DECODING NEURAL CIRCUITS MODULATING BEHAVIORAL RESPONSES TO AVERSIVE SOCIAL CUES

A Dissertation

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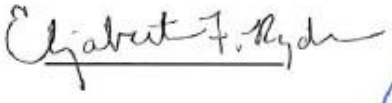

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Preface

Octopamine succinylated #9, osas#9, was discovered and published in June of 2013. Less than two months later I joined the lab and took “osas#9” under my wing. When I started there was three things known about osas#9: 1) The small ascaroside is produced by starved larval stage 1 (L1) animals, 2) starved *C. elegans* respond aversively to the compound, and 3) starved animals subjected to the compound plus *E. coli*, no longer avoid osas#9. Now, five years later, we have developed an extensive model for the underlying circuitry driving response and modulation to osas#9. Of course, I say we because it was a group effort, involving discussion with peers, input from collaborators, and assistance from undergraduates. And, of course, guidance by the principal investigator, Jagan Srinivasan. A special thanks is warranted to all of the JS lab members, present and past, who helped achieve the goals of this dissertation. But it was not just the compound that was new, Jagan was a new faculty member, our lab manager was straight out of undergraduate school, and I was a first generation college student starting graduate school. To say the least, it was an adventure.

In particular, it would be impossible not to individually mention Laura Innarelli (Aurillio), whom I began my PhD journey with. She started as Jagan’s lab manager just weeks before I arrived (and sadly left before my final year). Throughout four years she was pivotal in keeping the lab running smoothly, from ordering essentials to prepping daily media. She went above and beyond that with discussions and edits, and more importantly, friendship. After one year, Doug Reilly joined the lab, who along with Laura offered many laughs and… distractions. But daily crosswords and memes made for a great and productive environment. I especially would like to thank Doug for taking the

time to read through this dissertation and offer edits and comments. This last year, Liz DiLoreto took over as lab manager and kept things running like a well-oiled machine. All of the members, from volunteers to graduate students, made for a fun environment over the years.

With Jagan operating a new lab, no senior lab members, and three lab locations (two relocations over the course of my time here), it was quite the memorable experience. This meant many trial and error scenarios over the years, from molecular cloning techniques to setting up new equipment and developing new assays for the lab. One thing that was constant though, “The Fix”, which is a burger bar responsible for keeping us all satiated (maybe a bit too much) and bonding outside of lab to fulfill Jagan’s desire to have a lab that felt more like a group of friends with a common goal rather than a workplace.

After a few months of performing avoidance assays with a mouth pipette (I know) I was becoming quite concerned with how the next few years would pan out. Little did I know just how quickly, and simultaneously, I would be exposed to a plethora of techniques and investigative strategies making for a fulfilled graduate career. One thing I did not anticipate learning, especially so quickly, was communication with leading PI’s from the field. Of course, if you know Jagan, this is his strategy: jump in, get the experience, and learn. He connected me to many PIs, led to two important collaborations resulting in authorships, and most importantly - taught me to be confident in disseminating my knowledge to superiors and peers in person or via email. I will never forget the first oral presentation at a local worm meeting (just 9 months in), where he said before-hand “don’t worry, there will be a Nobel Laureate in the audience”. But this sort of urging and pressure

has resulted in scientific growth in not only knowledge, but in communication. Thank you Jagan.

Lastly, I would like to thank my friends and family who supported me throughout the rollercoaster that is grad school. During my time as a PhD student, I got married, bought a house, adopted a dog, and had a wonderful child. It goes without saying that balancing everything was not always easy. With that I would like to dedicate this dissertation to my anchors, my wife Katherine and son Yareev.

Contents

Abstract

Understanding how the human brain functions on a molecular and cellular level is nearly impossible with current technology and ethical considerations. Utilizing the small nematode, *Caenorhabditis elegans*, and its innate behavioral responses to olfactory social cues, we can begin to unravel the mechanisms underlying social behavior. This is made possible given that innate behaviors are crucial for survival, and therefore hardwired into the genome of organisms. This allows for genetic-level analysis of neural circuitries driving behavior. Studying the neuronal mechanisms underlying *C. elegans*' behavioral responses to social cues will not only assist in our overall understanding of how the brain perceives stimuli to enact a behavioral response at the cellular and molecular level, but also our understanding as to how the nervous system properly integrates information to enact social behavioral responses: mis-integration and social abnormalities are commonalities seen in many neuropsychiatric disorders, and these studies will provide fruitful insights into the defects observed in these disorders. Lastly, by comparing the perception of several different types of social chemicals, we can further our understanding of neural coding strategies for the various behaviors crucial for survival. Chapter One of this thesis orients the reader to social, innate behavior, and the usefulness of *C. elegans* as a tool for understanding behavioral coding. Chapter Two explores and establishes the required components of a socially aversive pheromone, providing insight into signaling evolution and co-option of biological machineries. Chapter Three examines how multiple, competing stimuli are integrated to modulate behavioral output, furthering our understanding of molecular and cellular integration and decision making within the nervous system. Chapter Four highlights the importance of predator

pressure, and provides insights into circuit strategies of redundant and promiscuous networks of threat detection. Lastly, Chapter Five considers the implications of these findings as a whole, in the perspective of evolutionary strategies leading to neuronal coding of different behavioral outputs. Taken together, this dissertation aimed to fill the void in our understanding of social behavior neural circuitries, and how integration governed at the molecular and cellular level of the nervous system affects those behaviors.

1 Introduction

In the opening chapter of this dissertation, I seek to orient the reader to the importance of innate behavior in itself, but also as a tool for deciphering how the brain encodes information and enacts a behavioral response. To elucidate the underpinnings of brain function, I utilized the small nematode *C. elegans,* which allows for characterizing behavioral circuits in response to stimuli at the molecular and cellular level. The primary reagents I used are social chemical compounds, or semiochemicals, detected by the *C. elegans* amphid chemosensory neurons. In the first part of this chapter, I introduce innate behavior, semiochemical signaling, olfaction, and extensively the sensory neurons and signal transduction pathways necessary for understanding behavior at the molecular and cellular level utilizing *C. elegans.* The second part of chapter 1 is a published review on mating pheromones underlying *C. elegans* behavior. This section highlights and familiarizes the reader to one extensively studied aspect of *C. elegans* social behavior as a tool for understanding behavior. In conclusion, I point out that much of the social behavior in *C. elegans* has been studied in regards to sex-specific modulation of intra-organismal semiochemicals, and much can be learned from comparing these data sets to the less studied intra- and inter-organismal social behaviors underlying aversive responses.

1 A .1 Innate animal behavior

How organisms interact with their surroundings is of the utmost importance for survival at the individual level and as a species. As such, those behaviors critical for life have been selected for throughout evolution, and are encoded within the genome as innate responses. These intrinsic, unlearned behaviors include actions such as: reflexes, taxis, fixed action patterns, courtship displays, and more. Ultimately, this myriad of different intrinsic behaviors serves purposes that can be generalized into four groups necessary for survival; foraging, defense and escape, reproduction, and for some life histories, care for offspring. Immediately apparent is that nearly all of these behaviors have an inherent social component that involves communication between two or more individuals. Innate communication and behavioral response between individuals can be utilize any of the senses: auditory, olfactory, gustatory, tactile, or visual (1). In 1973, the Nobel Prize in Physiology and Medicine was awarded to three pioneers in the field of animal behavior, who characterized, empirically, social innate behavior: Karl von Frisch, for deciphering the waggle dance of the honey bee (used for communicating foraging sites to conspecifics); Nikolaas Tinbergen and Konrad Lorenz, for characterizing innate behaviors in fish and birds in relation to aggression, courtship rituals, and care for young (2).

Humans display several innate social behaviors important for survival. For example, behaviors of newborns, such as sucking and rooting, grasping, moro, and crossed extension reflexes, all promote survival (3). Classical ethologists were largely focused on observable, innate behaviors, as doing so allowed for concrete characterizations. For example, fixed action patterns are innate behaviors that consist of

an external stimulus that elicits a behavioral response in all individuals of a species that typically is carried out to completion. A 1939 hallmark study by Lorenz and Tinbergen characterized the innate behavior of Graylag geese retrieving an egg that is out of the nest (4). Upon external stimulus of seeing an egg out of the nest, the animal will extend its neck, and use her bill to pull the egg back to the nest. Furthermore, if the egg is removed, the goose will continue to pull backwards with her bill (4). With the advent of modern technologies in science, these innate behaviors offer powerful tools for ethologists to understand how behavior is coded in the genome and neural circuitries. As such, organisms displaying innate behaviors with accessible genomes and nervous systems have become paramount in our goal of understanding the molecular and cellular bases for behavior.

Nudibranchia, or molluscan sea slugs, have been a powerful toll in understanding the neural mechanisms underlying innate behavior. When tube feet of predatory sea stars stimulate the sea slug, *Tritonia diomedea,* the slug responds by initiating strong, rhythmic ventral-dorsal flexions (5). Upon stimulation, the escape swim response is carried out to completion via a central pattern generator (CPG). The nature of CPGs, which require no feedback or innervation once triggered, allows for examination of these circuits *in vitro* (5). This, coupled with the large size of the neurons, allows for electrophysiological studies of the neural communication underlying the innate behavior (5). Applying these techniques throughout swimming and non-swimming *Nudibranchia* allows for insights on the evolution of behaviors and the underlying circuits (6, 7). These studies have revealed how differential expression in homologous neurons gives rise to divergent neural circuitries, driving homologous behaviors (6, 7).

A.11 Semiochemical Signaling and Innate behavior

Social chemical communication is ancient and ubiquitous, and as such is an alluring avenue to study (8). Furthermore, deciphering neural circuits underlying innate social chemical communication provides a powerful tool for furthering our understanding of how our brains organize, process, and act on social information. Social communication via chemical signaling within or between organisms that results in a change in the receiver is known as semiochemical signaling (8, 9). This can further be broken down by communication between species (allelochemicals) or within a species (pheromones) (8, 9).

Allelochemicals can further be broken down based on cost/benefit relationship between the emitter and receiver (Table A1). Allomones are beneficial to the emitter and detrimental to the receiver (10). One example of this are prey emitting signals to deter predators, such as the earwig emitting sulfide compounds that have a rotting flesh odor to deter predators (11). On the other hand, a predator may emit a chemical to attract prey, such as the bolas spider or Venus flytrap (12, 13). Another class of allelochemical, synomones, are beneficial to both the emitter and receiver (10). An example of these compounds can be found in the symbiotic relationship between sea anemones and clown fish, wherein clown fish are attracted to compounds released by the sea anemone (14). In turn, the organisms protect each other from predators and the anemone gains nutrients from fish waste (8). Lastly, there are kairomones, a type of allelochemical that benefits the receiver at the cost of the emitter (10). One type of kairomone detection is known as eavesdropping, where a predator “eavesdrops” on prey signals unintended for them. One such example is predatory beetles eavesdropping on the aggregation pheromones of

their prey, the bark beetle (15, 16). Strikingly, one predatory beetle, *T. formicarius* is capable of eavesdropping on 22 compounds emitted by bark beetles, as well their food sources (15). Other examples of a kairomones benefits the prey at the cost of the predator, such as signals present in predator urine, feces, or fur that are detected by prey, and promote behaviors that increase survival fitness (17, 18). For example, compounds present in predatory cat urine results in aversive responses in rodents (19, 20) (Fig. A1A).

Compounds underlying chemical communication between conspecifics, or members of the same species, are known as pheromones, a term which was coined by Karlson and Luscher in 1959 after the discovery of the silk moth sex attractant, bombykol (8, 21, 22) (Fig. A1B,C). Pheromones have since been found across kingdoms, spanning from single-cell to multicellular organisms (8, 23, 24). Mammalian pheromones have been found to play many roles. For example, in rodents, there is evidence that regulation of endocrine status and induction of innate behaviors related to mating, nurturing, fighting, and fear responses, are controlled by pheromones (25). Pheromones can relay negative information about the environment as well: rats that are stressed release compounds (4-methylpentanal and hexanal) that result in conspecifics displaying an increase in acoustic startle reflex and anxious behaviors (26). Contrarily, pheromones can also transmit favorable information about the environment, such as ant trails leading conspecifics to a foraging site (27) (Fig. A1C).

Table A1: Table depicting the classification of chemical compounds emitted and received by organisms of different species.

Inter-species chemical communication		Cost-/Benefit+	
Classification		Emitter	Reciever
Allelomones	Allomone	+	-
	Kairomone	-	+
	Synonome	+	+

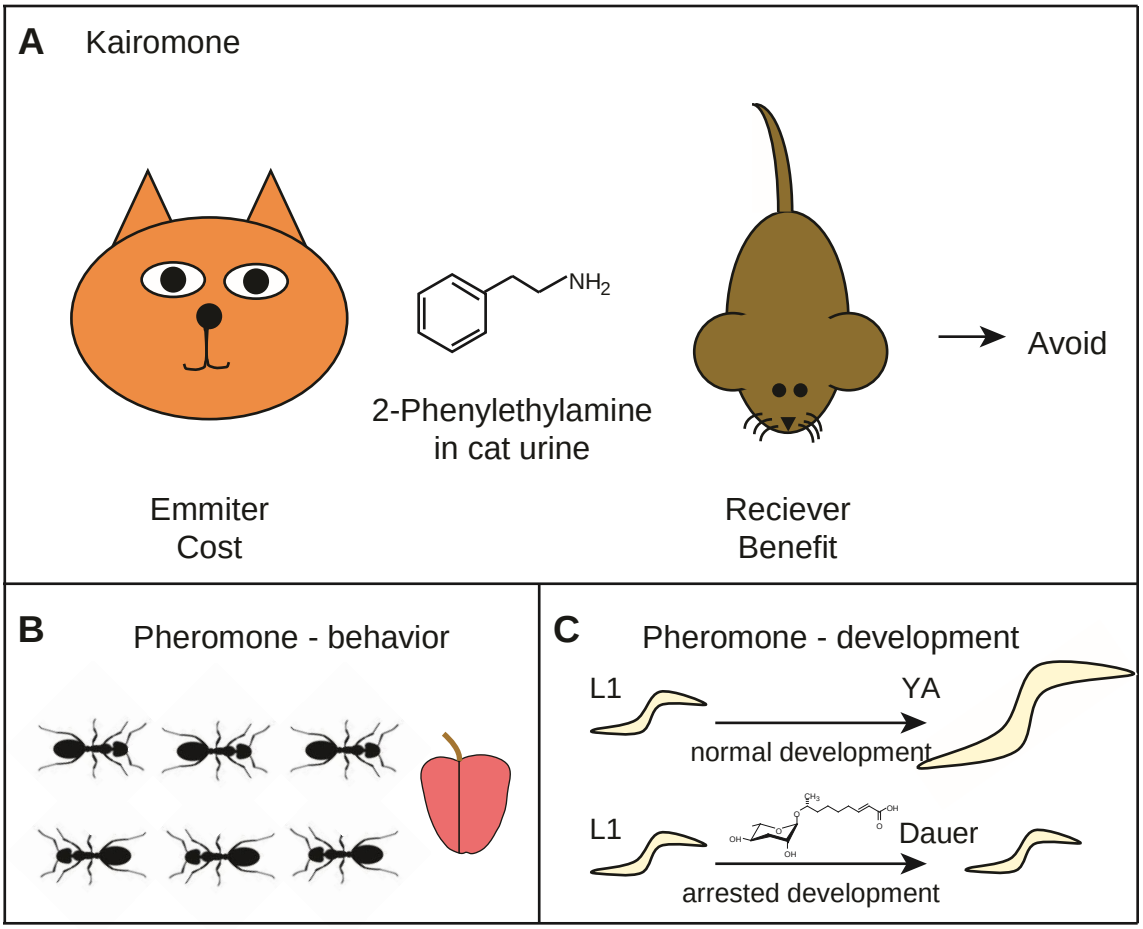


Figure A1: Semiochemicals can affect behavior and development. **A-B)** Semiochemicals with an immediate behavioral effect are known as releaser effects. **A)** The subgroup of allelomones known as kairomones are signals between species. The detection of the signal benefits the receiver at the cost of the emitter. The shown example demonstrates how predatory cat (emitter) urine can contain compounds detected by prey (receiver). The receiver benefits in this case because it detects the odor of a predator and becomes vigilant. Alternatively, a predator may eavesdrop on a signal produced by prey and have an easier time locating them. **B)** Pheromones are signals between conspecifics, or organisms of the same species. In this example, a pheromone trail left by ants assists conspecifics in food location and efficient foraging. **C)** Semiochemicals can also have

long term effects, known as primer effect. For example shown in panel C is a primer effect of pheromone signaling in *C. elegans.* Detection of particular ascarosides (ascr#3 shown) can result in formation of a developmentally persistent life stage known as dauer.

It is likely that humans also utilize pheromones, although the complex nature of human behavior and chemical secretions have made identification of any one pheromone difficult (28). One argument for pheromone signaling in humans is of the ability of newborns to sense breast milk (29-32). Neonates placed prone on their mothers after birth have been observed to locate and feed from an unwashed breast versus a washed one (31). It is thought that the chemical detection promoting neonatal location of the nipple may be a reflection of the mothers amniotic fluid, and increased areolar pH post-birth (30, 33). More intriguingly, newborns have the ability to differentiate between their biological mother's milk and that of an unfamiliar mother (29). Likewise, mothers given scents of babies are able to correctly identify the scent of their own child (34). These phenomena are likely due to unique, signature-like odors, rather than direct social communication. One set of promising, as yet unidentified compound(s), acting as a mammalian pheromone(s), are those secreted in the axillary region that elicit menstrual synchronization (35). As such, the role of social communication via chemical compounds, or semiochemical signaling, in humans remains largely unknown. Three regions of the mammalian olfactory system recognize these semiochemicals, the main olfactory epithelium (MOE) (which is also present in humans), the vomeronasal organ (VNO), and the Grueneberg ganglion (GG) (17, 18, 25). Related tissues in humans are thought to also recognize pheromones, which further complexes the elucidation of human sensation of pheromones as these organs are non-functional in humans following after *in utero* development (36).Much remains to be understood about how chemical social cues govern animal behavior, from sensation to behavioral enactment.

1 A.2 Olfaction

Proper interaction with the environment is crucial for the survivability of a species. As such, the major sensory systems have evolved to coordinate such appropriate response behaviors. One of the most ancient and ubiquitous systems is that of olfaction, or smell. While difficult to appreciate the human sense of smell due to our visually dominated orientation, it is actually the most discerning sense humans possess, with the ability to discriminate at least a trillion different scent combinations (37). Thus, elucidating mechanisms underlying chemical communication will provide insights on brain function; from the coordination of neural circuits to elicited behavioral responses. The importance of understanding olfaction to grow our understanding of brain function is showcased by the 2004 Nobel Prize in Physiology or Medicine being awarded to Linda B. Buck and Richard Axel for pioneering our understanding of chemoreceptors and olfactory organization (38).

Due to the importance of olfaction and its widespread nature, it is no surprise that olfactory systems are strikingly similar across different phyla (39). Despite similarities, there are notable differences as well (39, 40). At the most basic level, all olfactory systems at the primary level detect odors via olfactory receptors, which in turn manipulate activity of the first-order neurons they are expressed in, relaying information downstream to brain circuits that perceive and enact odor-driven responses (25, 39, 40). Whereas the physiology of olfaction is similar and conserved, the structural organization of these systems displays variation (39, 41). The three main models used for olfaction are those of mice (mammals), *D. melangoster* (insects), and *C. elegans* (nematodes) (39, 40).

The initiation of olfaction begins with detection of chemical cue via a receptor present in a sensory neuron. The most obvious similarity which spans from invertebrates to vertebrates is that most olfactory receptors are G Protein-Coupled Receptors (GPCRs) (39, 40). However, and not unexpectedly, GPCRs used by different organisms are not derived from the same family or evolutionary origins (40). Mammals and nematodes both have olfactory receptors in the rhodopsin-like superfamily, whereas insects utilize a very distantly related group of GPCRs in odor detection (40). Through genomic analyses it has become clear that the number of GPCR olfactory receptors also varies drastically in different animals. Humans have an estimated 400 olfactory receptors, compared to the potential 1300 in *C. elegans* (39, 42). However, the diminished number of receptors does not translate to a diminished repertoire of odor recognition, e.g. humans can detect at least a trillion olfactory stimuli (37). Furthermore, receptors are not necessarily tuned narrowly for one stimulus. Insects, with only 62 odorant receptors, show a wide ability to sense odors, with some receptors responding to up to 30 different olfactory stimuli (40). Lastly, olfactory receptors are not exclusively GPCRs, as guanylate cyclase homologues and four-transmembrane receptors have also been found to detect chemical cues in nematodes and mammals, respectively (40, 43, 44).

Receptor expression and number of sensory neurons are the principal differences present in primary olfaction. Both mice and fruit flies predominately express one receptor in each sensory neuron (39, 40) (Fig. A2A,C). Although the dominant organization is the one-neuron-one-receptor rule in mammals, recent evidence suggests that a group of sensory neurons in the mouse olfactory “necklace” subsystem of the main olfactory epithelium (MOE) can express multiple receptors in a single neuron (43). This aligns with

the strategy observed in *C. elegans*, wherein each chemosensory neuron expresses many receptors: they have roughly a thousand olfactory receptors coded for in the genome and only 12 amphid chemosensory neurons (39, 40, 45) (Fig. A2B,D).

Although the strategies of receptor expression may differ, all olfactory receptors are present in the cilia of primary bipolar sensory neurons (39) (Fig. A2E). Meaning, that each olfactory sensory neuron, regardless of organism, has: 1) a long dendritic process that terminates into a fluid-filled cavity with pores open to the environment, and 2) an axonal projection extending to the central nervous system (39) (Fig. A2E). Interestingly, aquatic animals also utilize a fluid-filled cavity for cilia, suggesting the composition of the fluid is as important for olfaction as its role in preventing desiccation (39). Indeed, there are enzymes in the fluid capable of regulating olfactory signals in insects, crustaceans, and mammals (39).

After receptor activation, signal transduction is similar across organisms as well, in that heterotrimeric G proteins relay messages by regulating intracellular secondary messengers (39, 45). The two primary pathways are cyclic nucleotide and phosphoinositide signaling, which regulate membrane excitability to relay signals. These signals go on to activate (or inhibit) second-order neurons. A commonality at the primary level of olfaction is the ability of sensory neurons to be finely tuned by interneurons to modify sensory response and the relayed signal (46, 47).

The primary olfactory sensing neurons of mice and fruit flies converge onto glomeruli, present in the olfactory bulbs and antennal lobes, respectively (39, 40) (Fig. A2C). Each glomerulus consists of olfactory sensory neurons expressing a single

receptor (40). The exception is the glomeruli of the necklace subsystem in mice, in which sensory neurons express more than one receptor (43). *C. elegans* do not exhibit glomeruli – this is likely due to the small number of neurons in the animal (302 in total), and the very simple “brain” (nerve ring) (40). Projection neurons in fruit flies, and mitral cells in mammals, project from single glomeruli to higher processing centers (40) (Fig. A2C). *C. elegans*, primary sensory neurons directly contact the nerve ring, which functions as a higher processing center (Fig. A2D). The common elements of *C. elegans* olfaction, coupled with its transparency, tractability, and “simple” nervous system, make it an ideal organism for studying how odors drive behavior. Understanding how odors give rise to behavior will provide key insights into neural circuitries underlying brain functionalities.

