K., M. Rothe, S. Zheng, N. Bhatla, C. L. Pender, R. Menzel, and H. R. Horvitz. 2013. Cytochrome P450 drives a HIF-regulated behavioral response to reoxygenation by *C. elegans*. *Science.* **341:** 554–558.
49. Spector, A. A. 2009. Arachidonic acid cytochrome P450 epoxygenase pathway. *J. Lipid Res.* **50 (Suppl.):** S52–S56.
50. Campbell, W. B., and I. Fleming. 2010. Epoxyeicosatrienoic acids and endothelium-dependent responses. *Pflugers Arch.* **459:** 881–895.
51. McGiff, J. C., and J. Quilley. 1999. 20-HETE and the kidney: resolution of old problems and new beginnings. *Am. J. Physiol.* **277:** R607–R623.
52. Campbell, W. B., and J. R. Falck. 2007. Arachidonic acid metabolites as endothelium-derived hyperpolarizing factors. *Hypertension.* **49:** 590–596.
53. Zou, A. P., J. T. Fleming, J. R. Falck, E. R. Jacobs, D. Gebremedhin, D. R. Harder, and R. J. Roman. 1996. 20-HETE is an endogenous inhibitor of the large-conductance $Ca^{(2+)}$-activated K^+ channel in renal arterioles. *Am. J. Physiol.* **270:** R228–R237.
54. Chen, B., P. Liu, H. Zhan, and Z. W. Wang. 2011. Dystrobrevin controls neurotransmitter release and muscle Ca(2+) transients by localizing BK channels in *Caenorhabditis elegans*. *J. Neurosci.* **31:** 17338–17347.
55. Chiang, J. T., M. Steciuk, B. Shtonda, and L. Avery. 2006. Evolution of pharyngeal behaviors and neuronal functions in free-living soil nematodes. *J. Exp. Biol.* **209:** 1859–1873.
56. Wang, Z. W., O. Saifee, M. L. Nonet, and L. Salkoff. 2001. SLO-1 potassium channels control quantal content of neurotransmitter release at the *C. elegans* neuromuscular junction. *Neuron.* **32:** 867–881.
57. Agbor, L. N., M. T. Walsh, J. R. Boberg, and M. K. Walker. 2012. Elevated blood pressure in cytochrome P4501A1 knockout mice is associated with reduced vasodilation to omega-3 polyunsaturated fatty acids. *Toxicol. Appl. Pharmacol.* **264:** 351–360.
58. Lauterbach, B., E. Barbosa-Sicard, M. H. Wang, H. Honeck, E. Kargel, J. Theuer, M. L. Schwartzman, H. Haller, F. C. Luft, M. Gollasch, et al. 2002. Cytochrome P450-dependent eicosapentaenoic acid metabolites are novel BK channel activators. *Hypertension.* **39:** 609–613.
59. Morin, C., M. Sirois, V. Echave, E. Rizcallah, and E. Rousseau. 2009. Relaxing effects of 17(18)-EpETE on arterial and airway smooth muscles in human lung. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **296:** L130–L139.
60. Yang, W., V. R. Tuniki, S. Anjaiah, J. R. Falck, C. J. Hillard, and W. B. Campbell. 2008. Characterization of epoxyeicosatrienoic acid binding site in U937 membranes using a novel radiolabeled agonist, 20-^{125}I-14,15-epoxyeicosa-8(Z)-enoic acid. *J. Pharmacol. Exp. Ther.* **324:** 1019–1027.
61. Chen, Y., J. R. Falck, V. L. Manthati, J. L. Jat, and W. B. Campbell. 2011. 20-Iodo-14,15-epoxyeicosa-8(Z)-enoyl-3-azidophenylsulfonamide: photoaffinity labeling of a 14,15-epoxyeicosatrienoic acid receptor. *Biochemistry.* **50:** 3840–3848.