WORMS GET THE MUNCHIES: ENDOCANNABINOID MODULATION OF FEEDING AND CHEMOSENSATION IN C. ELEGANS

by

ANASTASIA LEVICHEV

A DISSERTATION

Presented to the Department of Biology
and the Division of Graduate Studies of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

June 2022

DISSERTATION APPROVAL PAGE

Student: Anastasia Levichev

Title: Worms Get the Munchies: Endocannabinoid Modulation of Feeding and Chemosensation in C. Elegans

This dissertation has been accepted and approved in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Biology Department by:

Patrick Phillips	Chairperson
Shawn Lockery	Advisor
Cristopher Niell	Core Member
Matthew Smear	Core Member
Ulrich Mayr	Institutional Representative

and

Krista Chronister	Vice Provost for Graduate Studies

Original approval signatures are on file with the University of Oregon Division of Graduate Studies.

Degree awarded June 2022

DISSERTATION ABSTRACT

Anastasia Levichev

Doctor of Philosophy

Department of Biology

June 2022

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The ability of *Cannabis sativa* to stimulate appetite has been known for centuries. This effect results from the action of plant-derived cannabinoids at cannabinoid receptors in the brain where they mimic natural ligands called endocannabinoids. The endocannabinoid system contributes to many physiological functions in the body, including energy homeostasis. Although cannabinoid signaling is widely conserved across the animal kingdom, the degree of functional conservation, particularly in non-mammals remains an open question. This work explores the role of the endocannabinoid system in modulating food intake across species, with particular focus on the nematode worm *C. elegans.* Exposure to anandamide, an endocannabinoid common to nematodes and mammals, selectively increases *C. elegans*' consumption of nutritionally superior, highly palatable food without a concomitant increase in consumption of non-palatable food—a pattern of altered preferences analogous to that of mammals in response to cannabinoids. Anandamide's effect on feeding requires the worm's cannabinoid receptor NPR-19. Moreover, the NPR-19 receptor can be replaced by human CB_1 receptor, indicating a robust functional homology between the function of the endocannabinoid systems of mammals and nematodes. Anandamide's effect requires a single pair of primary chemosensory neurons, AWC, whose response to anandamide is sufficient to explain its effects on food preference. These

findings establish a surprising degree of conservation in appetitive behaviors and establish *C. elegans* as model system in which to investigate the cellular and molecular basis of endocannabinoid system function.

This dissertation includes previously unpublished coauthored material.

CURRICULUM VITAE

NAME OF AUTHOR: Anastasia Levichev

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene, Oregon

University of Bordeaux, Bordeaux, France

Charité Medical University, Berlin, Germany

University of California San Diego, San Diego, California

DEGREES AWARDED:

Doctor of Philosophy, Biology, 2022, University of Oregon

Master of Science, Neuroscience, 2014, University of Bordeaux

Bachelor of Science, Neuroscience and Physiology, 2012, UC San Diego

AREAS OF SPECIAL INTEREST:

Neuroscience

Pharmacology

PROFESSIONAL EXPERIENCE:

Graduate Employee/Teaching Fellow, University of Oregon, 2016 – 2022

GRANTS, AWARDS, AND HONORS:

Murdock Seed Grant, MJ Murdock Charitable Trust, 2021

William R Sistrom Memorial Scholarship, University of Oregon, 2019

Poster Recognition Award, *C. elegans* Neuronal Development, Synaptic Function, And Behavior Meeting, 2018

Special Opportunity travel Award, University of Oregon, 2018

PUBLICATIONS:

Tallafuss A, Stednitz SJ, Voeun M, Levichev A, Larsch J, Eisen J, Washbourne P. EGR-1 is necessary for forebrain dopaminergic signaling during social behavior. *ENeuro*. 9(2).

Guo L, Weems JT, Walker WI, Levichev A, Jaramillo S. (2019) Choice-selective neurons in the auditory cortex and its striatal target encode reward expectation. *J Neurosci*. 2585-18.

Hotter B, Ostwaldt A, Levichev-Connolly A, Rozanski M, Audebert H, Fiebach J. (2015). Natural course of total mismatch and predictors for tissue infarction. *Neurology* 85(9):770-5.

In Review:

Levichev A, Faumont S, Berner RZ, Purcell Z, Lockery SR. The conserved endocannabinoid anandamide modulates olfactory sensitivity to induce hedonic feeding in *C. elegans*. *Elife*.

ACKNOWLEDGMENTS

I wish to express sincere appreciation to Shawn Lockery for his scientific guidance and mentorship. Additionally, I would like to thank Serge Faumont for training me when I first joined the lab, talking through ideas (however half-baked), helping with troubleshooting of experiments and equipment, and in general knowing how to fix anything that is broken in the lab. I would also like to thank Kathy Chicas-Cruz and Jonathan Millet for making transgenic worm lines (no matter how many tries it took), discussions about experiments, and general emotional support over the past five years. My dissertation advisory committee has also been instrumental in this work, providing ideas, support, and encouragement. Finally, I would like to thank Nick Cady, Lucy Barton, Kara Zappitelli, Brittany Carter, Erik Toraason, Abbey Lasseigne, and so many others who have provided much needed distractions, laughs, words of encouragement, and general support throughout my PhD.

This dissertation is dedicated to my parents, Kirill and Marina Levichev

Now you can tell everyone your daughter is a doctor

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Introduction

To survive, animals must take into account their internal hunger state and availability of food in the environment, and then make decisions about whether and how much to feed. Energy balance is achieved when animals successfully replenish the calories they use. The complex integration of internal and external cues is thought to be achieved, in part, by signaling of the endocannabinoid system, which appears to reorient energy balance towards energy storage by increasing lipid production and accumulation (Piazza et al., 2007). The endocannabinoid system's involvement in feeding and appetite has been known for centuries, with users of the plant *Cannabis sativa* reporting increased appetite, colloquially known as "the munchies." Despite the obvious therapeutic potential of cannabinoid receptor agonists and antagonists in treating disorders of under- and over-eating, respectively, the mechanism and pathways underlying the endocannabinoid system's influence on feeding and appetite have not been fully elucidated. With the identification of cannabinoid receptors and natural ligands between the late 1980s and late 1990s (Devane et al., 1988; Michelle Glass & Northup, 1999; Matsuda et al., 1990), research has started to elucidate the endocannabinoid system's specific appetite effects and interaction with feeding (homeostatic) and reward (hedonic) pathways in both the brain and peripheral tissues to modulate food intake. It has now been shown in several species, from nematodes to humans, that the endocannabinoid system mediates food intake (Bellocchio et al., 2006; Cota et al., 2006; Fride et al., 2005; He et al., 2021; Isabel Matias & Di Marzo, 2007; Oakes et al., 2017; D. Osei-Hyiaman et al., 2006). However, cannabinoid receptor agonists have been shown to cause different effects on feeding in different species. Chapter I of this

dissertation will summarize what is known so far about the endocannabinoid system's role in feeding regulation and feeding decisions across species, how it interacts with various signaling systems in the body to modulate these decisions and delineate evolutionarily conserved motifs of cannabinoid system's role in energy balance maintenance. Chapter II will discuss what is currently known about the endocannabinoid system of *C. elegans,* and its effects on the physiology and behavior of the organism. Chapter III will present co-authored work showing that stimulation of the endocannabinoid system in *C. elegans* causes changes in feeding analogous to those observed in humans and other mammals and propose a mechanism for this effect. The work in Chapter III was conducted with collaboration from S. Faumont, R. Z. Berner, Z. Purcell, and S. R. Lockery. Lastly, Chapter IV will provide a summary and conclusions for this work.

The Endocannabinoid System

The plant *Cannabis sativa* has been used by humans for thousands of years (Ren et al., 2019). Its effects include euphoria, appetite stimulation, sedation, analgesia, altered perception, and impairment in cognition, memory, and motor control. The source of these effects are phytocannabinoids, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), which act by mimicking endogenous ligands called endocannabinoids. To date, there have been at least 5 endogenous molecules identified as endocannabinoids: N-arachidonoylethanolamine (AEA), 2-arachidonoylglycerol (2-AG), noladin ether, virodhamine, and *N*-arachidonoyldopamine (NADA). These molecules are derived from long-chain polyunsaturated fatty acids (N-Acylethanolamides) and have affinity for cannabinoid receptors.

The first identified and best studied endocannabinoids are AEA and 2-AG. AEA is synthesized from N-acyl-phosphatidylethanolamine (NAPE) by a NAPE-selective phospholipase

D enzyme (NAPE-PLD) (Okamoto et al., 2005) and is broken down by fatty acid amide hydrolase (FAAH) (Deutsch et al., 2002). 2-AG is synthesized by two diacylglycerol lipases, DAGLα and DAGLβ (Tiziana Bisogno et al., 2003) and catabolized by monoglyceride lipase (MAGL, monoacylglycerol lipase (Dinh et al., 2002) and cyclooxygenase 2 (COX2, prostaglandin-endoperoxide synthase) (Kozak et al., 2003).

Phytocannabinoids and the endocannabinoids they mimic act at the G-protein coupled receptors of the $G_{i/o}$ family, CB_1 and CB_2, as well as several non-canonical receptors such as the transient receptor potential vanilloid 1 receptor (TRPV1) (Zygmunt et al., 1999) and GPR55 (Baker et al., 2006; Johns et al., 2007). It was originally believed that the CB_1 receptor is expressed predominantly in the brain, whereas the CB_2 receptor is expressed in peripheral cells and tissues derived from the immune system (reviewed in Ameri, 1999). However, the CB_1 receptor has also been found in peripheral tissues, such as the cardiovascular, reproductive, and gastrointestinal systems (Croci et al., 1998; Pertwee, 1997, 2001; Szabo et al., 2001; Wagner et al., 2001), whereas the CB_2 receptor has been detected in microglia (Ashton et al., 2006; Carrier et al., 2004) and neurons (Gong et al., 2006; Skaper et al., 1996).

The endocannabinoid system is highly conserved across the animal kingdom. In addition to mammals, endocannabinoids or the genes encoding their precursors have been found in fish and birds (Ho et al., 2017; McPartland et al., 2001; Salzet et al., 2000; Valenti et al., 2005), as well as invertebrates, including hydra (De Petrocellis et al., 1999a), sea urchin (Tiziana Bisogno et al., 1997), leech (Isabel Matias et al., 2001), mussels (Mosca et al., 2021), snails (Lemak et al., 2007), Drosophila (Elphick & Egertová, 2005), and nematodes (Lehtonen et al., 2008). The CB_1 and CB_2 are thought to have occurred due to a gene duplication in a common ancestor of extant vertebrates, and thus can be found in the genomes of non-mammalian tetrapod vertebrates, such

as amphibians and birds, and in bony fish (e.g., zebrafish) (Elphick, 2012; Elphick & Egertová, 2005). Genes encoding the CB_1 and CB_2 receptors have been found in invertebrates that are most closely evolutionarily related to vertebrates, but not in non-chordates (McPartland et al., 2006; McPartland et al., 2006a; McPartland & Glass, 2003). The lack of CB_1/CB_2 receptors in non-chordates initiated debate regarding the functionality of the endocannabinoid systems of certain species, such as the model organisms Drosophila and *C. elegans* (McPartland et al., 2001; McPartland et al., 2006; McPartland & Glass, 2003). Nevertheless, administration of cannabinoids produces behavioral and physiological effects in many species without obvious CB_1/CB_2 homologs, indicating that these endocannabinoids exhibit affinity for different receptors in certain species (De Petrocellis et al., 1999b; Fezza et al., 2003; Lucanic et al., 2011; Sepe et al., 1998; Tortoriello et al., 2021). Indeed, the *C. elegans* receptor NPR-19, which was previously dismissed as a potential cannabinoid receptor due to low similarity to the CB_1/CB_2 receptors (Elphick & Egertová, 2001; McPartland et al., 2001), was shown to have binding affinity for both AEA and 2-AG in a heterologous expression system (Oakes et al., 2017).

Synaptic Regulation by the Endocannabinoid System

In the central nervous system, CB_1 receptors are specifically localized on presynaptic terminals and axons (Freund et al., 2003). Unlike classical neurotransmitters such as acetylcholine and GABA, endocannabinoids are synthesized ‘on demand’ and released immediately after their production. Upon membrane depolarization, postsynaptic neurons synthesize and release endocannabinoids, which act retrogradely on presynaptic CB_1 receptors (Kreitzer & Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson & Nicoll, 2001). Activation of the pre-synaptic CB_1 receptors by endocannabinoids leads to inhibition of pre-synaptic voltage-gated

Ca^{2+} channels, which suppresses further release of neurotransmitters from the pre-synaptic cell (Schlicker & Kathmann, 2001). This can occur at glutamatergic (Shen et al., 1996), cholinergic (Gifford et al., 1997), noradrenergic (Schlicker et al., 1997), and GABAergic synapses (Szabo et al., 1998), leading to endocannabinoid modulation of both excitatory and inhibitory neurotransmission throughout the nervous system. Specifically, retrograde endocannabinoid signaling has been shown to mediate activity-dependent long-term depression of both glutamatergic (Gerdeman et al., 2002; Haj-Dahmane & Shen, 2010) and GABAergic synaptic transmission (Chevaleyre & Castillo, 2003; Marsicano et al., 2002) throughout the brain.

Although most of what we know about endocannabinoid function at the synaptic level comes from mammalian studies, several studies have shown that its action is highly conserved across the animal kingdom. Endocannabinoid modulation of excitation and inhibition has also been shown in the lamprey, in which 2-AG is synthesized post-synaptically and act in a retrograde manner to inhibit both excitatory and inhibitory neurotransmission in a CB_1-dependent manner (Kettunen et al., 2005; Kyriakatos & El Manira, 2007; Pérez et al., 2009; Song et al., 2012). Additionally, CB_1-mediated long-term depression has also been observed in the zebra finch (Thompson & Perkel, 2011). Therefore, despite a limited number of studies to date, it appears that retrograde modulation of excitatory and inhibitory transmission by endocannabinoids is evolutionarily conserved, at least among vertebrates.

Endocannabinoid System Role in Physiological Functions

Distribution of cannabinoid receptors appears to be conserved among vertebrates, with birds (Alonso-Ferrero et al., 2006; Soderstrom & Tian, 2006; Stincic & Hyson, 2008), fish (Cottone et al., 2005; Lam et al., 2006), and amphibians (Cesa et al., 2001; Hollis et al., 2006),

which is reflected in its conservation of function across species. CB_1 receptors are expressed in feeding areas, stress response areas, reward areas, and olfactory areas. Indeed, the endocannabinoid system plays a myriad of important roles in the body, spanning cell migration during development (Song & Zhong, 2000), axon regeneration (Pastuhov et al., 2012), reward seeking (Arnold, 2005; reviewed in Wang et al., 2003), response to stress (reviewed in Viveros et al., 2005), response to nociception and pain (reviewed in Walker & Huang, 2002), immune function (reviewed in Salzet et al., 2000), and energy balance (more on this below). This range of effects is not surprising given that cannabinoid receptors are found in virtually every brain structure of the mammalian brain, including the neocortex, hippocampus, cerebellum, amygdala, thalamus, basal ganglia, and brainstem, and most other tissues, such as muscles, glands, and immune cells. Further, the effect of cannabinoids on the body depends on the receptor they bind; the CB_1, CB_2, TRPV1, and GPR55 receptors may act together, competitively, or in opposite directions to modulate the multitude of physiological effects of cannabinoids, and this interplay may be dependent on the location and distribution of these receptors both in the central nervous system and in peripheral tissues.

Cannabinoid Modulation of Feeding

Cannabinoid-Induced Hyperphagia

Anecdotally, consumption of *Cannabis sativa* is often reported to increase appetite and feeding (Allentuck & Bowman, 1942; Haines & Green, 1970; Halikas et al., 1971; Tart, 1970). While usage of *Cannabis* traces back nearly 5000 years, with written record of its effects dating back to 2737 B.C.E. (Lemberger, 1980), empirical studies on the effect of *Cannabis* and its main psychoactive molecule, Δ^9-THC, on feeding in humans are sparse. Early studies of the effects of

Cannabis found increased caloric consumption in subjects who either ingested (Hollister, 1971) or smoked (Abel, 1971; Foltin et al., 1986) *Cannabis* compared to placebo controls, irrespective of subjects' knowledge of their control or experimental status. A longitudinal study on the effect of *Cannabis* over years of use also found increased food intake following consumption of *Cannabis* (Halikas et al., 1985). Further, cancer patients receiving Δ^9-THC as an antiemetic reported increased appetite and food intake (Ekert et al., 1979; Lemberger et al., 1982). Though it is hard to dissociate true appetite stimulation from increased food intake due to alleviation of nausea and vomiting in cancer patients, treatment with Dronabinol, a synthetically made Δ^9-THC, has also been associated with increased percent body fat and improved appetite in patients with AIDS (Beal et al., 1995, 1997; Struwe et al., 1993) and Alzheimer's (Volicer et al., 1997). Other studies have also found substantial weight gain in disease-free *Cannabis* users (Greenberg et al., 1976; Williams & Himmelsbach, 1946). More recently, it has been shown that a polymorphism of the CNR1 gene, which encodes the CB_1 receptor, affects susceptibility to anorexia nervosa (Siegfried et al., 2004), and that plasma levels of AEA are significantly increased in people with eating disorders (Monteleone et al., 2005). Together, these findings suggest that endocannabinoid signaling is an important modulator of feeding, and eating disorders, of both over- and under-eating, are associated with a dysregulation of this system.

The effects of phytocannabinoids on appetite are not limited to humans. Rats, dogs, and sheep exposed to Δ^9-THC show increased feeding (Huy et al., 1975; Koch, 2001; McLaughlin et al., 1979; Van Den Broek et al., 1979; Wiley et al., 2005; Williams et al., 1998a; Williams & Kirkham, 2002). Further, exposure to not only phytocannabinoids but also endocannabinoids cause increased appetite and feeding. Mice (Hao et al., 2000), rats (Gómez et al., 2002; Williams & Kirkham, 1999, 2002), and fish (Piccinetti et al., 2010; Valenti et al., 2005) exposed to AEA

display increased food consumption, whereas mice lacking the CB_1 receptor have reduced food intake (Cota et al., 2003; Di Marzo et al., 2001).

Additionally, fasting and feeding have been shown to increase and decrease, respectively, both peripheral and central endocannabinoid levels, as well as expression of CB_1 receptors in fish (Cottone et al., 2009; Piccinetti et al., 2010; Valenti et al., 2005), rats (Vilches-Flores et al., 2010), and mice (DiPatrizio et al., 2015; Hanuš et al., 2003; Kirkham et al., 2002). Like in people with eating disorders, animals fed high-calorie diets exhibit dysregulation of the endocannabinoid system, with lower CB_1 expression, altered endocannabinoid levels, and increased expression of catabolic enzymes (Argueta & DiPatrizio, 2017; Di Marzo et al., 2008; Díaz-Rúa et al., 2020; Gamelin et al., 2016; Ramírez-López et al., 2016; Ramírez-López et al., 2016; Starowicz et al., 2008).

Nevertheless, contradictory findings of the effect on cannabinoids on feeding have also been published. For example, synthetic CB_1 agonist HU210, was shown to produce a dose- and time-dependent decrease in food intake and loss of body weight in rats (Giuliani et al., 2000), and a few studies have shown that treatment with Δ^9-THC either has no effect (Graceffo & Robinson, 1998) or causes a decrease in food intake (Drewnowski & Grinker, 1978). In invertebrates, the way by which cannabinoids affect feeding is just starting to emerge, but both phyto- and endocannabinoids produce an inhibitory effect on food intake by Drosophila (He et al., 2021). AEA and 2-AG were also shown to inhibit food intake in *C. elegans* (Oakes et al., 2017), but the effect of endocannabinoids on feeding has proven to be more complex than initially reported, causing an increase in feeding on certain foods and a decrease in feeding on others (see Chapter III). In mammals, most studies that have reported that CB_1 agonist administration decreases feeding have generally used high doses of these agonists (Salamone et

al., 2007), which have been shown to decrease food intake, whereas lower doses increase food intake (Bellocchio et al., 2010; Díaz-Rúa et al., 2020; Valenti et al., 2005). A mechanism for this has now been elucidated, where the action of CB_1 receptors causes a hypophagic effect through inhibition of GABAergic transmission and a hyperphagic effect through modulating excitatory transmission (Bellocchio et al., 2010).

With the knowledge that cannabinoid receptor agonists induce feeding came the potential of harnessing the endocannabinoid system to suppress feeding and promote weight loss. CB_1 antagonists, such as AM4113 (Salamone et al., 2007), and inverse agonists, which inhibit constitutive receptor activity, such as SR141716 (Rimonabant) (Rinaldi-Carmona et al., 1994), AM1387 (McLaughlin et al., 2006), and AM251 (Gatley et al., 1996) were shown to be suppress feeding and lead to reduced body weight in rodents (Chen et al., 2004; Colombo et al., 1998; Freedland et al., 2000; Fride et al., 2001; Hildebrandt et al., 2003; McLaughlin et al., 2003, 2005; 2006; Shearman et al., 2003; Sink et al., 2008; Tallett et al., 2007; Thornton-Jones et al., 2005; Verty et al., 2004; Werner & Koch, 2003; Wiley et al., 2005; Williams & Kirkham, 1999).

Rimonabant was also shown to be effective in humans, causing significant weight loss and decreased waist circumference after 12 months of use (Christensen et al., 2007; Després et al., 2005; Pi-Sunyer et al., 2006; Van Gaal et al., 2008; Van Gaal et al., 2005). However, CB_1 inverse agonists are associated with negative side effects, such as nausea in both rats (McLaughlin et al., 2005) and humans (Després et al., 2005; Pi-Sunyer et al., 2006; Van Gaal et al., 2008; Van Gaal et al., 2005), vomiting (Després et al., 2005; Pi-Sunyer et al., 2006; Van Gaal et al., 2008; Van Gaal et al., 2005), diarrhea (Pi-Sunyer et al., 2006; Van Gaal et al., 2005), and increased incidence of mood disorders (Christensen et al., 2007; Pi-Sunyer et al., 2006; Van Gaal

et al., 2008; Van Gaal et al., 2005). These adverse side effects are serious enough that rimonabant was not approved by the US Food and Drug Association and taken off the European market by 2008. Neutral antagonists of the CB_1 receptor have been proposed as an alternative to inverse agonists and suggested to suppress feeding without causing some of the more severe adverse effects (Gardner & Mallet, 2006; Janero, 2012; Sink et al., 2008; S. J. Ward & Raffa, 2011). Further, peripherally restricted antagonists that cannot cross the blood-brain barrier have also been proposed (O'Keefe et al., 2014; Pavón et al., 2006, 2008; Shrinivasan et al., 2012), but show lower efficacy in promoting weight loss than rimonabant, suggesting that central CB_1 receptors are a critical player in the endocannabinoid system's modulation of feeding. Finally, partial CB_1 agonists (Ohlsen & Pilowsky, 2005) and mixed CB_1 antagonist/CB_2 agonists have been proposed as well (LoVerme et al., 2009).

Cannabinoid-Mediated Increased Desire for Food

Increased consumption of food can stem from two independent changes in behavior: increased motivation to obtain food ("wanting") and increased enjoyment of food ("liking") (Berridge, 1996). One objective measure of "wanting" food, widely used in animal studies, is the willingness to exert energy to obtain food. Rimonabant and other CB_1 antagonists have been shown to reduce willingness of animals to work for food, whereas CB_1 agonists increase this willingness (Gallate et al., 1999; Gallate & McGregor, 1999; Marcello Solinas & Goldberg, 2005; Thornton-Jones et al., 2005). Further, mice lacking the CB_1 receptor show decreased motivation to obtain food (Sanchis-Segura et al., 2004). Another measure of "wanting" food used in animal studies involved quantifying latency to feed. Administration of Δ^9-THC, as well as AEA and 2-AG, reduces the latency of animals to feed (Kirkham & Williams, 2001), and in the

clinical trials for rimonabant, participants reported reduced desire to eat and decreased food cravings (Blundell et al., 2006). Together, these studies show that activation of the cannabinoid receptors increases the motivation to obtain food.