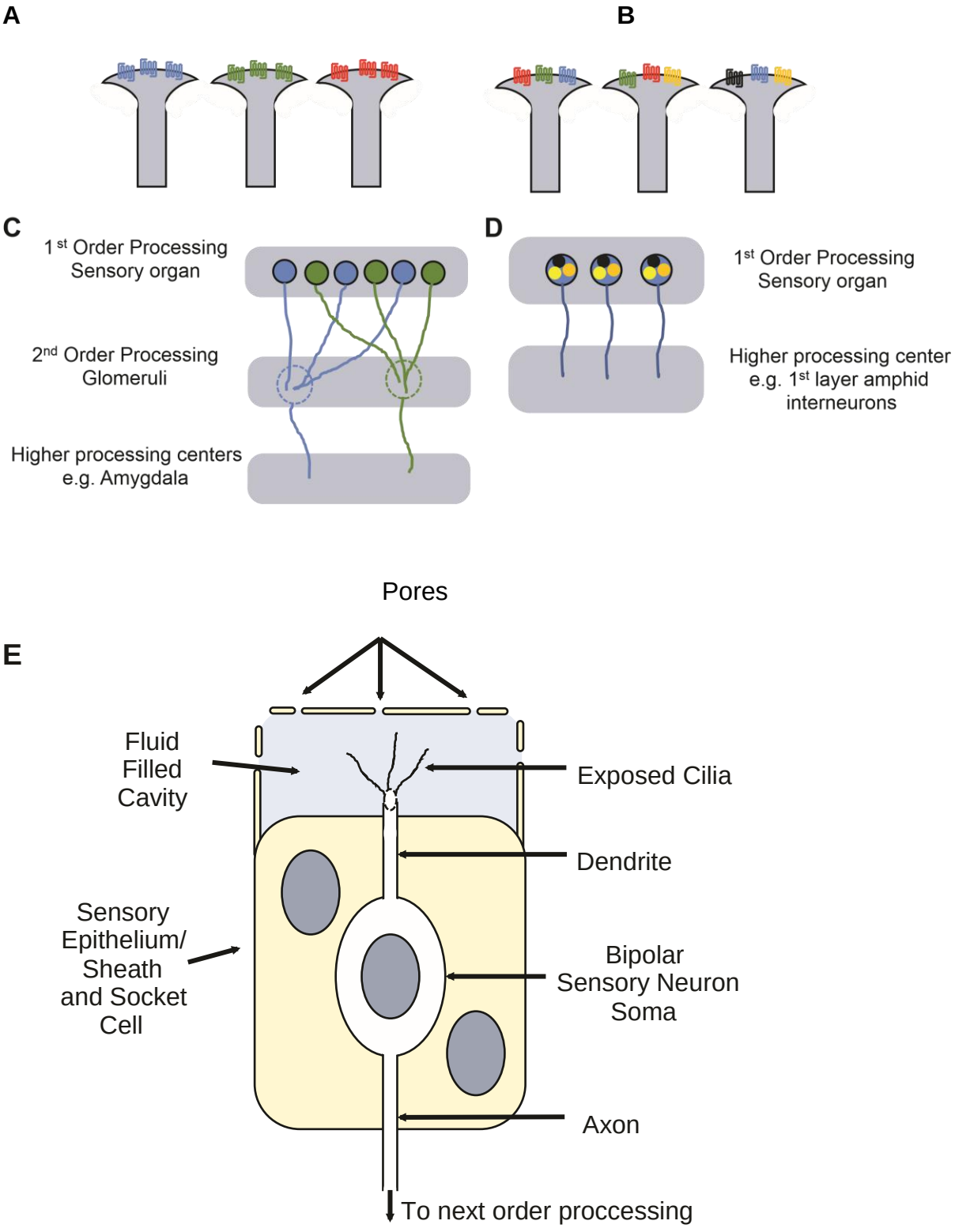


Figure A2: Olfactory systems and strategies. **A)** Mammals and fruit flies predominately show a one-receptor to one-neuron expression strategy. **B)** *C. elegans* expressed many

olfactory receptors on one sensory neuron. **C)** Mammals and fruit flies show first, second, and third order processing of olfactory cues. The first level is the olfactory receptor expressing neurons, which those of a common receptor converge as glomeruli in the second order processing station (antennal lobe or olfactory bulbs). A single neuron then transmits information from the glomerulus to higher order processing stations. **D)** *C. elegans* display a different strategy were one neuron expressing many receptors is directly innervated into higher processing centers of the animal. **E)** All olfactory neurons are bipolar: extending a dendrite to a fluid filled cavity with pores open to the environment and projecting an axon to higher order processing stations.

1 A.3 *Caenorhabditis elegans* as a Model

Due to the complex molecular profile of human secretions and the complex nervous system mediating human behavior – as well as the ethical considerations of studying humans directly – in order to better understand social chemical communication, from the biosynthesis of compounds to sensory cells to the molecular machinery underlying the physiological response to the cue, it is best to turn to a model organism. Ideal qualities would include: an organism that demonstrates robust social behavior to pheromones, is easily cultured, is genetically tractable, and has a simple, well studied nervous system. The small nematode, *Caenorhabditis elegans*, stands out in its relevant affordances for understanding molecular and cellular control of the reception of social cues (48).

C. elegans have a short, three day life cycle from egg to egg laying adult, with four developmental larval stages (L1-L4), and two stages of developmental arrest, L1 arrest and dauer (49). Animals will enter the alternative, environmentally persistent states under conditions of overcrowding, heat, or starvation, and resume development upon return to favorable conditions (50-52). Due the resistance of the L1 larva, animals at this stage can be cryogenically frozen for long term storage (53, 54). In addition to these persistent states, *C. elegans* eggs are resistant to bleach, allowing for easy decontamination and/or synchronization of cultures (49). On top of these qualities, the animals' small size and hermaphroditic nature make them particularly amenable to maintenance. Furthermore, *C. elegans* has a well annotated genome, a completely defined physical connectome of all 302 neurons in the hermaphrodite, and the fate of every cell has been mapped from the zygote to fully aged adult (55-58). Additionally, the worm displays robust behaviors,

is transparent (Fig. A3A,B), and susceptible to fluorescent proteins (Fig. A3C,D), allowing for rapid forward and reverse genetic screens to identify cells and genes involved in development, behavior, and physiological responses.

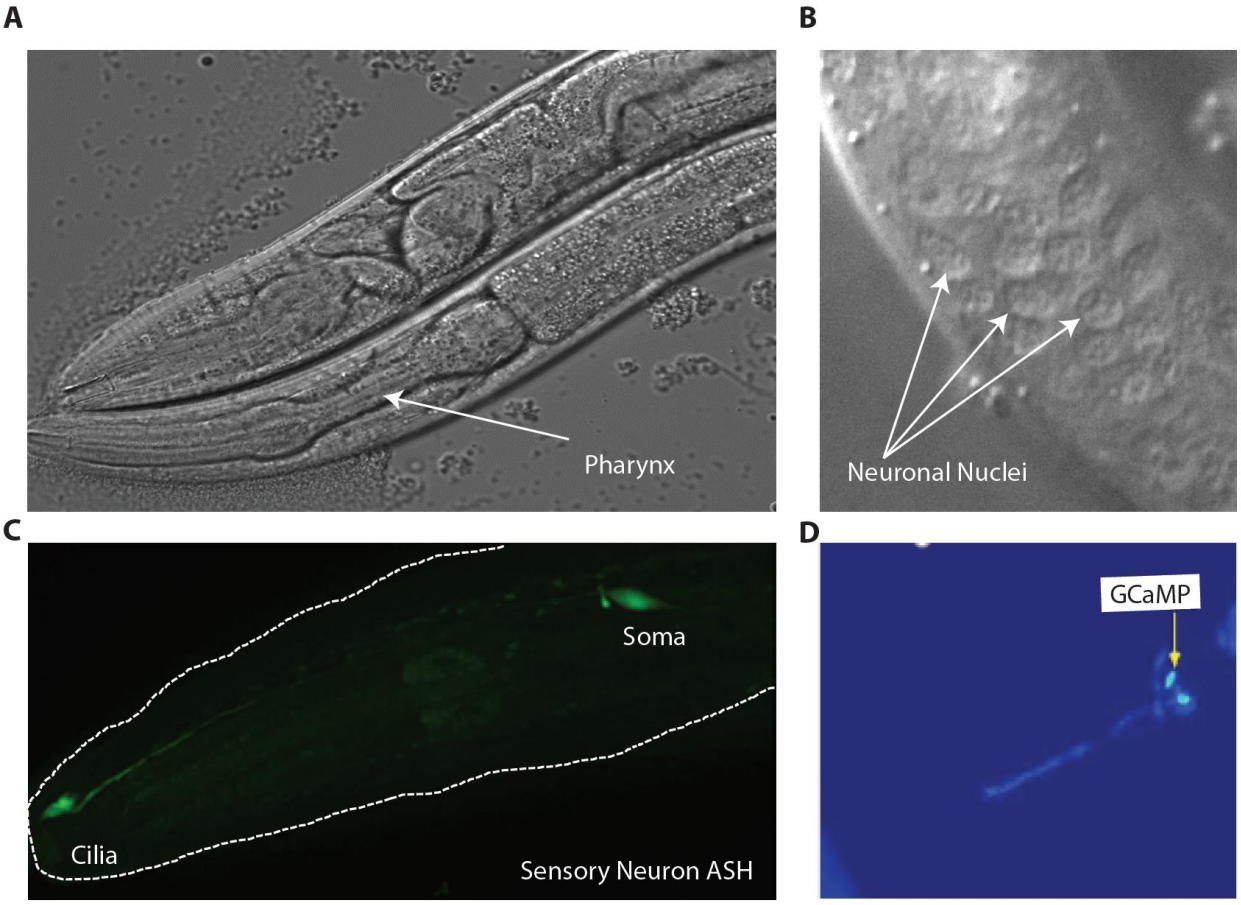


Figure A3: *C. elegans* are genetically tractable, transparent organisms. **A)** Tissues such as the pharynx (shown), gonadal arm, and intestines can be seen and observed for abnormalities. **B)** Due to the animals transparency and eutelic cell lineage, we can observe specific neuronal nuclei and perform laser microsurgeries. **C)** Due to the animals susceptibility to genetic manipulation and transparency, fluorescent reporters can be injected into the animal to observe cellular localization. **B)** In addition to reporters, similar techniques can be used to observe calcium dynamics in response to stimuli in specific neurons as a readout of neuronal activity.

Importantly, for betterment of our understanding of how underlying social odor pathways are able to dictate behavior, *C. elegans* must demonstrate robust behaviors in response to pheromones. Indeed, nematodes respond to a class of secreted compounds known as ascarosides. These compounds consist of a base ascarylose sugar base, with a fatty-acid derived side chain of varying lengths and substituent groups (59-61). Depending on the physiological state, developmental stage, diet, and sex of the animal, different variations of the ascaroside structure, ranging from side chain saturation to moiety addition on the base sugar, are released from the animal (59, 60, 62). This modularity serves as an alphabet for social communication, where the receiving conspecific is observed to undergo different physiological or behavioral changes upon sensation (63-71). Paramount to understanding social communication is the elucidation of chemosensory mechanisms and the circuitries coding perception.

A.31 Chemosensation in C. elegans

Chemosensory Organ Structures

C. elegans hermaphrodites have 302 neurons, of which 32 (16 types) are likely chemosensory, as they have ciliated endings and are exposed to the environment (45, 55, 72). These neurons extend their dendritic processes into either the amphid, phasmid, or labial organs and are supported and exposed to the environment by mucous-like glial cells, termed socket and sheath cells (45, 55, 72). Cilia either protrude through a pore created by the support cells, or can be embedded within them (45, 55, 72).

There are two amphid pores at the anterior tip of the animal, and each contains one of a pair of the twelve different sensory neurons, eleven of which are chemosensory (ADF, ADL, ASE, ASG, ASH, ASI, ASJ, ASK, AWA, AWB, and AWC) (45, 72). The

nomenclature of these neurons indicates the type of cilia and class of neuron. The first letter refers to the organ, e.g. “A” stands for amphid, whereas the second letter reveals if the cilia consists of a single rod (S), a double rod (D), or a winged, branch shaped cilia (W) (73). The last letter is to identify the class, or pairs of bilaterally symmetrical neurons. All of the aforementioned amphid sensory neurons extend through the socket cell channel with the exception of the wing neurons, which are embedded in the sheath cell (45, 72).

The phasmid organ consists of two pores similar to the amphid chemosensory organ, but located at the tail end of the animal, with external openings posterior to the anus (72, 73). Moreover, the phasmid is much smaller than the amphid organ and consists of only two sensory neurons, PHA and PHB, following the a similar nomenclature (Phasmid, “Ph”, and class “A or B”) (45, 72, 73). The inner labial neurons (IL1 and IL2) surround the mouth of *C. elegans*, and IL2 neurons are exposed to the environment, whereas IL1 neurons are embedded in the sub-cuticle (73).

Chemosensory neuron function

Much information has been elucidated regarding the 11 chemosensory neurons in the amphid sensory organs. For each class of neuron, at least one associated role has been characterized. Three primary methods are currently employed for elucidation of a neurons role in behavior: laser and genetic ablations; cell-specific rescues and knockdowns; and calcium imaging for physiological responses (72). Broadly, the roles can be grouped into three categories: attraction/chemotaxis, avoidance, and dauer regulation (45, 72). Interestingly, some sensory neurons seem to be hardwired in their response, such as AWA, AWB, and ASH (74, 75). On the other hand, other neurons, such as ADF and ASK may be involved in all three categories (45, 46, 76).

One neuron may be involved in all three categories, such as ADF in sex-specific attraction or avoidance to the pheromone ascr#3, and inhibition of dauer formation (76-78). Also unique to the ADF sensory neurons are that they are the only serotonergic amphid sensory neurons under normoxic conditions, and serotonin has been shown to inhibit dauer formation (79, 80).

In addition to ADF, ASG and ASI inhibit dauer entry (77, 78). ASG has additional roles in salt chemotaxis under hypoxic conditions and, in normoxic conditions, a minor role in the absence of ASE sensory neurons (80, 81). Just as ASG plays a minor role in chemotaxis in the absence of ASE, so does ASI (81). However, ASI has been found to be involved much more extensively in dauer regulation. DAF-7, a transforming growth factor beta (TGF-β) like ligand, is typically only expressed, and secreted by ASI sensory neurons when there is favorable food conditions and low levels of dauer pheromones (82, 83). The presence of DAF-7 signaling prevents dauer formation, as animals lacking *daf-7* result in constitutive dauer formation, even in the presence of favorable conditions (83). Interestingly, this signaling pathway also regulates fat storage and feeding rates (82). Thus, ASI also plays a role in energy balance. In addition to the ASI sensory neurons' role in dauer inhibition, the chemoreceptors SRG-36 and DAF-37 are required in ASI sensory neurons for dauer induction by ascr#5 and ascr#2, respectively (75, 84). Additionally, the sensory pair also contributes to regulating avoidance, roaming, and dwelling behaviors (85-89). These studies indicate that ASI is a key player in accessing nutrient availability in the environment to dictate the appropriate developmental response.

ASK, like the aforementioned neurons, has multiple roles, and is involved in attraction, avoidance, and dauer regulation (77, 89, 90). ASK plays a role in avoidance to

ascr#2, #3, and #5 (46, 67, 84). Specifically, ASK plays a primary role in avoidance to ascr#2, with this being dependent upon expression of the GPCR, DAF-37 (84). To a lesser extent, ASK mediates aversive responses when the ASH neurons are missing (91, 92). Additionally, ASK plays a role in attraction to ascarosides ascr#3 and icas#3, in concentration dependent manners (67, 68). Moreover, ASK, dependent on the social status of the animal, is required for hermaphrodite aversion to combinations of ascr#2, ascr#3, and ascr#5 (46, 93). Attraction to the amino acid lysine has also been shown regulated through ASK (81).

The last sensory neuron involved in dauer regulation is ASJ (77, 78). While less is known about this sensory neuron and what it may be detecting to induce dauer formation, it has been demonstrated that this chemosensory neuron promotes avoidance behavior in response to metabolites secreted by the pathogenic bacteria, *P. aeruginosa* (94). Interestingly, upon detection of secondary metabolites of *P. aeruginosa*, DAF-7 is produced in not only ASI, but ASJ as well (94). This production is necessary in ASJ for wild-type avoidance to the pathogenic bacteria (94).

The remaining chemosensory neurons (ADL, ASE, ASH, AWA, AWB, and AWC) are not involved in dauer regulation, and tend to have one characteristic response: either avoidance or attraction (45). ADL, ASH, and AWB are stereotypic aversive driving neurons, whereas AWA, AWC, and ASE are characteristically attractive (45). ADL drives chemosensory avoidance to ascr#3 in hermaphrodites through the hub-and-spoke model of RMG and NPR-1 modulation (46, 93). Furthermore, this avoidance response is increased when animals are starved (95). In addition to pheromone cues, it is likely that

ADL, along with the ASH sensory neurons, detects aversive chemical signals from food, assisting in the promotion of social feeding (96).

ASH sensory neurons are unique among the amphid sensory neurons in that they are polymodal – driving aversive responses from chemo-, osmo-, and mechanical stimuli (91, 92, 96-106). These stimuli include, but are not limited to: quinine, 1-octanol, glycerol, SDS, and copper (87, 91, 92, 107). This nociceptive pair is also required for mechano-response to nose touch (100). Intriguingly, stimulation of ASH always results in avoidance behavior, suggesting it to be a hardwired, invariant response. However, divergent signaling transduction pathways and synaptic targets give rise to same behavioral phenotype. For example, response to mechanosensation requires the IP_3 receptor, ITR-1 – but responses to osmotic stimuli do not (108). Moreover, the post-synaptic targets between the two modes of stimulation differ, as only nose touch and 1-octanol, and not osmotic stress, requires the glutamate receptor, GLR-1 (107, 109). Despite these differences in signaling pathways, stimulation of ASH sensory neurons always drives avoidance behavior.

It is probable that these different pathways arose to allow for finely tuning aversive response by input to ASH neurons themselves, such as by altering sensitivity based on the presence of food or cross-inhibition (87, 107). Different post-synaptic targets of ASH, such as the first layer amphid sensory neurons and command interneurons, also likely evolved to allow for adjusting the avoidance response to specific stimuli with respect to internal states and external conditions. Indeed, the first layer amphid interneurons integrate information from ASH, AWC, and ASE sensory neurons to adjust avoidance with respect to multiple sensory inputs (105, 110, 111). When off food, glutamate

signaling from AWC sensory neurons stimulates the AIB interneurons, and peptidergic signaling (NLP-9) from ASI prevents ASER (right ASE neuron) glutamate signaling from inhibiting AIB (105, 110). Ultimately, this signaling enhances the 1-octanol avoidance response (110) (Fig. A4).

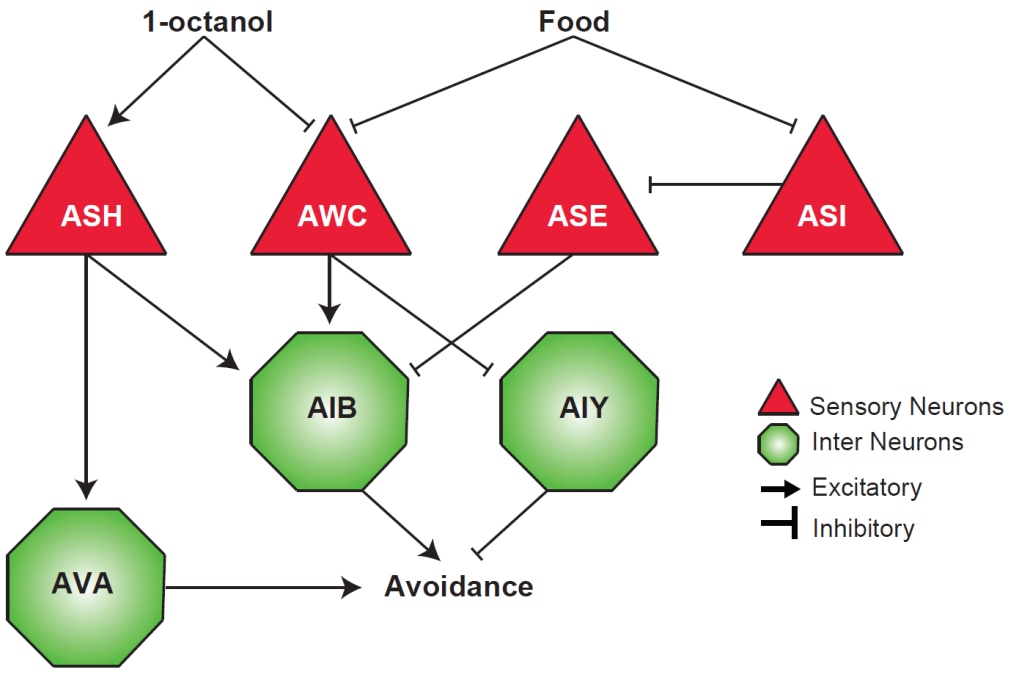


Figure A4: Integration of sensory inputs allows for finely tuning aversive behavior. Depicted here is multisensory integration of the repellent 1-octanol and attractive food cues. 1-octanol alone results in sustained reversal behavior. When food is detected simultaneously, the information is integrated by the first layer and command interneurons which tune the aversive response to be a shorter non-committed reversal followed by continued forward motion. Figure made from data presented in Summers *et al*. 2015, and Hapiak *et al.* 2013.

The last sensory pair characteristic of driving avoidance responses are the AWB chemosensory neurons. Like ASH, these neurons have a role in 1-octanol avoidance (74, 107). However, the role of AWB sensory neurons in 1-octanol avoidance is minor, and auxiliary to that of ASH detection, and is also dependent upon starvation (off food) (74, 107). The role of AWB in avoidance response to 2-nonanone is dramatically more robust than 1-octanol, and taken together, indicates that AWB drives aversive responses (74). Interestingly, this finding led to the use of stereotyped neurons for supporting the identification of receptors associated with ligands. It was found that expression of the GPCR, ODR-10, which is required for attractive behavior to the odor diacetyl in AWA, could drive avoidance behavior if ectopically expressed in AWB (74). This "reprogramming" of neurons to confirm receptor-ligand relationships has been used since to link icas#9 and ascr#5 to the receptors SRX-43 and SRG-36/37, respectively (75, 86).

AWA and AWC neurons preferentially code for attractive behaviors in response to volatile odors. The role of these chemosensory neurons was originally characterized through laser ablation studies and observation of defective chemotaxis to a variety of volatile compounds (112). Of the seven representative compounds tested (50 out of 121 volatiles were found to be attractive), six were found to be sensed by either AWA or AWC (112). AWA was found to be primarily required for chemotaxis towards diacetyl and pyrizine, whereas AWC sensory neurons are required for isoamyl alcohol, benzaldehyde, and butanone (112). AWA and AWC show redundancy in response to the remaining representative compound, trimethylthiazole (112). Interestingly, unlike the majority of the bilaterally symmetric amphid sensory pairs, AWC exhibits asymmetric function that arises from random, but coordinated, differential calcium signaling during development (113).

As a result, one of the AWC sensory neurons expresses the GPCR, STR-2, and is referred to as AWC^{on}, whereas the other neuron in the pair does not (AWC^{off}) (113). Since this characterization, the pair has been found to differentially sense attractants, and loss of asymmetry inhibits the ability to discriminate and respond to odors properly (114, 115).

The ASE sensory neurons are asymmetric as well. However, unlike AWC sensory neurons, the left and right functions are consistent in all animals (113, 116). The right ASE neuron, ASER, exclusively expresses the guanylyl cyclases GCY-1, GCY-2, GCY-4, GCY-5, and GCY-22; whereas GCY-6, GCY-7, GCY-14, and GCY-20 proteins are only present in the left, ASEL neuron (116, 117). The asymmetric fate of the pair shows functional disparity as well, ASER is responsible for attraction to potassium, bromine, iodine, and chloride; while ASEL detects sodium and magnesium (44, 118). It is not so surprising the guanylyl cyclases are responsible for asymmetric ASE detection of certain salts, given the differential expression of nine of these proteins within this sensory pair (44, 117). Overall, this asymmetry allows for discrimination of various salts to finely tune the chemotactic behavioral response (44).

Phasmids/Labial neurons

Predominately, functional characterization of these chemosensory neurons has focused on the role of the amphid sensory neurons. The phasmid neurons have recently been characterized as polymodal nociceptors that are stimulated by chemical and mechanical cues (119). Behaviorally, the phasmid neurons are the primary sensory neurons required for mechanosensation of harsh anal touch, and integrate information along with the amphid sensory neurons (primarily ASH) to create a head-tail map of repellents in the environment (91, 120). In this map, the amphid neurons dominate in

driving avoidance responses, but the phasmids antagonize the response, and fine-tune it (91).

Even less is known about the function of the labial neurons. To date, the only known role for IL2 neurons neurons is orchestrating nictation, a behavior that consists of lifting the anterior end of the body (standing on tail), and moving the head in all three dimensions (121).

A.32 G protein signaling

The nervous system of *C. elegans* shares many conserved pathways with vertebrates. Namely, the regulation and release of neuromodulators that interact with G protein-coupled receptors (GPCRs) and ligand gated channels (122). Heterotrimeric signaling in *C. elegans* functions to transduce signals in the same manner as vertebrates. A GPCR, containing seven transmembrane domains, rests within the plasma membrane, and is associated with three G protein subunits (Gα, Gβ, and Gγ) when inactivated. In the inactivated state, guanine diphosphate (GDP) is complexed with the Gα subunit (Fig. A5). Upon ligand binding, conformational changes in the receptor result in a guanine triphosphate (GTP) replacing the GDP on the α subunit (Fig. A5). Thus, the GPCR functions as a guanine exchange factor upon activation by a ligand. This exchange in turn dissociates the GPCR, Gα subunit, and the Gβγ complex. The dissociated subunits then proceed to activate downstream effectors, resulting in secondary messenger flux and further signal propagation (Fig. A5). The signal is ceased by the Gα subunits inherent ability to hydrolyze GTP back to GDP, upon which, the subunits re-associate back to the heterotrimeric state. Additional proteins, such as regulators of G protein signaling (RGS),

GTPase-activating proteins (GAPs), and GPCR kinases (GRKs) can modify the signal, and even trigger downstream signaling without ligand binding.

Within the Gα subunits, there are four families, based on sequence similarities and physiological function: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ (123-125). The canonical pathways of these subunits are described here, and are portrayed in (Fig. A5). The $G\alpha_s$ and $G\alpha_i$ families signal to adenylyl cyclases in either stimulatory or inhibitory manners. Stimulation of adenylyl cyclase increases cyclic adenosine monophosphate (cAMP) conversion from adenosine triphosphate (ATP), which in turn serves as a secondary messenger activating various downstream effectors, such as protein kinase A and cyclic nucleotide gated channels (123-125) (Fig. A5). $G\alpha_q$ subunits act via a different pathway, stimulating the production of inositol triphosphate (IP_3) and diacylglycerol (DAG) through hydrolysis of phosphatidylinositol bisphosphate (PIP_2) by phospholipase C – β isoform (PLC-β) (123-125). IP_3 and DAG act as secondary messengers to release intracellular calcium and activate protein kinase C (PKC) (123-125) (Fig. A5). Lastly, the $G\alpha_{12}$ family regulates the activity of the GTPase, Rho, indirectly through Rho-guanine exchange factors (123-125).

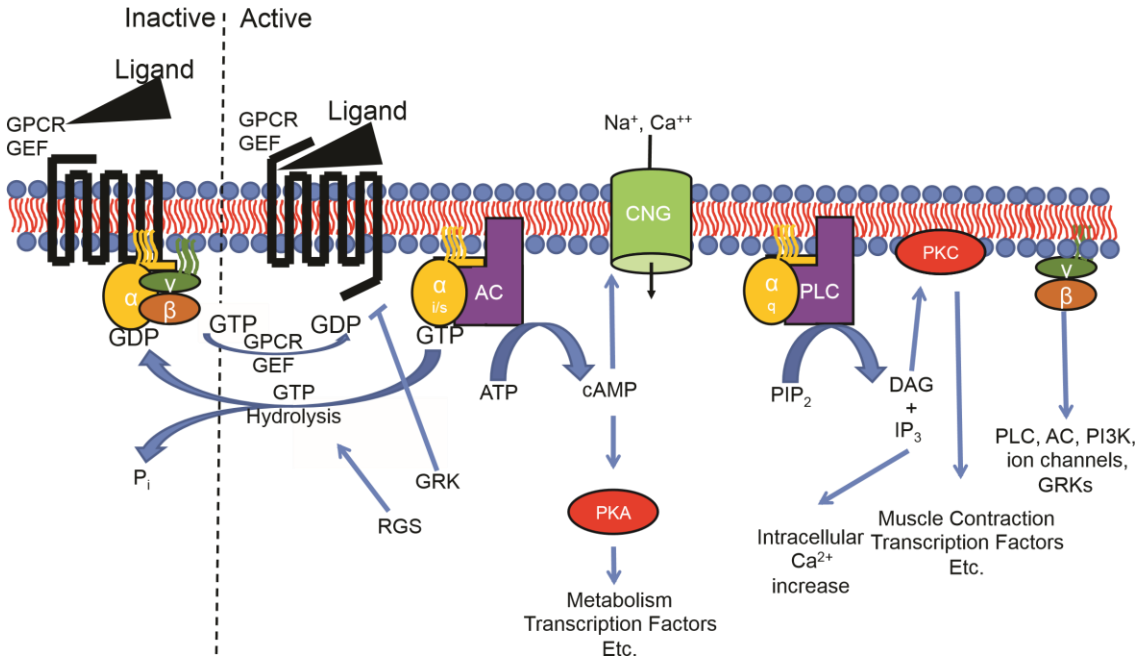


Figure A5: Summary of G protein signaling pathways. Left of vertical dotted line: Inactive state of a G protein-coupled receptor (GPCR), thus heterotrimeric G proteins (Gα, Gβ, and Gγ) are associated with the receptor. Right of vertical dotted line: Upon activation of the GPCR by a ligand, it acts as a guanine exchange factor and GTP replaces GDP, resulting in dissociation of the heterotrimeric proteins. The subunits go on to modulate secondary messengers based on their type: e.g. Gαi/s is shown which regulates adenylyl cyclase and Gαq is shown which modulates phospholipase c activity. The cessation of the signal occurs by natural hydrolysis of GTP back to GDP, and can be modulated by regulators for G proteins signaling (RGS). Gαq subunits are reverted back to heterotrimeric proteins in the same way depicted as Gαi/s shown, but omitted, along with Gα12 for simplicity.

C. elegans shares clear homology with mammalian G protein signaling molecules and regulators, and consists of 21 Gα subunits and two each of the Gβ and Gγ subunits (124). In each of the Gα families there is one orthologous gene: EGL-30 (G_q) GOA-1 (G_i), GSA-1 (G_s), and GPA-12 (G_{12}) (124). The Gβ subunits, GPB-1 and GPB-2, share amino acid similarity with mammals, and GPB-1 is required for *C. elegans* viability (124, 126). Interestingly, GPB-2 is a homolog of the mammalian $Gβ_5$, which is unique among mammalian Gβ proteins in several ways; namely, sharing with GPB-2 the ability to interact with RGS proteins (124, 126). Lastly, the Gγ subunits, GPC-1 and GPC-2 are not orthologous to mammalian subunits (124, 127). GPC-2 is ubiquitous and required with GPB-1 for proper development, whereas GPC-1 is expressed solely in the sensory neurons (124, 127). As for regulators of G protein signaling, GRK-1 and GRK-2 are homologous to the human GRK-5; and GRK-2 and GRK-3; respectively (128). RGS proteins, which enhance GTP hydrolysis, and thus terminate signaling, all contain a conserved RGS domain necessary for activating GTPase-activating proteins (GAP) (126) In *C. elegans*, EGL-10 is an orthologue of the human RGS7, containing sequence similarity that goes beyond the conserved domain (129).

Interestingly, the conserved Gα subunits are expressed ubiquitously and play major roles in locomotion and egg laying (127). Of the remaining *C. elegans* Gα subunits, 14 of them are almost exclusively expressed in subsets of sensory neurons (124, 127, 130).

Strikingly, roughly 5% of the *C. elegans* genome is encodes for GPCRs, with at least 500 (potentially 1300) being chemoreceptors (42, 45, 122). When considering the abundance of Gα subunits in the amphid sensory neurons, and the wealth of

chemoreceptors in *C. elegans*, there is reason to believe that the discrimination of different chemical compounds is based in varied receptor expression and signal transduction machinery. Indeed, we do see that different water-soluble deterrents utilize different primary Gα subunits (and presumable different GPCRs) to drive avoidance behaviors via the same neurons (92).

1 A.4 Conclusion

Given that humans can detect at least a trillion different odor combinations, it is likely we utilize pheromones to communicate social behavior (25, 37). However, little is known about pheromone detection in humans, and mammalian systems are limited in deepening our understanding of olfaction at the single-cell level (25). The innate social behaviors in *C. elegans* provide a powerful tool for unlocking the molecular and cellular machinery underlying neural circuits governing pheromone-elicited behaviors. Importantly, many aspects of *C. elegans* olfaction are homologous to mammalian olfaction, especially regarding signal transduction machinery.

To date, the majority of pheromone chemical communication studies with *C. elegans* have been concerned with attractive, aggregating, and dauer forming cues. As for social behavior, much has been elucidated regarding sensory circuits and signal transduction, but it has been primarily concerned with mate attraction and aggregation (see 1.2). There has been limited studies revealing the mechanisms of sex-specific pheromone elicited avoidance, but they have been focused on gender or social modulation, and not modulation by the environment or physiological state (46, 76, 93). Although physiological state has been extensively studied in 1-octonal aversion (131),

whose ecological importance can be debated given its absence in *C. elegans'* natural environment (132), it has not been studied in regards to social behavioral communication.

Throughout my doctoral research, I aimed to better our understanding of sensation and processing of non-sex-specific social aversive cues with respect to the animals' physiological state, and sensation of multiple stimuli on a molecular and cellular level. Deconstruction of these circuitries will bridge the gap in our understanding of brain function. Specifically, it will allow for comparison studies between the sensory strategies underlying evolutionary important social behaviors, ranging from reproduction to predatory aversion, potentiating our understanding of how the brain codes and integrates different social modalities.

1 B Chemical Mating Cues (Chute and Srinivasan 2014)

Chapter 1, part B is copyrighted material (doi: 10.1016/j.semcdb.2014.06.002). This section serves to introduce and discuss the importance of social communication in mate attraction in *C. elegans.* The published manuscript can be found in the addendum.

In this review, chemical signals that govern attraction and aggregation behavior in *C. elegans* will be discussed, from the existence and identification of these cues, to the neurons involved in the behavioral response. Specifically, mate attraction is dictated by specific glycosides and side chains of the dideoxysugar ascarylose, a class of molecules known as ascarosides. Intriguingly, modifications of the ascarosides can dictate different behaviors such as male attraction, hermaphrodite attraction, and dauer formation. In general, interactions between core sensory neurons such as ASK and sex-specific neurons like CEM are critical for detecting these small molecules. These data reveal the existence of a complex, synergistic, chemical mating cue system between males and hermaphrodites in *C. elegans*, thereby highlighting the importance of mate attraction in a primarily hermaphroditic population.

1 B.1 Introduction

How organisms interact with the environment is a fundamental question in the study of life. For instance, Darwin's theory of natural selection is based on the concept of the fittest organism passing on its favorable traits. Many of those traits are the ones which allow an organism to best interact with its environment by sensing their surroundings and responding appropriately, e.g. avoiding danger. An important environmental cue is the presence of chemical signals. For instance, detection of certain chemicals can direct animal locomotion, a phenomenon termed chemotaxis by the German botanist W. Pfeffer who observed sperm attraction to ova in ferns (133). Organismal behavior in response to chemicals has widely been studied with research ranging from oxygen directed attraction in Spirillum in 1901 (134), to the silk moth sex cue bombykol in 1959 (21), and to the first structural identity discovered in the well-known chemotactic ant trails (135). The abundant information being gathered made it necessary to further classify the various chemical signals present in the environment. The discovery of bombykol prompted Karlson and Luscher in 1959 to introduce the term "pheromone" (8). They defined pheromones as substances externally secreted by an organism that induce a specific behavior in another individual of the same species (22). If the pheromone is sex-specific, it is known as a sex pheromone, defined by Shorey as "chemicals produced by either males or females that stimulate one or more behavioral reactions in the opposite sex" (136). Though there have been many mating pheromones identified in a broad array of organisms such as insects (136, 137), fish (138), reptiles (139), amphibians (140), birds (141), nematodes (142, 143), and even humans (144, 145), it was not until 2002 that researchers showed evidence of a chemical cue involved in mate finding in the popular model organism

Caenorhabditis elegans. In this review we are interested in the sex pheromones produced by *C. elegans* and their effect on conspecific's behavior.

1 B.2 Evidence of pheromone mating cue(s) in *C. elegans*

In 2002, Simon and Sternberg demonstrated the presence of a *C. elegans* mating cue through several different bioassays consisting of sex-specific conditioned spots (Fig. B1b–d). The researchers used Cel-*unc-52* mutant hermaphrodites, which have an immobile phenotype, to condition specific spots on agar plates with hermaphrodite secretions. They quantified male behavior by looking at the response, attraction, and holding effects of the conditioned spots. To do this, researchers measured if the conditioned spot caused a response by quantifying reversals at a spot's edge (response assay, Fig. B1b), the time an organism spent in a conditioned area and the proximity to the spot in which the animal would stay (holding assay, Fig. B1c), and lastly, if the conditioned spot increased the rate of mate finding (attraction assay, Fig. B1d) (146). These data indicated an existence of a hermaphrodite secreted cue that attracts and holds males within close proximity, suggesting *C. elegans* hermaphrodites produce sex-specific mating cues (146). In a subsequent study, Lipton et al. produced data in agreement with Simon and Sternberg showing that there is a sex-specific chemical attractant secreted by the hermaphrodite. Lipton et al. used a leaving assay, which measures the time elapsed before *C. elegans* animals would leave a bacterial lawn (Fig. B1e). They found that isolated adult males tend to leave a bacterial lawn much faster than younger males and hermaphrodites, unless a hermaphrodite was placed on the lawn as well (147). Additionally, removing a hermaphrodite from the bacterial lawn causes males to quickly leave the food source in search of a mate (147). It is important to note that there

was no significant decrease in male leaving rates when in the presence of other males, and that the male did not need to be in direct contact with hermaphrodites to reduce leaving behavior. Thus, there must be a sex-specific signal from the hermaphrodite that keeps males on the bacterial lawn rather than mate seeking. Expectedly, males that were starved before the assay showed a reduced leaving rate presumably due to a physiological need for nutrition (147). Laser ablation of the gonads decreased male leaving behavior and increased hermaphrodite leaving behavior (147), suggesting that the cue is created and/or integrated by the sexually dimorphic gonadal system. Jamie White and coworkers in 2007 further demonstrated that the cue is secreted rather than present on the hermaphrodite cuticle (148). Spot bioassays were used to measure *C. elegans* chemotactic response, but the spots were conditioned with hermaphrodite liquid culture droplets (148), as opposed to conditioning techniques using the animal itself, as was performed in previous studies (146, 147). The authors found that *C. elegans* males would spend significantly more time in a region conditioned with hermaphrodite liquid culture droplets than they would in a control region. In addition, only sexually mature males are attracted to the mating signal (148) which coincides with results from the aforementioned study showing that sexually mature males have the highest leaving rate (147). Furthermore, overexpressing *fem-3* in neurons to masculinize the hermaphrodite nervous system (149), results in a hermaphrodite phenotype that responds as strongly as males to the attractant pheromone (148). This nervous system sex-reversal implies that the sex-specific mating cue response is primarily dependent upon the sex of the nervous system, and not the sex of the gonadal system. Despite aforementioned papers, a study by Chasnov et al. (150) produced contradicting results (150). They used a similar spot

attraction assay as White et al. with the addition of sodium-azide to the conditioned and control spots, paralyzing the worms in the spot they first enter (150) . Their chemotaxis experiments found that *C. elegans* hermaphrodites did not elicit attraction in *C. elegans* males, but females of other Caenohabditis species did (150). Thus, they concluded that the *C. elegans* hermaphrodites must have lost the ability to produce such molecular attractants (150). The authors also assayed for the involvement of the male-specific CEM neurons. They used mutant strains and laser ablated CEM neurons to demonstrate that proper CEM function is necessary for male attraction in their bioassay. While their results were in contrast with other studies about *C. elegans* hermaphrodites producing an attractant pheromone, their study is in agreement that the CEM neurons are required for male mating cue response and that only sexually mature males respond to the attractant pheromone cue (67, 148, 150).

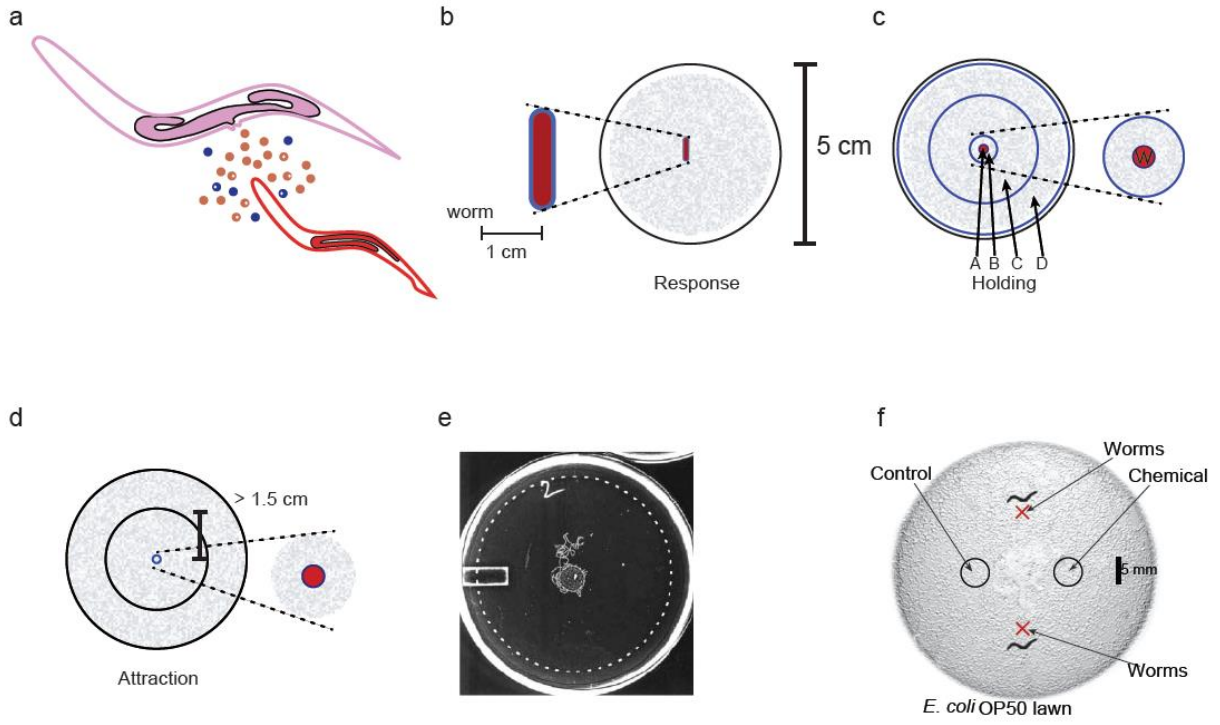


Fig. B1. Behavioral bioassays used to demonstrate presence of a *C. elegans* produced mating signal. (a) Illustration of sex-specific chemical attraction. (b) The response assay measured the time spent in a conditioned spot, as well as, the number of reversals associated with the spot once the male encountered the conditioned zone. (c) The holding assay measured time males spent on conditioned spot when placed directly on it. (d) The attraction assay measured the amount of time it took for the males to encounter the hermaphrodite when placed >1.5 cm away. (e) Lipton et al. used a leaving assay that compared the time elapsed until leaving a food lawn in isolation versus the presence of conspecifics. (f) Srinivasan, Kaplan et al. made use of a spot assay using discovered secreted molecules to measure time males spent in a control spot versus chemical spot.

1 B.3 Identification and characterization of male specific attractant cues

Srinivasan, Kaplan and coworkers used a new technique for isolating pheromones secreted by *C. elegans* (67). Using synchronized cultures of *C. elegans* grown in liquid media, the researchers generated worm conditioned water specific to each developmental stage. Through several washes of the worms, the final conditioned water confidently contained only *C. elegans* derived molecules secreted from the animals. The conditioned water, containing the secreted *C. elegans* metabolites, is referred to as the external metabolome, or the “exo-metabolome”. Metabolome refers to all the metabolites in *C. elegans*, and external specifically refers to the metabolites that are secreted. They found that the exo-metabolome from L4 and adult hermaphrodites elicited male chemoattraction using a spot based chemotaxis bioassay (Fig. B1f) (67). This assay measured time spent in the conditioned spot versus a control spot. The researchers then fractionated the exo-metabolome water using C18-reverse-phase solid-phase extraction chromatography, effectively separating the metabolites into different fractions. *C. elegans* males were then subjected to the different fractions, by means of the assay mentioned above, in order to hone in on what molecule(s) are responsible for male attraction. The assay revealed that combinations of fractions are required to reconstitute attraction levels similiar to the natural exo-metabolome (67). Nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography–mass spectrometry (LC–MS) analysis of the fractions resulting in a chemotactic response revealed the abundant presence of a class of molecules called ascarosides, specifically, ascaroside #2, #3, and #4 (abbreviated ascr) (Table B1). The nomenclature comes from the structure’s resemblance to the lipid derived molecules first identified in the parasitic nematode Ascaris lumbricoides in 1912

(151), and structurally elucidated in 1957 (152, 153). *C. elegans* male attraction was measured at different concentrations of ascr#2, ascr#3, and ascr#4, as well as a combinations of the three. It was found that the males displayed a characteristic bell shaped (normal distribution) response to ascr#2 and ascr#3, meaning males would not respond if the concentration was too low or high. The most potent response was a result of a mixture of the small molecules at their respective physiological levels, as determined by LC–MS, demonstrating that the molecules governing the male response are synergistic (67). Interestingly, ascr#1 and ascr#2 were previously identified as components of the dauer pheromone (69, 154). This suggests that the ascarosides are a molecular link between reproductive and developmental pathways of social signaling in *C. elegans*. Srinivasan, Kaplan et al. used exo-metabolome conditioned water of *daf-22* mutants to assay male behavior; *daf-22* has been shown to be required for formation of dauer inducing pheromones (155). The bioassays resulted in no male attraction effect, thus, confirming that the same small molecules, ascarosides, are involved in both male attraction and dauer formation (67). Additionally, in 2008, Butcher et al. found that the dauer response is based on synergism of the ascarosides (71), like the male attraction mating signal. Kaplan and coworkers were able to further demonstrate the link between development and reproduction. Different concentrations of the chemical cues were tested, showing that only *C. elegans* males were attracted to ascr#2 and ascr#3 at femtomolar concentrations. At high, dauer-forming concentrations, males were not attracted, and hermaphrodites were strongly deterred (67). Thus, the male specific chemical attractant at concentrations relevant to high population density, which we know is unfavorable to *C. elegans* based on dauer formation (51), repels hermaphrodites and

ceases to attract males (46, 67). In conjunction with other studies, this data suggests that that the effect of mating cues depends upon population (67) and food availability (147). Subsequent studies demonstrated that starved *C. elegans* hermaphrodites produce significantly more ascr#3 than fed hermaphrodites (62, 156). Again, high concentrations of ascr#3 do not attract males. It can be determined that the mating cues released by hermaphrodites are complex and stringently regulated, as is the male response to those molecules. Although ascr#2, #3, and #4 mixtures produced potent attraction, the combination did not reconstitute the same level of male attraction as the hermaphrodite exo-metabolome, suggesting the presence of other, unidentified, mate attraction molecules in the fractions tested by Srinivasan, Kaplan et al. in 2008. In order to identify novel compounds present in the hermaphrodite exo-metabolome not characterized by NMR and LC–MS, an unbiased metabolomics profiling technique termed Differential Analysis by 2D NMR Spectroscopy (DANS) (157) was used. To do this, the exo-metabolome of *daf-22* mutants was compared to the wild-type exo-metabolome (Fig. B2a) (66). This technique identified several previously missed ascarosides as well as the formerly identified mate signaling ascarosides (Fig. B2b). Using the spot assay from Srinivasan, Kaplan et al. (Fig. B1e), one of the four newly discovered ascarosides, ascr#8, was found to induce male attraction (Table B1) (66). Male *C. elegans* attraction was then measured in response to ascr#8 mixed with the other known mate signals. The inclusion of ascr#8 restored male attraction behavior to levels similar to the hermaphrodite exo-metabolome (66). All together the three strongest male attractant molecules when combined are ascr#2, ascr#3, and ascr#8. The strongest individually is ascr#3 (66, 67). These studies suggest several possibilities, which might explain the lack of a *C. elegans*

male specific mating cue secreted by *C. elegans* hermaphrodites observed by Chasnov et al. (150). Foremost, the discrepancy is likely related to the rather limited range of ascaroside concentrations observed to elicit attraction in *C. elegans* males. Ascr#3 and ascr#8 mainly induce male attraction at picomole amounts of 0.1–1 pmol and 1–10 pmol, respectively; concentrations outside of this range quickly taper off in their ability to attract males (66, 67). It is plausible that the experimental conditions of Chasnov et al. produced concentrations of the synergistic molecules that fell outside of the attraction behavioral range. This hypothesis is further supported by data that shows that hermaphrodite *C. elegans* secretions are dependent upon environmental factors such as nutritional state (156) and temperature [29]. Chasnov et al.'s experiments used overnight soaking of hermaphrodite *C. elegans*, which may have possibly resulted in a concentration of ascarosides too high for attraction. The authors also tested starved hermaphrodite extract for male attraction, however, starved hermaphrodites secrete significantly higher concentrations of ascr#3 in comparison to fed hermaphrodites (156), and high ascr#3 concentrations do not attract males (67). Further-more, the researchers collected the conditioned media at 25 ◦C and 30 ◦C. These temperatures are known to cause dauer inducing concentrations of ascarosides to be secreted (71). Hence, it seems that the conditions they tested, resulted in non-attractive concentrations of the male attracting chemical cues.