Another aspect of desire for food is "liking" of food. The orosensory rewarding properties of food (i.e., palatability), or "liking", have also been associated with endocannabinoids. In humans, consumption of highly palatable food, which is usually high in fat and sugar, is associated with elevated 2-AG levels in the plasma (Monteleone et al., 2005). In fact, circulating endocannabinoid levels increase when a person even *thinks* about a favorite food (Monteleone et al., 2016). And though "liking" of food is difficult to definitively assess in non-primate animals, indicators of "liking" food after cannabinoid administration have been observed in rodents. For example, rats increase the duration of licking bouts at spouts containing sucrose solution after administration of either AEA or Δ^9-THC (Higgs et al., 2003), a behavioral response that has previously been linked to palatability of food (Davis & Smith, 1992). Additionally, though mice lacking the CB_1 receptor show significantly decreased consumption of sucrose solution as compared to wildtypes, addition of quinine, a taste that is typically aversive, to the solution causes CB_1-null mice and wildtypes to consume the same amount of sucrose solution (Sanchis-Segura et al., 2004). This indicates that mice lacking the CB_1 receptor like sucrose and sucrose laced with quinine about the same amount, whereas wildtype mice like sucrose solution more than sucrose laced with quinine. Similarly, rimonabant has been shown to reduce sucrose consumption by increasing the length of pauses following reinforcement (Pério et al., 2001), which has been interpreted as antagonism of CB_1 signaling causing a reduction in the perceived palatability of sucrose. This suggests that the endocannabinoid system is specifically involved in mediating palatability, or "liking" of food.

In the most direct measure of perceived palatability, several studies showed that both Δ^9-THC and AEA administration increases the characteristic set of orofacial responses indicative of perceived palatability (Grill & Norgren, 1978) in response to intraoral infusion of sucrose solution in a CB_1-dependent manner (De Luca et al., 2012; Jarrett et al., 2005; Mahler et al., 2007), whereas the CB_1 inverse agonist AM251 reduces those responses (De Luca et al., 2012; Jarrett et al., 2007). Subsequently, Jarrett et al. also showed that Δ^9-THC administration decreases the orofacial responses indicative of rejection in response to quinine, indicating that cannabinoids increase the relative palatability of even aversive flavors (Jarrett et al., 2007). However, other studies have found that AEA (Mahler et al., 2007) or Δ^9-THC (De Luca et al., 2012) administration has no effect on orofacial responses indicative of rejection in response to quinine. The discrepancy possibly occurred due to the different degrees of CB_1 receptor activation by the agonists administered. Nevertheless, these data suggest that cannabinoids, acting on the CB_1 receptor, increase both the desire for food and the overall perceived palatability of food consumed.

Cannabinoid Modulation of Food Preferences

Consumption of *Cannabis* leads not only to generally increased feeding and appetite, but also to preferential desire for highly palatable foods, such as sweets (Allentuck & Bowman, 1942; Foltin et al., 1988; Halikas et al., 1971; Tart, 1970). In humans, cannabinoid-induced increase in caloric intake has been shown to specifically result from increased snacking on sweets between meals (Foltin et al., 1986). In rats, CB_1 receptor agonist CP55,940 was shown to increase motivation to obtain sucrose solution (Gallate et al., 1999), while mice lacking the CB_1

receptor consume less sucrose than wildtype mice (Poncelet et al., 2003; Sanchis-Segura et al., 2004).

While many studies have shown that cannabinoid signaling increases "wanting" and "liking" of palatable foods, most telling are the studies in which animals are presented with both highly palatable and less palatable food options, and show a selective, cannabinoid-induced increased preference for highly palatable food without a concomitant increase for less palatable food. In rats, Δ^9-THC (Brown et al., 1977; Koch & Matthews, 2001; Sofia & Knobloch, 1976) or 2-AG (DiPatrizio & Simansky, 2008) have been shown to induced significantly greater intake of sucrose solution and/or a high-fat diet as compared to standard laboratory chow. Conversely, CB_1 receptor antagonism with rimonabant or AM251 was shown to selectively decrease intake of palatable, sweet food in both rats (Arnone et al., 1997; DiPatrizio & Simansky, 2008; Mathes et al., 2008) and marmosets (Simiand et al., 1998), while intake of regular food remained the same. CB_1 receptor antagonism also selectively reduced the motivation for palatable food as compared to regular food (Droste et al., 2010; Ward et al., 2008). Notably, as in mammals, we have shown that *C. elegans* exposed to endocannabinoids also shows and increased preference for more palatable food when presented with both palatable and standard food options (see Chapter III), indicating that endocannabinoid system's involvement in food preference is conserved even in a species that diverged from a line leading to mammals more than 570 million years ago.

Studies showing that cannabinoid signaling affects consumption irrespective of food palatability have also been published. Experiments have shown that rimonabant decreases feeding on palatable food as well as bland chow in rodents (Rowland et al., 2001; Wierucka-Rybak et al., 2014), monkeys (Foltin & Haney, 2007), and humans (Heppenstall et al.,

2012). One explanation for differences in the effect of cannabinoids on palatable and non-palatable food consumption between studies is that baseline hunger and metabolic levels may cause nuances in the behavioral effects of cannabinoids. Because endogenous levels of endocannabinoids in brain region controlling appetite are higher in starved animals (Kirkham et al., 2002), pharmacological or genetic CB_1 impairment may have a stronger effect on the consumption of normal chow under these conditions (Colombo et al., 1998; Di Marzo et al., 2001; Kirkham et al., 2002). By contrast, specific effects of CB_1 antagonists on consumption of palatable foods have been shown in rodents fed ad libitum (Gallate et al., 1999). Nevertheless, the data generally supports the hypothesis that the endocannabinoid system promotes intake of highly palatable, high-calorie food.

Mechanisms for Cannabinoid Modulation of Feeding

Endocannabinoid Interaction with Hypothalamic Feeding Pathways

Though several brain regions are involved in the control of energy homeostasis, the ventromedial, dorsomedial, and lateral hypothalamus, together with arcuate and paraventricular nuclei of the hypothalamus, comprise the primary feeding control center of the brain (Horvath & Diano, 2004; Kennedy, 1966; Schwartz et al., 2000). The hypothalamus receives information about nutritional state through hormonal and nutrient signals and modulates food intake to maintain energy homeostasis.

In mammals (Kirkham et al., 2002) and fish (Díaz-Rúa et al., 2020), fasting and eating have been shown to increase and decrease levels of 2-AG in the hypothalamus, respectively, indicating a role for endocannabinoids in hypothalamic feeding pathways (Higuchi et al., 2012). In mammals (Mailleux & Vanderhaeghen, 1992), fish (Cottone et al., 2005), and frogs (Cesa et

al., 2001), the hypothalamus expresses CB_1 receptors, and administration of AEA, 2-AG, Δ^9-THC, or synthetic CB_1 receptor agonists into the hypothalamus stimulates feeding in a CB_1-dependent manner (Anderson-Baker et al., 1979; Jamshidi & Taylor, 2001; Kirkham et al., 2002; Kirkham & Williams, 2001; Koch et al., 2015). These results suggest that the effect of cannabinoids on feeding results at least partially from activation of hypothalamic CB_1 receptors.

The hypothalamus expresses both anorexigenic (appetite-reducing) and orexigenic (appetite-stimulating) neuropeptides, which work in concert to maintain energy balance. The endocannabinoid system is thought to interact with many of the appetite-related signaling factors in the hypothalamus (Cota et al., 2003). One of the most important signaling molecules of the hypothalamus is leptin, which reduces food intake by upregulating anorexigenic neuropeptides and downregulating the orexigenic factor neuropeptide Y (Friedman & Halaas, 1998; Stephens et al., 1995). Mutations in the leptin receptor have been linked to obesity (Chen et al., 1996). Interestingly, mice with defective leptin signaling, due to mutations in either the leptin gene or leptin receptor gene, have elevated hypothalamic levels of endocannabinoids (Di Marzo et al., 2001). Acute leptin treatment of both wildtype rats and mice with defective leptin signaling reduces AEA and 2-AG levels in the hypothalamus (Di Marzo et al., 2001; Jo et al., 2005; Malcher-Lopes et al., 2006). Additionally, mice lacking the CB_1 receptor do not exhibit the anorexigenic effect of leptin treatment (Cardinal et al., 2012), indicating that cannabinoid signaling is required for leptin's effect on feeding. CB_1 receptor expression has also been shown to increase with increased leptin levels, likely to compensate for lower endocannabinoid levels (Pagotto & Pasquali, 2005).

The endocannabinoid system exhibits complex interactions with several of the hypothalamic signaling molecules that are regulated by leptin. In rodents, lack of CB_1 receptor

signaling due to blockade with rimonabant (Arnone et al., 1997) or genetic mutations (Poncelet et al., 2003) reduces neuropeptide Y-induced food consumption and overall neuropeptide Y levels (Verty et al., 2009). Conversely, administration of AEA has been shown to increase neuropeptide Y levels (Piccinetti et al., 2010). This interaction suggests that endocannabinoids might interact with the leptin pathway downstream of neuropeptide Y, where its orexigenic effect requires CB_1 receptor signaling. This is further supported by the fact that rimonabant affects neuropeptide Y-deficient mice and wildtype mice similarly, causing both to decrease feeding by the same amount (Di Marzo et al., 2001). And though neuropeptide Y releasing neurons do not express CB_1 receptors (Cota et al., 2003), these receptors are found on GABAergic neurons that innervate them (Morozov et al., 2017). Therefore, it is possible that endocannabinoids promote feeding by increasing neuropeptide Y release through inhibition of inhibitory neurons upstream of neuropeptide Y neurons.

There are also CB_1 receptors on neurons that express leptin-regulated anorexigenic signals such as corticotropin-releasing hormone (CRH), proopiomelanocortin (POMC), and cocaine-and-amphetamine-regulated transcript (CART). Mice lacking CB_1 receptors have increased levels of CRH in the paraventricular nucleus and reduced levels of CART in dorsomedial and lateral hypothalamic areas (Cota et al., 2003). Further, elevated AEA levels are associated with a CB_1-dependent decrease in CART release (Osei-Hyiaman et al., 2005; Verty et al., 2009). In zebrafish, both knockdown of CB_1 and fasting lead to decreased CART expression in brain regions that coexpress CB_1 and CART (Nishio et al., 2012). Since both CRH and CART are known to inhibit feeding, the differential effect of cannabinoid signaling on concentrations of these factors in different parts of the hypothalamus shows the complexity of the interaction between the endocannabinoid system and feeding circuits.

POMC neurons also interact with endocannabinoids in modulation of feeding. CB_1 receptors are found on both POMC neurons (Morello et al., 2016) and neurons that make synaptic connections to POMC neurons (Koch et al., 2015). Though POMC neurons are usually anorexigenic, CB_1 activation has been shown to increase POMC neuron activity but selectively increase β-endorphin release from these neurons (Koch et al., 2015), which would promote *hyper*phagia. POMC neurons have also been shown to release endocannabinoids continuously under basal conditions. The released of endocannabinoids inhibits GABA release onto POMC neurons in a CB_1-dependent manner but may also inhibit glutamate release onto the POMC neurons (Hentges et al., 2005), adding to the complexity of the interaction of the endocannabinoid system with hypothalamic feeding circuits, and possibly explaining why higher concentrations of CB_1 agonists may lead to an inhibition, rather than a stimulation of feeding.

Stimulation of both POMC and CART neurons by leptin is known to cause the release of melanocortins, which cause a decrease in food intake (Fan et al., 1997). There is evidence that CB_1 receptors in the hypothalamus are located downstream of melanocortin receptors, as CB_1 antagonism with rimonabant decreases the orexigenic effects of the melanocortin receptor-4 antagonism (Verty et al., 2004). Further, antagonism of the melanocortin receptor-4 leads to increased endocannabinoid levels, suggesting that melanocortins might reduce feeding by inhibiting endocannabinoid signaling (Matias et al., 2008).

Finally, endocannabinoids interact with several orexigenic signals other than leptin, such as orexin, agouti-related protein (AGRP), and melanocyte-concentrating hormone (MCH). CB_1 strongly colocalizes with orexin-1 receptors in the arcuate and paraventricular nuclei of the hypothalamus, at both the pre-synaptic and post-synaptic levels (Hilairet et al., 2003). CB_1 receptors are also expressed on both glutamatergic and GABAergic neurons that synapse

onto orexin-1 receptor expressing neurons, which also coexpress 2-AG (Morello et al., 2016), indicating retrograde signaling from orexin-1 expressing neurons to the neurons that impinge on them. CB_1 receptor antagonism with rimonabant inhibits the hyperphagic effects of orexin A, confirming the interaction between orexin and endocannabinoid signaling (Crespo et al., 2008; Hilairet et al., 2003). Indeed, activation of the orexin-1 receptor promotes 2-AG synthesis and release (Jäntti et al., 2013; Morello et al., 2016; Tung et al., 2016; Turunen et al., 2012). Retrograde activation of CB_1 receptors by post-synaptically released 2-AG inhibits the inhibitory effects of the GABAergic neurons onto orexinergic neurons, facilitating the release of orexin, and leading to an increase in feeding. It has now also been shown that CB_1 receptors and orexin-1 receptors also function as heterodimers (Ellis et al., 2006; Imperatore et al., 2016; Jäntti et al., 2014; Ward et al., 2011), activation of which causes 2-AG biosynthesis (Imperatore et al., 2016). Further, orexin-1-mediated 2-AG release has now been linked to disinhibition of dopaminergic neurons in the ventral tegmental area, which mediates reward and is involved in cannabinoid modulation of feeding (more on this below) (Tung et al., 2016).

CB_1 receptors have been shown to be located on the presynaptic terminals of GABAergic cells that synapse onto MCH-releasing neurons (Huang et al., 2007). As MCH release induces feeding (Rossi et al., 1997), activation of these CB_1 receptors by cannabinoids would decrease GABA release, which would disinhibit MCH neurons and likely contribute to the observed increase in eating after cannabinoid intake. Together, these findings indicate that the endocannabinoid system modulates homeostatic feeding circuits of the hypothalamus, and that activation of the hypothalamic endocannabinoid system stimulates feeding by increasing orexigenic signals and decreasing anorexigenic signals.

Endocannabinoid Interaction with Cortical Reward Pathways

Selective increase in preference for highly palatable foods after cannabinoid exposure implicates an interaction between the endocannabinoid system and reward areas of the brain in the modulation of feeding. Corticolimbic structures, including the mesolimbic dopaminergic pathway, which connects the ventral tegmental area to the nucleus accumbens, and the ventral pallidum are major players in the processing of reward, motivation, and pleasure (reviewed in Spanagel & Weiss, 1999). These brain regions are also highly interconnected with the hypothalamus and play a crucial role in feeding (Castro et al., 2015; reviewed in Spanagel & Weiss, 1999). In fact, both feeding and stimulation of the hypothalamus increase dopaminergic signaling in the nucleus accumbens (Hernandez & Hoebel, 1988b, 1988a; Radhakishun et al., 1988), particularly in fasted animals (Aitken et al., 2016). Further, mice that cannot synthesize dopamine show decreased food consumption and die of starvation by 4 weeks of age (Szczypka et al., 1999). However, corticolimbic areas seem to be particularly involved in mediating food palatability, as restoration of dopamine production in the nucleus accumbens of mice lacking dopaminergic signaling restores feeding on palatable food but not on regular chow (Szczypka et al., 2001). In humans, fMRI studies show that reward regions of the brain, including the nucleus accumbens, activate when people are presented with pictures of high-calorie foods as compared to pictures of low-calorie foods (Schur et al., 2009). In rodents, palatable food cues have been found to enhance dopamine release in the nucleus accumbens (Aitken et al., 2016; McCutcheon et al., 2012). Therefore, the reward regions of the brain also mediate the rewarding properties and hedonic value of food.

Endocannabinoids and their receptors are found on dopaminergic neurons that project to the nucleus accumbens (Bisogno et al., 1999; Glass et al., 1997; Hermann et al., 2002). Fasting

has been shown to increase levels of AEA and 2-AG in the limbic forebrain (Kirkham et al., 2002), whereas injection of endocannabinoids or synthetic cannabinoid receptor agonists into the nucleus accumbens has been shown to induce hyperphagia in rats fed ad libitum in a CB_1-dependent manner (Kirkham et al., 2002; Mahler et al., 2007; Soria-Gómez et al., 2007). Endocannabinoid signaling in the nucleus accumbens appears to specifically mediate food palatability. In support of this, injection of AEA into the nucleus accumbens increases the number of orofacial responses indicative of perceived palatability in responses to intra-oral infusions of sweet solutions (Mahler et al., 2007). Additionally, CB_1 receptors in the nucleus accumbens become downregulated in rats that consume large amounts of palatable food (Harrold et al., 2002), likely due to increased stimulation of these receptors by endocannabinoids released upon consumption of palatable food.

To mediate food palatability, cannabinoids appear to interact with the dopaminergic neurons that project to the nucleus accumbens. *In vitro* studies have shown that cannabinoid receptor agonists enhance dopamine synthesis (Bloom, 1982; Navarro et al., 1993) and inhibit dopamine reuptake (Banerjee et al., 1975; Bloom et al., 1977; Hershkowitz & Szechtman, 1979; Poddar & Dewey, 1980). *In vivo* studies indicate that systemic administration of AEA (Marcello Solinas et al., 2006) or Δ^9-THC (De Luca et al., 2012) enhances dopaminergic signaling within the nucleus accumbens, a hallmark of reward and behavior reinforcements (Nicola et al., 2005). Additionally, the release of dopamine in the nucleus accumbens provoked by presentation of palatable food is eliminated when CB_1 receptor are blocked by rimonabant (Melis et al., 2007). Interestingly, dopamine inhibits synthesis of AEA and 2-AG in the limbic forebrain (Patel et al., 2003). This suggests that the endocannabinoid system acts upstream of dopamine release within an inhibitory feedback loop and interacts specifically with mesolimbic dopamine signaling that

influences the rewarding properties of food. However, this interaction is complex as CB_1 receptors are found on dopaminergic neurons in the nucleus accumbens (Tsou et al., 1998; Winters et al., 2012), as well as GABAergic and glutamatergic neurons that synapse onto dopaminergic neurons of both the nucleus accumbens and the ventral tegmental area (reviewed in Lupica et al., 2004). CB_1 receptors are also colocalized with dopamine type 1 and 2 receptors (Hermann et al., 2002). Thus, endocannabinoids may modulate dopamine signaling directly, but also indirectly, by disinhibiting dopaminergic neurons in the ventral tegmental area and nucleus accumbens. It was also recently shown that GABAergic neurons from the nucleus accumbens project to and inhibit GABAergic neurons in the lateral hypothalamus, which leads to an inhibition of feeding behavior (O'Connor et al., 2015). This projection from the nucleus accumbens is inhibited by endocannabinoids acting on CB_1, which promotes feeding (Thoeni et al., 2020). To add to the complexity of the interaction between the endocannabinoid and dopaminergic signaling systems, there is also evidence that dopamine and endocannabinoids form heterodimers of dopamine type 2 and CB_1 receptors (Khan & Lee, 2014; Przybyla & Watts, 2010). Therefore, endocannabinoids and dopamine may act together to inhibit dopamine release from synaptic terminals by dual activation of their receptors, which strongly attenuates further dopamine release (Everett et al., 2021).

The opioid system has also been implicated in the mediation of food reward. Opioid receptor agonists can increase food intake, whereas antagonist decrease food intake (reviewed in Valbrun & Zvonarev, 2020). Additionally, in humans, opioid receptor antagonists decrease perceived palatability of foods (Drewnowski et al., 1992; Yeomans & Gray, 1996). As with AEA, injection of opioid receptor agonist morphine into the nucleus accumbens increases orofacial responses indicative of liking in response to sucrose solutions (Peciña & Berridge,

2000), whereas systemic opioid receptor antagonist administration decreases the perceived hedonic properties of sucrose solution (Parker et al., 1992). Studies have now shown a link between the opioid and endocannabinoid systems in feeding modulation. For example, the cannabinoid-induced increased feeding and preference for palatable food is sensitive not only to CB_1 receptor antagonists but also the opioid receptor antagonist, naloxone (Gallate & McGregor, 1999; Williams & Kirkham, 2002). Further, administering opioid antagonists together with CB_1 antagonists causes additive effects on the expected inhibition of feeding (Chen et al., 2004; Kirkham & Williams, 2001; Rowland et al., 2001). Mice lacking the CB_1 receptor are not only insensitive to cannabinoid receptor agonists but also have reduced sensitivity to opioids (Ledent et al., 1999), and administration of cannabinoids decreases opioid withdrawal symptoms (Vela et al., 1995; Yamaguchi et al., 2001). It has been hypothesized that the endocannabinoid system modulates food palatability by activating opioid pathways specifically within the reward circuitry of the brain. In support of this, it has been shown that co-administration of opioid antagonist naloxone into the nucleus accumbens blocks AEA's effect on orofacial "liking" expression toward sucrose solution (Mitchell et al., 2018). Administration of Δ^9-THC stimulates release of β-endorphins in the ventral tegmental area and nucleus accumbens (Solinas et al., 2004), which has previously been associated with feeding on highly palatable food (Dum et al., 1983; reviewed in Mercer & Holder, 1997). Further, the mu-opioid receptor forms a functional heterodimer with the CB_1 receptor, indicating a possibility of activation of CB_1 or opioid receptors by both endocannabinoids and opioid molecules (Hojo et al., 2008). Together, these studies suggest an intricate interaction between the endocannabinoid and opioid systems in mediating food reward.

A putative link between the endocannabinoid and opioid systems in mediating food palatability is through the actions of cholecystokinin (CCK). CCK is as satiety signal and acts as an anti-opioid peptide (Wiesenfeld-Hallin et al., 1999). CCK releasing neurons have been shown to express the CB_1 receptor (Marsicano & Lutz, 1999; Tsou et al., 1999) and cannabinoids acting on the CB_1 receptor have been shown to inhibit CCK release (Beinfeld & Connolly, 2001). This could result in enhanced activation of opioid pathways, leading to an increase in the rewarding properties of food. Overall, these observations imply that cannabinoids affect the motivation to feed via activation of both the endocannabinoid and opioid systems.

Endocannabinoid Interaction with Serotonin

Serotonin (5-HT) is known to be involved in both reward and food intake regulation (reviewed in Simansky, 1996), and coexpression of serotonin and CB_1 receptors in reward areas (the striatum, caudate putamen, and nucleus accumbens) suggests an interaction between the serotoninergic and the endocannabinoid signaling systems (Hermann et al., 2002). CB_1 receptors and serotonin $5\text{-}HT_{2C}$ receptors have been shown to both be involved in motivated feeding behavior, and synergistically modulate motivation for palatable food (Ward et al., 2008). Diet restriction in mice causes a significant decrease 5-HT in the hypothalamus, whereas administration of AEA causes significant increases in the concentrations of 5-HT in the hypothalamus (Hao et al., 2000). In other areas, activation of the CB_1 receptors has been shown to inhibit 5-HT release (Egashira et al., 2002; Merroun et al., 2009; Nakazi et al., 2000), whereas CB_1 receptor antagonism causes an increase in 5-HT levels (Aso et al., 2009; Darmani et al., 2003; Merroun et al., 2009; Tzavara et al., 2003). However, simultaneous administration of cannabinoid receptor antagonists and dexfenfluramine, a drug that induces 5-HT release, leads to

additive but not synergistic effects on reducing food intake (Rowland et al., 2001), indicating that the endocannabinoid and serotonergic pathways that affect feeding are working through parallel but independent mechanisms.

Endocannabinoid Modulation of Sensory Systems

An important feature of proper energy balance is integration of internal hunger cues with external food cues. Visual, gustatory, and olfactory cues can all play a role in driving an animal toward or away from potential food sources, and the internal hunger state of an animal can modulate sensory responses to either promote or inhibit feeding to maintain energy balance. Sensory areas and the hypothalamus are highly interconnected, and changes in energy or metabolic state affects the activity of both areas. For example, humans (Jansen et al., 2003; Pastor et al., 2016; Richardson et al., 2004) and rodents (Aimé et al., 2014; Thanos et al., 2008) suffering from obesity or eating disorders show altered olfactory perception. Further, nutritional state has been shown to change the levels of hypothalamic neuropeptides in the olfactory bulb (Prud'homme et al., 2009; Saito et al., 1981; Scallet et al., 1985; Shibata et al., 2008) and the overall activity of cells in the olfactory bulb (Apelbaum & Chaput, 2003; Daumas-Meyer et al., 2018; Wu et al., 2020). This change in peptide levels may underly changes in olfactory detection (Aimé et al., 2012, 2014; Julliard et al., 2007; Prud'homme et al., 2009), which has been shown to be modulated depending on energy state (Aimé et al., 2007; O'Doherty et al., 2000; Pager et al., 1972).