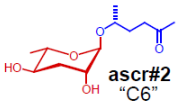
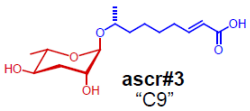
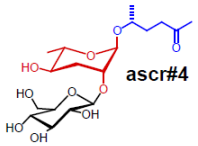
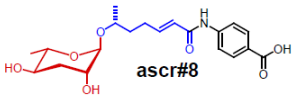
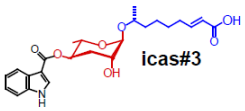
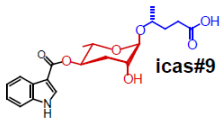
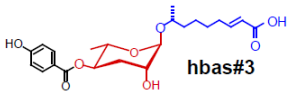
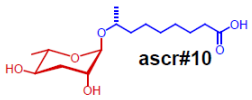
Molecule	Function	Reference
 ascr#2 "C6"	**Male attraction** * Synergy with ascr#3, ascr#4 and ascr#8	Srinivasan, Kaplan et al., 2008, Pungaliya et al , 2009
 ascr#3 "C9"	**Male attraction** * Synergy with ascr#2, ascr#4 and ascr#8	Srinivasan, Kaplan et al., 2008, Pungaliya et al , 2009
 ascr#4	**Male attraction** * Synergy with ascr#2 and ascr#3	Srinivasan, Kaplan et al., 2008,
 ascr#8	**Male attraction** * Strongest attraction in synergy with ascr#2 and ascr#3	Pungaliya et al , 2009
 icas#3	**Hermaphrodite aggregation**	Srinivasan, von Reuss et al. 2012
 icas#9	**Hermaphrodite aggregation**	Srinivasan, von Reuss et al. 2012
 hbas#3	**Hermaphrodite aggregation**	von Reuss et al. 2012
 ascr#10	**Hermaphrodite attraction**	Izrayelit et al., 2012

Table B1. Summary of the important mating cues discovered in *C. elegans*. Ascaroside structures display remarkable diversity; blue marks moiety derived from lipids, red marks the dideoxysugar ascarylose, and black the additional moiety and their function as mating cues. Adapted from ref. (158)

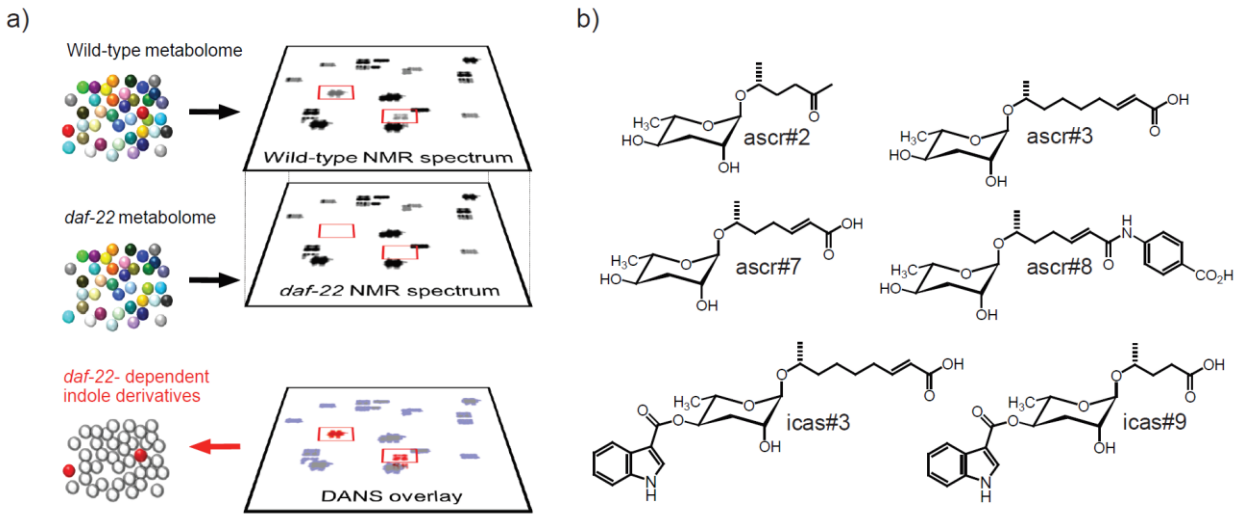


Fig. B2. (a) Comparison of wild type exo-metabolome to *daf-22* exo-metabolome using differential analysis by 2D-NMR spectroscopy (DANS) to identify ascarosides secreted by hermaphrodite *C. elegans*, (b) structures identified by DANS method.

Adapted from Ref. (66).

1 B.4 Chemical cues attracting hermaphrodites

1 B.41 *Hermaphrodite produced hermaphrodite attractants*

Until 2012 there was no evidence of a sex pheromone that attracted *C. elegans* hermaphrodites. However, a new class of ascaroside molecules, containing an indole moiety, was discovered by Butcher et al. and were found to induce dauer formation (70). More recently, Srinivasan et al. identified several indole ascarosides by means of DANS and MS, between wild type and *daf-22 C. elegans* hermaphrodites (Fig. B2) (68). They termed these molecules indole carboxy ascarosides, or icas. The ascarosides were found to be modified by the addition of a tryptophan derived moiety to the ascarylose (Table B1) (68). Out of the five icas's discovered, icas#3 was found to be the most prevalent, and produced at a level 10–40 fold less than its non-indole form, ascr#3. Using spot chemoattraction bioassays they demonstrated that at physiological levels, icas#3 and icas#9 attracted hermaphrodites only and induced aggregation, with icas#3 eliciting the strongest effect (68). Interestingly, icas#3 is a competing signal with ascr#3. Ascr#3 deters hermaphrodites at high concentrations but at low concentrations has no affect (67), whereas icas#3 has no affect at high concentrations but attracts at low concentrations (68). The attraction effect of icas#3 is voided if there are signals of high population density via ascr#3. When population density is low, hermaphrodites are drawn to each other and aggregate by detection of icas#3. It is important to note that icas#1, icas#3, and icas#9 do attract males at high concentrations, but not at low concentrations (68). Recently, another potent hermaphrodite produced hermaphrodite attractant has been found. In 2012, von Reuss et al., discovered a robust attraction molecule using an altered HPLC–MS/MS analysis (156). Yet again it was an altered ascaroside #3, this time with a

hydroxybenzoyl at the four carbon of the sugar, termed hbas#3 (Table B1) (156). Hbas#3 was found to be an extremely attractive pheromone that elicits behavioral affects in hermaphrodites at a mere 0.001 fmol concentration (156), compared to 10 fmol for icas#3 (68).

1 B.42 *Males also produce hermaphrodite attraction cues*

In 2012, Izrayelit et al. found a pheromone produced by *C. elegans* males [38]. HPLC–MS was used to unveil the composition of *C. elegans him-5* males exo-metabolome similiar to studies on *C. elegans* hermaphrodites (68, 156). They found several key differences between wild type hermaphrodites and *him-5* males. Males secreted significantly less ascr#3 (64), a primary male attractant (66-68), and significantly more icas#3 (64), a primary hermaphrodite attractant (68), than wild type hermaphrodites. A previously understudied molecule, ascr#10, was the dominant component of the male exo-metabolome. It is present in a significantly higher concentration in *him-5* males than in hermaphrodites, whereas ascr#3 concentrations are significantly lower (64). Although the only difference in ascr#10 from ascr#3 is a saturated carbon chain (Table B1), the effects are significant. Ascr#10 does not attract males at any concentration, and yet heavily attracts hermaphrodites, even at levels as low as 1 attomole (64). Ascr#3 provokes no response from hermaphrodites at 1 pmol but actually deters them at 10 pmol (67). Additionally, increasing the density of males altered the secretion levels of ascr#3 and ascr#10; doubling the amount of males in a given space led to a near four-fold increase in ascr#10 secretion and less ascr#3 secretion (64). Contrarily, hermaphrodites' secretion rates did not change (156), which is to be expected since the pheromones are

known to be secreted constitutively (51). Albeit, there is evidence that there is at least one mating cue secreted by the hermaphrodite that does vary (159).

1 B.43 Unidentified chemical mating signals produced by hermaphrodites

In 2011, it was shown that hermaphrodites could successfully reproduce with males for a week after self-sperm depletion (160). If the self-depleted hermaphrodite is still able to reproduce with males for nearly a third of its lifespan, it would make sense that the nematode evolved to further attract males at this time point to increase diversity of offspring. Morsci et al. investigated if there is, in fact, variation in male attraction to hermaphrodites before and after self-sperm depletion. They found that when less sperm is present in the hermaphrodite, males were more likely to attempt to mate (159). Male attraction to old age hermaphrodites in comparison to the first day of maturity is three times higher in *pkd-2* sensory defective mutants and was not dependent upon the known male attractant ascarosides (159). This indicates that there is possibly, yet another mating signal produced and another response pathway. Based on the nature of Morsci and colleague's assay, it needs to be determined if the attraction increase at late age was due to secretions or a molecule present on the hermaphrodite cuticle.

1 B.44*. Genetic and neuronal regulation of sex-specific chemical signaling*

The existence of several sex-specific chemical attractants in *C. elegans* indicates receptor and signaling pathway differences between the hermaphrodites and males. While characterizing the mating cues and their behavioral affects, researchers have discovered both genes and neurons required for the chemical mating signals. White et al. were amongst the first to identify proteins and neurons required for male attraction behavior. Using reverse genetics and their attraction bioassay, the researchers found that

the transient receptor potential vanilloid (TRPV) channel OSM-9 was necessary for normal levels of attraction. Further screening found that a double mutant, *osm-9; tax-4*, showed no attraction to the hermaphrodite conditioned spots (148), and yet, a *tax-4* mutation alone did not reduce attraction. Similarly, OCR-1 and OCR-2 mutations alone did not show a defect in attraction, but the double mutation defective behavior resembled levels seen in the OSM-9 mutation (148) . These results demonstrate the complexity involved in chemical mate attraction signaling in *C. elegans* and support later findings that male attraction behavior is governed by synergy amongst several ascarosides (67). White et al. then examined attraction behavior after ablating both the CEM neurons and neurons known to express *osm-9* in L4 males. They found that the two sensory neurons AWA and AWC were required for normal attraction behavior, as well as the male specific CEM neurons (148). This finding is in agreement with Chasnov et al. and Srinivasan, Kaplan et al., who both have identified the CEM neurons as being required for male attraction (67, 150). When either the AWA, AWC, or CEM neurons are ablated at an earlier developmental stage (L3) there is no impairment of attraction behavior unless all three are removed (148). Likewise, single genetic mutations lacking functional AWA, AWC, or CEM neurons show no impairment but triple mutants do not show attraction behavior (148). Together, these data demonstrates the neurons' ability to compensate if an alteration is made before the L4 stage. In addition to the CEM requirement, Srinivasan, Kaplan et al. found that the sensory ASK neurons are also necessary for male attraction behavior (67). Specifically, they found the ASK neuron is required for response to ascr#3. AWA and AWC do not appear to be required for ascr#3 attraction behavior. Furthermore, the researchers found that *osm-3* and *osm-6* mutants are defective in response to ascr#3

(67). These data suggests that the synergistic attractant molecules act not through one sensory neuron, but through ASK, AWA, and AWC neurons and also require OSM-3, OSM-6, and OSM-9 (67, 148). The sex-specific response proved to still be more complex than synergy amongst molecules and their pathways. Macosko et al. demonstrated that *npr-1* expression in the interneuron RMG governs the ASK response to pheromone attractant cues (46). Loss-of-function *npr-1* strains show higher RMG activity and elicit an attraction response in hermaphrodites comparable to males (46). This study demonstrates that hermaphrodites are capable of responding to the sex-specific male attractants, but may have mechanisms for suppressing the behavior. It has since been shown that hermaphrodites have the same core neurons required for attraction by ablating the neurons AWC, AWA, and ASK in *daf-7* hermaphrodites (161). White and Jorgenson used reverse genetics to identify hermaphrodites with a phenotype that displayed attraction behavior and found that *daf-7* hermaphrodites demonstrated this behavior (161). Thus *daf-7* is likely involved in repressing this behavior in wild type hermaphrodites. To further test the role of DAF-7 in repressing attraction, the researchers ablated the DAF-7 expressing ASI neuron which resulted in hermaphrodite attraction behavior but only when perturbed during development (161). This suggests that ASI is needed during development to repress male neuron circuitry formation. Furthermore, by genetically causing the release of TGF-β in a *daf-7* mutant, which resulted in the normal repressed attraction in hermaphrodites, they demonstrated that the role of inhibiting attraction in hermaphrodites by ASI is a result of TGF- β release in the *daf-7* pathway (161). A study by Barrios et al. further showed sex-specificity in core neurons shared by both genders. They found that the interneuron AIM required *pdfr-1* and pdf-1 for male

mate searching behavior, but defective or overexpressed *pdfr-1* and *pdf-1* in hermaphrodites did not result in mate searching behavior (162). This demonstrates a difference in downstream connectivity resulting in sexually dimorphic behavior in a shared gender neuron (162). The importance of the sensory neuron ASK for attraction behavior has been well documented by researchers. In addition to its role in male attraction behavior, ASK is involved in hermaphrodite attraction and aggregation behavior. Srinivasan et al. demonstrated, by means of ablation, that ASK is required for the attraction behavior elicited by icas#3 in hermaphrodites (68). However, instead of RMG being downstream of ASK, as is the case for male attraction (46), the interneuron AIA is necessary for hermaphrodite attraction behavior (68). Thus, the *npr-1* level of expression is not important in the hermaphrodite attraction behavior to icas#3.

1 B.5 Conclusions and Future directions

The last decade has seen evidence of the presence of sex pheromones in *C. elegans* and the structural elucidation of these chemical signals. These signals incorporate a complex communication system, having both synergistic elements and sex-specific neuronal circuits governing the response (Fig. B3). These signals are modular in nature and mediate several behaviors. Behavioral responses are not only concentration dependent, but also depend on the current physiological state of the animal. Hence this small-molecule library represents a metabolic link between diverse life history traits such as development and reproduction. In light of all the recent discoveries, there is still much to learn about this ancient form of communication. For instance, how many more ascarosides are present, and what are their functions? What are the genes and proteins involved in signal transduction of these ascarosides? It is predicted that roughly half of *C.*

elegans 1000+ G protein coupled receptors (GPCRs) are located in chemosensory neurons (40, 45). Of those roughly 500 receptors, how many are involved in mate signaling? Given the structural identity of the mating cues, characterizing the specific receptors for each type of ascaroside is an essential first step toward understanding the molecular control underlying ascaroside sensation. Some of these questions have begun to be answered. For instance, Kim et al. found that *srbc- 64* and *srbc-66* are involved, but not exclusively, in perception of ascr#1, ascr#2, and ascr#3 by means of a genetic screen of dauer formation (90). Another receptor, specific to ascr#2, has been deter-mined to be a heterodimer between *daf-37* and *daf-38* (84). These receptor candidates have been shown not to be involved in male attraction through spot bioassays (Chute and Srinivasan, unpublished results). Hence, though these receptors are known to be involved in sensing these molecules, there seems to be specific receptors for different biological processes. We are currently in the process of elucidating the molecular players governing male attraction and the neuronal networks underlying these conserved behaviors. At an organismal level, the synergistic interactions of these signals result in stereotyped behavioral outputs. Accordingly, the organism must recruit different signaling pathways, suggesting the presence of a complex molecular machinery to enact these behaviors. Hence, future studies will focus on understanding the chemoreceptors, their neuronal locations, downstream components and the neural circuits involved in transduction of mating signals. Therefore, a systems-level approach is essential in unveiling the signaling architecture of these signals. This information is crucial in our quest of understanding how an organism locates mates and survives in its natural habitat.

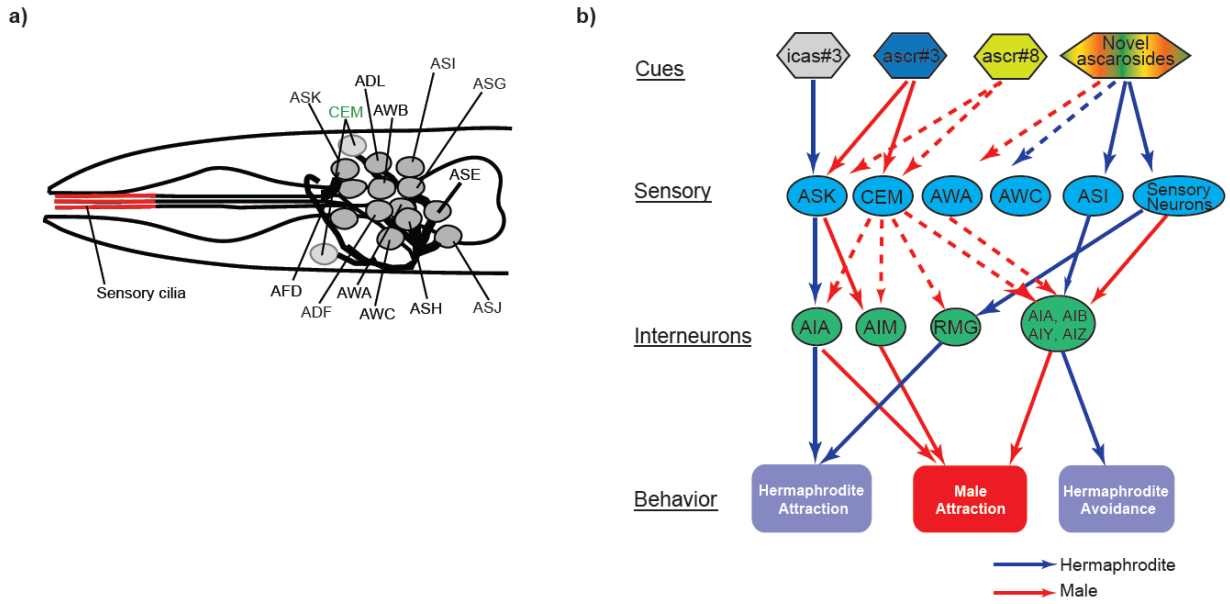


Fig. B3. Neural responses to *C. elegans* chemical mating signals. (A) Sensory neuroanatomy of *C. elegans* indicating different sensory neurons including the male-specific CEM neurons (green). Most of these sensory neurons have sensory cilia that extend to the tip of the nose, thereby sensing the environment. (B) Complex sensory transduction of ascarosides. Different derivatives are sensed by either core sensory neurons or sex-specific neurons. The red and blue lines indicate male signaling and hermaphrodite signaling respectively. The dotted red or blue line indicates a probable pathway that might be used to sense the signals.

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2 Primary Detection of osasa#9

This chapter focuses on understanding the sensation of octopamine succinylated ascaroside #9. This compound is released exclusively by starved, larval stage 1 animals, and results in avoidance in starved conspecifics. In this chapter I pose the ecological significance of this molecule and characterize the minimum requirements needed of the nervous system to drive the behavioral response. Part “A” of this chapter concerns itself with the receptor and sensory neuron required for the osas#9 response and has been submitted to eLife. Part 2 highlights a few preliminary studies in which can inform and lead future work. Together, this work provides the foundation for which the circuit governing the behavior can be built, and ultimately, allows to begin elucidating how starvation state may be modulating this response. It also provides critical findings necessary for building the model circuit of multisensory integration of osas#9 and *E. coli* extract in Chapter Three, and provides for a comparison between a socially aversive pheromone and a predatory kairomone (Chapter Four).

2 A Co-option of neurotransmitter signaling for inter-organismal communication in *C. elegans*

Biogenic amine neurotransmitters play a central role in metazoan biology, and both their chemical structures and cognate receptors are evolutionarily conserved. Their primary roles are in intra-organismal signaling, whereas biogenic amines are not normally recruited for communication between separate individuals. Here, we show that in *C. elegans*, a neurotransmitter-sensing G protein-coupled receptor, TYRA-2, is required for avoidance responses to osas#9, an ascaroside pheromone that incorporates the neurotransmitter octopamine. Neuronal ablation, cell-specific genetic rescue, and calcium imaging show that *tyra-2* expression in the nociceptive neuron ASH is necessary and sufficient to induce osas#9 avoidance. Ectopic expression in the AWA neuron, which is generally associated with attractive responses, reverses the response to osas#9, resulting in attraction instead of avoidance behavior, confirming that TYRA-2 partakes in sensing osas#9. The TYRA-2/osas#9 signaling system thus represents an inter-organismal communication channel that evolved via co-option of a neurotransmitter and its cognate receptor.

2 A.1 Introduction

Inter-organismal communication occurs in several forms across the animal kingdom, both within and between species: prairie dogs use audio alarm calls to signal danger to conspecifics (1), birds display ornate visual cues and dances to attract mates (2), and honeybees dance to signal food location (3). Less apparent, though ancient and ubiquitous across all kingdoms of life, is chemical communication, which underlies social responses driven by chemosensation (4-7). Social chemical communication requires both intra- and inter- organismal signaling. First, a chemical cue is released into the environment by one organism that is then detected by specific receptors in another organism. Upon sensation, intra-organismal signaling pathways, e.g. neurotransmitter signaling, are activated that ultimately coordinate a social response.

Neurotransmitter monoamines such as dopamine, serotonin, tyramine and octopamine serve diverse functions across kingdoms (8). The associated signaling pathways often rely on highly regulated compound biosynthesis, translocation, either by way of diffusion or through active transport, and finally perception by dedicated chemoreceptors. Many neurotransmitters are perceived via G protein-coupled receptors (GPCRs); in fact, there appears to be a close relationship between GPCR diversification and neurotransmitter synthesis in shaping neuronal systems (9). Notably, the most common neurotransmitters share similar behavioral functions across phyla, for example, serotonin is commonly involved in regulating food responses (10-12). Other neurotransmitters, such as tyramine and octopamine, are only found in trace amounts in vertebrates, and in invertebrates act as adrenergic signaling compounds (13-15).

The nematode *Caenorhabditis elegans* affords many advantages for studying social chemical communication and neuronal signaling, namely, the animal's tractability, well-characterized nervous system, and social behavioral responses to pheromones (16, 17). *C. elegans* secretes a class of small molecules, the ascaroside pheromones, which serve diverse functions in inter-organismal chemical signaling (18-20). As a core feature, these molecules include an ascarylose sugar attached to a fatty acid-derived side chain that can be optionally decorated with building blocks from other primary metabolic pathways (21). Ascaroside production, and thus the profile of relayed chemical messages, is strongly dependent on the animal's sex, life stage, environment, and physiological state (22-25). Depending on their specific chemical structures and concentration, the effects of ascaroside signaling vary from social (e.g. attraction to icas#3) to developmental (e.g. induction of dauer by ascr#8) (Fig. 1A) (25-28). Furthermore, different combinations of these ascarosides can act synergistically to elicit a stronger behavioral response than one ascaroside alone, such as male attraction to ascr#2, ascr#3, and ascr#4 (19). Several GPCRs have been identified as chemoreceptors of ascaroside pheromones, such as SRX-43 in ASI in dwelling behavior and DAF-37 in ASK in hermaphrodite repulsion (29-33).

Recently, an ascaroside, named osas#9, that incorporates the neurotransmitter octopamine was identified (22). Osas#9 is produced in large quantities specifically by starved L1 larvae and elicits aversive responses in starved, but not well fed conspecifics (22). The dependency on starvation of both its production and elicited response suggests osas#9 relays information on physiological status and unfavorable foraging conditions. However, it is unknown how osas#9 is perceived and drives starvation-dependent

behavioral responses. Based on the unusual incorporation of a monoamine neurotransmitter building block in osas#9, we asked whether other components of monoamine signaling pathways have been recruited for inter-organismal signaling via osas#9. Here, we show that TYRA-2, an endogenous trace amine receptor, is required for the perception of osas#9, demonstrating co-option of a neurotransmitter and a neurotransmitter receptor for inter-organismal communication.