It has been shown that the endocannabinoid system interplays with not only homeostatic and hedonic centers, as discussed earlier, but also with sensory systems to modulate feeding. Particularly, studies have shown that taste perception, which results from a combination of

olfactory and gustatory signaling, is modulated by endocannabinoids. CB_1 receptors have been found in the olfactory structures of amphibians (Cesa et al., 2001; Hollis et al., 2006), fish (Son & Ali, 2022), rodents (Pettit et al., 1998; Egertová & Elphick, 2000; Hutch et al., 2015; Moldrich & Wenger, 2000; Tsou et al., 1998), and dogs (Freundt-Revilla et al., 2017), showing a remarkable level of conservation across vertebrates. Endocannabinoids are also found throughout the olfactory system (Hutch et al., 2015; Wang et al., 2019) and fasting increases their levels in those areas (Breunig et al., 2010). Activation of CB_1 receptors in olfactory areas has been shown to increase odor sensitivity in tadpoles (Breunig et al., 2010) and mice (Soria-Gómez et al., 2014) but is not required for normal olfaction (Hutch et al., 2015). In *C. elegans*, AEA administration tunes olfactory neuron responses to food in a way that promotes feeding on more palatable foods (see Chapter III). In humans, surprisingly, Δ^9-THC was shown to impair performance on olfactory tests (Walter et al., 2014), but still mitigate some chemosensory alterations in cancer patients (Brisbois et al., 2011). The differences in findings between those two studies may arise from the large discrepancy in the dose of Δ^9-THC administered (20mg in the Walter et al. study vs 2.5mg in the Brisbois et al. study). It is plausible that higher doses inhibit olfactory perception whereas lower doses facilitate it.

Nevertheless, animal studies indicate that cannabinoids may stimulate food intake and increase olfactory sensitivity by increasing excitatory activity of the olfactory bulb and decreasing inhibitory signals from the olfactory cortex to the olfactory bulb (Pouille & Schoppa, 2018; Soria-Gómez et al., 2014). As CB_1 receptors are expressed on axon terminals of glutamatergic neurons that project to inhibitory granule cells of the olfactory bulb, as well as on the GABAergic cells themselves, this increase in odor sensitivity likely occurs through CB_1 mediated inhibition of GABAergic granule cells, which disinhibits downstream mitral cells

(Wang et al., 2019). Additionally, endocannabinoids have also been shown to directly inhibit GABAergic periglomerular cells and cause a disinhibition of the tufted cells they synapse on (Wang et al., 2012). Mitral and tufted cells receive and process olfactory information in the glomeruli of the olfactory bulb, and are the main output of the olfactory bulb, so increased activity of mitral and tufted cells likely leads to increased sensitivity of glomeruli to sensory input and increased input of odor information to the olfactory cortex.

Gustatory sensitivity is also modulated by endocannabinoid signaling. Obese patients have increased salivary endocannabinoids (Matias et al., 2012). Further, animals lacking the CB_1 receptor exhibit decreased consumption of not only calorically dense foods, but also decreased consumption of the calorie-free sweetener saccharin (Sanchis-Segura et al., 2004). This implicates primary gustatory cells in endocannabinoid modulation of feeding, as hypothalamic and dopaminergic control of feeding should presumably preferentially stimulate high calorie feeding. In support of this, polymorphisms in the gene encoding the CB_1 receptor has been shown to alter sensitivity to sweet taste in humans (Umabiki et al., 2011), and activation of the CB_1 receptor in type II taste cells by AEA or 2-AG in mice increases gustatory responses to sweet taste without affecting responses to salty, sour, bitter, or umami compounds (Yoshida et al., 2010). The endocannabinoid system also appears to interact with leptin in modulating taste perception. Leptin has been shown to selectively suppress sweet taste responses in mice, whereas endocannabinoids oppose the action of leptin and enhance sweet taste sensitivity (Niki et al., 2010, 2015; Yoshida et al., 2013). Further, endocannabinoids may also modulate the taste of fat. Though rats that are sham fed fatty food exhibit increased endocannabinoid release in the gut but not the tongue (DiPatrizio et al., 2011), there is some preliminary evidence that endocannabinoids are involved in fat taste perception (Brissard et al., 2018). Additionally, CB_1

receptors have been found in the pontine parabrachial nucleus (PBN), which is located in the pons and is known to relay sensory information, including taste to forebrain structures, including the hypothalamus. PBN neurons that express calcitonin gene-related peptide have been shown to play a major role in appetite regulation (Campos et al., 2016). Injection of 2-AG into the PBN stimulates feeding on high-fat and sweet foods in a CB_1-dependent manner, while not affecting consumption of standard chow (DiPatrizio & Simansky, 2008). Together, these data indicate that gustatory endocannabinoid signaling likely contributes to increased preference for and feeding on calorically dense, highly palatable food.

Though CB_1 receptors and endocannabinoids are found in the retina (Straiker et al., 1999; Yazulla et al., 1999) and appear to modulate activity of retinal ganglion cells (Cécyre et al., 2013, 2020; Middleton et al., 2019; Middleton & Protti, 2011; Miraucourt et al., 2016; Ryskamp et al., 2014; Yoneda et al., 2013), there is no evidence, yet, that the endocannabinoid system interacts with the visual system to modulate feeding, though if such an interaction exists, it likely occurs at the level of visual processing rather than at the periphery.

Endocannabinoid Interaction with Gastrointestinal and Adipose Signals

Gastrointestinal and adipose signaling to the hypothalamus are crucial components of energy balance regulation. AEA and 2-AG and their receptors have been identified throughout the gastrointestinal tract (Buckley et al., 1998; Casu et al., 2003; Coutts et al., 2002; Croci et al., 1998; Facci et al., 1995; Griffin et al., 1997; Kulkarni-Narla & Brown, 2000; Mechoulam et al., 1995). CB_1 and CB_2 receptors have been shown to be upregulated in both experimental models (Izzo et al., 2001) and clinical manifestations of intestinal dysfunction, such as celiac disease (Battista et al., 2013; D'Argenio et al., 2007) and inflammatory bowel disease (Wright et al.,

2005). CB_1 activation by exogenous applications of either Δ^9-THC or AEA inhibit intestinal motility (Calignano et al., 1997; Izzo et al., 1999; Krowicki et al., 1999; McCallum et al., 1999; Shook et al., 1986) and peristalsis (Izzo et al., 2000). TRPV1, which binds endocannabinoids and is normally activated by spicy compounds such as capsaicin, high temperature, and low pH (Geppetti & Trevisani, 2004), is expressed on sensory neurons in all regions of the gastrointestinal tract, as well as enteric nerves and epithelial cells (Holzer, 2004), and has also been shown to be upregulated under inflammatory conditions in the gut (Holzer, 2004).

Expression of endocannabinoids and their receptors in the enteric nervous system, gastrointestinal system, and central nervous system suggest crosstalk between central and peripheral feeding systems in the modulation of energy balance. In support of this idea, it has been shown the gut endocannabinoid system undergoes changes in response to food deprivation (Gómez et al., 2002). Fasting causes an increase in AEA concentration in the small intestine (Gómez et al., 2002) and an upregulation of CB_1 receptor expression (Burdyga et al., 2004). By contrast, high-fat diets are associated with down-regulation of overall AEA levels and CB_1 receptor expression in the gut, likely as a compensatory mechanism for increased caloric intake (Di Marzo et al., 2008).

The endocannabinoid system also interacts with two important peripherally secreted hormones, ghrelin and insulin, that signal within the hypothalamus to modulate feeding. Ghrelin is an orexigenic hormone that is a critical player in feeding regulation. Ghrelin concentrations increase after food deprivation, and administration of ghrelin into the hypothalamus cause an increase in feeding (Wren et al., 2001). Interestingly, ghrelin has now been linked to increased motivation for food (“wanting”) through an interaction with dopaminergic signaling (Cone et al., 2015; Overduin et al., 2012; Simon et al., 2017). Increased ghrelin levels have been shown to

cause an increase in endocannabinoid levels as well (Kola et al., 2008), and the levels of ghrelin and endocannabinoids correlate during consumption of palatable food (Monteleone et al., 2012). Conversely, a decrease in gastric AEA levels or CB_1 receptor expression reduces gastric ghrelin secretion (Cani et al., 2004). Ghrelin's orexigenic effect has been shown to require the CB_1 receptor (Kola et al., 2008; Tucci et al., 2004), indicating that cannabinoids act downstream of ghrelin to increase appetite and motivation for food, and are required for ghrelin's orexigenic effects.

Insulin is a hormone released by pancreatic β-cells that acts as a satiety signal, regulates breakdown of macronutrients, and promotes glucose absorption from the bloodstream (reviewed in Figlewicz Lattemann & Benoit, 2009). It was speculated early on in cannabinoid research that the endocannabinoid system interacts with insulin, as Δ9-THC administration was shown to affect glucose metabolism in rats (Margulies & Hammer, 1991) and humans (Volkow et al., 1996). Indeed, central administration of CB_1 receptor agonists leads to reduced insulin signaling in the hypothalamus, which can lead to a dysregulation of glucose production and adipose tissue lipolysis (O'Hare et al., 2011; Scherer et al., 2012). Conversely, antagonism or knockout of the CB_1 receptor in the central nervous system leads to increased insulin activity in the hypothalamus (Bajzer et al., 2011; Mølhøj et al., 2010; O'Hare et al., 2011). As part of its actions a satiety signal, insulin has been shown to cause long-term depression of dopamine neurons in the ventral tegmental area (Figlewicz et al., 2003; Labouèbe et al., 2013). It was later shown that this effect is dependent on endocannabinoids, which inhibit glutamate release onto these dopaminergic neurons, leading to long-term depression (Liu et al., 2016). Paradoxically, this effect would lead to a reduction in the rewarding properties of food following feeding, and a

cessation of food intake, highlighting the endocannabinoid system's ability to both increase and decrease feeding and palatability of food.

Finally, endocannabinoid signaling also occurs in peripheral adipose tissues. Activation of CB_1 receptors in adipocytes or sympathetic fibers that innervate adipose tissue stimulates synthesis and release of leptin, which, as discussed earlier, leads to satiation and cessation of eating (Tam et al., 2012). Obese patients have decreased CB_1 receptor expression, increased 2-AG levels in visceral fat (Matias et al., 2008) and plasma (Blüher et al., 2006), and decreased FAAH expression in visceral fat, suggesting a negative-feedback regulation between endocannabinoids and their receptors and catabolic enzymes to maintain energy homeostasis (Blüher et al., 2006).

Conclusions and Future Directions

In summary, the endocannabinoid system has been shown to play an important role in the central and peripheral regulation of energy homeostasis across species (Bellocchio et al., 2006; Cota et al., 2006; Fride et al., 2005; Matias & Di Marzo, 2007; Osei-Hyiaman et al., 2006). At the behavioral level, activation of cannabinoid receptors increases "wanting" of food, "liking" of food, and the desire to feed on more palatable food. It achieves this by modulating each of the systems involved in energy balance and feeding.

Energy balance occurs through an intricate interplay between homeostatic feeding areas (the hypothalamus), hedonic feeding areas (corticolimbic dopaminergic areas), sensory input (visual, olfactory, and gustatory), and hormonal signaling, particularly from the gastrointestinal tract. Crosstalk between these regions allows for modulation of feeding as needed to maintain energy balance. Hedonic areas project to the hypothalamus to deliver information regarding the

palatability and hedonic value of food. Conversely, energy state information from the hypothalamus affects signaling in hedonic areas. Both hedonic and homeostatic areas are modulated by peripheral signals as well. Neurons of the hypothalamus receive input about hunger levels and food intake from the gut via leptin and ghrelin, and information about the availability and value of food in the environment from olfactory and the gustatory systems. The hypothalamus, in turn, regulates activity of neurons in sensory areas depending on energy state. Ghrelin signals from the gut also modulate dopaminergic neuron activity in hedonic areas. The endocannabinoid system is involved in the function of each of these systems, and some of the crosstalk between them.

In general, activation of the endocannabinoid system promotes food intake and energy storage. At the central level, the endocannabinoid system seems to regulate food intake by both hedonic and homeostatic pathways. During fasting, endocannabinoid levels increase in both homeostatic and hedonic brain regions, whereas administration of AEA to the hypothalamus (Jamshidi & Taylor, 2001) or 2-AG to the nucleus accumbens (Soria-Gómez et al., 2007) stimulates feeding in general, induces palatable food intake in particular, and increases motivation for food intake by acting on CB_1 receptors. Endocannabinoids also modulate hypothalamic signaling to the nucleus accumbens via orexinergic signaling. At the peripheral level, the endocannabinoid system regulates food intake by modulating sensory systems, gastrointestinal function, and secretion of hormones, once again accentuating feeding signals.

However, cannabinoids can cause an inhibition of food intake as well and have been shown to do so at high concentrations. This is due to the distribution and signaling pattern of the endocannabinoid system. Activation of CB_1 receptors, which are expressed on both glutamatergic or GABAergic terminals, can cause either excitation or inhibition of signaling. The

distribution of excitatory and inhibitory neurotransmission in the hypothalamus gets rewired at the synaptic level depending on energy state or in lean versus obese animals. Therefore, CB_1 receptor activation can modulate food intake differently (Crosby et al., 2011).

Nevertheless, the endocannabinoid system is an important player in feeding regulation and can be harnessed to both stimulate and inhibit food intake. However, the widespread expression of endocannabinoid receptors and their participation in a vast number of physiological functions has made modulation of feeding with simple receptor agonism and antagonism difficult. Since the discovery and characterization of cannabinoid receptors in 1990s, we have learned a lot about the function and distribution of these receptors. Our deeper understanding of the interplay between different areas that regulate feeding, as well as the participation of the endocannabinoid system in each of those areas, has helped shape the future of endocannabinoid system targeting therapies for feeding regulation, such as silent agonists and peripherally restricted antagonists. Additionally, much of the research regarding the endocannabinoid system's role in feeding has focused on the CB_1 and CB_2 receptors, and further exploration of the role of non-canonical receptors such as TRPV1 and GPR55 may identify novel pharmacological targets as well.

Finally, identification of an endocannabinoid system in non-mammalian generically tractable species, such as zebrafish, *C. elegans*, and Drosophila, will facilitate our advancements in this field, likely leading to a deeper understanding of endocannabinoid signaling and possible novel targets for pharmaceutical targeting. The next chapter will explore what is currently known about the endocannabinoid system of *C. elegans.* Recent advancements showing the presence of a functional endocannabinoid system in *C. elegans* that affects its behavior and physiology and has opened the door to further exploration of endocannabinoid system function.

CHARTER II: THE C. ELEGANS ENDOCANNABINOID SYSTEM

Introduction to *C. Elegans*

Caenorhabditis elegans is a free-living nematode worm that diverged from a line leading to mammals 500 million years ago. *C. elegans* has been found in North and South America, Europe, Africa, and Australia, as well as several islands, where it lives in decaying vegetation and consumes bacteria. Though initially characterized in the early 1900s (Honda, 1925; Maupas, 1900), much of the work that led to *C. elegans* becoming a commonly used model organism for research fields spanning evolution, aging, and neuroscience was done by Sidney Brenner in the 1960s and 1970s (Brenner, 1974). After that, *C. elegans* became the first multicellular organism to have its genome sequenced (*C. elegans* Sequencing Consortium, 1998), and has since had all of its 302 neurons characterized and their synaptic connections mapped (Cook et al., 2019; White et al., 1986). Several key advancements in biology have come from *C. elegans* research, including the mechanisms underlying apoptosis (Conradt & Xue, 2005) and gene silencing by small RNAs (Grishok, 2013).

C. elegans is a self-fertilizing hermaphrodite, though males do occur at low frequency (<1%) depending on genotype and environmental conditions (Hodgkin & Doniach, 1997; Teotónio et al., 2006). Within 3 days, fertilized eggs mature to gravid adults, which begin to lay eggs themselves. *C. elegans* has food preferences (Shtonda & Avery, 2006), responds to attractive and aversive stimuli (Bargmann & Horvitz, 1991; L'Etoile & Bargmann, 2000), and is capable of simple learning (Nishijima & Maruyama, 2017). Its neuronal signaling relies on many of the same neurotransmitters as mammals, including dopamine, serotonin, glutamate, and GABA. Further, *C. elegans* shares 65% of its genes with humans. Due to this, *C. elegans* is an attractive model organism in which to probe the cellular and molecular basis of simple

behaviors. Importantly, *C. elegans* was recently shown to have a functional endocannabinoid system (Oakes et al., 2017), opening the door to studying this complex signaling system and its effect on animal physiology in a simple, well-characterized, genetically tractable model organism.

The *C. Elegans* Endocannabinoid System

The endocannabinoids AEA and 2-AG and the CB_1 and CB_2 receptors have been found across vertebrate species. However, the lack of clear homologues of the CB_1/CB_2 receptor in invertebrates brought the existence of a functional endocannabinoid system in those species into question (Elphick & Egertová, 2001; McPartland et al., 2001). Phylogenetic analysis showed that the closest gene in the *C. elegans* genome to the CB_1 receptor is C02H7.2, which encodes the NPR-19 receptor. Additionally, *C. elegans* genes NPR-24 and NPR-32 are related to human GPR18 and GPR55 receptors, which have affinity for cannabinoids as well (Pastuhov et al., 2016). However, though NPR-19 clades with cannabinoid receptors (McPartland et al., 2006), it has low similarity to human CB_1 (Elphick & Egertová, 2001; McPartland & Glass, 2003), with substitutions at half of the motifs required for normal mammalian CB_1 function (McPartland et al., 2006). The authors argued that NPR-19 may be an ortholog of the gene encoding CB_1 that does not function as a cannabinoid receptor. Nevertheless, it was shown shortly thereafter that *C. elegans* neural tissues exhibit high-affinity binding of cannabinoid receptor agonists (McPartland et al., 2006), indicating that a receptor capable of binding cannabinoids exists in *C. elegans*.

Around the same time *C. elegans* was shown to exhibit cannabinoid receptor binding, it was also shown to express the synthetic and degradative enzymes for AEA and 2-AG (McPartland et al., 2006). Shortly thereafter, Lehtonen *et al.* showed that *C. elegans* and two

other nematode species, *Caenorhabditis briggsae* and *Pelodera strongyloides*, produce both 2-AG and AEA (Lehtonen et al., 2008). Despite several discoveries of endocannabinoid-induced physiological effects in *C. elegans* (described below), a receptor was not identified until 2016, when Pastuhov *et al.* showed that AEA affects axonal regeneration by acting on the NPR-19 and NPR-32 receptors (Pastuhov et al., 2016). Finally, definitive evidence that NPR-19 is a *C. elegans* endocannabinoid receptor came when both 2-AG and AEA were shown to have high binding affinity for NPR-19 in a heterologous expression system (Oakes et al., 2017). Further, 2-AG was also shown to activate the *C. elegans* α_{2A}-adrenergic-like octopamine receptor OCTR1 (Oakes et al., 2017). Together, these studies confirmed that *C. elegans* has all the components of an endocannabinoid system, which work together to mediate its physiology.

Endocannabinoid System Effect on Development

Development and Lifespan

Endocannabinoid signaling has been shown to play a role in *C. elegans* development and lifespan. Overexpression of *faah-1* in worms, an enzyme that degrades AEA and to a lesser degree 2-AG and several other N-Acylethanolamines (NAEs), causes a developmental delay that can be rescued by RNAi knockdown of *faah-1*. Further, concentrations of NAEs change throughout development, reaching their highest levels in the second larval stage (L2) and then declining into adulthood (Lucanic et al., 2011). Together, these data suggest that NAEs promote larval development.

Overexpression of *faah-1* also increases lifespan, whereas treatment with NAEs decreases lifespan (Lucanic et al., 2011). Further, overexpression of *nape-1* or *nape-2,* the synthetic enzymes for AEA, affects lifespan and growth, though the effect is complex and

temperature dependent (Harrison et al., 2014). Nevertheless, *nape-1* overexpression reduces the extended lifespan of the long-lived *daf-2* mutant worms (Harrison et al., 2014), indicating that endocannabinoid signaling is involved in development, growth, and lifespan of *C. elegans*.

Phytocannabinoids have also been shown to affect lifespan. Treatment with cannabidiol (CBD), a phytocannabinoid derived from the *Cannabis sativa* plant, has recently been shown to extend lifespan in C. elegans by nearly 20% (Frandsen & Narayanasamy, 2022; Land et al., 2021; Wang et al., 2021, 2022). The health of aged worms also appeared to be increased by CBD, as measured by late-life-stage activity (Land et al., 2021). Lifespan extension through CBD appears to occur due to an increased activity of autophagy pathways (Wang et al., 2022).

The Dauer Lifecycle

C. elegans can undergo two alternative life cycles. Upon hatching, if abundant food is available, it will progress through the four larval stages (L1-L4) and reach adulthood in 3 days. However, under stressful environmental conditions such as limited food supply, worms can shift to an alternate life cycle after the L1 larval state called dauer (Golden & Riddle, 1982). The fact that the peak in NAE levels occurs at L2, which coincides with the time at which a worm is committed to reproductive growth rather than entry into dauer, suggests that NAEs may be involved in the decision between the two alternate life cycles. Indeed, NAE levels of worms in the L1 stage are similar under dauer-inducing and normal conditions but are reduced in worms that have entered the dauer life cycle as compared to non-dauer worms (Lucanic et al., 2011). Additionally, overexpression of *nape-1* or *nape-2* suppresses the dauer life cycle in worms carrying the *daf-2* mutation, which causes a constitutive dauer phenotype (Harrison et al., 2014), whereas worms lacking AEA and 2-AG show an increased rate of dauer (Galles et al., 2018).

Administration of AEA and 2-AG also rescues normal development in another constitutive dauer mutant, *daf-7;fat-3* (Galles et al., 2018). Surprisingly, one group showed that CB_1 antagonists, rather than agonists, suppress the dauer phenotype and promotes normal development by acting though DAF-7 (TGF-β) and insulin-like peptides secreted from the ASI neurons (Reis-Rodrigues et al., 2016). Despite this contradictory finding, it appears that in general, endocannabinoid signaling suppresses dauer entry and promotes reproductive growth.

Endocannabinoid System Effect on Physiology

Energy Balance

The endocannabinoid system is a key player in energy balance in mammals and other vertebrates. Its involvement in dauer formation, which can be mediated by nutritional availability, and longevity, which has been linked to caloric restriction, suggests that it may be involved in energy balance regulation in *C. elegans* as well. In support of this, the effect of NAE signaling on lifespan has been shown to depend on food availability (Lucanic et al., 2011). Accordingly, both starved L1 worms and adult worms maintained under dietary restriction have reduced levels of NAEs, which increase to normal levels upon refeeding (Lucanic et al., 2011). *faah-1* overexpression is associated with lifespan extension in the presence of abundant food but not under conditions of optimal dietary restriction. Similarly, lifespan suppression due to increased NAE levels is minimal under conditions of abundant food, but is profound under optimal dietary restriction conditions (Lucanic et al., 2011). The lack of an additive effect of NAE signaling inhibition on lifespan under dietary restriction conditions provides strong evidence that lifespan extension resulting from reduced endocannabinoid levels is mediated through the same pathways as lifespan extension due to dietary restriction.

So far, there have been two feeding-related mechanisms shown to underly endocannabinoid system effects on development and lifespan. First, NAEs appear to act through the mTOR pathway, as NAEs suppress the extended lifespan of *rsks-1(ok1255)* mutants (Lucanic et al., 2011). The protein encoded by *rsks-1* is a target of mTOR signaling, which is known to mediate diet-dependent extension of lifespan (Vellai et al., 2003). Second, endocannabinoids affect cholesterol homeostasis. AEA and 2-AG have been shown to rescue the dauer phenotype resulting from cholesterol deficiency by stimulating cholesterol trafficking through mobilization of internal cholesterol pools (Galles et al., 2018). Together, although the field is still in its infancy, these studies suggest that the endocannabinoid system could be signaling nutrient availability, which promotes energy balance through choice of the correct life cycle.