2 A.2 Results

Aversive responses to osas#9 require the GPCR TYRA-2

Previous work showed that production of the ascaroside osas#9 (Fig. 1A) is starkly increased in starved L1 larvae and elicits avoidance behavior in starved young adult hermaphrodites using a behavioral drop test assay (Fig. 1B) (22). This starvation dependent response is reversible: when animals are starved for an hour, and then reintroduced to food for two hours, no avoidance behavior is observed (Fig. S1A). For the current study we tested a broader range of conditions. We found that osas#9 elicits avoidance regardless of sex or developmental stage of animals (Fig. 1C), and that osas#9 is active over a broad range of concentrations (fM - µM) (Fig. S1B). 1 µM osas#9 was used for the remainder of this study unless otherwise noted (Fig. 1D). Ascarosides such as the male attractant ascr#3 and aggregation ascaroside icas#3 show activity profiles that are similarly broad as that of osas#9, whereas others, such as the mating cue ascr#8, are active only within more narrow concentration ranges (26, 34, 35).

The chemical structure of osas#9 is unusual in that it includes the neurotransmitter octopamine as a building block (Fig. 1A). Because octopamine and the biosynthetically related tyramine play important roles in orchestrating starvation responses, we

investigated octopamine (*ser-3, ser-6, and octr-1*) and tyramine receptors (*tyra-2, tyra-3, ser-2, and ser-3*) for potential involvement in the osas#9 response (Fig. 2A) (36-40). We found that avoidance to osas#9 is largely abolished in a *tyra-2* loss-of-function (*lof*) mutant, whereas osas#9 avoidance was largely unaffected in the other tested neurotransmitter receptor mutants (Fig. 2A). We confirmed this phenotype was a result of the *lof* of *tyra-2* by testing a second *lof* allele of *tyra-2* (Fig. 2B), and by neuron-targeted RNAi (S2A,B) (41-43).

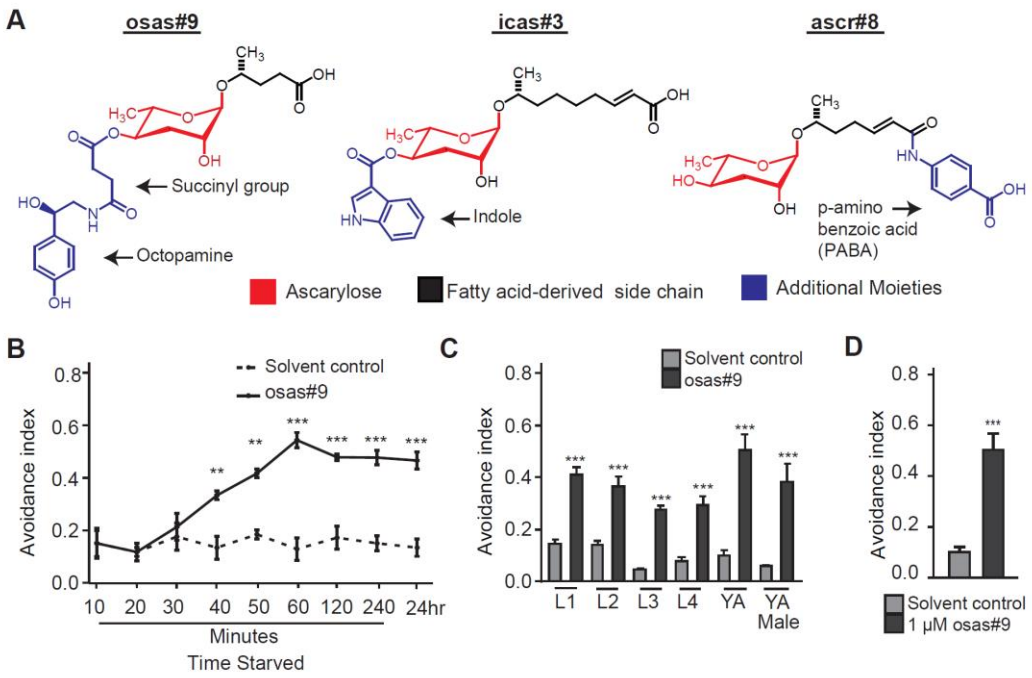

Figure 1. osas#9 is repulsive to starved animals. **A)** Structural and functional diversity of ascarosides. osas#9 is involved in avoidance, icas#3 attracts hermaphrodites and ascr#8 attracts males at low concentrations and induces dauer formation at high concentrations. **B)** Avoidance to osas#9 is dependent on the physiological state of *C. elegans*. Avoidance index of young adult (YA) wildtype (N2) animals in response to solvent control (SC) and 1 µM osas#9 after at different time points after removal from food. After 40 minutes of starvation, animals begin to avoid osas#9, and the response reaches a plateau at about 60 minutes, n ≥ 3 trials. Note for all other assays, unless otherwise stated, animals are starved for at least 60 minutes. **C)** All life stages of hermaphrodites and adult males avoid osas#9 when starved, n ≥ 4 trials. **D)** Avoidance index for starved young adult (YA) wildtype (N2) animals in response to the solvent control (SC) and to 1 µM osas#9, n = 8 trials. 1 µM osas#9 concentration was used in all other assays unless stated otherwise. Data presented as mean ± S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA

with Sidak's multiple comparison posttest, except for Fig 1D, where student's t-test was used. Asterisks displayed depict compared osas#9 avoidance response to respective solvent control.

TYRA-2 is a G protein-coupled receptor (GPCR) that has been shown to bind tyramine with high affinity and octopamine to a lesser extent (38). To exclude the possibility that *tyra-2* is necessary for avoidance behaviors in general, we subjected *tyra-2 lof* animals to three well-studied chemical deterrents, SDS, copper chloride ($CuCl_2$), and glycerol. No defects were found in the animals' ability to respond aversively to these deterrents (Fig. 2C). This indicates that *tyra-2* is specifically required for osas#9 avoidance and is not part of a generalized unisensory avoidance response circuit. Since the response to osas#9 is dependent on physiological state, we examined whether *tyra-2* transcript levels changed under starved versus fed conditions using RT-qPCR. Starved animals exhibited a nearly two-fold increase in *tyra-2* expression (Fig. S2C).

We then asked whether tyramine signaling is required for the osas-9 avoidance response as *tyra-2* is known to bind to tyramine (38). We assayed two *tdc-1 lof* mutants, which lack the ability to synthesize tyramine (44). We observed that the behavioral response to osas#9 was unaltered in animals lacking tyramine biosynthesis (Fig. 2D). This demonstrates that the function of TYRA-2 in osas#9 avoidance is independent of tyramine, suggesting that TYRA-2 may be involved in perception of a ligand other than tyramine to promote aversive response to osas#9.

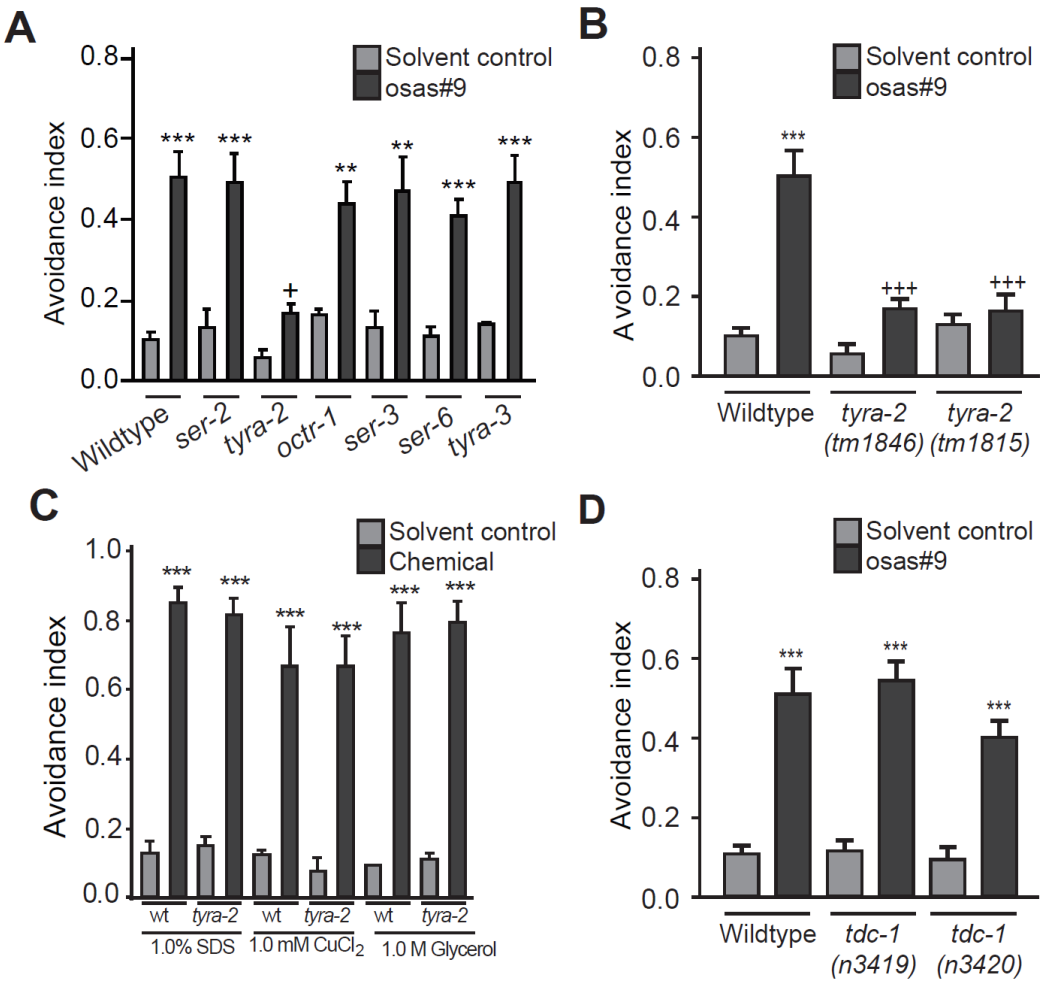

Figure 2. *tyra-2* is required for osas#9 aversive responses independent of tyramine. **A)** Screen for receptors required to mediate osas#9 avoidance. *tyra-2 lof* animals are defective in osas#9 avoidance response, n ≥ 4 trials. **B)** Two alleles of *tyra-2 lof* animals, *tm1846* and *tm1815*, are defective in osas#9 avoidance behavior, n ≥ 4 trials. *tyra-2*(*tm1846*) *lof* animals were used for the remainder of data presented in this manuscript. **C)** *tyra-2 lof* mutants showed no significant differences when subjected to known chemical deterrents, n ≥ 3 trials. **D)** osas#9 avoidance response is not dependent on endogenous tyramine. Two different alleles of *tdc-1 lof* animals, *n3419* and *n3420*, which lack tyramine biosynthesis, show normal response to osas#9, n ≥ 7 trials. Data presented as mean ±

S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest. Asterisks displayed without bar depict compared osas#9 avoidance to respective solvent control within groups. '+' signs represent same p value as asterisks but representing difference between osas#9 avoidance of a strain/conditions in comparison to wildtype.

tyra-2 is required in the ASH sensory neuron for physiological osas#9 response

We next asked where *tyra-2* is acting in the osas#9 aversion pathway. To determine the site of action of *tyra-2* in osas#9 avoidance, we designed a *tyra-2* translational fusion construct consisting of the entire genomic locus, including 2kb upstream, fused to GFP (p*tyra-2::*TYRA-2*::*GFP). We observed TYRA-2 expression in four sensory neurons: ASH, ASE, ASG, and ASI (Fig. 3A). These results are in agreement with previous expression studies on *tyra-2* localization (38) (Fig. 3A). We laser-ablated individual amphid sensory neurons to determine if a *tyra-2* expressing sensory neuron is required for the response. This revealed that ASH neurons are required for osas#9 response, whereas ablation of other neurons did not have a strong effect (Fig. 3B). We observed a slight reduction in the magnitude of the osas#9 aversive response in ASE- and ASI- laser-ablated animals (Fig. 3B); however, ASH/ASE and ASH/ASI double ablated animals did not differ in response from animals with ASH ablated alone, and ASE/ASI ablated animals did not differ from ASE or ASI alone (Fig. 3B). We then tested ASH, ASE, and ASI genetic ablation lines (45-48) and observed that at all tested concentrations, only ASH genetic ablation line resulted in complete abolishment of osas#9 avoidance (Fig. S3A,B,C). As with the laser ablation studies, we observed a slight decrease in osas#9 avoidance in ASE and ASI ablated animals (Fig. S3A,B,C) consistent with the findings for laser-ablated animals. Neurons not expressing *tyra-2* showed no defect in response to osas#9 (Fig. S3D). Our data implies that osas#9 is primarily sensed by ASH sensory neurons and that the ASE and ASI sensory neurons can potentially contribute by sensitizing ASH sensory neurons or by regulating downstream interneurons within the osas#9 response circuit.

To further elucidate the role of the ASH sensory neurons and TYRA-2 in osas#9 sensation, we utilized a microfluidic olfactory imaging chip that enables detection of calcium transients in sensory neurons (49, 50). We observed that, upon exposure to 1 µM osas#9, wildtype animals expressing GCaMP3 in the ASH sensory neurons exhibit robust increase in fluorescence upon stimulus exposure (Fig. 3C,D and Supplementary Video 1). Animals lacking *tyra-2* displayed no changes in fluorescence upon osas#9 exposure (Fig. 3C,D). These findings imply that *tyra-2* activity is necessary in ASH sensory neurons to sense and elicit osas#9 physiological responses.

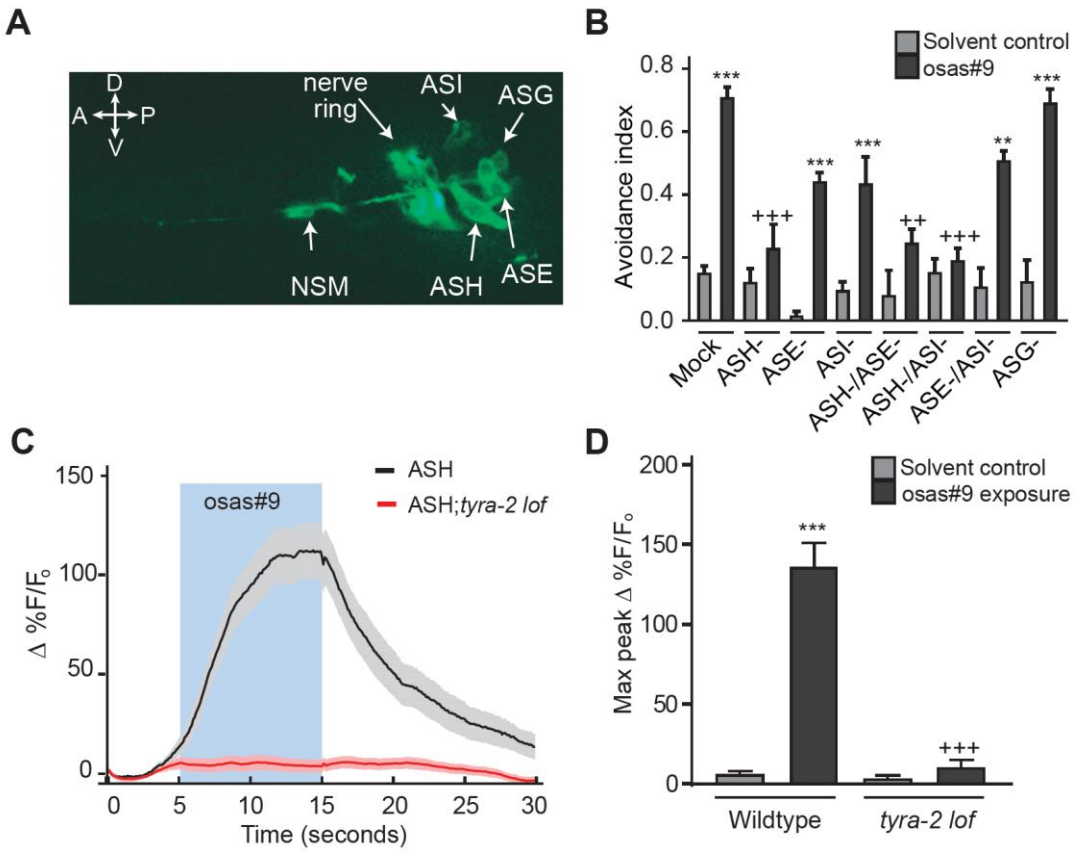

Figure 3. *tyra-2* expression in ASH sensory neurons is required for osas#9 response. **A)** Translational fusion consisting of 2kb upstream of the *tyra-2* gene and the entire *tyra-2* genomic locus was fused to GFP (*ptyra*-2::*tyra-2::*GFP) and injected in wildtype animals at 30 ng/µL revealing *tyra-2* expression in sensory neurons ASE, ASG, ASH, ASH, and NSM (40x magnification). **B)** Chemosensory neurons required for osas#9 response. Neurons expressing *tyra-2* reporter were ablated using laser microbeam. ASH neuronal ablations resulted in abolished response to osas#9 that was indistinguishable from solvent control. ASE and ASI ablated animals showed a reduced avoidance, but not to the extent of ASH neurons, n ≥ 3 trials with at least 10 ablated animals for each condition. **C,D)** Calcium dynamics of ASH neurons upon osas#9 exposure in a microfluidic olfactory chip. **C)** ASH::GCaMP3 animals (black) display a change in calcium transients when

exposed to osas#9. *tyra-2 lof* ASH::GCaMP3 animals (red) did not display a change in fluorescence upon stimulation with the chemical. Shaded blue region depicts time when animals were subjected to the stimulus, n = 10 animals, 30 pulses. **D)** Maximum fluorescence intensity before (solvent control) and during exposure to 1 µM osas#9. Data presented as mean ± S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest. Asterisks depict comparison between osas#9 and respective solvent control. '+' signs represent same p value as asterisks but representing difference between osas#9 avoidance of a strain/conditions in comparison to wildtype.

Given that tyramine and octopamine are known ligands of TYRA-2, we also tested whether these neurotransmitters elicit aversive responses in *C. elegans* (38). Previous studies have shown that both tyramine and octopamine inhibit serotonin food-dependent increases in aversive responses to dilute octanol via specific G protein-coupled receptors (40). Both biogenic amines exhibited aversive behaviors at non-physiological concentrations much higher than required for osas#9, 1 mM for tyramine and octopamine compared to 1 µM for osas#9 (Fig. S4A,B, S1B). Similarly, high concentrations of tyramine (1mM) elicited calcium transients in ASH::GCaMP3 but lower concentrations (1 µM) did not show calcium changes (Fig. S4C,D). Worms exposed to 1 mM octopamine displayed minimal change in calcium transients (Fig.S4C,D). These data show that the TYRA-2 receptor in the ASH sensory neurons is specifically involved in the avoidance response to osas#9. Tyramine or octopamine do not appear to be participating in the avoidance response, in agreement with the finding that tyramine biosynthesis is not required for avoidance to osas#9 (Fig. 2D).

tyra-2 expression confers the ability to sense osas#9

Since expression of *tyra-2* in the ASH sensory neurons is required for calcium transients in response to osas#9, we asked whether *tyra-2* expression in the ASH neurons is sufficient to rescue the osas#9 behavioral response in *tyra-2 lof* animals. Expression of *tyra-2* under the *nhr-79* promoter, which is expressed in the ASH and ADL neurons, fully restored osas#9 avoidance (Fig. 4A,B) (51). To test whether expression of *tyra-2* in the ADL neurons is required for the phenotypic rescue, we ablated the ADL neurons in the transgenic animals. Ablation of the ADL neurons did not affect avoidance to osas#9 (Fig. 4C). Additionally, injection of the *tyra-2* translational reporter into *tyra-2*

lof animals displayed sub-cellular localization in the ASH sensory cilia (Fig. 4D) and was observed to be functional as osas#9 aversion is rescued in these animals (Fig. 4E). These results affirm that the aversive behavioral response to osas#9 is dependent on *tyra-2* expression in the ASH neurons.

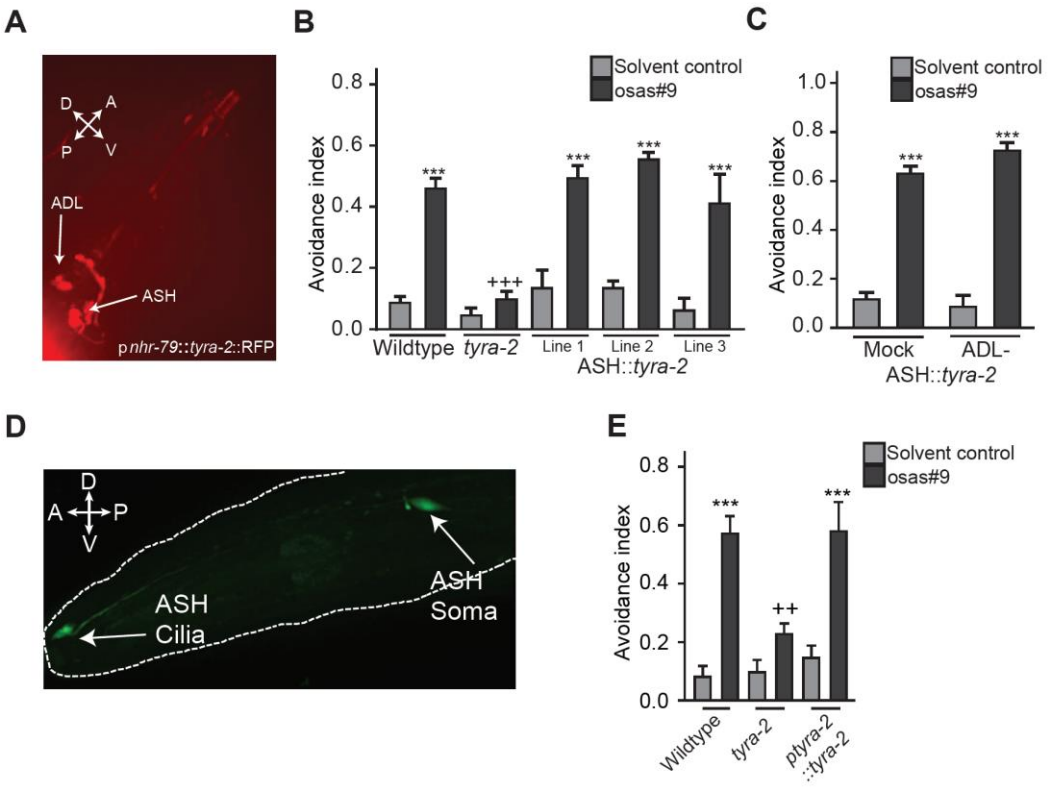

Figure 4. *tyra-2* expression is required in ASH sensory neurons for avoidance response to osas#9. **A)** A transcriptional rescue construct, p*nhr-79*::*tyra-2*::RFP exhibited expression of *tyra-2* in both ASH and ADL neurons (40x magnification). **B)** Rescue of *tyra-2* in ASH neurons fully reconstituted behavioral response to 1 µM osas#9, n ≥ 4 trials. **C)** Ablation of ADL neurons does not affect osas#9 avoidance in the rescue lines n≥4 trials. **D)** Sub cellular localization of *tyra-2*. A translational reporter of the entire *tyra-2* genomic locus (*ptyra*-2::*tyra-2::*GFP) was injected into *tyra-2 lof* animals at 1 ng/µL, revealing expression of the receptor in both soma and sensory cilia. (60x magnification). **E)** Expression of the translational reporter restores wildtype behavior in a *tyra-2 lof* background, n ≥ 5 trials. Data presented as mean ± S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest. Asterisks depict comparison between osas#9 and respective solvent control. '+' signs represent

same p value as asterisks but representing difference between osas#9 avoidance of a strain/conditions in comparison to wildtype.

Previous studies in *C. elegans* indicate that behavioral responses (such as aversion or attraction) elicited by an odorant are specified by the olfactory neuron in which the receptor is activated in, rather than by the olfactory receptor itself (31, 52). Therefore, we asked whether expression of TYRA-2 in AWA neurons, which are generally involved in attractive responses to chemical cues (53, 54) would switch the behavioral valence of osas#9, resulting in attraction to osas#9, instead of aversion. Misexpression of *tyra-2* in the AWA sensory neurons in a *tyra-2 lof* background did not result in avoidance of osas#9, in contrast to expression of *tyra-2* in the ASH neurons (Fig. 5A). We then performed a leaving assay to test for attraction to osas#9 in the worms expressing *tyra-2* in the AWA neurons. This assay involves the placement of animals into the center of a NGM agar plate where osas#9 is present and measuring the distance of animals from the origin in one-minute intervals (Fig. 5B). *tyra-2 lof* animals displayed osas#9 leaving rates equal to the solvent control (Fig. 5C, S5), whereas worms misexpressing *tyra-2* in the AWA neurons displayed osas#9 leaving rates lower than that for solvent controls, indicating attraction (Fig. 5C, S5). Furthermore, worms misexpressing *tyra-2* in the AWA neurons stayed significantly closer to the origin than either wildtype or *tyra-2 lof* animals when exposed to osas#9 (Fig. 5C, S5). We confirmed that ectopic expression of *tyra-2* in AWA sensory neurons did not alter the native chemosensory parameters of AWA neurons (Fig. S6A,B). Hence misexpression of *tyra-2* in AWA neurons resulted in reprogramming of these nematodes, promoting attraction to the normally aversive compound osas#9.