Additional evidence that the endocannabinoid system affects energy balance in *C. elegans* comes from studies showing that AEA and 2-AG affect feeding. *C. elegans* eats through rhythmic contractions of a pharynx. At high concentrations, 2-AG and AEA were shown to decrease pharyngeal pumping and feeding by acting on the NPR-19 receptor (Oakes et al., 2017). However, both assays were conducted in the absence of food. We showed that AEA affects feeding differently depending on the quality of food presented to the worms (see Chapter III). Worms exposed to AEA increase consumption of palatable, nutritionally dense food, and decrease consumption of less palatable food. This effect is mediated by the NPR-19 receptor, which can be functionally substituted by the human CB_1 receptor. These findings indicate that the endocannabinoid system affects feeding in *C. elegans* similarly to feeding in mammals, where it has been shown to promote feeding on calorically dense foods (see Chapter I).

Nociception

In mammals, cannabinoids have been shown to cause analgesic and antinociceptive effects (Hohmann et al., 1995; Martin et al., 1996; Sanders et al., 1979; Tsou et al., 1996). These effects also appear to be conserved in *C. elegans*. Administration of 2-AG or AEA inhibits aversive responses to noxious stimuli by acting on the NPR-19 receptor, which, again, can be functionally substituted by the CB_1 receptor (Oakes et al., 2017). 2-AG-mediated inhibition of nociception also requires the α_{2A}-adrenergic-like receptor OCTR-1 and a serotonin receptor SER-4 to modulate nociception (Oakes et al., 2017). In mammals, antinociceptive effects of endocannabinoid signaling have previously been linked to serotonergic (Aksu et al., 2018) and noradrenergic (Gutierrez et al., 2003; Khodayar et al., 2006; Tham et al., 2005) signaling (reviewed in Dogrul et al., 2012), showing a conserved mechanism for endocannabinoid modulation of nociception.

Locomotion

In humans, Δ9-THC consumption has been shown to cause impaired motor control (Kvålseth, 1977; Mclaughlin et al., 2000). In *C. elegans*, cannabinoid exposure inhibits locomotion (Oakes et al., 2017, 2019; Shrader et al., 2020). At high concentrations, exposure to 2-AG causes a "locomotory confusion" phenotype (Oakes et al., 2017) in which animals cannot effectively initiate and sustain normal forward/backward locomotion (Law et al., 2015). This effect has been shown to occur due to serotonin inhibition of locomotion (Law et al., 2015). 2-AG-mediated effects on locomotion do not require the NPR-19 receptor but require TRPV1 and TRPN-like channel signaling in serotonergic or dopaminergic neurons, respectively (Oakes et al., 2017, 2019). The phytocannabinoids CBD and cannabidivarin (CBDV) have also been shown to cause decreased locomotion though activity of the dopamine D2-like receptor DOP-3, but

interestingly, does not require dopamine release (Shrader et al., 2020). This suggests that cannabinoids may themselves activate DOP-3, though no direct evidence of this exists yet. While the exact mechanism for locomotion impairment in mammals has not been elucidated, the abundant expression of CB_1 receptors in the basal ganglia and the occurrence of changes in endocannabinoid transmission in the basal ganglia of people with motor disorders supports the involvement of the endocannabinoid is motor function (Fernández-Ruiz & González, 2005). Further, activation of TRPV1 by endocannabinoids has been shown to modulate motor activity in mammals (El Khoury et al., 2012; Lee et al., 2006). Therefore, there may be conservation of the endocannabinoid system's role in motor control between nematodes and mammals.

Endocannabinoid Modulation of Axonal Repair

It has been shown that AEA inhibits axon regeneration after injury. As in mammals, axon regeneration after injury in *C. elegans* is regulated by the p38 and c-Jun N-terminal kinase (JNK) mitogen activated protein kinase (MAPK) pathways (Nix et al., 2011; Pastuhov et al., 2012). In young adult worms, laser-severed axons initiate regeneration within 24 hours (Pastuhov et al., 2012). However, worms treated with AEA, lacking *faah-1*, or over-expressing *nape-1* have reduced axon regeneration (Pastuhov et al., 2012, 2016). Interestingly, the effect of cannabinoids on axon regeneration was specific to adults, as L4 animals did not exhibit reduced regeneration from either AEA or genetic deletion of *faah-1* (Pastuhov et al., 2012). Specifically, AEA acts as a repulsive signal to the growth cone of the injured axon, causing it to navigate away from the injury site (Pastuhov et al., 2016). The AEA-mediated inhibition of axon regeneration specifically depended on the NPR-19 and NPR-32 receptors (Pastuhov et al., 2016). Endocannabinoids have been shown to act as chemorepulsive axon guidance cues and regulate synaptogenesis and target selection in mammals and amphibians (Berghuis et al., 2005; Mulder

et al., 2008; Whalley, 2007), and interference with cannabinoid signaling during development has been shown to lead to neuronal miswiring in zebrafish (Zuccarini et al., 2019). This indicates that cannabinoid action as repulsive axon guidance cues is also conserved across species.

Summary and Conclusions

Though the field of cannabinoid research in *C. elegans* is young, a profound conservation of function of the endocannabinoid system between mammals and nematodes has already been highlighted. In vertebrates, the endocannabinoid system has been shown to be involved in nervous system development and wiring, nociception, motor control, and energy balance. In *C. elegans*, endocannabinoids appear to mediate all of these processes as well. Importantly, endocannabinoids signal nutrient availability in the environment, promoting feeding and cholesterol mobilization from internal reserves. In doing so, they inhibit dauer formation and signal for a normal lifespan. The next chapter (Chapter III) will further explore the endocannabinoid system's involvement in energy balance and describe our findings that activation of the endocannabinoid system of *C. elegans* leads to altered food preference, promoting intake of calorically dense food.

CHAPTER III: ENDOCANNABINOID MODULATION OF FEEDING IN C. ELEGANS

The work presented in this chapter was conducted by S. Faumont, R. Z. Berner, Z. Purcell, and myself. R. Z. Berner and Z. Purcell conducted T-Maze experiments, testing the effect of AEA on food preference in wildtype and mutant worms. S. Faumont conducted the electropharyngeogram experiments, testing the effect of AEA on food consumption. S. R. Lockery was the principal investigator for this study. I conducted all calcium imaging experiments and mapping of the NPR-19 receptor, analyzed the data, generated the figures, and wrote the first draft of the paper. The final Chapter III, including figures and text, was edited by S. Faumont, S. R. Lockery, and myself. This chapter has been submitted for publication.

Introduction

It has been known for centuries that smoking or ingesting preparations of the plant *Cannabis sativa* stimulates appetite (Abel, 1971; Kirkham & Williams, 2001). Users report persistent hunger while intoxicated, even if previously satiated. This feeling of hunger is often accompanied by a strong and specific desire for foods that are sweet or high in fat content, a phenomenon colloquially known as “the munchies” (Abel, 1975; Foltin et al., 1986, 1988; Halikas et al., 1971; Hollister, 1971; Tart, 1970). The effects of cannabinoids on appetite result mainly from Δ^9-tetrahydrobannabinol (THC), a plant-derived cannabinoid. THC acts at cannabinoid receptors in the brain where it mimics endogenous ligands called endocannabinoids, which include N-Arachidonoylethanolamine (AEA) and 2-Arachidonoylglycerol (2-AG). AEA and 2-AG are the best studied signaling molecules of the mammalian endocannabinoid system, which comprises the cannabinoid receptors CB_1 and CB_2, metabolic enzymes for synthesis and degradation of the endocannabinoids, and a variety of ancillary proteins involved in receptor

trafficking and modulation (Bauer et al., 2012; Fu et al., 2011; Jin et al., 1999; Kaczocha et al., 2009, 2012; Liedhegner et al., 2014; Martini et al., 2007; Oddi et al., 2009; Rozenfeld & Devi, 2008).

A large number of studies in laboratory animals have established a strong link between endocannabinoid signaling and energy homeostasis, defined as the precise matching of caloric intake with energy expenditure to maintain body weight (Cristino et al., 2014). Food deprivation increases endocannabinoid levels in the limbic forebrain, which includes the nucleus accumbens and hypothalamus, two brain regions that express CB_1 receptors and contribute to the appetitive drive for food (Kirkham et al., 2002). Systemic administration of THC or endogenous cannabinoids increases feeding (Williams & Kirkham, 1999). Similarly, micro-injection of cannabinoid receptor agonists or endocannabinoids directly into the nucleus accumbens also increases feeding (Deshmukh & Sharma, 2012; Mahler et al., 2007). Thus, the endocannabinoid system can be viewed as a short-latency effector system for restoring energy homeostasis under conditions of food deprivation (Cristino et al., 2014; Devane et al., 1988; Munro et al., 1993; Parker, 2017).

To respond effectively to an energy deficit, an animal should be driven both to seek food (*appetitive* behavior) and, once food is encountered, to maximize caloric intake (*consummatory* behavior). The endocannabinoid system is capable of orchestrating both aspects of this response simultaneously. With respect to appetitive behavior, CB_1 agonists reduce the latency to feed (Freedland et al., 2000; Gallate et al., 1999; Gallate & McGregor, 1999; Maccioni et al., 2008; McLaughlin et al., 2003; Salamone et al., 2007; Thornton-Jones et al., 2005) and induce animals to expend more effort to obtain a given food or liquid reward (Barbano et al., 2009; Freedland et al., 2000; Gallate et al., 1999; Guegan et al., 2013), whereas CB_1 antagonists have the opposite

effect (Freedland et al., 2000; Gallate et al., 1999; Gallate & McGregor, 1999; Maccioni et al., 2008; McLaughlin et al., 2003; Salamone et al., 2007; Thornton-Jones et al., 2005). With respect to consummatory behavior, studies in rodents show that administration of THC or endocannabinoids specifically alters food preferences in favor of palatable, calorically dense foods, such as those laden with sugars and fats, as opposed to laboratory pellets. For example, THC causes rats to consume larger quantities of chocolate cake batter without affecting consumption of simultaneously available laboratory pellets (Koch & Matthews, 2001). It also causes them to consume larger quantities of sugar water than plain water, and of dry pellets than watered-down pellet mash, which is calorically dilute (Brown et al., 1977). Administration of endocannabinoids, including microinjection into the nucleus accumbens, has similar effects, which can be blocked by simultaneous administration of CB_1 antagonists (Deshmukh & Sharma, 2012; Escartín-Pérez et al., 2009; Shinohara et al., 2009). CB_1 antagonists, administered alone, specifically suppress consumption of sweet and fatty foods in rats (Arnone et al., 1997; Gessa et al., 2006; Mathes et al., 2008) as well as in primates (Simiand et al., 1998), indicating that basal endocannabinoid titers can be regulated up or down to re-establish energy homeostasis.

There is considerable support for the hypothesis that animals treated with cannabinoids consume larger quantities of calorically dense foods because cannabinoids amplify the pleasurable or rewarding aspects of these foods. This phenomenon has been termed *hedonic amplification* (Castro & Berridge, 2017; Mahler et al., 2007), whereas the food-specific increase in consumption it engenders has been termed *hedonic feeding* (Edwards & Abizaid, 2016). Inferences concerning pleasurable and rewarding aspects of animal experience can be difficult to establish, but both THC and AEA specifically increase the vigor of licking at spouts delivering sweet fluids (Davis & Smith, 1992; Higgs et al., 2003). In a more direct measure of hedonic

responses, the frequency of orofacial movements previously shown to be associated with highly preferred foods can be monitored in response to oral delivery of a sucrose solution (Grill & Norgren, 1978). Injection of THC or a CB_1 antagonist respectively increases or decreases this frequency (Jarrett et al., 2005), suggesting that pleasure may have been increased by cannabinoid administration.

Cannabinoid effects on hedonic responses may be at least partially chemosensory in origin, including both taste (gustation) and smell (olfaction). With respect to gustation, a majority of sweet-sensitive taste cells in the mouse tongue are immunoreactive to CB_1, and a similar proportion shows increased response to saccharin, sucrose, and glucose following endocannabinoid administration (Yoshida et al., 2010, 2013). These effects are recapitulated in afferent nerves from the tongue (Yoshida et al., 2010), as administration of AEA or 2-AG specifically increases chorda tympani responses to sweeteners rather than NaCl (salt), HCl (sour), quinine (bitter), or monosodium glutamate (umami). With respect to olfaction, CB_1 receptors expressed in the olfactory bulb are required for post-fasting hyperphagia in mice, and THC decreases the threshold of food-odor detection during exploratory behavior (Soria-Gómez et al., 2014).

The high degree of conservation of the endocannabinoid system at the molecular level is well established (Elphick, 2012). Although CB_1 and CB_2 receptors are unique to chordates, there are numerous candidates for cannabinoid receptors in most animals. Furthermore, orthologs of the enzymes involved in biosynthesis and degradation of endocannabinoids occur throughout the animal kingdom. This degree of molecular conservation, coupled with the universal need in all organisms to regulate energy balance, suggests the hypothesis that hedonic amplification and

hedonic feeding are also widely conserved, but studies in animals other than rodents and primates appear to be lacking.

The present study tests the hypothesis that the hedonic effects of cannabinoids are conserved in the nematode *C. elegans*. This organism diverged from the line leading to mammals more than 500 million years ago (Raible & Arendt, 2004). Nevertheless, *C. elegans* has a fully elaborated endocannabinoid signaling system including: (i) a functionally validated endocannabinoid receptor NPR-19, which is encoded by the gene *npr-19* (Oakes et al., 2017); (ii) the endocannabinoids AEA and 2-AG, which it shares with mammals (Higgs et al., 2003; Lehtonen et al., 2008, 2011; Sugiura et al., 1995), (iii) orthologs of the mammalian endocannabinoid synthesis enzymes NAPE-PLD, and DAGL (Harrison et al., 2014), and (iv) orthologs of endocannabinoid degradative enzymes FAAH and MAGL (Y97E10AL.2 in worms) (Oakes et al., 2017). Endocannabinoid signaling in *C. elegans* is so far known to contribute to six main phenotypes: (i) axon navigation during regeneration (Pastuhov et al., 2012, 2016), (ii) lifespan regulation related to dietary restriction (Harrison et al., 2014; Lucanic et al., 2011) (iii) altered progression through developmental stages (Harrison et al., 2014; Reis-Rodrigues et al., 2016), (iv) suppression of nociceptive withdrawal responses (Oakes et al., 2017), (v) inhibition of feeding rate (Oakes et al., 2017), and (vi) inhibition of locomotion (Oakes et al., 2017, 2019). Despite considerable conservation between the *C. elegans* and mammalian endocannabinoid systems, to our knowledge the effects of cannabinoids on food preference in *C. elegans* have not been described.

The feeding ecology of *C. elegans* supports the possibility of hedonic feeding in this organism. *C. elegans* feeds on bacteria in decaying plant matter (Frézal & Félix, 2015). It finds bacteria by chemotaxis driven by a combination of gustatory and olfactory cues (Bargmann et

al., 1993; Bargmann & Horvitz, 1991). Bacteria are ingested through the worm's pharynx, a rhythmically active muscular pump that constitutes the animal's throat. Although *C. elegans* is an omnivorous bacterivore, different species of bacteria have a characteristic quality as a food source defined by the rate of growth of individual worms feeding on that species (Δ length/unit time). Hatchlings are naïve to food quality but in a matter of hours begin to exhibit a preference for nutritionally superior species (henceforth *favored*) over nutritionally inferior species (henceforth *non-favored*) (Shtonda, 2006).

Here we show that transient exposure of *C. elegans* to the endocannabinoid AEA simultaneously biases appetitive and consummatory responses toward favored food. With respect to appetitive responses, the fraction of worms approaching and dwelling on patches of favored food increases whereas the fraction approaching and dwelling on non-favored food decreases. With respect to consummatory responses, feeding rate in favored food increases whereas feeding rate in non-favored food decreases. Taken together, the appetite and consummatory manifestations of cannabinoid exposure in *C. elegans* imply increased consumption of favored food characteristic of hedonic feeding. We also find that AEA's effects require the NPR-19 cannabinoid receptor. Further, AEA's effects persist when *npr-19* is replaced by the human CB_1 receptor gene CNR1, indicating a high degree of conservation between the nematode and mammalian endocannabinoid systems. At the neuronal level, we find that under the influence of AEA, AWC, a primary olfactory neuron required for chemotaxis to food, becomes more sensitive to favored food and less sensitive to non-favored food. Together, our findings indicate that the hedonic effects of endocannabinoids are conserved in *C. elegans*.

Results

AEA Exposure Increases Preference for Favored Food

We pre-exposed well-fed, adult, wild type (N2 Bristol) worms to the endocannabinoid AEA by incubating them for 20 min at a concentration of 100 µM. Food preference was measured by placing a small population of worms at the starting point of a T-maze baited with patches of favored and non-favored bacteria at equal optical densities (OD_{600} 1), where optical density served as a proxy for bacteria concentration (see Materials and Methods; Fig. 1A). This assay is analogous to assays used in mammalian studies in which both palatable and standard food options are simultaneously available (Brown et al., 1977; Deshmukh & Sharma, 2012; Escartín-Pérez et al., 2009; J E Koch & Matthews, 2001; Shinohara et al., 2009). The number of worms in each food patch was counted at 15-minute intervals for one hour. At each time point, we quantified preference in terms of the index $I = (n_{\mathrm{F}} - n_{\mathrm{NF}})/(n_{\mathrm{F}} + n_{\mathrm{NF}})$, where n_{F} and n_{NF} are the number of worms in favored and non-favored food, respectively, and $I = 0$ indicates indifference between the two food types. We found that AEA exposure increased preference for favored food (Fig. 1B, C; Suppl. Table 1, line 2). This effect lasted at least 60 minutes without significant decrement (Fig. 1B; Suppl. Table 1, line 3-4) despite the absence of AEA on the assay plates. Thus, the amount of AEA absorbed by worms during the exposure period was sufficient to maintain the increased preference for favored food throughout the observation period.

A simple interpretation of the data in Fig. 1B, C is that AEA exposure specifically increases the relative attractiveness of favored food. However, an alternative interpretation is that AEA promotes the attractiveness of whichever food is already preferred under the baseline conditions of the experiment (AEA–). To test this possibility, we titrated the densities of favored

and non-favored food so that under baseline conditions neither food was preferred ($I \approx 0$; Fig. 1D, E; Suppl. Fig. 1A, B). Under these conditions, AEA still increased the preference for favored food (Suppl. Table 1, line 6, 10). This finding supports the hypothesis that AEA differentially affects accumulation based on food identity, not relative food density. In a similar experiment in which baseline preference was titrated approximately to zero, we tested the effect of AEA on preference for a different pair of favored and non-favored bacteria (Fig. 1F; Suppl. Fig. 1C). Once again, AEA caused a shift in the positive direction, indicating increased preference for favored food relative to baseline (Suppl. Table 1, line 14). We conclude that AEA's effect on preference is not specific to a particular pair of favored and non-favored bacteria. Taken together, the data in Fig. 1B-F show that AEA's ability to increase preference for favored food is not limited to a particular pair of foods or their relative concentrations.

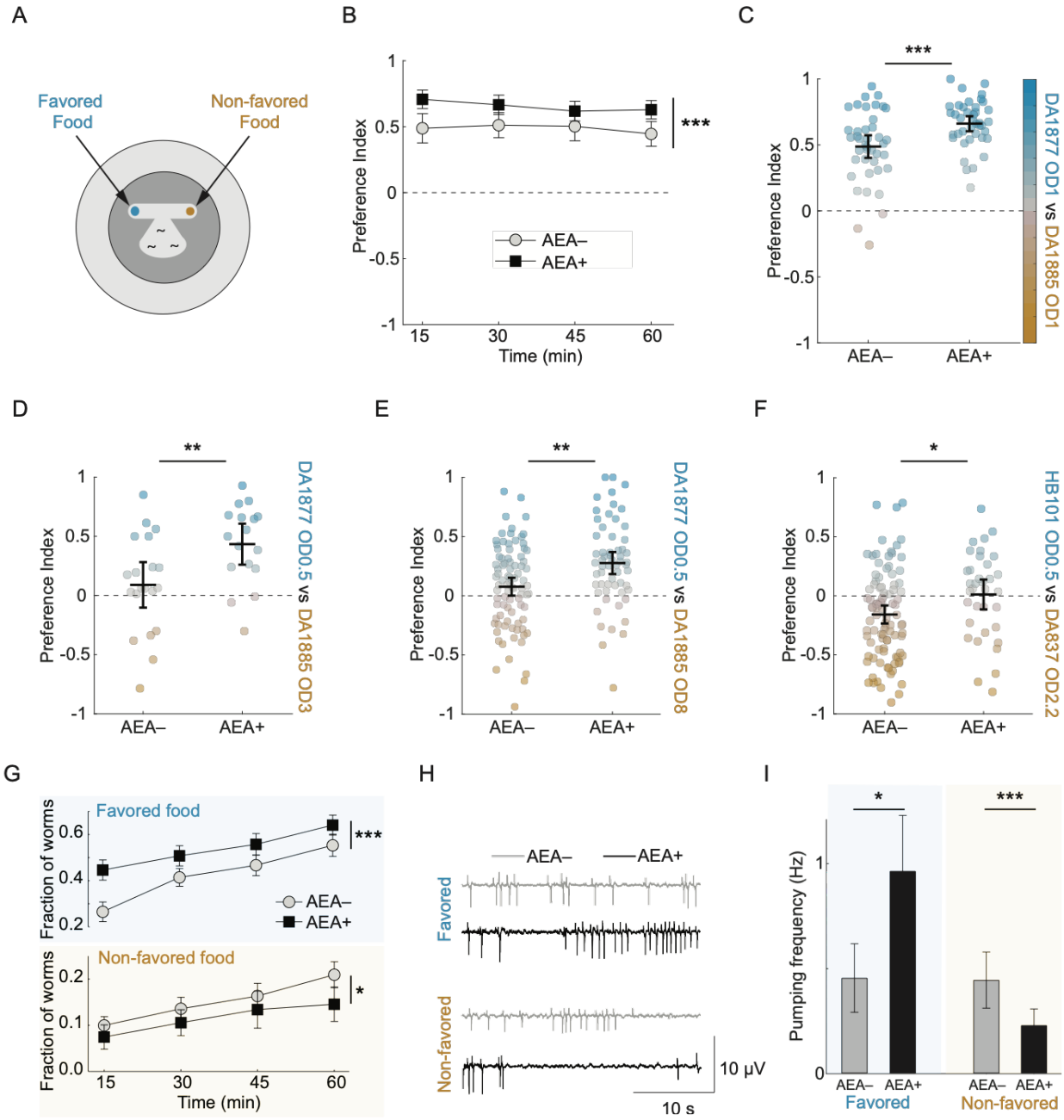


Fig 1. AEA-mediated hedonic feeding. A. Food preference assay. T-maze arms were baited with patches of favored (blue) and non-favored (orange) bacteria. **B**. Mean preference index (*I*) versus time for AEA-exposed animals (AEA+) and unexposed controls (AEA–), where $I > 0$ is preference for favored food, $I < 0$ is preference for non-favored food, and $I = 0$ is indifference (*dashed line*). Favored food, DA1877, OD 1; non-favored food, DA1885, OD 1. **C**. Summary of the data in **B**. Each dot is mean preference over time in a single T-maze assay. *Dot color* indicates preference index according to the color scale on the right. **D**, **E**. Effect of AEA on preference when baseline preference is at the indifference point (symbols as in **C**). For preference time courses, see Supp. Fig. 1. In **D**: Favored food, DA1877, OD 0.5; non-favored food, DA1885, OD 3. In **E**: favored food, DA1877, OD 0.5; non-favored food, DA1885, OD 8. **F**. Effect of AEA on preference for a different pair of favored and non-favored bacteria (symbols as in **C**). Favored food, HB101, OD 0.5; non-favored food, DA837, OD 2.2. For preference time course, see Supp. Fig. 1. **G**. Effect of AEA on fraction of worms in favored and non-favored food patches versus time. Same experiment as in panels **B**, **C**. **H**, **I**.

Effect of AEA on pharyngeal pumping in favored versus non-favored food. Favored food, DA1877, OD 0.8; non-favored food, DA1885, OD 0.8. **H** shows electrical recordings of four individual worms under the conditions shown. Each spike is the electrical correlate of one pump. Traces were selected to represent the population median pumping frequency in each condition. **I** shows mean pumping frequency in each condition. For statistics in **B-G** and **I,** see Supp. Table 1. Symbols: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., not significant. Error bars, 95% confidence interval.

In mammals, cannabinoid administration can differentially increase responses to favored versus non-favored food. Because worms in the T-maze assay could occupy foodless regions of the assay plate in addition to the food patches themselves, the increased accumulation in favored food could represent an increased appetitive response to favored food, a decreased appetitive response to non-favored food, or both. Further analysis revealed that AEA exposure increased the fraction of worms in favored food and decreased the fraction in non-favored food (Fig. 1G; Suppl. Table 1, line 18, 22). Thus, AEA exposure produces a bidirectional effect on appetitive responses to favored versus non-favored food, the net result of which is increased accumulation in favored food.

Are these food-specific appetitive responses accompanied by food-specific changes in consumption behavior? *C. elegans* swallows bacteria by means of rhythmic contractions of its pharynx, a muscular organ comprising its throat; each contraction is called a pump. We recorded pumping electrically in individual worms restrained in a microfluidic channel with integrated electrodes (Lockery et al., 2012; David M. Raizen & Avery, 1994). The channel contained either favored or non-favored food and pumping was recorded for 1 min following a 3 min accommodation period. Under these conditions, pumping rate is a reasonable proxy for the amount of food consumed because food concentration at this optical density is effectively constant. Unexposed worms pumped at equal frequencies in the presence of favored and non-favored species (Fig. 1H, I; Suppl. Table 1, line 25). However, under the influence of AEA, pumping frequency in favored food increased whereas pumping frequency in non-favored food

decreased (Fig. 1H, I; Suppl. Table 1, line 26-27). Thus, the effects of AEA exposure on food consumption mirror its bidirectional effects on accumulation shown in Fig. 1G.

Taken together, the results in Fig. 1 demonstrate clear similarities between the effects of cannabinoids on feeding behavior in nematodes and mammals in two key respects. First, AEA differentially alters *appetitive* responses to favored and non-favored food, causing more worms to accumulate in the former and fewer in the latter. Second, AEA differentially alters *consummatory* responses measured in terms of feeding rate, causing individual worms to consume more favored food and less non-favored food per unit time. The appetitive and consummatory effects of AEA, acting in concert, are consistent with a selective increase in consumption of favored food, which is phenomenologically analogous to hedonic feeding in mammals (Edwards & Abizaid, 2016).

AEA Differentially Modulates Chemosensory Responses to Favored and Non-Favored Food

In theoretical terms, accumulation in a food patch is determined by just two factors: entry rate and exit rate. Previous studies in *C. elegans* have shown that both rates can contribute to differential accumulation in one food versus another (Shtonda, 2006). Thus, AEA could modulate appetitive responses by acting on entry, exit rate, or both. Chemotaxis toward food patches is driven by olfactory neurons responding to airborne cues encountered at a distance (Bargmann et al., 1993; Bargmann & Horvitz, 1991). Thus, changes in entry rate might implicate changes in the function of olfactory neurons. A simple but powerful way to examine the contribution of entry rate is to spike food patches with a paralytic agent so worms that enter a patch cannot leave, thereby setting exit rate to zero. Under these conditions, if AEA exposure still modulates relative preference for favored versus non-favored food, then AEA must be differentially altering the entry rate into the two foods. To test this, we added sodium azide, a

paralytic agent commonly used to immobilize nematodes (Hart, 2006), to both food patches in the T-maze. We found that AEA still produced a marked increase in preference for favored food (Fig. 2A; Suppl. Table 2, line 2), showing that it differentially affects patch entry rates.

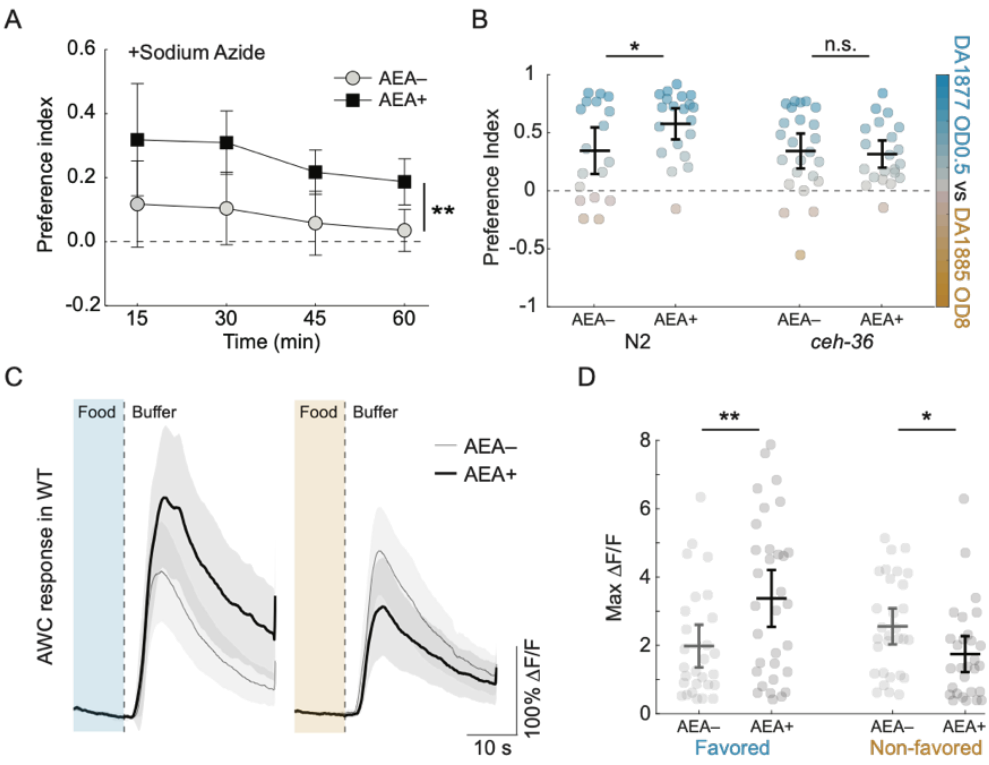


Fig 2. Chemosensory correlate of hedonic feeding. **A**. Mean preference index (*I*) versus time for AEA-exposed animals (AEA+) and unexposed controls (AEA–) when sodium azide was added to food patches. Favored food, DA1877, OD 0.5; non-favored food, DA1885, OD 3. **B**. Effect of AEA on preference in wild type (N2) and *ceh-36* mutants. Favored food, DA1877, OD 0.5; non-favored DA1885, OD 8. Each dot is mean preference in a single T-maze assay. **C**. Effect of AEA on the response of AWC neurons to the removal of favored or non-favored food. Each trace is average normalized fluorescence change ($\Delta F/F$) versus time. Favored food (blue), DA1877, OD 1; non-favored food (orange), DA1885, OD 1. **D**. Summary of the data in in C, showing mean peak $\Delta F/F$. For statistics in **A**-**D**, see Supp. Table 2. Symbols: *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. Error bars and shading, 95% confidence interval.

Having found that AEA alters food-patch entry rates, we next considered the possibility that AEA acts on olfactory neurons to produce the appetitive component of hedonic feeding. *C. elegans* senses food or food-related compounds by means of 11 classes of chemosensory neurons (two neurons/class), which have sensory endings in the anterior sensilla near the mouth (Bargmann et al., 1993; Zaslaver et al., 2015). We focused on the AWC class, a pair of olfactory neurons that responds directly to many volatile odors (Leinwand et al., 2015) and is required for

chemotaxis to them (Bargmann et al., 1993). To investigate whether AEA acts on AWC to alter food preference, we measured AEA's effect on preference in *ceh-36* mutants, in which AWC function is selectively impaired. This gene is expressed only in AWC and the gustatory neuron class ASE. *ceh-36* is required for normal expression levels of genes essential for chemosensory transduction, particularly in AWC (Koga & Ohshima, 2004; Lanjuin et al., 2003). Accordingly, *ceh-36* mutants are strongly defective in their chemotaxis responses to three food-related odorants that directly activate AWC (Lanjuin et al., 2003). Although ASE neurons are required for chemotaxis to at least one AWC-sensed odorant (Leinwand et al., 2015), they do not respond directly to these compounds; rather, they inherit their response via peptidergic signaling from AWC. Thus, loss of appetitive responses in *ceh-36* mutants can be attributed to AWC neurons.

In T-maze assays, we found a modest strain × AEA interaction ($p = 0.08$), and a significant effect of AEA in wild type animals which was absent in the mutants (Fig. 2B; Suppl. Fig. 2A, B; Suppl. Table 2, line 6, 10-11, 13). This finding indicates that AWC is required for the appetitive component of hedonic feeding. With respect to the consummatory component, whereas AEA exposure had no effect on pumping frequency of *ceh-36* null worms in non-favored food, it still increased pumping frequency in favored food, just as it did in wild type worms (Suppl. Fig. 3, Suppl. Table 5, line 1-2), indicating that *ceh-36* is partially required for the consummatory component of hedonic feeding. Taken together, these data suggest that AWC is required for the normal magnitude of both components of hedonic feeding.

AWC is activated by *decreases* in the concentration of food or food-related odors (Calhoun et al., 2015; Chalasani et al., 2007; Zaslaver et al., 2015). AWC can nevertheless promote *attraction* to food patches because its activation truncates locomotory head bends away from the odor source, thereby steering the animal toward the odor source. Additionally, its

activation causes the animal to stop moving forward, reverse, and resume locomotion in a new direction better aligned with the source; this behavioral motif is known as a pirouette (Pierce-Shimomura et al., 1999). To test whether AEA alters AWC sensitivity to favored and non-favored food, we compared AWC calcium transients in response to the removal of either type of food in wild type worms exposed to AEA, and in unexposed controls. In unexposed animals, AWC neurons responded equally to the removal of either food (Fig. 2C, D, Suppl. Table 2, line 21). However, exposure to AEA caused a dramatic change in food sensitivity, increasing AWC's response to the removal of favored food and decreasing its response to the removal of non-favored food (Fig. 2C, D, Suppl. Table 2, line 17, 19-20, 22). This bidirectional effect mirrors AEA's effect on both the appetitive and consummatory aspects of hedonic feeding (Fig. 1G, I) and is consistent with a model in which hedonic feeding is triggered at least in part by modulation of chemosensation in AWC neurons.

Dissection of Signaling Pathways Required for Hedonic Feeding

The NPR-19 receptor has been shown to be required for AEA-mediated suppression of withdrawal responses and feeding rate (Oakes et al., 2017). To test whether *npr-19* is required for hedonic feeding, we measured food preference in *npr-19* null mutants following exposure to AEA. Mutant worms failed to exhibit increased preference for favored food (Fig. 3A; Suppl. Fig. 2C, D; Suppl. Table 3, line 6-7). This defect was rescued by over-expressing *npr-19* under control of the native *npr-19* promoter (Fig. 3A; Suppl. Fig. 2C, E; Suppl. Table 3, line 11-12, 15-16, 18). We conclude that *npr-19* is required for the appetitive component of hedonic feeding. This defect was also rescued by over-expressing the human cannabinoid receptor CB_1 under the same promoter (Fig. 3A; Suppl. Fig. 2F; Suppl. Table 3, line 20-21, 24-25, 27). This finding

indicates a remarkable degree of conservation between the nematode and human endocannabinoid systems. With respect to the consummatory component of hedonic feeding, the role of *npr-19* was unclear: *npr-19* mutants worms exhibited only a partial phenotype which was not rescued by overexpression of either *npr-19* or CNR1 (Suppl. Fig. 3), despite evidence of rescue in a previous study (Oakes et al., 2017). Significant differences in experimental approach might explain this discrepancy (see Materials and Methods).

The forgoing results suggest a model of hedonic feeding in *C. elegans* in which activation of the NPR-19 receptor by AEA triggers a bidirectional change in AWC's food sensitivity (Fig. 2C, D) to induce the appetitive component of hedonic feeding. We therefore tested whether *npr-19* is required for AEA's effects on AWC. The effect of AEA on AWC's response to food was abolished in *npr-19* mutants (Fig. 3B, C, Suppl. Table 3, line 30, 33-34, 39, 42-43). This phenotype was partially rescued by over-expression of the human cannabinoid receptor CB_1 (Suppl. Fig. 4A, B, Suppl. Table 5, line 12, 15, 18, 22, 24). We conclude that the appetitive component of AEA-induced hedonic feeding requires both the NPR-19 receptor and AWC neurons.

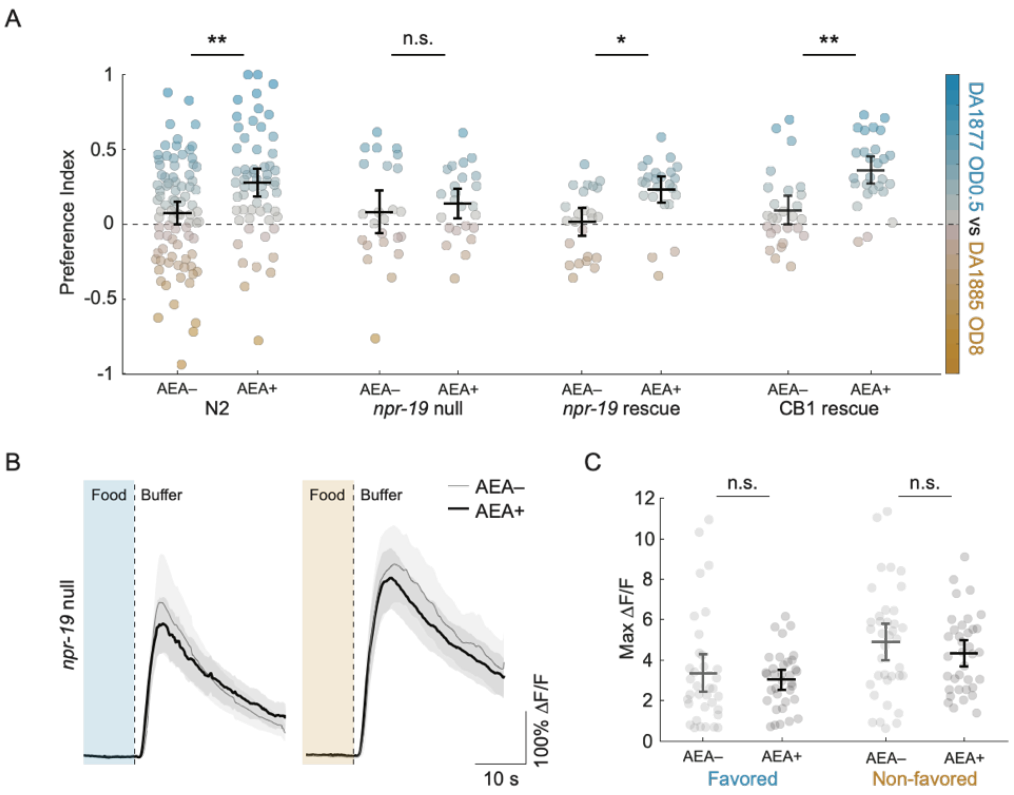


Fig 3. Requirement of NPR-19 for hedonic feeding and chemosensory modulation. A. Effect of AEA on preference in wild type worms (N2) and the indicated genetic background. Favored food, DA1877, OD 0.5; non-favored food, DA1885, OD 8. Each dot is mean preference over time in a single T-maze assay. *Dot color* indicates preference index according to the color scale on the right. **B**. Effect of AEA on the response of AWC neurons to the removal of favored or non-favored food in *npr-19* mutants. Each trace is average normalized fluorescence change ($\Delta F/F$) versus time. Favored food (blue), DA1877, OD 1; non-favored food (orange), DA1885, OD 1. **C**. Summary of the data in **B**, showing mean peak $\Delta F/F$. For statistics in **A**-**C**, see Supp. Table 3. Symbols: *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. Error bars and shading, 95% confidence interval.

In perhaps the simplest model of AEA's effect on AWC, NPR-19 is expressed in AWC, and activation of NPR-19 produces the observed bidirectional modulation of sensitivity to favored and non-favored food. To test this model, we characterized the *npr-19* expression pattern. This was done by expressing a p*npr-19*::GFP transgene together with either p*cho-1*::mCherry or p*eat-4*::mCherry, two neuronal markers whose expression pattern has been thoroughly characterized (Pereira et al., 2015; Serrano-Saiz et al., 2013). We observed expression of *npr-19* in body wall muscles together with an average of 29 neuronal somata in the head and 8 in the tail (Fig. 4A, Suppl. Table 6). Using positional cues in addition to the markers, we identified 28 of the GFP-positive somata, which fell into 15 neuron classes (Table 1). These

classes could be organized into four functional groups: sensory neurons (URX, ASG, AWA, and PHC), interneurons (RIA, RIM, and LUA), motor neurons (URA and PDA), and pharyngeal neurons (M1, M3, MI, MC, I2, and I4). Although AWC could be identified in every worm by its characteristic position in the p*eat-4*::mCherry expressing strain, GFP expression was never observed in this neuron class. Our expression data, together with the absence of significant *npr-19* expression in AWC in RNA sequencing experiments based on the *C. elegans* Neuronal Gene Expression Map & Network (CeNGEN) consortium (Hammarlund et al., 2018), suggests that AWC does not express *npr-19*. These findings are inconsistent with a direct action of AEA on AWC neurons mediated by the NPR-19 receptor.

The *npr-19* expression pattern supports at least two indirect models of AEA's effect on AWC. In one model, AWC inherits its sensitivity to AEA from AEA-sensitive synaptic pathways that involve classical neurotransmitters. In the other model, AWC inherits its sensitivity from signaling pathways that rely on neuromodulators. Results presented below suggest that the effect of AEA on AWC depends on neuromodulators rather than classical neurotransmitters.

Function	Identity of *npr-19*::GFP+ neurons	*eat-4*::mCherry expression	*cho-1*::mCherry expression	Cell body position and morphology	CenGen *npr-19* expression	Transmitters	*unc-31 expression*
Pharyngeal	M3 L/R	*				Glu, FLP-18, NLP-3	
	MI	*			*	Glu	*
	MC L/R			*	*	Ach, FLP-21	*
	I2 L/R	*				Glu, NLP-3, NLP-8	*
	I4			*	*	NLP-3, NLP-13	*
	M1			*	*	Ach, NLP-3	*
Sensory	PHC L/R	*			*	Glu	
	URX L/R		*		*	Ach, FLP-8, FLP-10, FLP-11, FLP-19	*
	ASG L/R	*			*	Glu, 5HT, FLP-6, FLP-13, FLP-22, INS-1	*
	AWA L/R			*	*	INS-1	*
Interneuron	RIA L/R	*				Glu	*
	RIM L/R	*			*	Glu, Tyr	
	LUA L/R	*			*	Glu, NLP-13, PDF-1	
Motor	URA D/V L/R		*		*	ACh	*
	PDA		*		*	ACh	*

Table 1. *npr-19*-expressing neurons. The *npr-19* expression pattern was characterized by expressing a p*npr-19*::GFP transgene together with either p*cho-1*::mCherry or p*eat-4*::mCherry, respectively labeling previously identified cholinergic and glutamatergic neurons (Pereira et al., 2015; Serrano-Saiz et al., 2013). GFP-positive neurons that expressed neither of the markers were identified by position and morphology, and confirmed by cross-reference to CeNGEN expression data showing *npr-19*. Also shown are neurotransmitter identity (Loer and Rand, 2016; Altun, 2011) and *unc-31* expression (CeNGEN) of each identified neuron class. See also Supp. Table 6.

Synaptic pathways utilizing classical neurotransmitters can be sensitive to cannabinoids in a variety of ways. In one common endocannabinoid signaling motif, endocannabinoids act as retrograde signals released by a postsynaptic neuron to suppress neurotransmitter release by binding to cannabinoid receptors on presynaptic terminals. This motif could render AWC-related synaptic pathways sensitive to AEA. To determine whether this motif may be present in *C. elegans*, we searched the *C. elegans* connectome for the anatomical substrate of retrograde signaling: synaptically coupled pairs of neurons in which the *presynaptic* neuron expressed *npr-19* and the *postsynaptic* neuron expressed genes that encode key synthesis enzymes for AEA, in particular, *nape-1* and *nape-2*, the *C. elegans* orthologs of the mammalian gene NAPE-PLD. The

set of presynaptic, *npr-19*-expressing neurons was limited to the six non-pharyngeal neuron classes in the head, where AWC is located (ASG, AWA, RIA, RIM, URA, URX). We found that these six classes are presynaptic to 42 different *nape-1*,*2*-expressing neurons. Approximately half of these neurons receive synaptic input from more than one *npr-19* expressing neuron such that there are 74 coupled pairs fitting the necessary (but not sufficient) anatomical and gene-expression criteria for retrograde AEA signaling. In 14 of these coupled pairs, the postsynaptic neuron is directly presynaptic to AWC, opening the possibility that AWC inherits its AEA sensitivity synaptically.

To test whether classical synaptic pathways render AWC sensitive to AEA, we imaged AWC activity in worms with a null mutation in *unc-13*, the *C. elegans* homolog of Munc13, which is required for exocytosis of the clear-core synaptic vesicles that contain classical neurotransmitters (Richmond et al., 1999). We found that AEA's effect on food sensitivity in *unc-13* mutants was essentially the same as in wild type worms (Fig. 4B, C; Suppl. Table 4, line 3, 6-7, 9, 13, 15-16, 18). This result makes it unlikely that AWC inherits its AEA sensitivity from synaptic pathways that involve classical neurotransmitters.

We next tested a model in which AEA causes the release of neuromodulators that act on AWC. Most neuromodulatory substances, such as neuropeptides and biogenic amines, are released by exocytosis of dense-core vesicles (Devine & Simpson, 1968; Probert et al., 1983). In mammals, presynaptic terminals that both contain dense-core vesicles and are immunoreactive for the cannabinoid receptor CB_1 are a recurring synaptic motif in several brain regions including the CA1 and CA3 of the hippocampus, prefrontal cortex, and basolateral amygdala (Fitzgerald et al., 2019; Takács et al., 2014). To determine whether this motif may be present in *C. elegans*, we used gene expression data (Hammarlund et al., 2018) to search for *npr-19*-expressing neurons

that also express *unc-31*, the *C. elegans* ortholog of human CADPS/CAPS, which is required for calcium-regulated dense-core vesicle fusion (Speese et al., 2007). We found that most of the *npr-19*-expressing neurons identified in our study (11 out of 15, Table 1) also express *unc-31*. This result indicates that the anatomical substrate for cannabinoid-mediated release of neuromodulators exists in *C. elegans*.