Finally, we tested whether ectopic expression of *tyra-2* in the ADL neurons, which have been shown to detect aversive stimuli (55-58), results in a behavioral response to osas#9. For this purpose, we ablated the ASH neurons in the p*nhr-79*::*tyra-2* strain, in

which *tyra-2* is expressed in the ASH and ADL neurons. We found that these ASH ablated animals still avoid osas#9, similar to ADL ablated worms from this rescue line (Fig. 5D). Ablation of both the ASH and ADL neurons in this strain abolished the avoidance response (Fig. 5D). This implies that mis-expression of *tyra-2* in the ADL neurons confers the ability of this neuron to drive avoidance to osas#9. Taken together, results from both misexpression experiments (AWA and ADL neurons) demonstrate that TYRA-2 is necessary and sufficient to elicit osas#9-dependent behaviors.

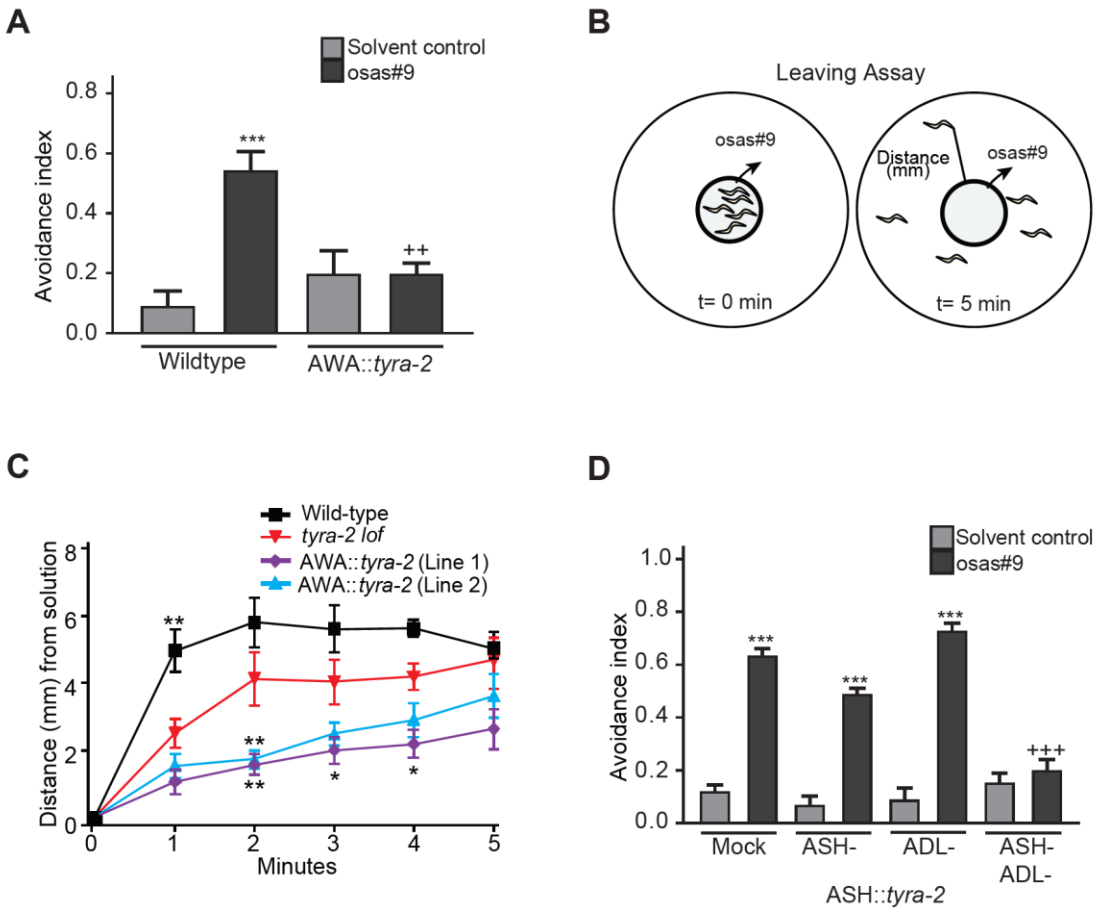

Figure 5. Ectopic expression of *tyra-2* confers the ability to respond to osas#9. **A)** Animals with reprogrammed AWA sensory neurons in *tyra-2 lof* background do not avoid osas#9, n ≥ 4 trials. **B)** Schematic illustration of the leaving assay to measure osas#9 attraction. (See material and methods for detailed description). **C)** Wildtype, *tyra-2 lof,* and AWA::*tyra-2* lines were subjected to 10 pM osas#9 in the leaving assay. Wildtype animals left the osas#9 solution spot quicker than the *tyra-2 lof* animals, whereas the misexpression lines remained closer to osas#9, n ≥ 3 trials. **D)** Misexpression of *tyra-2* in ADL neurons confers avoidance behavior in response to osas#9. *nhr-79* promoter driving *tyra-2* in ASH and ADL sensory neurons rescues osas#9 avoidance. Ablation of ASH neurons in this line resulted in avoidance behavior to osas#9. Ablation of both ASH and

ADL neurons in this line completely abolished avoidance, n ≥ 3 trials. Data presented as mean ± S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest. Asterisks depict comparison between osas#9 and respective solvent control. '+' signs represent same p value as asterisks but representing difference between osas#9 avoidance of a strain/conditions in comparison to wildtype.

Gα protein gpa-6 is necessary in ASH sensory neurons for osas#9 avoidance

Since expression of the *tyra-2* GPCR is required in ASH neurons for osas#9 response, we sought to identify the Gα subunit necessary for osas#9 avoidance. Eight of the 21 Gα proteins are expressed in subsets of neurons that include the ASH sensory pair (*gpa-1, gpa-3, gpa-6, gpa-11, gpa-13, gpa-14, gpa-15,* and *odr-3*) (59-61). We tested mutants for each of those eight Gα subunits for their response to osas#9, (Fig. 6A) and found that gpa-*6 lof* animals do not avoid osas#9 (Fig. 6A). To determine whether *gpa-6* is necessary in ASH sensory neurons to mediate osas#9 responses, we expressed *gpa-6* using p*nhr-79* in the ASH neurons in a *gpa-6 lof* background. These animals displayed wildtype behavior when tested for osas#9 avoidance (Fig. 6B). To characterize cellular and sub-cellular localization of the *gpa-6* Gα subunit, we created a full-length RFP translational fusion of the entire *gpa-6* locus including 4kb upstream. We detected *gpa-6* expression in the soma of AWA and ASH sensory neurons (Fig. 6C), in agreement with previous studies (60). However, in addition to ASH soma localization, the translational fusion revealed presence of *gpa-6* in ASH cilia (Fig. 6C). Behavioral rescue by *gpa-6* expression specifically in the ASH neurons and its ciliary localization, support that this Gα subunit functions in mediating osas#9 avoidance.

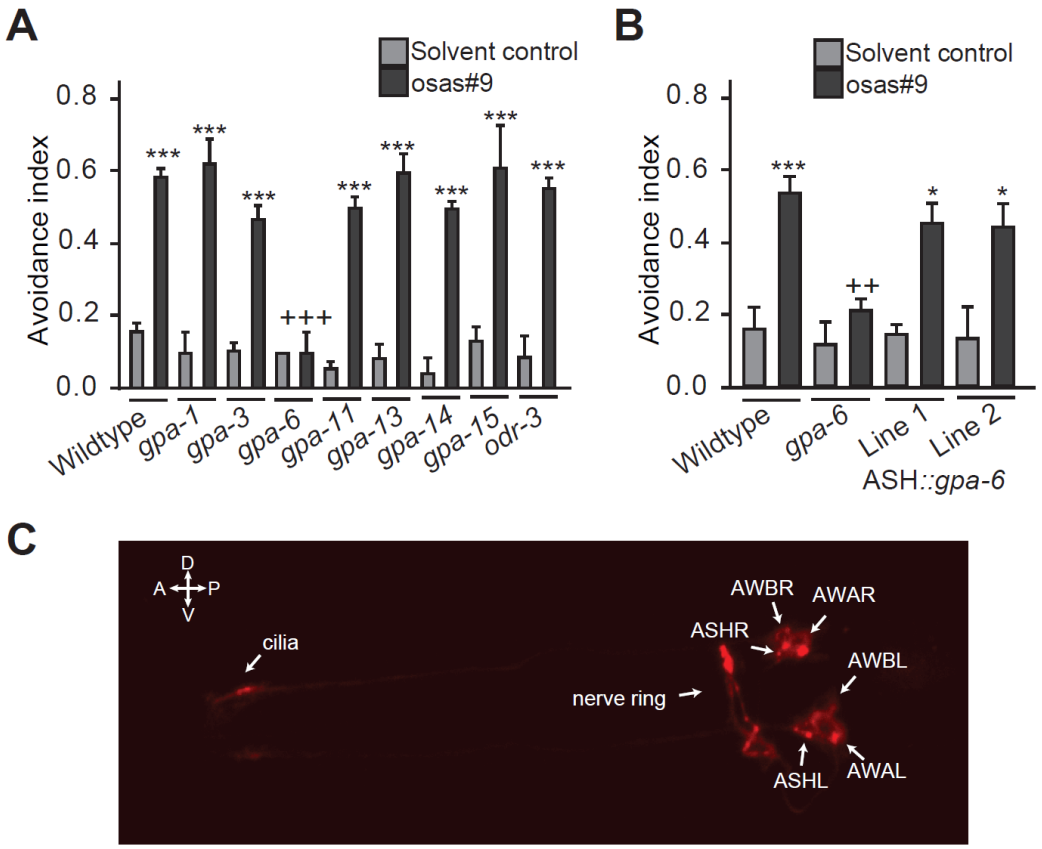

Figure 6. GPA-6 functions in ASH sensory neurons to mediate osas#9 response. **A)** Screen of mutations in Gα subunits resulted in identification of the Gαsubunit *gpa-6*, which were defective in their avoidance response to osas#9, n ≥ 3 trials. **B)** Expression of *gpa-6* in ASH neurons using *nhr-79* promoter reconstituted avoidance response similar to wildtype animals, n ≥ 3 trials. **C)** *gpa-6* localizes to the soma and cilia in ASH neurons. Translational fusion of the entire *gpa-6* genomic region displayed localization of the subunit to the soma of AWA, AWB, and ASH neurons. In addition, we also observed ciliary localization in ASH neurons (40x magnification). Data presented as mean ± S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest. Asterisks depict comparison between osas#9 and respective solvent control. '+'

signs represent same p value as asterisks but representing difference between osas#9 avoidance of a strain/conditions in comparison to wildtype.

2 A.3 Discussion

How does a worm survive in changing environmental and physiological conditions? Given *C. elegans*' complex ecology and a boom and bust lifestyle, worms need to make frequent adaptive developmental and physiological choices (62). The octopamine-derived pheromone osas#9, secreted in large quantities by L1 larvae under starvation conditions, appears to promote dispersal away from unfavorable conditions (Fig. 7).

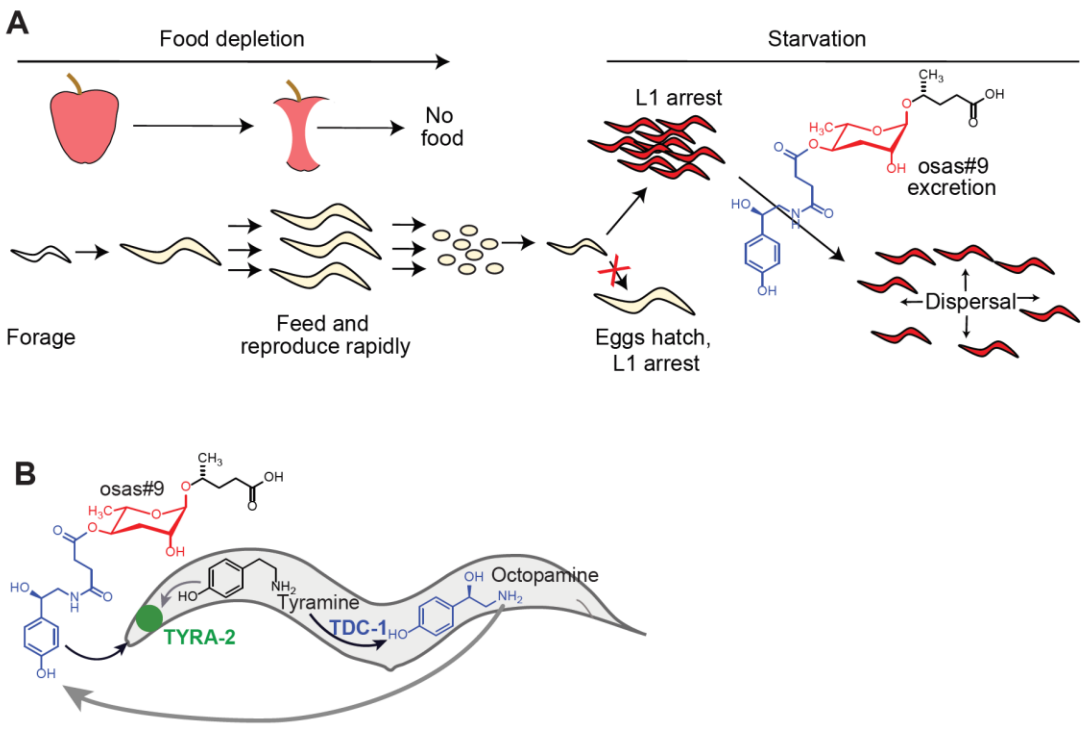

Figure 7. osas#9 serves as a dispersal cue in *C. elegans*. **A)** An animal navigating its environment encounters a food source, and offspring grow and reproduce rapidly, eventually depleting the food. Eggs hatch on depleted food patch and halt development as L1 arrest animals. L1 arrest animals secrete the aversive compound, osas#9 assisting in dispersal away from unfavorable conditions. **B)** Inter-organismal signaling coopts neurotransmitter signaling in *C. elegans*. The G protein-coupled receptor *tyra-2,* which senses tyramine is also required for sensing the biogenic metabolite osas#9 derived from octopamine, to mediate avoidance behavior.

Here we show that this pheromone is detected by the GPCR *tyra-2*, a canonical neurotransmitter receptor that is expressed in the ASH sensory neurons. To our knowledge this is the first instance in which a “repurposed internal receptor” partakes in pheromone perception. Similar to osas#9 biosynthesis, *tyra-2* transcript levels are increased in starved animals (Fig. S2C). Notably, octopamine, the distinguishing structural feature of osas#9, has been implicated in responses to food scarcity in invertebrates, including insects (13, 63, 64), *C. elegans* (36, 65-70), and molluscs (71, 72). These findings indicate that worms navigate adverse environmental conditions in part via social communication channels that employ signaling molecules and receptors derived from relevant endocrine signaling pathways.

Previous studies have identified several GPCRs involved in ascaroside (ascr) perception: *srbc-64*, *srbc-66* (ascr#1,2,3) (33); *srg-36*, *srg-37* (ascr#5) (31); *srx-43*, *srx-44* (icas#9) (29, 30); *daf-37* (ascr#2), *daf-38* (ascr#2,3,5) (32). These studies demonstrate that GPCRs involved in ascaroside perception may act as heterodimers (32). TYRA-2 has previously been shown to contain the conserved $Asp^{3.32}$ required for amine binding, allowing the receptor to bind tyramine with high affinity, and octopamine to a lesser extent (38). In contrast, osas#9 lacks the basic amine, and instead has an amide as well as an acidic sidechain. These chemical considerations suggest that TYRA-2 may facilitate osas#9 perception by interacting with another GPCR that directly binds to osas#9. However, by ectopically expressing *tyra-2* in ADL and AWA neurons , we were able to elicit responses characteristic to each neuron (Fig. 5). These data show that the response to osas#9 depends on the neuron *tyra-2* is expressed in, providing additional support for direct involvement of TYRA-2 in chemosensation of osas#9. Alternatively, a different

receptor that directly interacts with TYRA-2 and is expressed in the ASH, ADL, and AWA neurons could bind osas#9.

Our data suggests that ASE and ASI sensory neurons may regulate ASH sensitivity during osas#9 avoidance serving as modulators at the sensory level, similar to previously observed cross inhibition of ASI and ASH neuronal activity in avoidance to copper, and decision making based on physiological state (73, 74). Alternatively, these neurons could be interacting with ASH neuronal targets in the osas#9 response, strengthening or dampening the relayed signal, possibly through peptidergic or aminergic signaling to establish the functional circuit. Recent studies have shown that *tyra-2* is necessary for binding tyramine in a RIM-ASH feedback loop in multisensory decision making (75). Animals lacking TYRA-2, or the tyramine biosynthetic enzyme TDC-1, crossed a 3M fructose barrier towards an attractant, diacetyl, faster than wildtype *C. elegans.* This demonstrated the endogenous role of tyramine binding to TYRA-2 increasing avoidance in multisensory threat tolerance (75); however, our results show that tyramine signaling is not involved in the response to osas#9. It will be interesting to elucidate the role other neurons or tissues and neuromodulatory signaling have in shaping the osas#9 response. Such modulation of the osas#9 response circuitry remains to be investigated.

Our findings demonstrate that TYRA-2, a member of a well conserved family of neurotransmitter receptors, functions in chemosensation of osas#9, a neurotransmitter-derived inter-organismal signal. Typically, neurotransmitter signaling is intra-organismal, facilitating cell-to-cell communication. This involves the highly regulated biosynthesis of specific chemical compounds, e.g. biogenic amines, their translocation (either by way of

diffusion or through active transport), and, finally, perception by dedicated chemoreceptors (76). This mode of communication is strikingly similar to pheromone communication between organisms, as it involves highly specific production and reception of ligands for communication. As evolution is opportunistic, it stands to reason that some machinery from intra-cellular signaling would be utilized for inter-organismal signaling. Indeed, co-option has been hinted at before, in both the trace amine associated receptor (TAAR) and formyl peptide receptor-like (FPRL) receptor classes, both of which are involved in inter-organismal signaling (77-80). Of the TAARs, only TAAR1 and TAAR2 have been found to have endogenous roles: TAAR1 in mammalian CNS, and both TAAR1 and TAAR2 in leukocyte migration (78, 81). Additionally, TAAR2 mRNA has been detected in mouse olfactory epithelium, suggesting it may be involved in both intra-and inter-organismal signaling (77). However, no odor molecules have been linked to TAAR2 in the olfactory epithelium.

How key innovations in metazoan complexity could have evolved from pre-existing machineries is of great interest (82). Our findings demonstrate that the tyramine receptor TYRA-2 functions in chemosensation of osas#9, a neurotransmitter-derived inter-organismal signal, thus revealing involvement of both neurotransmitter biosynthesis and neurotransmitter reception in intra- and inter-organismal signaling. Therefore, evolution of an inter-organismal communication channel co-opted both a small molecule, octopamine, and the related receptor TYRA-2, for mediating starvation-dependent dispersal in *C. elegans* (Fig. 7), suggesting that such co-option may represent one mechanism for the emergence of new inter-organismal communication pathways.

2 A.4 Methods

Avoidance drop test

In this assay, the tail end of a forward moving animal is subjected to a small drop (~5 nl) of solution, delivered through a hand-pulled 10 µl glass capillary tube. The solution, upon contact, is drawn up to the amphid sensory neurons via capillary action. In response, the animal either continues its forward motion (scored as “no avoidance response”), or displays an avoidance response within four seconds (83). The avoidance response is characterized by a reversal consisting of at least one half of a complete “head swing” followed by a change in direction of at least 90 degrees from the original vector. For quantitative analysis, an avoidance response is marked as a “1” and no response as a “0”. The avoidance index is calculated by dividing the number of avoidance responses by the total number of trials. Each trial is done concurrently with osas#9, diluted in DIH_2O, and a solvent control. Osas#9 was synthesized by methods in Artyukhin et al. 2013 (22).

Integrated mutant strains and controls are prepared using common M9 buffer to wash and transfer a plate of animals to a microcentrifuge tube where the organisms are allowed to settle. The supernatant is removed and the animals are resuspended and allowed to settle again. The supernatant is again removed and the animals then transferred to an unseeded plate. After 1 hour, young adult animals are subjected to the solvent control and the chemical of interest at random with no animal receiving more than one drop of the same solution. Refed animals were transferred to a seeded plate with M9 buffer, and after the allotted time, transferred to an unseeded plate and tested after 10 minutes.

Ablated and extrachromosomal transgenic animals and controls are gently passed onto an unseeded plate and allowed to crawl around. They are then gently passed to another unseeded plate to minimalize bacterial transfer. Ablated animals are tested three times with the solvent control and solution of interest with 2 minute intervals between drops (83).

Strains and Plasmids

tyra-2 rescue and misexpression plasmids were generated using MultiSite Gateway Pro Technology and injected into strain FX01846 *tyra-2*(*tm1846*) with co-injection marker p*elt-2*;mCherry by Knudra Transgenics. The promoter attB inserts were generated using PCR and genomic DNA or a plasmid. The *tyra-2* insert was isolated from genomic DNA using attB5*ggcttatccgttgtggagaa* and attB2*ttggcccttccttttctctt.* PDONR221 p1-p5r and PDONR221 P5-P2 donor vectors were used with attB inserts. The resultant entry clones were used with the destination vector pLR305 and pLR306.

AWA::*tyra-2* misexpression*:* For AWA expression, a 1.2 kb *odr-10* promoter was isolated from genomic DNA using primers attB1*ctcgctaaccactcggtcat* and attB5r*gtcaactagggtaatccacaattc.* Entry clones were used with destination vector pLR305 resulting in p*odr-10::tyra-2:: RFP* and co-injected with p*elt-2*::mCherry into FX01846.

ASH::*tyra-2* rescue*:* For ASH expression, a 3 kb *nhr-79* promoter was isolated from genomic DNA using primers attB1*gtgcaatgcatggaaaattg* and attB5r*atacacttcccacgcaccat.* Entry clones were used with destination vector pLR306 resulting in p*nhr-79::tyra-2::RFP* and co-injected with p*elt-2*::mCherry into FX01846.

ASH::*gpa-6* rescue*:* For ASH expression, a 3 kb *nhr-79* promoter was isolated from genomic DNA using primers attB1*gtgcaatgcatggaaaattg* and attB5r*atacacttcccacgcaccat. gpa-6* was isolated from genomic DNA using primers attB5 cgtctctttcgtttcaggtgtat and attB2 tattttcaaagcgaaacaaaaa. Entry clones were used with destination vector pLR304 resulting in p*nhr-79::gpa-6::RFP* and co-injected with p*unc-122*::RFP into NL1146.

Translational fusions*: tyra-2::*GFP fusions were created by PCR fusion using the following primers to isolate 2kb p*tyra-2* with its entire genomic locus from genomic DNA: A) *atgttttcacaagtttcaccaca,* A nested) *ttcacaagtttcaccacattacaa,* and B with overhang) *AGTCGACCTGCAGGCATGCAAGCT gacacgagaagttgagctgggtttc.* GFP primers as described in WormBook (84). The construct was then co-injected with p*elt-2*::mCherry into both N2 and FX01846.

*gpa-6::*RFP was generated by adding the restriction sites, AgeI and KpnI, to isolate 4kb p*gpa-6* and the entire *gpa-6* locus from genomic DNA using primers: acatctggtacccctcaatttcccacgatct and acatctaccggtctcatgtaatccagcagacc. RFP::*unc-54*, ori, and AMPr was isolated from p*unc-122*::RFP plasmid by PCR addition of the restriction sites AgeI and KpnI with primers: acatctaccggt ATGGTGCGCTCCTCCAAG and ttaataggtaccTGGTCATAGCTGTTTCCTGTG. After digestion and ligation, the clone was injected into N2 with co-injection marker p*unc-122*::GFP.