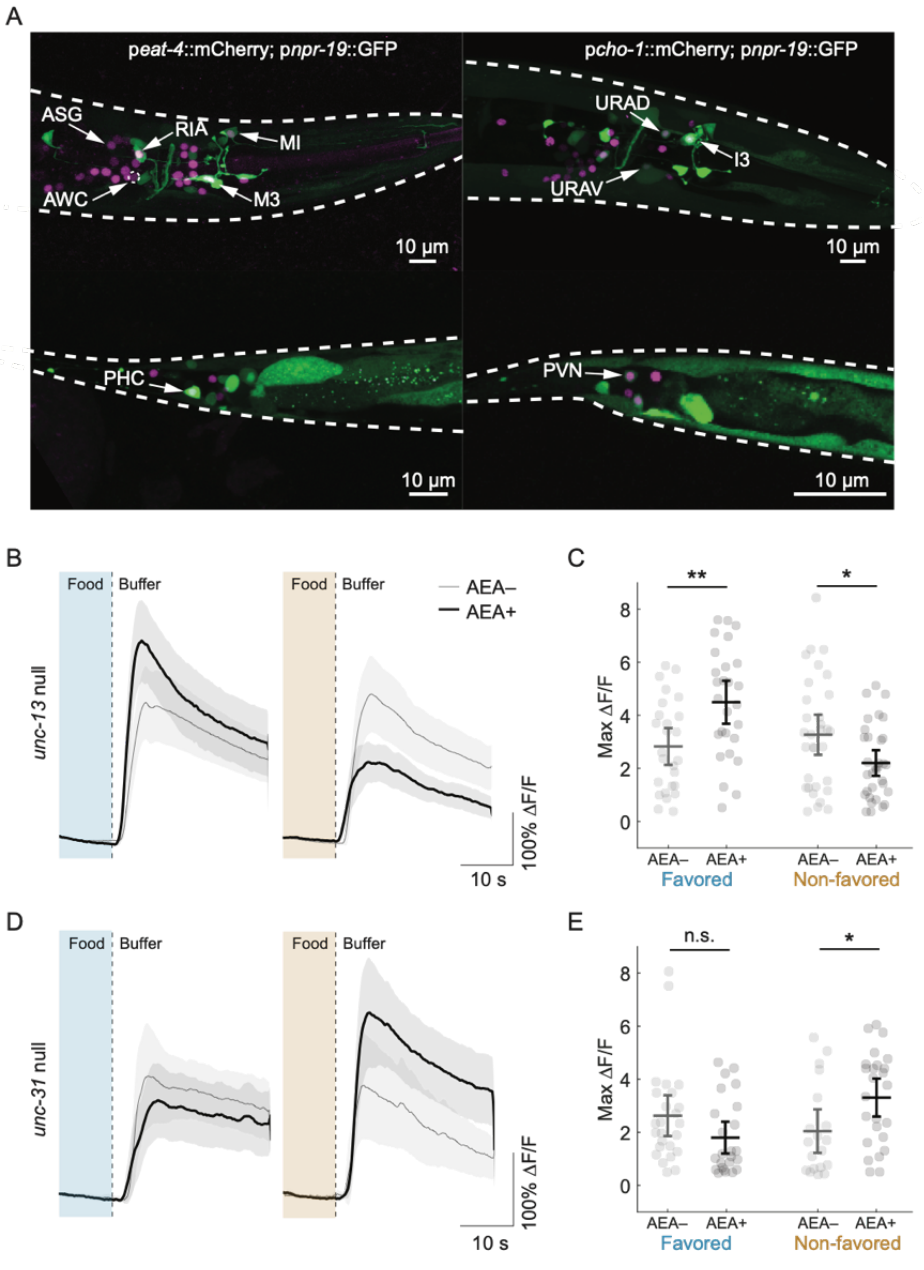


Fig 4. Genetic pathways underlying AEA-mediated AWC modulation. **A**. Expression pattern of *npr-19*. Green cells express *npr-19*. *Left*, magenta indicates expression of *eat-4*, a marker for glutamatergic neurons. *Dashed circle*, the soma of AWC, which is glutamatergic. *Right*, magenta indicates expression of *cho-1*, a marker for cholinergic neurons. *Top*, *bottom*, head and tail expression, respectively. **B**. Effect of AEA on the response of AWC neurons to the removal of favored or non-favored food in *unc-13* mutants. Each trace is average normalized fluorescence change ($\Delta F/F$) versus time. Favored food (blue), DA1877, OD 1; non-favored food (orange), DA1885, OD 1. **C**. Summary of the data in **B**, showing mean peak $\Delta F/F$. **D**. Effect of AEA on the response of AWC neurons to the removal of favored or non-favored food in *unc-31* mutants. Each trace is average normalized fluorescence change ($\Delta F/F$) versus time. Favored food (blue), DA1877, OD 1; non-favored food (orange), DA1885, OD 1. **E**. Summary of the data in **D**, showing mean peak $\Delta F/F$. For statistics in **B**-**E**, see Supp. Table 4. Symbols: *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. Error bars and shading, 95% confidence interval.

To test this version of the indirect model, we recorded from AWC in an *unc-31* deletion mutant. If AEA's effect on AWC were solely the result of neuromodulation mediated by *unc-31*, one would expect this mutation to phenocopy *npr-19* null: exhibiting no AEA effects on AWC responses. This appeared to be the case for the response to favored food, in which there was no effect of AEA (Fig. 4D, E; Suppl. Table 4, line 21, 24-25, 27). AWC responses to non-favored food were still modulated by AEA (Fig. 4D, E; Suppl. Table 4, line 31, 33, 36), but they were increased rather than decreased. The fact that AEA's modulation of AWC food sensitivity is severely disrupted in *unc-31* mutants supports a model in which NPR-19 receptors activated by AEA promote the release of dense-core vesicles containing modulatory substances that act on AWC.

Discussion

In mammals, administration of THC or endocannabinoids induces hedonic feeding, meaning an increase in consumption of calorically dense, palatable foods. The present study provides two converging lines of evidence in support of the hypothesis that cannabinoids induce hedonic feeding in *C. elegans*. First, AEA can differentially alter accumulation in favored and non-favored food, causing a larger proportion of worms to accumulate in the former and a smaller proportion in the latter (Fig. 1G). Individual worms tend to exit, explore, and re-enter food patches multiple times over the time scale of our experiments (Shtonda, 2006). Thus, these proportions are mathematically equivalent to the average fraction of time that an individual worm spends feeding on each type of food. Furthermore, worms given an inexhaustible supply of food, feed at a constant rate for at least six hours (Izquierdo et al., 2021), far longer than

observation times in this study. Combining these two observations, we can infer that for *C. elegans*, differential accumulation results in differential consumption. Second, AEA differentially alters feeding rate, causing worms to feed at a higher rate in preferred food and a lower rate in non-preferred food (Fig. 1I). Thus, the effect of AEA on feeding rate amplifies its effect on fraction of time feeding in favored and non-favored food patches. The result of this amplification is increased consumption of favored food in a manner consistent with hedonic feeding. We conclude that hedonic feeding is conserved in *C. elegans*.

Our findings confirm and extend previous investigations concerning the role of the endocannabinoid system in regulating feeding in *C. elegans*. The endocannabinoids AEA and 2-AG were previously shown to reduce pumping frequency in animals feeding on nutritionally inferior food (Oakes et al., 2017). We now show that this reduction is part of a broader pattern in which pumping rate on superior food increases and pumping on inferior food decreases. Additionally, we have confirmed that *npr-19* is expressed in a limited number of neurons including the inhibitory pharyngeal motor neuron M3 and the sensory neuron URX. We extend these results by identification of 13 additional *npr-19* expressing neurons including sensory neurons, interneurons, and motor neurons. Of particular interest is the detection of *npr-19* expression in five additional pharyngeal neurons. Thus, 6 of the 20 neurons comprising the pharyngeal nervous system are potential sites for endocannabinoid mediated regulation of pumping rate. It is notable that these six neurons include the motor neuron MC, which is hypothesized to act as the pacemaker neuron for rhythmic pharyngeal contractions (Avery & Horvitzt, 1989; Raizen et al., 1995), and M3, which regulates pump duration (Avery, 1993). It will now be important to tackle the question of how pumping rate is modulated in different directions for favored and non-favored foods.

To date, only a small number of studies have examined the effects of cannabinoids on feeding and food preference in invertebrates. Early in evolution, the predominant effect may have been feeding inhibition. Cannabinoid exposure shortens bouts of feeding in Hydra (De Petrocellis et al., 1999a). Larvae of the tobacco hornworm moth *Manduca Sexta* prefer to eat leaves containing lower rather than higher concentrations of the phytocannabinoid cannabidiol (Park et al., 2019). In adult fruit flies (*Drosophila melanogaster*), pre-exposure to phyto- or endocannabinoids (AEA and 2-AG) for several days before testing reduces consumption of standard food. On the other hand, in side-by-side tests of sugar-yeast solutions with and without added phyto- or endocannabinoids, adult fruit flies prefer the cannabinoid-spiked option. The picture that emerges from these studies is that whereas the original response to cannabinoids may have been feeding suppression, through evolution the opposite effect arose, sometimes in the same organism. As we have shown, *C. elegans* exhibits both increases and decreases in feeding responses under the influence of cannabinoids and does so in a manner that would seem to improve the efficiency of energy homeostasis by promoting consumption of nutritionally superior food and depressing consumption of nutritionally inferior food. At present there is no evidence in mammals for bidirectional modulation of consumption, but our results, together with the logic of homeostasis, predict that such an effect may exist under certain conditions.

Although administration of cannabinoids causes hedonic feeding in *C. elegans* and mammals, there are notable differences in how it is expressed. One experimental design commonly used in mammalian studies is to measure consumption of a single test food, which is either standard lab food or a more palatable food. In such experiments, consumption of both food types is increased (Williams et al., 1998b; Williams & Kirkham, 1999). The analogous experiment in the present study is the experiment of Fig. 1I, in which consumption (inferred

from pumping rate) was measured in response to either favored or non-favored food. We found that consumption of favored food increases as in mammalian studies whereas, in contrast, consumption of non-favored food decreases. A second experimental design commonly used in mammalian studies is to measure consumption of standard and palatable foods when the two foods are presented together. In this type of experiment, cannabinoids increase consumption of palatable food, but consumption of standard food is unchanged (Brown et al., 1977; Deshmukh & Sharma, 2012; Escartín-Pérez et al., 2009; Koch & Matthews, 2001; Shinohara et al., 2009). Cannabinoid receptor antagonists produce the complementary effect: reduced consumption of palatable food with little or no change in consumption of standard food. The analogous experiments in the present study are the T-maze assays in which maze arms are baited with favored and non-favored food. We find that following cannabinoid administration, consumption of favor food increases whereas consumption of non-favored food decreases.

Thus, considering both experimental designs, the effects of cannabinoid exposure on consumption in *C. elegans* are bidirectional, whereas in mammals they are not. It is conceivable that a bidirectional response is advantageous in that it produces a stronger bias in favor of superior food than a unidirectional response, raising the question of why bidirectional responses have not been reported in mammals. There are, of course, considerable differences in the feeding ecology of nematodes and mammals; perhaps mammals evolved under a different set of constraints under which unidirectional responses are the better strategy. On the other hand, differences in experimental procedures may explain the absence of bidirectional responses. For example, in mammalian studies in which the two foods are presented together, standard and palatable foods are placed in close proximity within a small cage, with the result that there is essentially no cost in terms of physical effort for the animal to switch from one feeding location

to the other. It is conceivable that increasing the switching cost (Salamone et al., 1994) could lead to a differential effect on consumption in mammals.

We propose the following model of differential accumulation on food leading to hedonic feeding in *C. elegans*. The model focusses on the olfactory neuron AWC, which is necessary and sufficient for navigation to the source of food-related odors (Kocabas et al., 2012) and exhibits bidirectional modulation by AEA. Calcium imaging shows that AWC is activated by food removal, regardless of whether favored or non-favored food is removed (Fig. 2C)(Chalasani et al., 2007). Previous studies have demonstrated that exogenous activation of AWC triggers two previously described behavioral motifs known to contribute to locomotion oriented toward attractive odors. First, its activation truncates bends of the head and neck that occur during the worm's normal sinusoidal locomotion (Kocabas et al., 2012). This means that each time a body bend moves the head away from an odor source, AWC will activate, and this bend will be truncated. Over time, successive truncations of bends in the wrong direction steer the animal in the right direction: toward the odor source; this widely conserved behavioral motif is known as *klinotaxis* (Fraenkel & Gunn, 1961). Second, activation of AWC causes the animal to stop moving forward, reverse, and resume locomotion in a new direction that is better aligned with the food odor source (Gordus et al., 2015; Gray et al., 2005); this behavioral motif is known as a *pirouette* (Pierce-Shimomura et al., 1999). Both motifs not only promote navigation toward a patch of food, but also promote retention in a patch. For example, a pirouette initiated when the worm's head protrudes beyond the food-patch boundary will return the worm into the patch. We find that AEA exposure increases AWC's response to the removal of favored food (Fig. 2C). In the proposed model, this effect both accentuates klinotaxis and increases the probability of pirouettes caused by locomotion away from the odor source. The net result is enhanced approach

to, and retention in, patches of favored food. Conversely, we also find that AEA exposure decreases responses to removal of non-favored food. This effect weakens klinotaxis and decreases pirouette probability, resulting in diminished approach and retention in non-favored food. The result of these two processes is increased or decreased accumulation, respectively, in patches of favored and non-favored food.

The requirement for *ceh-36* in rendering *C. elegans* food preferences sensitive to AEA (Fig. 2B) suggests that AWC neurons provide a necessary link between AEA and hedonic feeding. However, this experiment does not have statistical power sufficient to rule out contributions from other chemosensory neurons. Of particular interest are two chemosensory neurons AWA and ASG, both of which express *npr-19* (Table 1) and are required for chemotaxis (Bargmann et al., 1993; Bargmann & Horvitz, 1991). It will now be important to map cannabinoid sensitivity across the entire population or food-sensitive odors to understand how cannabinoids alter the overall chemosensory representation of favored and non-favored foods.

Cannabinoids have been observed to modify chemosensitivity at several levels in mammals. Both AEA and 2-AG amplify the response of primary chemosensory cells, such as the sweet-taste cells in the tongue (Yoshida et al., 2010, 2013), which may help to explain increased consumption of sweet foods and liquids. Cannabinoids can also increase the sensitivity of the mammalian olfactory system as measured during food-odor exploration (Heinbockel & Straiker, 2021; Nogi et al., 2020; Edgar Soria-Gómez et al., 2014). We observed an analogous effect in *C. elegans*, in that AEA alters the sensitivity of a primary chemosensory neuron, AWC. In unexposed worms, AWC is equally sensitive to favored and non-favored food, suggesting it cannot detect a difference in the odors released by the two food types. However, in remarkable alignment with the observed bidirectional changes in food preference in worms exposed to AEA,

this neuron becomes more sensitive to favored food and less sensitive to non-favored food, therefore acquiring the ability to discriminate between the odors of these foods.

AEA's effect on AWC appears to be indirect. Our results are consistent with a model in which AEA activates NPR-19 receptors to promote release of dense-core vesicles containing neuromodulators that act on AWC. This model is supported by evidence in *C. elegans* that 2-AG, which is capable of activating NPR-19, stimulates widespread release of serotonin (Oakes et al., 2017, 2019); thus, NPR-19 activation seems capable of promoting dense-core vesicle release. Additionally, AWC expresses receptors for biogenic amines, and it responds to neuropeptides released by neighboring neurons (Chalasani et al., 2010; Leinwand & Chalasani, 2013), suggesting that it has postsynaptic mechanisms for responding to neuromodulation. Identification of one or more neuromodulators responsible for AEA's effect on AWC, together with their associated receptors, will be an important step in answering the question of how AEA causes differential changes in food-odor sensitivity.

Our results establish a new role for endocannabinoids in *C. elegans*: the induction of hedonic feeding. There is general agreement that the endocannabinoid system and its molecular constituents offer significant prospects for pharmacological management of health, including eating disorders and substance abuse (Parsons & Hurd, 2015). Clear parallels between the behavioral, neuronal, and genetic basis of hedonic feeding in *C. elegans* and mammals establish the utility of this organism as a new genetic model for the investigation of molecular and cellular basis of these and related disorders.

Materials and Methods

Strains

Animals were cultivated under standard conditions (Brenner, 1974) using *E. coli* OP50 as a food source. Young adults of the following strains were used in all experiments:

Experiment	Strain	Genotype
Reference strain	N2, Bristol	Wild type
Preference and feeding assays	FK311	*ceh-36*(ks86)
	RB1668	*npr-19*(ok2068)
	XL324	ntIS1701[*npr-19*::CNR1::gfp-*npr-19*(1.1);*unc-122*::RFP]
	XL325	ntIS1702[*npr*-19::*npr-19*::gfp-*npr-19*(1.1)]
Calcium imaging	XL322	ntIS1703[*str-2*::GCaMP6::wCherry;*unc-122*::dsRed]
	XL327	*unc-13*(e51);ntIs1703[*str-2*::GCaMP6::wCherry;*unc-122*::dsRed]
	XL326	*unc-31*(e928);ntIs1703[*str-2*::GCaMP6::wCherry;*unc-122*::dsRed]
	XL346	*npr-19*(ok2068);ntIs1912[*str-2*::GCaMP6::wCherry;*unc-122*::dsRed]
npr-19 expression pattern	XL334	otIs544[cho-1::SL2::mCherry::H2B+pha-1(+)];ntIS19114[npr-19::GFP1.1;unc-122::dsred]
	XL335	ntIS19114[npr-19::GFP1.1;unc-122::dsred];otIs518[eat-4::SL2::mCherry::H2B+pha-1(+)]

Bacteria

The following streptomycin-resistant bacterial strains were used in this study: DA1885 (*Bacillus simplex*), DA1877 (*Comamonas* sp.), *E. Coli* HB101, and *E. Coli* DA837. Bacteria were grown overnight at 37ºC in presence of 50 mg/ml streptomycin, concentrated by centrifugation, rinsed three times with either M9 medium (for EPG experiments) or A0 buffer (for behavioral/imaging experiments; $MgSO_4$ 1 mM, $CaCl_2$ 1 mM, HEPES 10 mM, glycerol to 350 mOsm, pH 7), and resuspended to their final concentration. Concentration was defined as optical density at 600 nm (OD_{600}), as measured with a DSM cell density meter (Laxco, Bothell, WA, USA). All measurements were performed on samples diluted into the linear range of the instrument (OD 0.1-1). Previous experiments determined that $OD_{600} = 1$ corresponds to

approximately 2.35×10^9 and 2.00×10^9 colony forming units/mL of *Comamonas* and *Simplex*, respectively.

Animal preparation

Worms were washed five times in M9 for EPG experiments or A0 buffer (see above) for behavioral/imaging experiments. Worms were then incubated for 20 minutes with either background solution alone or background solution + 300 μM (electropharyngeogram experiments) or 100 μM (behavioral assays and calcium imaging experiments) Arachidonoylethanolamide (AEA, Cayman chemical, Ann Arbor, MI, USA). The incubation time and relatively high concentration reflects the low permeability of the *C. elegans* cuticle to exogenous molecules (Rand & Johnson, 1995; Sandhu et al., 2021).

Behavioral assays

Freshly poured NGM agar plates were dried in a dehydrator for 45 minutes at 45°C. A maze cut from foam sheets (Darice, Strongsville, OH, USA) using a laser cutter was placed on each plate (Fig. 1A). Maze arms were seeded with 4.5 μl of bacteria. Animals were deposited at the starting point of the maze by liquid transfer and a transparent plastic disc was placed over the maze to eliminate air currents; 12 plates were placed on a flatbed scanner and simultaneously imaged every 15 minutes (Mathew et al., 2012; Stroustrup et al., 2013). The number of worms in the two patches of food and the region between them was counted manually and a preference index I calculated as: $I = (n_{\mathrm{F}} - n_{\mathrm{NF}})/(n_{\mathrm{F}} + n_{\mathrm{NF}})$, where n_{F} is the number of worms in the favored food patch, and n_{NF} is the number of worms in the non-favored food patch. Worms that did not leave the starting point were excluded. For experiments involving mutants, a cohort of N2 animals was run in parallel on the same day. Data from statistically indistinguishable N2

cohorts were pooled where possible. In some experiments, a paralytic agent (sodium azide, NaN_3, 3 µl at 20 mM), was added to each food patch to prevent animals from leaving the patch of food after reaching it. Sodium azide diffuses through the agar over time and its action is not instantaneous. These two characteristics resulted in some worms becoming paralyzed around rather than in the patch of food, as they stop short of the patch or escape the patch briefly before becoming paralyzed. To account for these effects all worms within 5mm of the end of the maze's arm, rather than on food, were used when calculating preference index.

Electropharyngeograms

Pharyngeal pumping was measured electrophysiologically (Lockery et al., 2012) using a ScreenChip microfluidic system (InVivo Biosystems, Eugene, OR, USA). Briefly, following pre-incubation as described above, worms were loaded into the worm reservoir of the microfluidic device which was pre-filled with bacterial food (OD_{600} = 0.8) ±AEA 300 µM; this food density was chosen to reduce possible ceiling effects on pumping rate modulation by AEA. To record voltage transients associated with pharyngeal pumping (David M. Raizen & Avery, 1994)., worms were transferred on at a time from the reservoir to the recording channel of the device such that the worm was positioned between a pair of electrodes connected to a differential amplifier. Worms were given three minutes to acclimate to the channel before and recorded for one minute. Mean pumping frequency was extracted using custom code written in Igor Pro (Wavemetrics, Lake Oswego, OR, USA).

Calcium imaging

After pre-incubation with buffer or buffer +AEA (see: animal preparation), worms were immobilized in a custom microfluidic chip and presented with alternating 30-second epochs of buffer and bacteria (either *B. Simplex* or *Comamonas sp.* at OD_{600} 1, at a flow rate of 100 µl/min) for 3 minutes. Optical recordings of GCaMP6-expressing AWC neurons were performed on a Zeiss Axiovert 135, using a Zeiss Plan-Apochromat 40× oil, 1.4 NA objective, a X-Cite 120Q illuminator, a 470/40 excitation filter, and a 560/40 emission filter. Neurons were imaged at 3-10 Hz on an ORCA-ERA camera (Hamamatsu). Images were analyzed using custom code written in MATLAB: the change in fluorescence in a hand-drawn region of interest that contained only the soma and neurite. Data were normalized to the average fluorescence F_0 computed over the 15 second interval before the first food stimulus. We computed normalized fluorescence change as $\Delta F(t)/F_0$, where $\Delta F(t) = F(t) - F_0$; following convention, we refer to this measure as "$\Delta F/F$." For comparison of treatment groups, we used the peak amplitude of post-stimulus $\Delta F/F$. In some animals, AWC appeared not to respond to the food stimulus, regardless of treatment group. To classify particular AWC neurons as responsive or non-responsive, we obtained the distribution of peak $\Delta F/F$ values in control experiments in which the stimulus channel contained no food; responsive neurons were defined as those whose peak $\Delta F/F$ value exceeded the 90th percentile of this distribution. Critically, the percentage of non-responders did not vary between AEA-treated animals and (25.46% vs 22.49%, respectively; $\chi^2(1,759) = 0.699$, $p = 0.4031$).

Expression profile for npr-19

Worms were immobilized with 10 mM sodium azide (NaN_3) and mounted on 5% agarose pads formed on glass slides. Image stacks (30-80 images) were acquired using a Zeiss confocal microscope (LSM800, ZEN software) at 40X magnification. Identification of neurons was done based on published expression profiles of the p*cho-1*::mCherry (Pereira et al., 2015) and p*eat-4*::mCherry (Serrano-Saiz et al., 2013) transgenes in *C. elegans*. Individual neurons were identified by mCherry expression and the relative positions of their cell bodies; *npr-19* expression was visualized using a p*npr-19*::GFP transgene. Co-expression of GFP and mCherry was assessed by visual inspection using 3D image analysis software Imaris (Oxford Instruments). Representative images (Fig. 4A) are maximum intensity projections of 30-80 frames computed using ImageJ software (Collins, 2007). Expression of the NPR-19 receptor was widespread in body wall muscles, but restricted to 29 neurons in the head (27 - 31, 95% confidence interval, n = 20 worms imaged) and 8 neurons in the tail (7.8 - 8.5, 95% confidence interval, n = 22 worms imaged) (Suppl. Table 6). Overall, 28 of the *npr-19*-expressing neurons co-localized with either *cho-1* or *eat-4*, whereas ~9 did not co-localize with either marker. The identity of the latter cells was ascertained based on cell body position and morphology, and verified by *npr-19* expression (threshold = 2) as reported in the *C. elegans* Neuronal Gene Expression Map & Network (CeNGEN) consortium database (Hammarlund et al., 2018).

Statistics

A detailed description of statistical tests used, their results, and their interpretation is presented in Supplemental Tables 1-5. Data were checked for normality with a Kolmogorov-Smirnov test.

1) *Number of replicates*. The minimal sample size for the T-maze assays were based on pilot experiments which showed an acceptable effect size with ~10 replicates per experimental condition. Similarly, the minimal number of replicates for EPG experiments and imaging experiments were based on previously published data in which mutants/treatments could be distinguished with ~10 replicates.
2) *Effect sizes*. Effect sizes were computed as follow: Cohen's d for t-tests, partial eta-squared for ANOVAs, and $|z|/\sqrt{n}$ for Mann-Whitney test, where z is the z-score and n is the number of observations.
3) *Behavioral experiments (T-mazes).* Preference indices were analyzed using a two-factor ANOVA with repeated measures (effect of AEA × effect of time, with time as a repeated measure). For easier presentation, an average index across the four time-points was calculated and displayed (Fig. 1C-F, 2B, 3A). All time-series are nonetheless available for inspection in Fig. 1A, 2A and Supplemental Fig. 1 and 2. The effect of AEA was deemed significant if main effect of AEA was significant in the ANOVA. Averaging the four time points in a series would only be problematic if there was a non-ordinal interaction AEA × time. Inspection of ANOVA results and time series reveal that the only AEA × time interaction in Fig. 1E is ordinal and minimal. In cases where the effect of time was important (Fig. 2A) or the interaction AEA × time was meaningful (Fig. 1G) the time series of preference indices was presented. The comparison of preference indices between N2 and mutants relied on a two-factor ANOVA (effect of strain × effect of AEA). The average preference index across the four time-points was used for the comparison. In addition to an ANOVA, planned comparisons were incorporated in the experimental design using t-tests and focusing on four scientifically relevant contrasts:

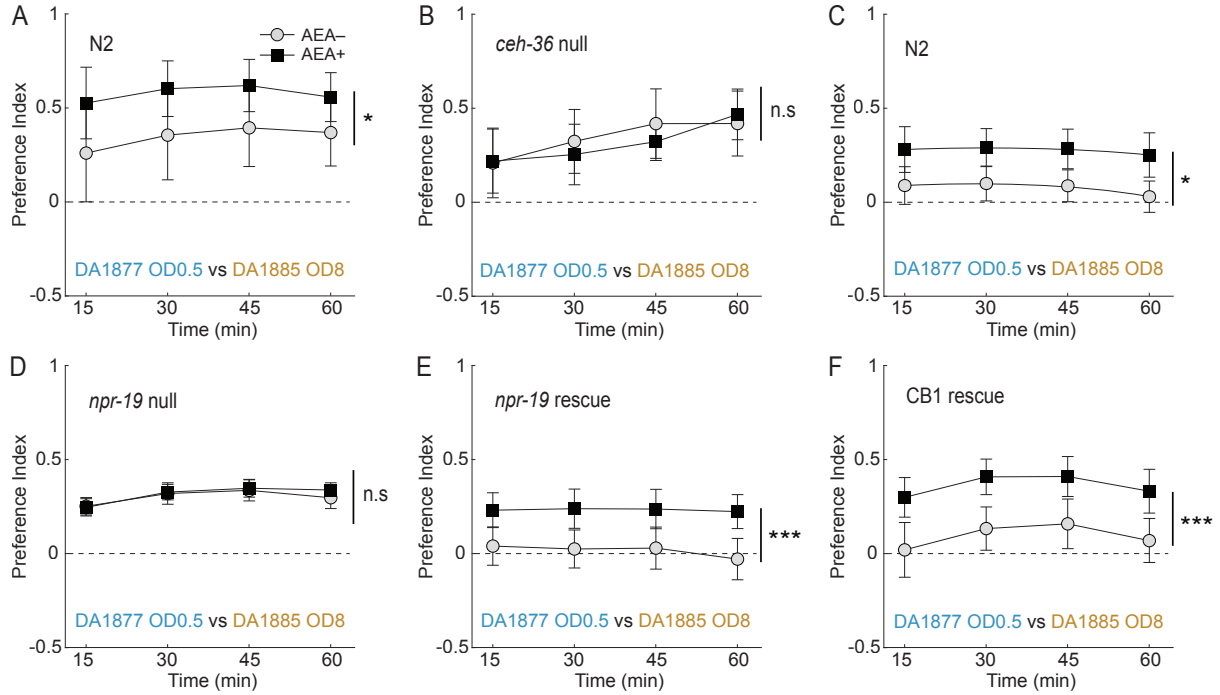
(1) mutants, AEA– vs AEA+; (2) N2, AEA– vs AEA+; (3) AEA–, mutants vs N2; (4) AEA+, mutants vs N2.

4) *Electropharyngeograms*. As the data were not normally distributed in most of the cohorts, a non-parametric test (Mann-Whitney) was used to compared pumping frequencies between strains/treatments.

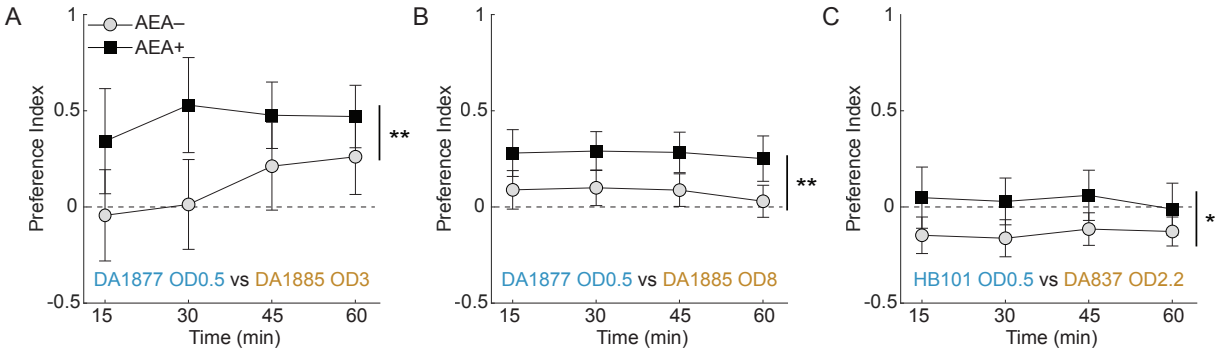
5) *Calcium imaging*. Peak ΔF/F was used as the primary measure. A two-factor ANOVA (effect of AEA × effect of bacteria type) was used to assess the effect of AEA on AWC responses. Planned t-tests were focused on four contrasts: (1) favored food, AEA– vs AEA+; (2) non-favored food, AEA– vs AEA+; (3) AEA-, favored food vs non-favored food; (4) AEA+, favored food vs non-favored food. For comparisons between N2 and mutants, a two-factor ANOVAs (effect of AEA × effect of strain) was performed for each of the bacteria type (favored and non-favored) and followed by four contrasts (t-tests): (1) mutants, AEA– vs AEA+; (2) N2, AEA– vs AEA+; (3) AEA–, mutants vs N2; (4) AEA+, mutants vs N2.

6) *Multiple comparisons.* No correction for multiple comparisons was applied in *t*-tests used in pair-wise comparisons of means in multifactor experiments as the experimental design in this study relied on a small number (3 per condition) of planned (a priori), rather than unplanned (a posteriori), scientifically relevant contrasts (Keppel & Zedeck, 1989).

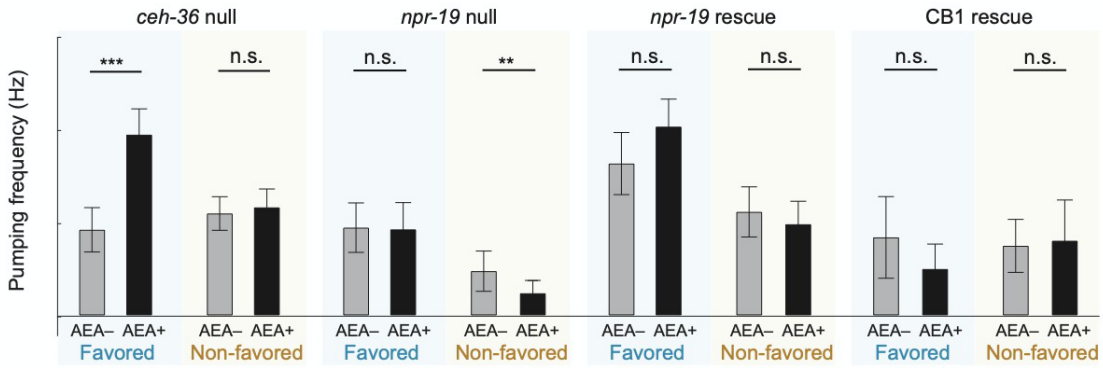
Supplemental material



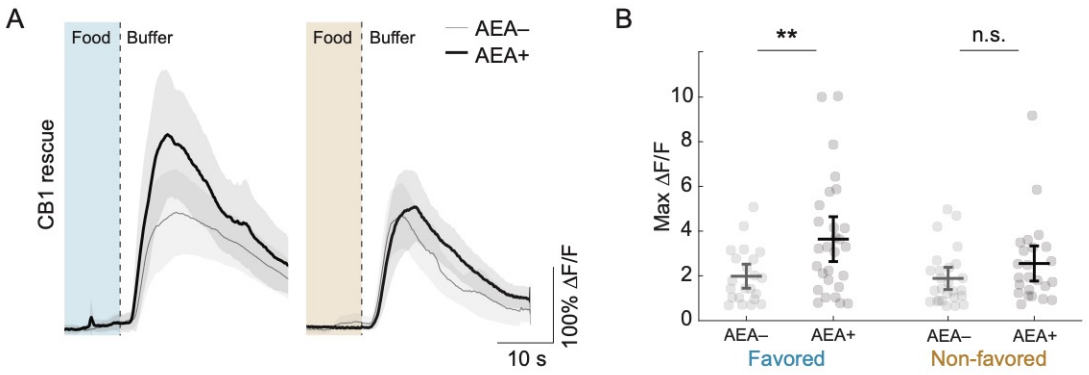
Supp. Fig 1. Effect of baseline preference and bacteria identity on preference time course. Mean preference index (*I*) versus time for AEA-exposed animals (AEA+) and unexposed controls (AEA–), where $I > 0$ is preference for favored food, $I < 0$ is preference for non-favored food, and $I = 0$ is indifference (*dashed line*). **A**. Time course, Fig. 1D. **B**. Time course, Fig. 1E. **C**. Time course, Fig. 1F. For statistics in **A**-**C**, see Supp. Table 1. Symbols: *, $p < 0.05$; **, $p < 0.01$; n.s., not significant. Error bars, 95% confidence intervals.



Supp. Fig 2. Effect of genetic background on preference time course. Mean preference index (*I*) versus time for AEA-exposed animals (AEA+) and unexposed controls (AEA–), where $I > 0$ is preference for favored food, $I < 0$ is preference for non-favored food, and $I = 0$ is indifference (*dashed line*). **A**. Time course, Fig. 2B, N2. **B**. Time course, Fig. 2B, *ceh-36*. **C**. Time course, Fig. 3A, N2. **D**. Time course, Fig. 3A, *npr-19* null. **E**. Time course, Fig. 3A, *npr-19* rescue. **F**. Time course, Fig. 3A, CB_1 rescue. **A**-**F**. For statistics, see Supp. Tables 2, 3. Symbols: *, $p < 0.05$; ***, $p < 0.001$; n.s., not significant. Error bars, 95% confidence intervals.



Supp. Fig 3. Effect of AEA on pharyngeal pumping frequency in different genetic backgrounds. Mean pumping frequency in favored and non-favored food is shown for or AEA-exposed animals (AEA+) and unexposed controls (AEA–). Favored food, DA1877, OD 0.8; non-favored food, DA1885, OD 0.8. For statistics, see Supp. Table 5. Symbols: **, $p < 0.01$; ***, $p < 0.001$. Error bars, 95% confidence intervals.



Supp. Fig 4. CB_1 partial rescue of AEA sensitivity in AWC neurons. A. Effect of AEA on the response of AWC neurons to the removal of favored or non-favored food in *npr-19* mutants in which CB_1 was overexpressed under control of the *npr-19* promoter. Each trace is average normalized fluorescence change ($\Delta F/F$) versus time. Favored food (blue), DA1877, OD 1; non-favored food (orange), DA1885, OD 1. **B**. Summary of the data in **A**, showing mean peak $\Delta F/F$. For statistics in **A**-**B**, see Supp. Table 5. Symbols: **, $p < 0.01$. Error bars or shading, 95% confidence intervals.

Line	Figure	Condition	Narrative	Test	Measure	Units of replication	Number of replicates	Statistic	p-value	Significance	Condition 1 avg +/-CI	Condition 2 avg +/-CI	Effect size	Note
1	2A	T-maze, + sodium azide Favored (DA1877) OD 0.5 Non-Favored (DA1885) OD 3 AEA– vs AEA+	AEA increases preference for favored food in presence of azide (main effect of AEA). The effect of time reflects a drop in preference over time in both AEA– and AEA+ conditions.	Two-factor ANOVA, repeated measures	Preference index over time	Assay plate (16-135 animals/plate)	*n=12 (AEA–)* *n=12 (AEA+)*							
2				Main effect of AEA				F(1,22)= 11.71	0.002	**	0.08 ± 0.09 (AEA–)	0.26 ± 0.07 (AEA+)	0.35	
3				Main effect of time				F(3,22)= 3.70	0.016	*				
4				Interaction, AEA × time				F(3,66)= 0.26	0.146					
5	2B, Suppl. Fig 2A,B,	T-maze Favored (DA1877) OD 0.5 Non-Favored (DA1885) OD 8 *ceh-36* vs N2 AEA– vs AEA+	*ceh-36* is necessary for the effect of AEA on food preference. A moderate interaction is accompanied by a clear effect of AEA in N2, an absence of effect in *ceh-36* as well as a clear difference between the two strains in the presence of the drug.	Two-factor ANOVA	Preference	Assay plate (17-123 animals/plate)	*n=86 (N2 AEA–)* *n=59 (N2 AEA+)* *n=24 (ceh-36 AEA–)* *n=21 (ceh-36 AEA+)*							Same N2 data as in Fig. 1E
6				Main effect of strain				F(1,79)= 3.27	0.074					
7				Main effect of AEA				F(1,79)= 1.98	0.164					
8				Interaction, AEA × strain				F(1,79)= 3.15	0.080					
9				Planned comparisons, t-test										
10				N2, AEA– vs AEA+				t(79)= -2.16	0.034	*	0.34 ± 0.20 (AEA–)	0.58 ± 0.13 (AEA+)	0.67	
11				*ceh-36* , AEA– vs AEA+				t(79)= -0.27	0.787		0.34± 0.15 (AEA–)	0.32 ± 0.12 (AEA+)		
12				AEA–, N2 vs ceh-36				t(79)= 0.02	0.981		0.34 ± 0.20 (N2)	0.34± 0.15 (*ceh-36*)		
13				AEA+, N2 vs *ceh-36*				t(79)= 2.53	0.013	*	0.58 ± 0.13 (N2)	0.32 ± 0.12 (*ceh-36*)	-1.0	
14	2D	AWC calcium imaging N2 Favored (DA1877) OD 1 vs non-favored (DA1885) OD 1 AEA– vs AEA+	AEA increases and decreases AWC response to favored and non-favored food, respectively. AWC responses to favored and non-favored are not different in the absence of AEA. Although main effects are non-significant, further analysis of the significant interaction reveals opposing effects of AEA on AWC response to favored and non-favored food.	Two-factor ANOVA	ΔF/F	individual worm	*n= 28 (Favored, AEA –)* *n= 32 (Favored, AEA+)* *n= 30 (Non-favored, AEA –)* *n= 29 (Non-favored, AEA+)*							
15				Main effect of bacteria				F(1,115)= 3.17	0.078					
16				Main effect of AEA				F(1,115)= 0.89	0.349					
17				Interaction, AEA × bacteria				F(1,115)= 11.98	0.001	***				
18				Planned comparisons, t-test										
19				Favored AEA– vs AEA+				t(58)= -2.68	0.010	**	1.98 ± 0.62 (AEA–)	3.38 ± 0.83 (AEA+)	0.34	
20				Non-favored AEA– vs AEA+				t(57)= -2.23	0.030	*	2.56 ± 0.53 (AEA–)	1.75 ± 0.53 (AEA+)	-0.4	
21				AEA– Favored vs Non-favored				t(56)= 1.45	0.152		1.98 ± 0.62 (Favored)	2.56 ± 0.53 (Non-favored)		
22				AEA+ Favored vs Non-favored				t(59)= -3.30	0.002	**	3.38 ± 0.83 (Favored)	1.75 ± 0.53 (Non-favored)	0.4	

Supplemental Table 1. Statistics for Fig. 1 and Supp. Fig. 1. Experimental conditions and comparisons tested are described in column 3. Stars in the Significance column indicate significance levels: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Effect sizes were computed as described in Materials and Methods and 95% confidence intervals were used as a dispersion measure.

Line	Figure	Condition	Narrative	Test	Measure	Units of replication	Number of replicates	Statistic	p-value	Significance	Condition 1 avg +/-CI	Condition 2 avg +/-CI	Effect size	Note
1	1B, 1C	T-maze Favored (DA1877) OD 1 Non-Favored (DA1885) OD 1 AEA– vs AEA+	AEA increases preference for favored food (main effect of AEA)	Two-factor ANOVA, repeated measures	Preference index over time	Assay plate (7-117 animals/plate)	*n=41 (AEA–)* *n=40 (AEA+)*							
2				Main effect of AEA				F(1,79)= 11.00	0.001	***	0.49 ± 0.09 (AEA–)	0.66 ± 0.06 (AEA+)	0.12	
3				Main effect of time				F(3,79)= 1.73	0.162					
4				Interaction, AEA × time				F(3,237)= 1.10	0.351					
5	1D Supp. Fig 1A	T-maze Favored (DA1877) OD 0.5 Non-Favored (DA1885) OD 3 AEA– vs AEA+	AEA increases preference for favored food when there is no baseline preference for either food (main effect of AEA). The main effect of time reflects an slight increase in preference observed after 15-30 min.	Two-factor ANOVA, repeated measures	Preference index over time	Assay plate (16-135 animals/plate)	*n=20 (AEA–)* *n=17 (AEA+)*							
6				Main effect of AEA				F(1,35)= 7.58	0.009	**	0.09 ± 0.19 (AEA–)	0.43 ± 0.17 (AEA+)	0.18	
7				Main effect of time				F(3,35)= 4.10	0.009	**				
8				Interaction, AEA × time				F(3,105)= 2.00	0.118					
9	1E Supp. Fig 1B	T-maze Favored (DA1877) OD 0.5 Non-Favored (DA1885) OD 8 AEA– vs AEA+	AEA increases preference for favored food when there is no baseline preference for either food (main effect of AEA). The mild interaction time X AEA reflects a slight drop in preference after 45 min in the AEA- group.	Two-factor ANOVA, repeated measures, interaction	Preference index over time	Assay plate (7-76 animals/plate)	*n=86 (AEA–)* *n=59 (AEA+)*							
10				Main effect of AEA				F(1,143)= 11.16	0.001	**	0.08 ± 0.08 (AEA–)	0.28 ± 0.09 (AEA+)	0.07	
11				Main effect of time				F(3,143)= 1.15	0.329					
12				Interaction, AEA × time				F(3,429)= 0.11	0.043	*				
13	1F Supp. Fig 1C	T-maze Favored (HB101) OD 0.5 Non-Favored (DA837) OD 2.2 AEA– vs AEA+	AEA increases preference for favored food in a different pair of bacteria (main effect of AEA).	Two-factor ANOVA, repeated measures	Preference index over time	Assay plate (12-117 animals/plate)	*n= 96 (AEA–)* *n=35 (AEA+)*							
14				Main effect of AEA				F(1,129)= 5.26	0.023	*	-0.16 ± 0.08 (AEA–)	0.01 ± 0.17 (AEA+)	0.04	
15				Main effect of time				F(3,129)= 0.70	0.448					
16				Interaction, AEA × time				F(3,387)= 0.63	0.402					
17	1G	T-maze Favored (DA1877) OD 1 AEA– vs AEA+	AEA increases the fraction of worms in favored food (main effect of AEA). The effect of time reflects the progressive accumulation of worms in food. The interaction is ordinal.	Two-factor ANOVA, repeated measures	Fraction of worms in favored food	Assay plate (7-117 animals/plate)	*n=41 (AEA–)* *n=40 (AEA+)*							Same data as in 1B
18				Main effect of AEA				F(1,79)= 23.57	0.000	***	0.42 ± 0.03 (AEA–)	0.54 ± 0.03 (AEA+)	0.22	
19				Main effect of time				F(3,79)= 75.42	0.000	***				
20				Interaction, AEA × time				F(3,237)= 3.80	0.011	*				
21	1G	T-maze Non-Favored (DA1885) OD 1 AEA– vs AEA+	AEA decreases the fraction of worms in non-favored food (main effect of AEA). The effect of time reflects the progressive accumulation of worms in food.	Two-factor ANOVA, repeated measures	Fraction in non-favored food	Assay plate (7-117 animals/plate)	*n=41 (AEA–)* *n=40 (AEA+)*							Same data as in 1B
22				Main effect of AEA				F(1,79)= 4.74	0.033	*	0.15 ± 0.03 (AEA–)	0.11 ± 0.02 (AEA+)	0.06	
23				Main effect of time				F(3,79)= 32.05	0.0000	***				
24				Interaction, AEA × time				F(3,237)= 1.74	0.1596					
25	1I	Electropharyngeogram AEA– favored (DA1877) OD 0.8 vs non-favored (DA1885) OD 0.8	AEA– pumping frequency does not differ between favored and non-favored food.	Mann-Whitney	Frequency of pumps in EPG recordings	Individual worm	*n=67 (AEA–)* *n=124 (AEA+)*	U= 2280	0.412		0.46 ± 0.17 (favored)	0.45 ± 0.14 (non-favored)		
26	1I	Electropharyngeogram Favored (DA1877) OD 0.8 AEA– vs AEA+	AEA increases pumping in presence of favored food.	Mann-Whitney	Frequency of pumps in EPG recordings	Individual worm	*n=67 (AEA–)* *n=67 (AEA+)*	U= 1667.5	0.010	*	0.46 ± 0.17 (AEA–)	0.98 ± 0.27 (AEA+)	0.55	
27	1I	Electropharyngeogram Non-Favored (DA1885) OD 0.8 AEA– vs AEA+	AEA decreases pumping in presence of non-favored food.	Mann-Whitney	Frequency of pumps in EPG recordings	Individual worm	*n=74 (AEA–)* *n=124 (AEA+)*	U= 3196.5.	0.000	***	0.45 ± 0.14 (AEA–)	0.23 ± 0.08 (AEA+)	-0.43	

Supplemental Table 2. Statistics for Fig. 2 and Supp. Fig. 2 A, B. Experimental conditions and comparisons tested are described in column 3. Stars in the Significance column indicate significance levels: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Effect sizes were computed as described in Materials and Methods and 95% confidence intervals were used as a dispersion measure.