(See Supplementary Table 1-3 for details on strains, plasmids, and primers used in this study.)

RNA interference

RNAi knockdown experiments were performed by following the RNAi feeding protocol found at Source Bioscience (https://www.sourcebioscience.com/products/life-sciences-research/clones/rnai-resources/c-elegans-rnai-collection-ahringer/). The RNAi clones (F01E11.5, F14D12.6, and empty pL4440 vector in HT115) originated from the Vidal Library (85), were generously provided by the Ambros Lab at UMASS Medical School. We observed that RNAi worked best when animals were cultured at 15ºC. We used the *nre-1(hd20);lin-15B(hd126)* (VH624) strain for the RNAi studies as it has been previously shown to be sensitive to neuronal RNAi (42, 43).

Laser ablations

Laser ablations were carried out using DIC optics and the MicroPoint laser system following the procedures as outlined in Fang-Yen *et al.* 2012 (86, 87). Ablated animals were assayed 72 hours later, at the young adult stage. All ablated animals were tested in parallel with control animals that were treated similarly as ablated animals but were not exposed to the laser microbeam.

Imaging

Translational fusion animals were prepared for imaging by mounting them to a 4% agar pad with 10 mM levamisole on a microscope slide as outlined in O'Hagen and Barr 2016 (88). Animals were imaged using a Nikon Multispectral Multimode Spinning Disk Confocal Microscope, courtesy of Dr. Kwonmoo Lee at Worcester Polytechnic Institute or a Zeiss LSM700 Confocal Microscope, courtesy of the Department of Neurobiology at University of Massachusettes Medical School, Worcester.

Calcium imaging was perfomed by using a modified olfactory chip as described in Reilly et al 2017 (49, 50). A young adult animal was immobilized in a PDMS olfactory chip with its nose subject to a flowing solution. Animals were imaged at 40x magnification for 30 seconds, and experienced a 10 second pulse of osas#9 in between the solvent control. Each animal was exposed to the stimulus three times. Soma fluorescence from GCaMP3 was measured using ImageJ. Background subtraction was performed for each frame to obtain the value ΔF. Change in fluorescence ($\Delta F/F_0$) was calculated by dividing the ΔF value of each frame by F_0. F_0 was calculated as the average ΔF of 10 frames prior to stimulus exposure (50).

RT-qPCR

RNA was isolated from individual animals, either freshly removed from food or after four hours of starvation using Proteinase K buffer as previously published (89). cDNA was subsequently synthesized using the Maxima H Minus First Strand cDNA Synthesis Kit. iTaq Universal SYBR Green Supermix was used for amplification with the Applied Biosystem 7500 Real Time system. Primer efficiency was determined to be 97.4% for *tyra-2* primers (GAGGAGGAAGAAGATAGCGAAAG, TGTGATCATCTCGCTTTTCA) and 101.8% for the reference gene *ama-1* (GGAGATTAAACGCATGTCAGTG, ATGTCATGCATCTTCCACGA) using the equation 10^(-1/slope)-1. Technical replicates with large standard deviations and trials with a Ct within 5 cycles of the negative control (no reverse transcriptase used in prep) were removed from analyses.

Locomotion

Speed*:* Five animals were gently transferred to a 35mm plate and filmed for 20 minutes. Videos were generated using the Wormtracker system by MBF Bioscience. Videos were then analyzed and average speed was computed using software WormLab4.1 (MBF Bioscience, Williston, VT USA).

Chemoattraction

Diacetyl chemotaxis assays were carried out as previously published, with slight modifications (53). 10 animals were placed in the center of a 35mm plate, equidistant from two spots, one containing 1 µl of solvent control and the other 1 µl of 10^{-2} diacetyl. Both spots contained sodium azide for anesthetizing animals that entered the region. After 45 minutes, the chemotaxis index was calculated by subtracting the number of animals in the solvent control from the number of animals in the solution of interest and divided by the total number of animals.

Leaving Assay

The leaving assay consisted of the use of 60 mm culture plates containing standard NGM agar. A transparency template that included a 6mm diameter circle in the center was attached to the underside of the NGM plate. One hour before running the assay, young adult animals were passed on to an unseeded plate and allowed to starve for one hour. 100 µl of *E. coli* OP50 liquid culture was spread onto a separate NGM assay plates. These plates were allowed to dry at 25^{o}C without a lid for one hour. After an hour of incubation, 4 µl of either solvent control or 10 pM osas#9 was pipetted onto the agar within the center circle outlined on the template. 10 animals were gently passed into the

center circle and their movement was recorded. At one minute intervals, the distance the animals traveled from the origin was measured using ImageJ.

Statistical analysis

Statistical tests were run using Graphpad Prism. For all figures, when comparing multiple groups, ANOVAs were performed followed by Sidak's multiple comparison test. When only two groups were compared, a Student's t-test was used (Figure 1D, S2C). When comparing different strains/conditions, normalized values of osas#9 avoidance index response relative to the respective solvent control were used. This was done to account for any differences in baseline response to solvent control for the respective genotypes, laser ablations, or physiological conditions. When normalizing fold change of osas#9 response to solvent control response for the avoidance assay within a strain/condition, data was first log transformed so a fold change could still be calculated for control plates that had a "0" value. For avoidance assays, statistical groups were based on the number of plates assayed, not the number of drops/animals. For calcium imaging, averages were calculated by obtaining the max peak value before and during exposure to the chemical of interest for each trial.

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Neurobiology department and M. Gorczyca for assistance and use of confocal microscope; V. Ambros, Dana-Farber Cancer Institute, and BioScience Life Sciences for Vidal library RNAi clones; A. Maurya and Piali Sengupta for technical suggestions; D. Vargas Blanco for RT-qPCR guidance; the Srinivasan lab, Rick Komuniecki, Michael Nitabach and Nitabach lab and S. Chalasani for critical comments on the manuscript; A. Warty for contribution to glycerol assays. This work was supported in by grants from the NIH (R01DC016058 to J.S. and GM113692 and GM088290 to FCS and GM084491 to MJA).

Author Contributions

CDC performed the molecular biology, ablations, and behavioral assays. CDC and LD performed calcium imaging. CDC and VC performed the RNAi behavioral assays. YZ synthesized osas#9. H Choi helped in confocal microscopy of transgenic strains. DR generated strains from MA lab. CDC and JS wrote the manuscript with input from FCS and MJA.

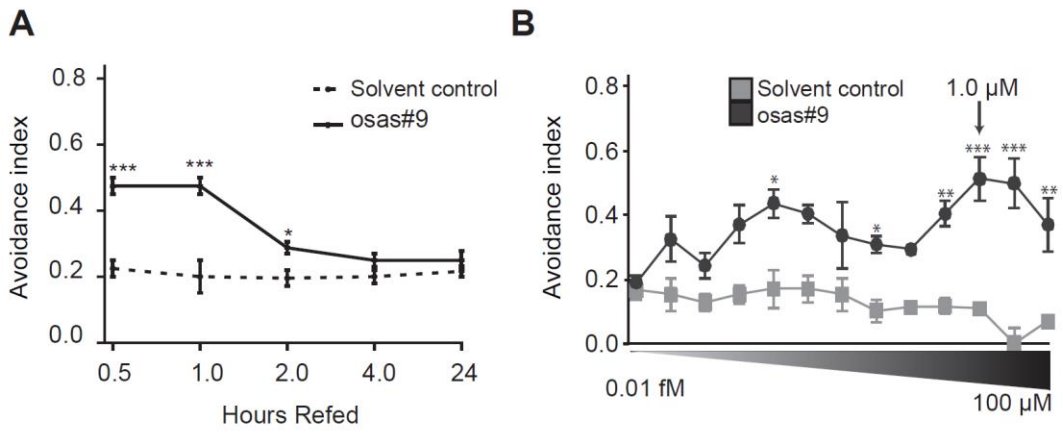

Figure S1. A) Attenuation of osas#9 avoidance response by *E. coli* OP50. Animals reintroduced to *E. coli* OP50 for two hours exhibited an attenuated response to osas#9, n≥3 trials. **B)** osas#9 exhibits avoidance response over a broad range of concentrations (fM - µM) in YA wildtype animals, n≥3 trials. Data presented as mean ± S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest. Asterisks depict comparison between test solution and respective solvent control.

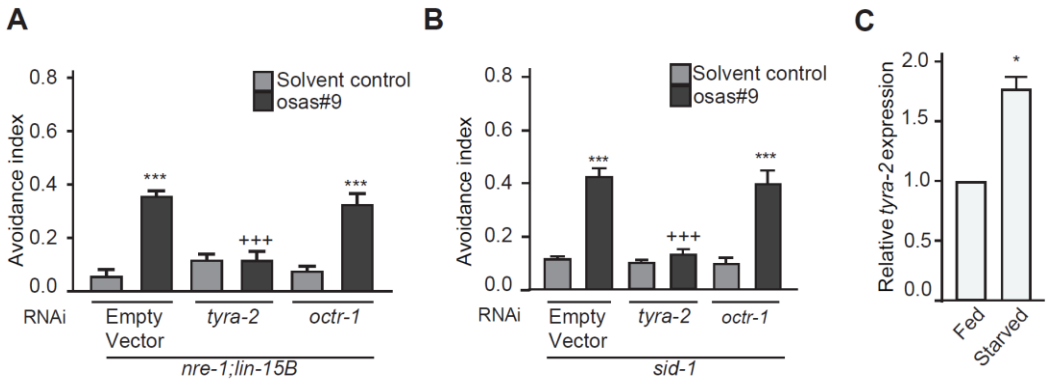

Figure S2. A-B) *tyra-2* RNAi knockdown results in loss of avoidance to osas#9. Animals cultured at 15ºC and fed *tyra-2* RNAi clones were defective in response to osas#9 in two different RNAi sensitive backgrounds A) *nre-1(hd20) lin-15B(hd126),* n≥10. B**)** *sid-1(pk3321),* n≥3. **C)** Physiological state dependence of expression of *tyra-2* receptor. RT-qPCR analysis of fed versus starved animals indicates that starved animals upregulate *tyra-2* nearly two-fold. Data shown is the ratio of endogenous *tyra-2* messenger RNA to *ama-1* messenger RNA from three independent RT-qPCR experiments (See materials and methods for more details), n=3. Data presented as mean ± S.E.M;*P<0.05, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest, except for Fig S2C where student's t-test was used. Asterisks depict comparison between test solution and respective solvent control. '+' signs represent same p value as asterisks but representing difference between osas#9 avoidance of a strain/conditions in comparison to wildtype.

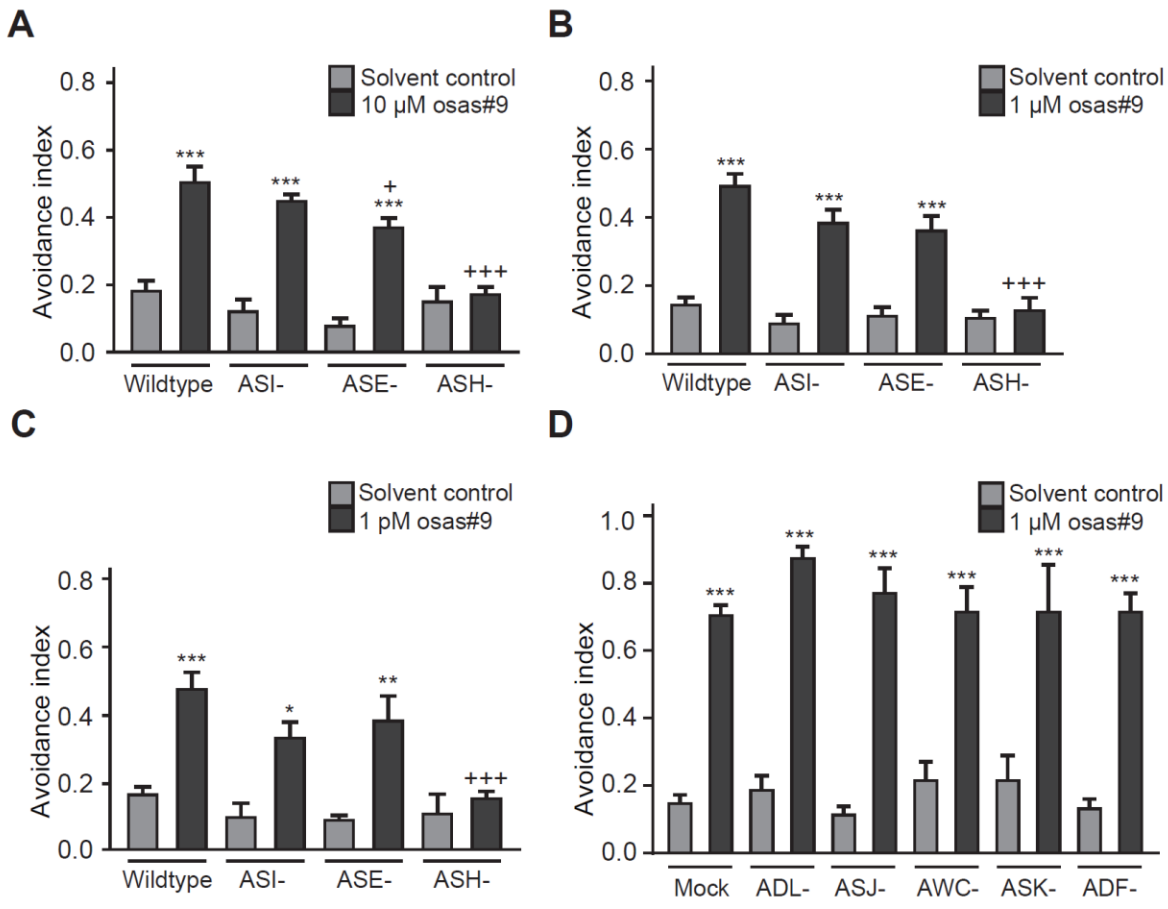

Figure S3. Role of different sensory neurons in osas#9 avoidance behavior. **A-C)** Genetically ablated ASH, ASI and ASE neurons were tested for their response to various concentration of osas#9, n≥3 trials. **D)** Sensory neurons not required for osas#9 avoidance. Note that ADL is not required for osas#9 avoidance. All ablated animals were tested with at least 10 animals with the exception of ADF-, which is 7 animals. Data presented as mean ± S.E.M; *P<0.05, **P<0.01, ***P<0.001, one factor ANOVA with Sidak's multiple comparison posttest. Asterisks depict comparison between test solution and respective solvent control. '+' signs represent same p value as asterisks but representing difference between osas#9 avoidance of a strain/conditions in comparison to wildtype.

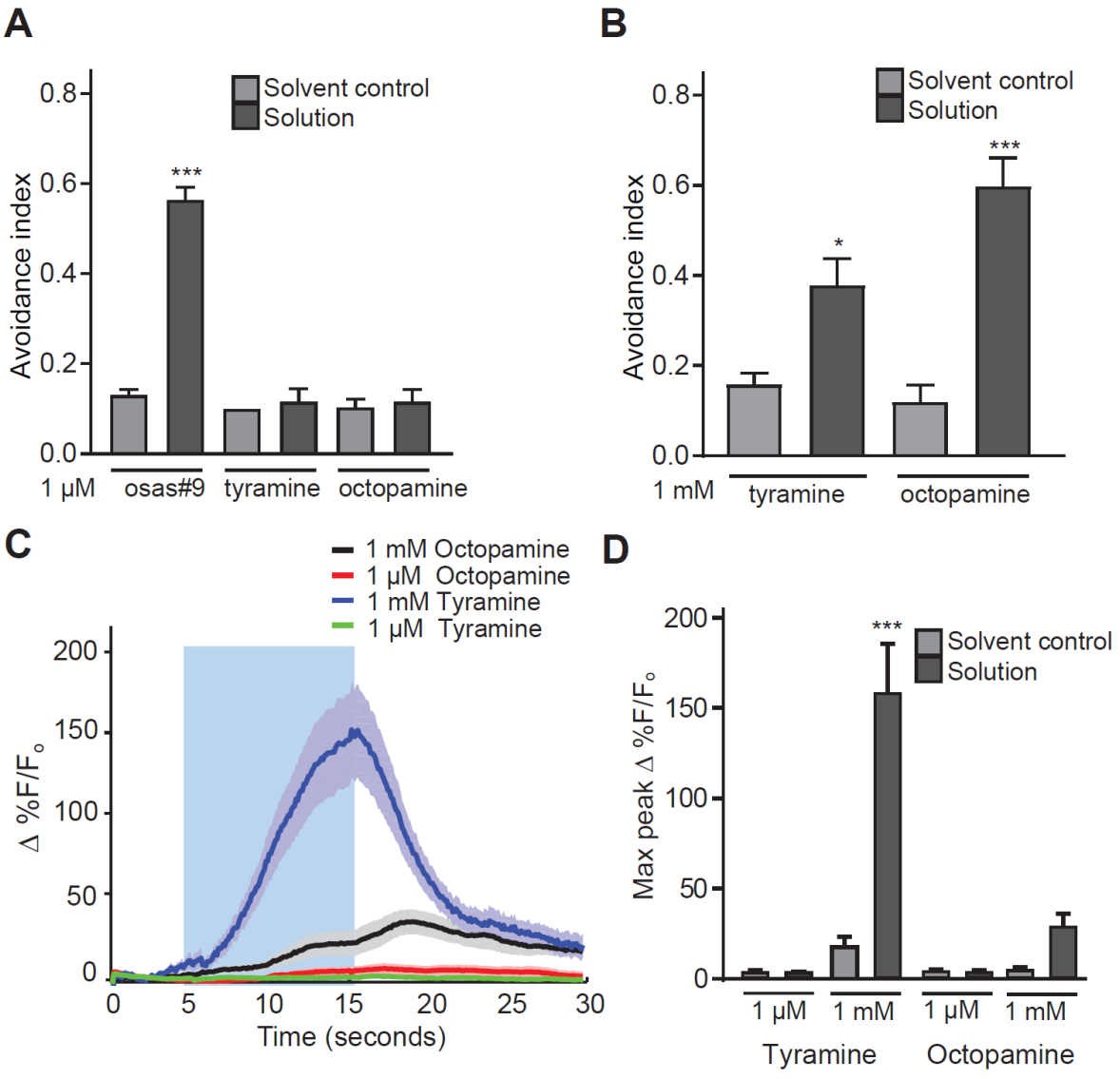

Figure S4. Tyramine and octopamine elicit avoidance at high concentrations. **A)** Animals do not display avoidance to 1 µM tyramine or octopamine, in contrast to osas#9, n≥3 trials. **B)** Tyramine and octopamine result in aversive responses of wildtype animals at higher concentrations, n≥5 trials. **C,D)** Calcium dynamics in ASH sensory neurons upon exposure to tyramine and octopamine. Tyramine exposure resulted in a significant increase in calcium transients in ASH at concentrations of 1 mM, n≥10. Data presented as mean ± S.E.M; *P<0.05, **P<0.01, one factor ANOVA with Sidak's multiple

comparison posttest. Asterisks depict compared solution of interest avoidance response to the solvent control.

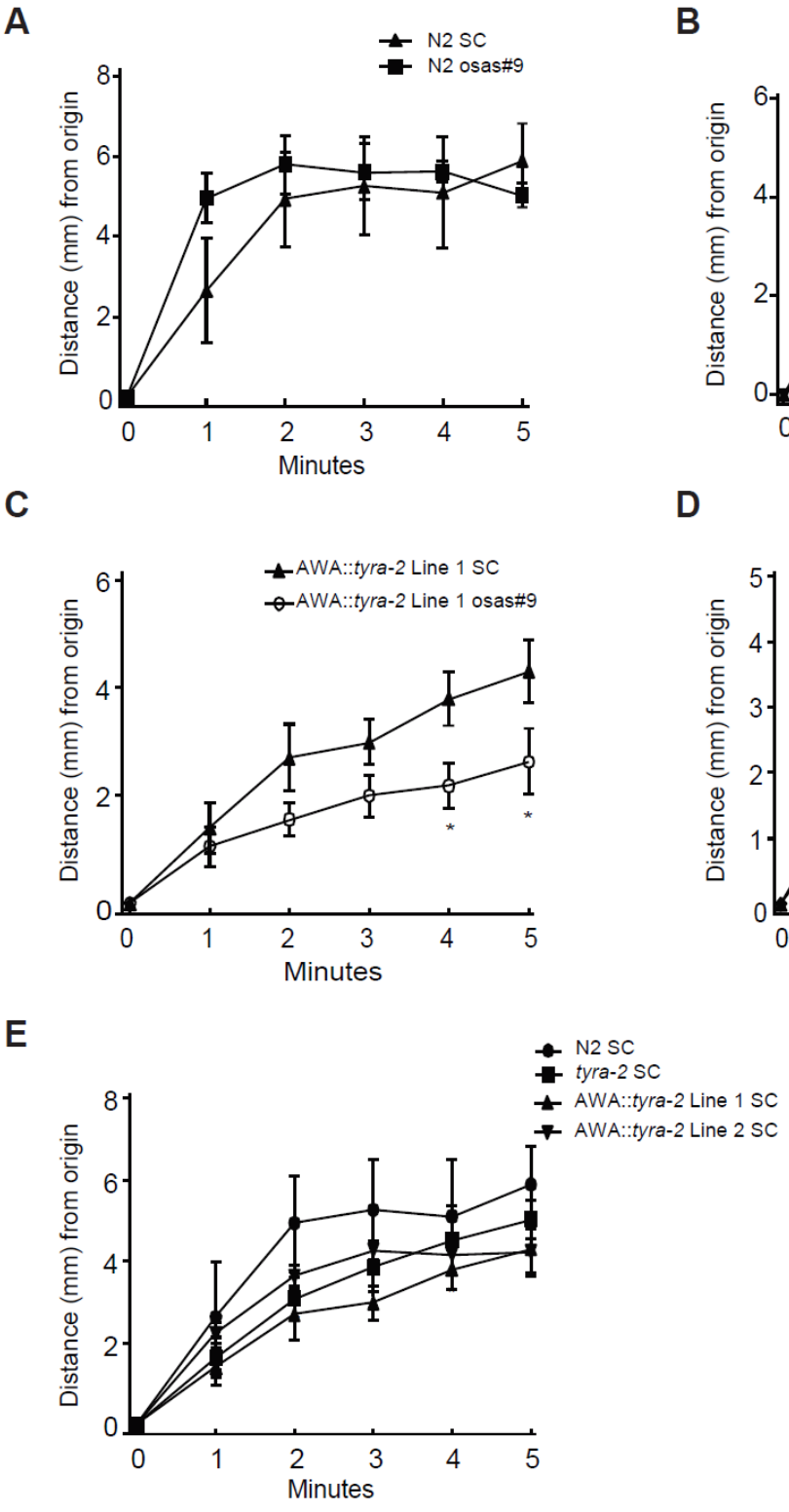
A
N2 SC
N2 osas#9
Distance (mm) from origin
Minutes
B
tyra-2 SC
tyra-2 osas#9
Distance (mm) from origin
Minutes
C
AWA::tyra-2 Line 1 SC
AWA::tyra-2 Line 1 osas#9
Distance (mm) from origin
Minutes
D
AWA::tyra-2 Line 2 SC
AWA::tyra-2 Line 2 osas#9
Distance (mm) from origin
Minutes
E
N2 SC
tyra-2 SC
AWA::tyra-2 Line 1 SC
AWA::tyra-2 Line 2 SC
Distance (mm) from origin
Minutes

Figure S5. Leaving rates for animals expressing *tyra-2* ectopically in AWA neurons are slower than both wildtype and *tyra-2 lof* animals at 10 pM osas#9. **A)** Wildtype, n≥3 trials. **B)** *tyra-2*, n=6 trials. **C,D)** Two different lines of AWA::*tyra-2* display slower leaving rates at 10 pM osas#9. n≥6 trials, Line 1 and n≥7 trials, Line 2. **E)** Comparison of solvent control for all strains in leaving assay. None of the animals varied in their response, n≥3 trials. Data presented as mean ± S.E.M; *P<0.05.