Line	Figure	Condition	Narrative	Test	Measure	Units of replication	Number of replicates	Statistic	p-value	Significance	Condition 1 avg +/-CI	Condition 2 avg +/-CI	Effect size	Note
1	3A, Supp. Fig 2C, D	T-maze Favored (DA1877) OD 0.5 Non-Favored (DA1885) OD 8 *npr-19* null vs N2 AEA– vs AEA+	*npr-19* is necessary for the effect of AEA on food preference. Although the ANOVA indicates an effect of AEA, that effect is restricted to N2 in t-tests.	Two-factor ANOVA	Preference index over time	Assay plate (7-85 animals/plate)	*n=86 (N2 AEA–)* *n=59 (N2 AEA+)* *n=24 (npr-19 null AEA–)* *n=24 (npr-19 null AEA+)*							Same N2 data as in Fig. 1E
2				Main effect of strain				F(1,189)= 1.29	0.257					
3				Main effect of AEA				F(1,189)= 5.15	0.024	*				
4				Interaction, AEA × strain				F(1,189)= 1.58	0.210					
5				Planned comparisons, t-test										
6				N2, AEA– vs AEA+				t(189)= -3.49	0.001	**	0.08 ± 0.08 (AEA–)	0.28 ± 0.09 (AEA+)	0.56	
7				*npr-19* null, AEA– vs AEA+				t(189)= 0.59	0.559		0.08 ± 0.14 (AEA–)	0.14 ± 0.1 (AEA+)		
8				AEA–, N2 vs *npr-19* null				t(189)= -0.09	0.931		0.08 ± 0.08 (N2)	0.08 ± 0.14 (*npr-19* null)		
9				AEA+, N2 vs *npr-19* null				t(189)= 1.66	0.100		0.28 ± 0.09 (N2)	0.14 ± 0.1 (*npr-19* null)		
10	3A, Supp. Fig 2C, E	T-maze Favored (DA1877) OD 0.5 Non-Favored (DA1885) OD 8 *npr-19* rescue vs N2 AEA– vs AEA+	*npr-19* expression rescues the effect of AEA in *npr-19* mutants. A significant main effect of AEA is reflected in a significant effects of AEA in both N2 and *npr-19* rescue in t-tests. Moreover the effect of AEA is similar in both strains (t-test: AEA+,N2 vs *npr-19* rescue).	Two-factor ANOVA	Preference index over time	Assay plate (7-76 animals/plate)	*n=86 (N2 AEA–)* *n=59 (N2 AEA+)* *n= 24(npr-19 rescue AEA–)* *n= 24(npr-19 rescue AEA+)*							Same N2 data as in Fig. 1E
11				Main effect of strain				F(1,189)= 0.92	0.339					
12				Main effect of AEA				F(1,189)= 14.58	0.000	***				
13				Interaction, AEA × strain				F(1,189)= 0.02	0.878647					
14				Planned comparisons, t-test										
15				N2, AEA– vs AEA+				t(189)= -3.63	0.000	***	0.08 ± 0.08 (AEA–)	0.28 ± 0.09 (AEA+)	0.56	
16				*npr-19* rescue, AEA– vs AEA+				t(189)= 2.30	0.022	*	0.02 ± 0.09 (AEA–)	0.23 ± 0.09 (AEA+)	1.13	
17				AEA–, N2 vs *npr-19* rescue				t(189)= 0.81	0.421		0.08 ± 0.08 (N2)	0.02 ± 0.09 (*npr-19 rescue*)		
18				AEA+,N2 vs *npr-19* rescue				t(189)= 0.56	0.578		0.28 ± 0.09 (N2)	0.23 ± 0.09 (*npr-19 rescue*)		
19	3A, Supp. Fig 2C, F	T-maze Favored (DA1877) OD 0.5 Non-Favored (DA1885) OD 8 CB1 rescue vs N2 AEA– vs AEA+	CB1 expression rescues the effect of AEA in *npr-19* mutants. A significant main effect of AEA is reflected in significant effects of AEA in both N2 and CB1 rescue in t-tests. Moreover, the effect of AEA is similar in both strains (t-test: AEA+, N2 vs CB1 rescue).	Two-factor ANOVA	Preference index over time	Assay plate (4-150 animals/plate)	*n=86 (N2 AEA–)* *n=59 (N2 AEA+)* *n= 27(CB1 rescue AEA–)* *n= 27(CB1 rescue AEA+)*							Same N2 data as in Fig. 1E
20				Main effect of strain				F(1,195)= 0.97	0.325					
21				Main effect of AEA				F(1,195)= 19.88	0.000	***				
22				Interaction, AEA × strain				F(1,195)= 0.41	0.521					
23				Planned comparisons, t-test										
24				N2, AEA– vs AEA+				t(195)= -3.61	0.000	***	0.08 ± 0.08 (AEA–)	0.28 ± 0.09 (AEA+)	0.56	
25				CB1 rescue, AEA– vs AEA+				t(195)= 3.00	0.003	**	0.09 ± 0.1 (AEA–)	0.36 ± 0.09 (AEA+)	1.13	
26				AEA–, N2 vs CB1 rescue				t(195)= -0.25	0.803		0.08 ± 0.08 (N2)	0.09 ± 0.1 (*CB1 rescue*)		
27				AEA+, N2 vs CB1 rescue				t(195)= -1.123	0.263		0.28 ± 0.09 (N2)	0.36 ± 0.09 (*CB1 rescue*)		
28	3C	AWC calcium imaging Favored (DA1877) OD 1 *npr-19* null vs N2 AEA– vs AEA+	AEA no longer modulates AWC rersponse to favored food in *npr-19* mutants. There is no main effect of AEA or effect of AEA in t-tests. In absence of AEA, the response of AWC is elevated relative to N2 controls.	Two-factor ANOVA	$\Delta F/F$	individual worm	*n= 28 (N2, AEA–)* *n= 32 (N2, AEA+)* *n= 35 (npr-19, AEA+)* *n= 35 (npr-19, AEA+)*							Same N2 data as in Fig. 2C
29				Main effect of strain				F(1,126)= 1.67	0.198					
30				Main effect of AEA				F(1,126)= 1.60	0.208					
31				Interaction strain × AEA				F(1,126)= 5.42	0.022	*				
32				Planned comparisons, t-test										
33				*npr-19* AEA– vs AEA+				t(68)= -0.63	0.532		3.36 ± 0.90 (AEA–)	3.04 ± 0.47 (AEA+)		
34				N2 AEA– vs AEA+				t(58)= -2.67627	0.010	**	1.98 ± 0.62 (AEA–)	3.38 ± 0.83 (AEA+)	0.34	
35				AEA– N2 vs *npr-19*				t(61)= 2.51	0.015	*	1.98 ± 0.62 (N2)	3.36 ± 0.90 (*npr-19*)	0.26	
36				AEA+ N2 vs *npr-19*				t(65)= -0.71	0.480		3.38 ± 0.83 (N2)	3.04 ± 0.47 (*npr-19*)		
37	3C	AWC calcium imaging Non-Favored (DA1885) OD 1 *npr-19* mutants vs N2 AEA– vs AEA+	AEA no longer modulates AWC rersponse to non-favored food in *npr-19* mutants. There is no main effect of AEA or effect of AEA in t-tests. In absence of AEA, the response of AWC is elevated relative to N2 controls.	Two-factor ANOVA	$\Delta F/F$	individual worm	*n= 30 (N2, AEA–)* *n= 29 (N2, AEA+)* *n= 37 (npr-19, AEA+)* *n= 36 (npr-19, AEA+)*							Same N2 data as in Fig. 2C
38				Main effect of strain				F(1,128)= 50.22	0.000	***				
39				Main effect of AEA				F(1,128)= 3.79	0.054					
40				Interaction strain × AEA				F(1,128)= 0.13	0.721					
41				Planned comparisons, t-test										
42				*npr-19* AEA– vs AEA+				t(71)= -1.02	0.310		4.90 ± 0.87 (AEA-)	4.33 ± 0.63 (AEA+)		
43				N2 AEA– vs AEA+				t(57)= -2.23	0.030	*	2.56 ± 0.53 (AEA–)	1.75 ± 0.53 (AEA+)	-0.42	
44				AEA– N2 vs *npr-19*				t(65)= 4.55	0.000	***	2.56 ± 0.53 (N2)	4.90 ± 0.87 (*npr-19*)	0.47	
45				AEA+ N2 vs *npr-19*				t(63)= 6.31	0.000	***	1.75 ± 0.53 (N2)	4.33 ± 0.63 (*npr-19*)	0.89	

Supplemental Table 3. Statistics for Fig. 3 and Supp. Fig. 2 C-F. Experimental conditions and comparisons tested are described in column 3. Stars in the Significance column indicate significance levels: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Effect sizes were computed as described in Materials and Methods and 95% confidence intervals were used as a dispersion measure.

Line	Figure	Condition	Narrative	Test	Measure	Units of replication	Number of replicates	Statistic	p-value	Significance	Condition 1 avg +/-CI	Condition 2 avg +/-CI	Effect size	Note
1	4C	AWC calcium imaging Favored (DA1877) OD 1 *unc-13* vs N2 AEA– vs AEA+	In *unc-13* mutants, AEA increases AWC response to favored food in a manner similar to that seen in N2. The significant main effect of strain reflects slightly elevated AWC responses in *unc-13* compared to N2 controls, both at baseline and in AEA-treated animals.	Two-factor ANOVA	ΔF/F	individual worm	*n= 27 (unc-13, AEA–) n= 27 (unc-13, AEA+) n= 28 (N2, AEA–) n= 32 (N2, AEA+)*							
2				Main effect of strain				F(1,109)= 6.650	0.011	*				
3				Main effect of AEA				F(1,109)= 17.031	0.000	***				
4				Interaction, AEA × strain				F(1,109)= 0.134						
5				Planned comparisons, t-test										
6				*unc-13* AEA– vs AEA+				t(51)= 3.22	0.002	**	2.83 ± 0.66 (AEA–)	4.49 ± 0.77 (AEA+)	0.47	
7				N2 AEA– vs AEA+				t(58)= -2.68	0.010	**	1.98 ± 0.62 (AEA–)	3.38 ± 0.83 (AEA+)	0.34	
8				AEA– N2 vs *unc-13*				t(52)= 1.87	0.067		1.98 ± 0.6 (N2)	2.83 ± 0.66 (*unc-13*)		
9				AEA+ N2 vs *unc-13*				t(57)= 1.97	0.054		3.38 ± 0.8 (N2)	4.49 ± 0.77 (*unc-13*)		
10	4C	AWC calcium imaging Non-Favored (DA1885) OD 1 *unc-13* vs N2 AEA– vs AEA+	In *unc-13* mutants, AEA decreases AWC response to non-favored food in a manner similar to that seen in N2. The significant main effect of strain reflects slightly elevated AWC responses in unc-13 compared to N2 controls, both at baseline and in AEA-treated animals.	Two-factor ANOVA	ΔF/F	individual worm	*n= 32 (unc-13, AEA–) n= 33 (unc-13, AEA+) n= 30 (N2, AEA–) n= 29 (N2, AEA+)*							
11				Main effect of strain				F(1,120)= 3.94	0.050	*				
12				Main effect of AEA				F(1,120)= 10.80	0.001	**				
13				Interaction, AEA × strain				F(1,120)= 0.20	0.658					
14				Planned comparisons, t-test										
15				*unc-13* AEA– vs AEA+				t(63)= -2.42	0.019	*	2.56 ± 0.5 (AEA-)	2.2 ± 0.47 (AEA+)	-0.34	
16				N2 AEA– vs AEA+				t(57)= -2.23	0.030	*	2.56 ± 0.53 (AEA–)	1.75 ± 0.53 (AEA+)	-0.4	
17				AEA– N2 vs *unc-13*				t(60)= 1.58	0.119		2.56 ± 0.5 (N2)	3.27 ± 0.72 (*unc-13*)		
18				AEA+ N2 vs *unc-13*				t(60)= 1.31	0.197		1.75 ± 0.5 (N2)	2.2 ± 0.47 (*unc-13*)		
19	4E	AWC calcium imaging Favored (DA1877) OD 1 *unc-31* vs N2 *AEA– vs AEA+*	In *unc-31* mutants, AEA no longer increases AWC response to favored food. The interaction AEA × strain reflects the effect of AEA on N2 and its absence in *unc-31*.	Two-factor ANOVA	ΔF/F	individual worm	*n= 25 (unc-31, AEA–) n= 24 (unc-31, AEA+) n= 28 (N2, AEA–) n= 32 (N2, AEA+)*							
20				Main effect of strain				F(1,99)=1.98	0.163					
21				Main effect of AEA				F(1,99)=1.22	0.271					
22				Interaction, AEA × strain				F(1,99)=9.54	0.003	**				
23				Planned comparisons, t-test										
24				*unc-31* AEA– vs AEA+				t(47)= -1.75	0.087		2.62 ± 0.73 (AEA–)	1.8 ± 0.57 (AEA+)		
25				N2 AEA– vs AEA+				t(58)= -2.68	0.010	**	1.98 ± 0.62 (AEA–)	3.38 ± 0.83 (AEA+)	0.34	
26				AEA– N2 vs *unc-31*				t(51)= 1.34	0.187		1.98 ± 0.6 (N2)	2.62 ± 0.73 (*unc-31*)		
27				AEA+ N2 vs *unc-31*				t(54)= -3.15	0.003	**	3.38 ± 0.8 (N2)	1.8 ± 0.57 (*unc-31*)	-0.40	
28	4E	AWC calcium imaging Non-Favored (DA1985) OD 1 *unc-31* vs N2 AEA– vs AEA+	In *unc-31* mutants, AEA increases AWC response to non-favored food, which is the opposite of its effect in N2 controls.	Two-factor ANOVA	ΔF/F	individual worm	n= 19 (unc-31, AEA–) n= 25 (unc-31, AEA+) n= 30 (N2, AEA–) n= 29 (N2, AEA+)							
29				Main effect of strain				F(1,99)= 0.13	0.717					
30				Main effect of AEA				F(1,99)= 3.78	0.055					
31				Interaction, AEA × strain				F(1,99)= 11.26	0.001	**				
32				Planned comparisons, t-test										
33				*unc-31* AEA– vs AEA+				t(42)= 2.42	0.020	*	2.56 ± 0.5 (AEA-)	3.31 ± 0.68 (AEA+)	0.43	
34				N2 AEA– vs AEA+				t(57)= -2.23	0.030	*	2.56 ± 0.53 (AEA–)	1.75 ± 0.53 (AEA+)	-0.4	
35				AEA– N2 vs *unc-31*				t(47)= -1.1	0.281		2.56 ± 0.5 (N2)	2.04 ± 0.76 (*unc-31*)		
36				AEA+ N2 vs *unc-31*				t(52)= 3.61	0.001	***	1.75 ± 0.5 (N2)	3.31 ± 0.68 (*unc-31*)	0.64	

Supplemental Table 4. Statistics for Fig. 4. Experimental conditions and comparisons tested are described in column 3. Stars in the Significance column indicate significance levels: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Effect sizes were computed as described in Materials and Methods and 95% confidence intervals were used as a dispersion measure.

Line	Figure	Condition	Narrative	Test	Measure	Units of replication	*Number of replicates*	Statistic	p-value	Significance	Condition 1 avg ± CI	Condition 2 avg ± CI	Effect size	Note
1	Supp. Fig 3	Electropharyngeogram Favored (DA1877), *ceh-36* null AEA– vs AEA+	AEA increases pumping in presence of favored food in *ceh-36*.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	n=76 (AEA–) n=53 (AEA+)	U= 888.5	0.000	***	0.9 ± 0.28	1.91 ± 0.23	0.62	
2	Supp. Fig 3	Electropharyngeogram Non-Favored (DA1885), *ceh-36* null AEA– vs AEA+	AEA has no effect on pumping in presence of non-favored food in *ceh-36*.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	*n=90 (AEA–)* *n=88 (AEA+)*	U= 3894	0.849		1.08±0.18 (AEA–)	1.14±0.19 (AEA+)		
3	Supp. Fig 3	Electropharyngeogram Favored (DA1877), *npr-19* null AEA– vs AEA+	AEA has no effect on pumping in presence of favored food in *npr-19* mutants.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	n=86 (AEA–) n=77 (AEA+)	U= 3137.5	0.562		0.93 ± 0.26 (AEA–)	0.91 ± 0.28 (AEA+)		
4	Supp. Fig 3	Electropharyngeogram Non-Favored (DA1885), *npr-19* null AEA– vs AEA+	AEA decreases pumping rate in *npr-19* mutants in presence of non-favored food.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	*n=44 (AEA–)* *n=56 (AEA+)*	U= 804.5	0.003	**	0.47 ±0.21 (AEA–)	0.23 ± 0.14 (AEA+)	-0.30	
5	Supp. Fig 3	Electropharyngeogram Favored (DA1877), *npr-19* rescue AEA– vs AEA+	AEA has no effect on pumping in presence of favored food in *npr-19* rescue worms.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	n=76 (AEA–) n=95 (AEA+)	U= 3074.5	0.097		1.60 ± 0.32 (AEA–)	1.99 ± 0.29 (AEA+)		
6	Supp. Fig 3	Electropharyngeogram Non-Favored (DA1885), *npr-19* rescue AEA– vs AEA+	AEA has no effect on pumping in presence of non-favored food in *npr-19* rescue worms.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	*n=67 (AEA–)* *n=67 (AEA+)*	U= 2222.5	0.920		1.09 ±0.27 (AEA–)	0.96±0.24 (AEA+)		
7	Supp. Fig 3	Electropharyngeogram, Favored (DA1877), CB1 rescue AEA– vs AEA+	AEA has no effect on pumping in presence of favored food in CB1 rescue worms.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	n=32 (AEA–) n=28 (AEA+)	U= 388.5	0.384		0.82 ± 0.43 (AEA–)	0.49 ±0.26 (AEA+)		
8	Supp. Fig 3	Electropharyngeogram Non-Favored (DA1885), CB1 rescue AEA– vs AEA+	AEA has no effect on pumping in presence of non-favored food in CB1 rescue worms.	Mann-Whitney	Frequency of pumps in EPG recordings	individual worm	*n=52 (AEA–)* *n=25 (AEA+)*	U= 637	0.889		0.73 ±0.28 (AEA–)	0.79 ±0.43 (AEA+)		
9														
10	Supp. Fig 4B	AWC calcium imaging Favored (DA1877) OD 1 CB1 rescue vs N2 AEA– vs AEA+	CB1 expression restores AEA sensitivity in *npr-19* mutants in response to favored food. N2 and CB1 rescue are not different (no main effect of strain), and a significant effect of AEA (main effect of AEA) is present. In t-tests, AEA has a signifcant effect on AWC response in both strains to the same extent (no difference in contrast: AEA+, N2 vs CB1 rescue).	Two-factor ANOVA	ΔF/F	individual worm	n=22 (CB1 rescue, AEA–) n=28 (CB1 rescue, AEA+) n=28 (N2, AEA–) n=32 (N2, AEA+)							Same N2 data as in Fig. 2C
11				Main effect of strain				F(1,106)= 0.23	0.629					
12				Main effect of AEA				F(1,106)= 14.84	0.000	**				
13				Interaction, AEA × strain				F(1,106)= 0.11	0.740					
14				Planned comparisons, t-test										
15				CB1 rescue AEA– vs AEA+				t(48)= 3	0.005	**	1.99 ± 0.51 (AEA–)	3.64 ± 0.96 (AEA+)	-0.38	
16				N2 AEA– vs AEA+				t(58)= -2.68	0.010	**	1.98 ± 0.62 (AEA–)	3.38 ± 0.83 (AEA+)	0.34	
17				AEA– N2 vs CB1 rescue				t(48)= 0.02	0.988		1.98 ± 0.62 (N2)	1.99 ± 0.51 (*CB1 rescue*)		
18				AEA+ N2 vs CB1 rescue				t(58)= 0.42	0.674		3.38 ± 0.8 (N2)	3.64 ± 0.96 (*CB1 rescue*)		
19	Supp. Fig 4B	AWC calcium imaging Non-Favored (DA1885) OD 1 CB1 rescue vs N2 AEA– vs AEA+	CB1 expression does not restores AEA sensitivity in *npr-19* mutants in response to non-favored food. The interaction reflects the effect of AEA in N2 and its absence in CB1 rescue.	Two-factor ANOVA	ΔF/F	individual worm	n=26 (CB1 rescue, AEA–) n=24 (CB1 rescue, AEA+) n=30 (N2, AEA–) n=29 (N2, AEA+)							Same N2 data as in Fig. 2C
20				Main effect of strain				F(1,105)= 0.03	0.859					
21				Main effect of AEA				F(1,105)= 0.22	0.638					
22				Interaction, AEA × strain				F(1,105)= 14.74	0.011	*				
23				Planned comparisons, t-test										
24				CB1 rescue AEA– vs AEA+				t(48)= 1.48	0.146		1.89 ± 0.5 (AEA-)	2.55 ± 0.74 (AEA+)		
25				N2 AEA– vs AEA+				t(57)= -2.23	0.030	*	2.56 ± 0.53 (AEA–)	1.75 ± 0.53 (AEA+)	-0.4	
26				AEA– N2 vs CB1 rescue				t(54)= -1.89	0.064		2.56 ± 0.5 (N2)	1.89 ± 0.47 (*CB1 rescue*)		
27				AEA+ N2 vs CB1 rescue				t(51)= 1.76	0.085		1.75 ± 0.5 (N2)	2.55 ± 0.74 (*CB1 rescue*)		

Supplemental Table 5. Statistics for Supp. Fig. 3, Supp. Fig. 4. Experimental conditions and comparisons tested are described in column 3. Stars in the Significance column indicate significance levels: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Effect sizes were computed as described in Materials and Methods and 95% confidence intervals were used as a dispersion measure.

		Number of GFP positive cells			
		Head			Tail
Worm #	1	28	Worm #	1	7
	2	22		2	9
	3	33		3	10
	4	30		4	9
	5	28		5	9
	6	33		6	8
	7	28		7	9
	8	29		8	8
	9	36		9	7
	10	26		10	9
	11	19		11	8
	12	26		12	7
	13	36		13	9
	14	35		14	8
	15	34		15	7
	16	29		16	9
	17	32		17	8
	18	26		18	7
	19	26		19	8
	20	27		20	7
	21			21	10
	22			22	8
Mean ± 95% CI		29.2 ± 2.1	Mean ± 95% CI		8.2 ± 0.4

Supplemental Table 6. Counts of *npr-19*-expressing neurons in the head and tail. Number of p*npr-19*::GFP positive neurons present in the head (n = 20 worms), or the tail (n = 22 worms).

CHARTER IV: CONCLUSIONS AND FUTURE DIRECTIONS

C. elegans is a millimeter long, free-living nematode with only 302 neurons. Despite its simplicity, it has proven to be a valuable model system in which to probe the cellular and molecular basis of behavior. Over the last 80 years of *C. elegans* research, we have learned that many aspects of mammalian physiology and behavior are conserved in this simple nematode, including the roles of several signaling systems. We have only recently learned that *C. elegans* has a functional endocannabinoid system, but since this discovery, many key functions of the endocannabinoid system have proven to be conserved in nematodes. Among these functions are the inhibition of motor control and nociception and ability to promote food intake and shift preference toward calorically dense, palatable food—a phenomenon known as the munchies in humans.

The ability of the endocannabinoid system to modulate feeding has made it an attractive target for pharmaceutical weight regulation. However, the widespread distribution of cannabinoid receptors throughout the body makes it difficult to modulate feeding in isolation, without affecting all of the other physiological functions this system affects. To best take advantage of this system to regulate food intake, we need to uncover where the endocannabinoid system acts to modulate feeding. In mammals, as endocannabinoids and their receptors are found centrally in feeding and reward areas of the brain, and peripherally in gustatory and olfactory epithelium, the gastrointestinal tract, and adipose cells, and because many of these regions are highly interconnected, dissecting the individual contribution of endocannabinoid action in these areas to the munchies is a complex task. Identification of a functional endocannabinoid system in *C. elegans* has allowed us to probe the central and peripheral mechanisms that contribute to this phenomenon in a simple, well-characterized organism.

One of the mechanisms shown to contribute to the munchies in mammals is cannabinoid-mediated tuning of chemosensory neurons to promote sweet and fat food intake. *C. elegans* has well-characterized chemosensory neurons, and several of these neurons are known to play a crucial role in feeding, attraction to food-related odors, and navigation to food. We showed that endocannabinoid exposure tunes the activity of the olfactory neuron, AWC, to promote approach to and dwelling in highly palatable food, mirroring the effect of cannabinoids on mammalian olfactory neurons.

Nevertheless, there is still a lot to learn about cannabinoid modulation of feeding in *C. elegans*. Since AWC does not express the NPR-19 receptor, it must inherit the cannabinoid signal from an upstream neuron. Future work should attempt to map the full neural circuit involved in cannabinoid modulation of feeding, from the NPR-19 expressing neuron upstream of AWC to the motor neurons involved in navigation to and from food and pharyngeal neurons involved in consumption of food. Further, we mapped the *C. elegans* cannabinoid receptor, NPR-19, to several sensory and gustatory neurons, such as AWA and AGS, which likely contribute to AEA's effect on food preference. The compact nervous system of *C. elegans* allows us to study the effect of a ligand on the activity of all neurons simultaneously. Future work should explore the effect of AEA on the activity of all neurons in response to different types of foods to better understand the role of different signaling systems in cannabinoid-mediated modulation of feeding.

Finally, we observed expression of the NPR-19 receptor throughout the gut. In mammals, CB_1 receptors in the gastrointestinal tract contribute to feeding decisions through secretion of orexigenic and anorexigenic factors that modulate hypothalamic activity. Future work in *C. elegans* should explore the contribution of NPR-19 receptor signaling in the gut to feeding

decisions. Though the field of cannabinoid research in *C. elegans* is still young, great strides have been made in establishing this nematode as a model organism for cannabinoid studies due to the many functional homologies between *C. elegans* and mammal that have already been established. This has opened the door to deeper exploration and isolation of mechanisms underlying the endocannabinoid system's effect on physiology and behavior, and will hopefully lead to great advancements in this field.

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