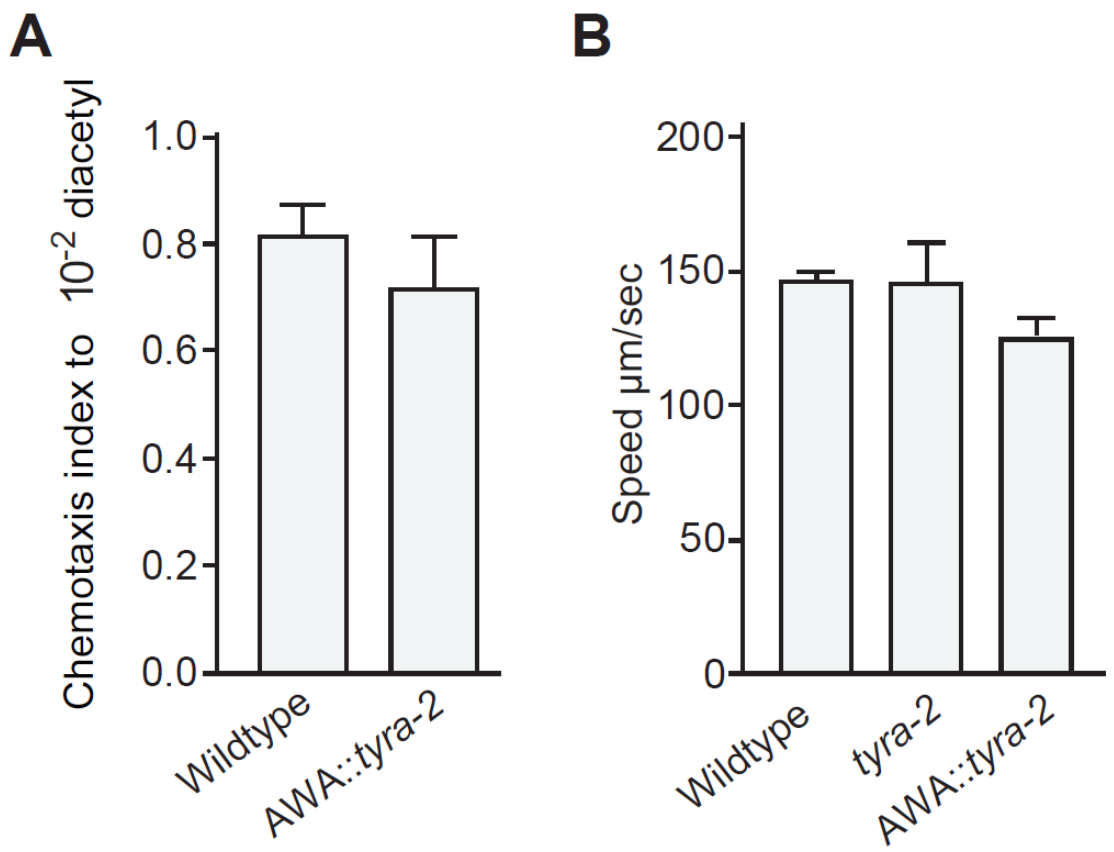

Figure S6. Ectopic expression of *tyra-2* in AWA neurons does not affect AWA-specific behaviors. **A)** Chemotaxis to 10^{-2} diacetyl was unaffected by AWA::*tyra-2,* n≥7. **B)** Locomotory behaviors were unaltered in AWA::*tyra-2* animals. Wildtype, *tyra-2 lof*, and AWA::*tyra-2* speeds are not statistically different, n≥3 trials. Data presented as mean ± S.E.M.

Video S1. Video of ASH::GCaMP3 animal being stimulated with 1 µM osas#9. osas#9 presented to animal when red dot appears on screen. Blue is low level of fluorescence and red is high fluorescence level.

Table S1. List of Strains

Source	Strain	Genotype (allele)	Avoid osas#9?
Ambros	NL3321	sid-1 (pk3321)	yes
Alkema	QW42	*tyra-2* (tm1815)	no
Alkema	MT13113	*tdc-1 (n3419)*	yes
Alkema	QW569	*octr-1* (ok371)	yes
Alkema	QW284	*tdc-1* (n3420)	yes
Alkema	CX11839	*tyra-3* (ok325)	yes
Alkema	QW1853	*tyra-2* (tm1846);worEx14[p*tyra-2* ::*tyra-2* ::GFP @ 1ng/µL]	yes
Bargmann	CX10979	N2;KyEx2865 [p*sra-6* ::GCAMP3 @ 100 ng/µL])	n.d.
CGC	CB1489	*him-8* (e1489)	yes
CGC	NL332	*gpa-1* (pk15)V.	yes
CGC	NL335	*gpa-3* (pk35)V.	yes
CGC	NL1146	*gpa-6* (pk480)X.	no
CGC	NL787	*gpa-11* (pk349)II.	yes
CGC	NL2330	*gpa-13* (pk1270)V.	yes
CGC	NL788	*gpa-14* (pk347)I.	yes
CGC	NL797	*gpa-15* (pk477)I.	yes
CGC	CX2205	*odr-3* (n2150)V.	yes
CGC	PR672	*che-1* (p672) I.	yes
Iino	JN1713	Is[*sra6* p::mCaspI]	no
Komuniecki	FX01846	*tyra-2* (tm1846)	no
Komuniecki	OH313	*ser-2* (pk1357)	yes
Komuniecki	DA1774	*ser-3* (ad1774)	yes
Schwarz	VH624	rhIs13 [unc-119::GFP + dpy-20(+)] V; nre-1(hd20) lin-15B(hd126) X.	yes
Srinivasan	JSR19	*tyra-2* (tm1846);worEx12[pLR306_p*nhr-79 _tyra-2*]	yes
Srinivasan	JSR23	N2;worEx13[p*tyra-2* ::*tyra-2* ::GFP @ 30ng/µL]	n.d.
Srinivasan	JSR45	*tyra-2* (tm1846);worEx15[pLR305_p*odr-10 _tyra-2*]	no
Srinivasan	JSR47	*tyra-2* (tm1846);worEx15[pLR305_p*odr-10 _tyra-2*]	no
Srinivasan	JSR50	*tyra-2* (tm1846) ;KyEx2865 [p*sra-6* ::GCAMP3 @ 100 ng/µL])	n.d.
Srinivasan	JSR72	*gpa-6* (pk480)X.; WorEx19 (p*nhr-79* ::*gpa-6* ::RFP @30ng/ul; punc-122::RFP)	yes
Srinivasan	JSR86	*gpa-6* (pk480)X.; WorEx19 (p*nhr-79* ::*gpa-6* ::RFP @30ng/ul; p*unc-122* ::RFP)	yes
Srinivasan	JSR88	N2; WorEx20 [p*gpa-6* ::*gpa-6* ::RFP::*unc-54* @ 5ng/ul]	n.d.
Srinivasan	JSR89	QW1853; WorEx20 [p*gpa-6* ::*gpa-6* ::RFP::*unc-54* @ 5ng/ul]	n.d.
Sternberg	PY7505	oyIs84 [*gpa-4* p::TU#813 + *gcy-27* p::TU#814 + *gcy-27* p::GFP + *unc-122* p::DsRed]	yes
Suo	VN280	*ser-6* (2146)	yes

Table S2. List of Plasmids

Source	Construct	Notes
Fire vector kit	pPD95_75	GFP
Rene Garcia	pLR305	destination vector
Rene Garcia	pLR306	destination vector
Rene Garcia	pLR304	destination vector
Srinivasan	JSR#CDC8	*gpa-6* entry clone
Srinivasan	JSR#CDC9	ASH::*GPA-6*
Srinivasan	JSR#CDC2	*tyra-2* entry clone
Srinivasan	JSR#CDC1	p*nhr-79* entry clone
Srinivasan	JSR#CDC4	p*odr-10* entry clone
Srinivasan	JSR#CDC3	ASH::TYRA-2
Srinivasan	JSR#CDC6	AWA::TYRA-2
Srinivasan	JSR#CDC24	*pgpa-6::gpa-6::RFP::unc-54*
Vidal Library	F01E11.5	*tyra-2* RNAi clone
Vidal Library	F14D12.6	*octr-1* RNAi clone
Vidal Library	pL4440	Empty RNAi clone

Table S3. List of Primers

Purpose	Gene	Primer	Sequence
tyra-2 ASH rescue construct	*tyra-2* (from genomic)	F	attB5 *ggcttatccgttgtggagaa*
		R	attB2 *ttggcccttccttttctctt*
tyra-2 ASH rescue	*pnhr-79* (from genomic)	F	attB1 *gtgcaatgcatggaaaattg*
		R	attB5r *atacacttcccacgcaccat*
tyra-2 translational fusion (PCR)	*tyra-2* (from genomic)	F	*atgttttcacaagtttcaccaca*
		F nested	*ttcacaagtttcaccacattacaa*
		R (w overhang in caps)	*AGTCGACCTGCAGGCATGCAAGCT gacacgagaagttgagctgggtttc*
	GFP (pPD95_75)	as found on wormbook chapter: reporter gene fusion	
AWA *tyra-2* misexpression	*podr-10* (from genomic)	F	attB1 *ctcgctaaccactcggtcat*
		R	attB5r *gtcaactagggtaatccacaattc*
RT-qPCR	*ama-1*	F	GGAGATTAAACGCATGTCAGTG
		R	ATGTCATGCATCTTCCACGA
	tyra-2	F	GAGGAGGAAGAAGATAGCGAAAG
		R	TGTGATCATCTCGCTTTTCA
gpa-6 ASH rescue	*gpa-6* (from genomic)	F	attB5 cgtctctttcgtttcaggtgtat
		R	attB2 tattttcaaagcgaaacaaaaa
gpa-6 translational fusion (plasmid)	p*gpa-6*::*gpa-6* (from genomic)	F	acatctggtacccctcaatttccacgatct
		R	acatctaccggtctcatgtaatccagcagacc
	unc-122::RFP	F	acatctaccggtATGGTGCGCTCCTCCAAG
		R	ttaataggtaccTGGTCATAGCTGTTTCCTGTG

2 B Additional behavioral parameters of osas#9

In addition to part A of Chapter Two, additional preliminary information was obtained for understanding primary sensation of osas#9 and the mechanisms of the circuit. At the transduction level we have identified the importance of TYRA-2 and GPA-6 for driving osas#9 behavior via ASH sensory neurons. Herein, we show further data on the relationship between osas#9 and TYRA-2 through calcium imaging of the reprogrammed AWA sensory neurons and further look at signal propagation by investigating the G protein regulators and channels. Furthermore, we analyze several potential neuromodulators shaping the circuit, including DAF-7 and neuropeptide signaling. Lastly, we explore two additional behavioral paradigms involving osas#9: developmental memory and choice.

2 B.1 TYRA-2 and osas#9 signaling

Previous studies in *C. elegans* have revealed that behavioral responses provoked by an odorant are specified by the nature of the sensory neuron in which the receptor is activated in (52). Utilizing this strategy, in Chapter Two, part A, we designed a *tyra-2* misexpression line that ectopically expressed *tyra-2* in the AWA sensory neurons in a *tyra-2 lof* background. We found that animals with reprogrammed sensory neurons displayed attractive behavior, a characteristic of AWA function (53), rather than the typical aversive response to osas#9. Furthermore, this technique has been used in conjunction with calcium dynamics to show stimulation of reprogrammed neurons (29, 31). Therefore, we hypothesized that only reprogrammed AWA sensory neurons with TYRA-2 would show calcium transients upon exposure to osas#9. As such, we measured calcium transients using GCaMP2.2b expressed in AWA neurons in both wild-type and

AWA::TYRA-2 animals. Upon osas#9 exposure, we observed hyperpolarization in AWA::TYRA-2 animals only, whereas no change was observed in wildtype animals expressing GCaMP2.2b (Fig. B1A,B). As depolarization of AWA sensory neurons upon diacetyl stimulation results in suppressed turning behavior (90), we asked whether hyperpolarization of AWA by osas#9 increases reversals in AWA::*tyra-2* mis-expression animals. We analyzed animal behavior during the leaving assay and found that AWA::TYRA-2 animals show nearly a two-fold increase in reversals when exposed to osas#9 compared to *tyra-2* mutant and wild-type animals (Fig. B1C). Our findings suggest that in worms ectopically expressing TYRA-2, osas#9 perception results in hyperpolarization of this neuron, increasing reversal frequency, resulting in attraction to osas#9.

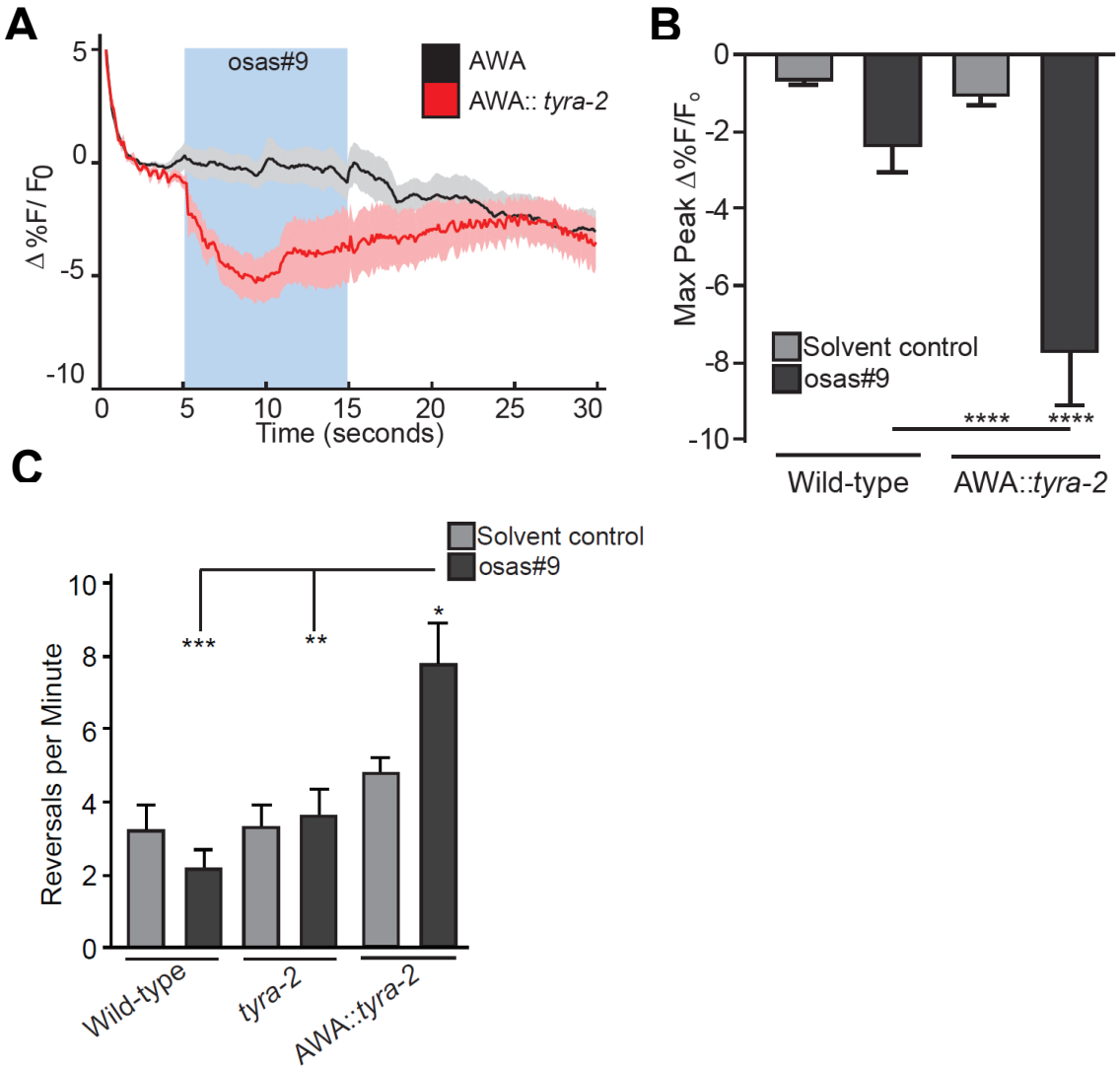

Figure B1. Reprogramming AWA sensory neurons confers behavioral and physiological changes to osas#9 **A,B)** Calcium dynamics of AWA neurons upon osas#9 exposure. **A)** AWA::*tyra-2*::GCaMP2.2b animals (red) show hyperpolarization when exposed to 1 µM osas#9 in a microfluidic olfactory chip. AWA::GCaMP2.2b animals (black) did not display a change in fluorescence upon stimulation. Shaded blue region depicts time when animals were subjected to the stimulus, n=10 animals. **B)** Maximum peak fluorescence

before (solvent control) and during exposure to 1 µM osas#9 was plotted from the data shown in A for statistical comparison. Without *tyra-2,* no change in calcium transients is seen in AWA, n=10 animals. **C)** AWA::*tyra-2* animals have an increased reversal rate in comparison to both wild-type and *tyra-2 lof* animals in 10 pM osas#9, n≥3. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups, asterisks with bars depict comparison between groups.

In addition to looking at heterotrimeric protein involvement in osas#9 sensation, we asked if G protein regulators may be involved in osas#9 sensation. Previously the Regulator of G protein Signaling (RGS) protein RGS-3 and the G protein-coupled receptor kinase (GRK) protein GRK-2 have been shown to be involved in aversive responses (91, 92). As such, we subjected *rgs-3* and *grk-2 lof* animals to osas#9. Animals lacking these proteins were not deterred by the ascaroside, indicating the necessity of these regulators in signal transduction of osas#9 (Fig. B2). The vanilloid transient receptor potential channel (TRPV) OSM-9 in *C. elegans* is required for nociceptive signal transduction in ASH sensory neurons (57, 93-95). Therefore, we investigated *osm-9 lof* mutants and a cell-sepcific rescue of *osm-9* in ASH. We found that aversion to osas#9, like other repellents, required OSM-9 in ASH sensory neurons (Fig. B3). Taken together with the results of Chapter Two, part A, osas#9 transduction occurs in ASH sensory neurons via activation of the GPCR TYRA-2, and requires the Gα subunit GPA-6, the regulators RGS-3 and GRK-2, and the cation channel OSM-9.

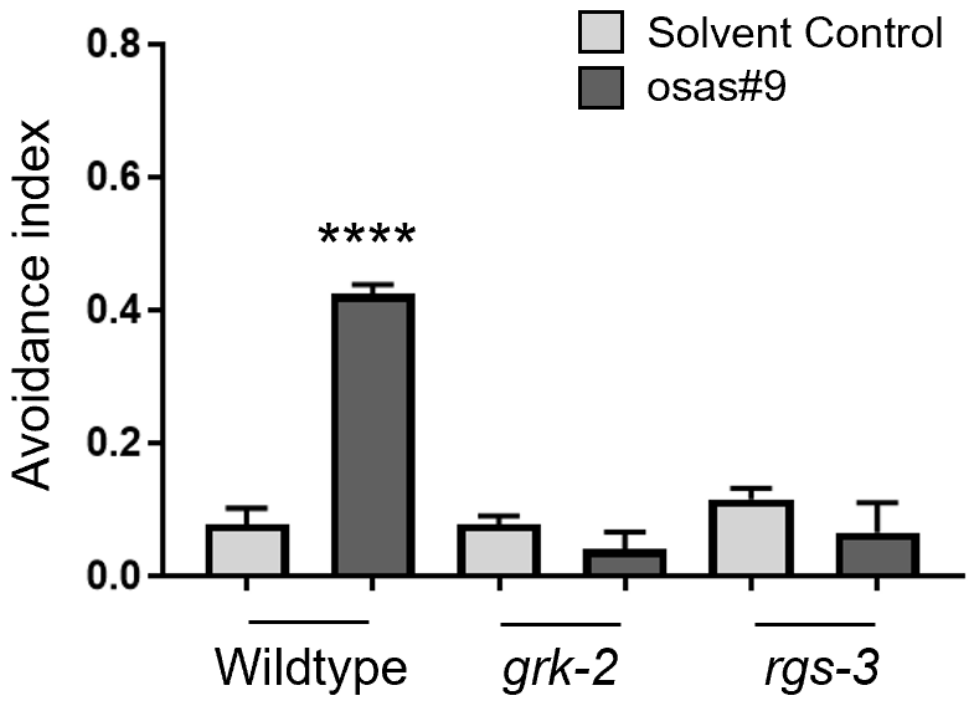

Figure B2. G protein regulators are required for osas#9 aversion. Animals with null mutations in *rgs-3* and *grk-2* are unable to avoid osas#9. n≥3. Data presented as mean ± S.E.M; **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups.

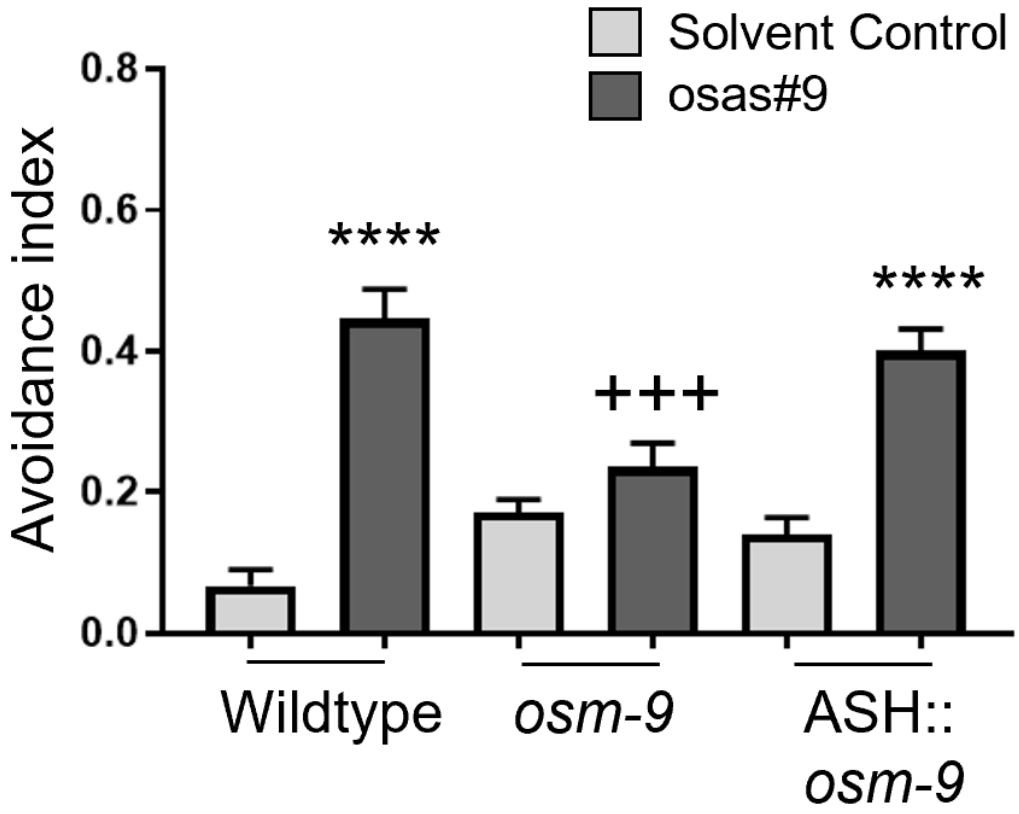

Figure B3. ASH requires OSM-9 for osas#9 response. OMS-9 rescued in ASH neurons is sufficient to reconstitute wild-type behavior in response to 1 µM osas#9, n≥4. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups, "+" signs denote comparison to wildtype.

2 B.2 Signaling compounds necessary for osas#9 response.

In addition to the necessity of ASH sensory neurons for osas#9 sensation, our data in Chapter Two, part A, indicate that other sensory neurons, namely ASI and ASE, may have a role in establishing the neural circuitry underlying osas#9 aversion. ASI sensory neurons display a broad role in *C. elegans* behavior and development. The sensory pair contributes to regulating avoidance, foraging behaviors, and dauer formation (29, 31, 32, 73, 74, 96, 97). Unique to ASI sensory neurons under normal conditions is the secretion of a transforming growth factor beta (TGF-β) like ligand, DAF-7, when there are favorable environmental conditions, including food availability (66, 98). The removal of food results in decreased DAF-7 signaling which, in turn, results in the animals' physiology preparing for starvation conditions (66). We hypothesized that DAF-7 may be necessary for proper osas#9 avoidance. Interestingly, we found that starved animals lacking DAF-7 no longer displayed avoidance to osas#9 (Fig. B4A), implying that low levels, and not complete eradication of the signaling may be necessary.

In addition to targeting the role ASI signaling may be having on the circuit, we tested mutations in TAX-2 and TAX-4 cyclic nucleotide gated channels, as they are expressed broadly in the amphid sensory neurons, but not in the ASH pair (99). We found that TAX-2, TAX-4, and TAX-2;TAX-4 double mutants all had defective behavioral response to osas#9 (Fig. B4B), indicating that although ASH may be necessary for the response, other sensory neurons are contributing to the circuit.

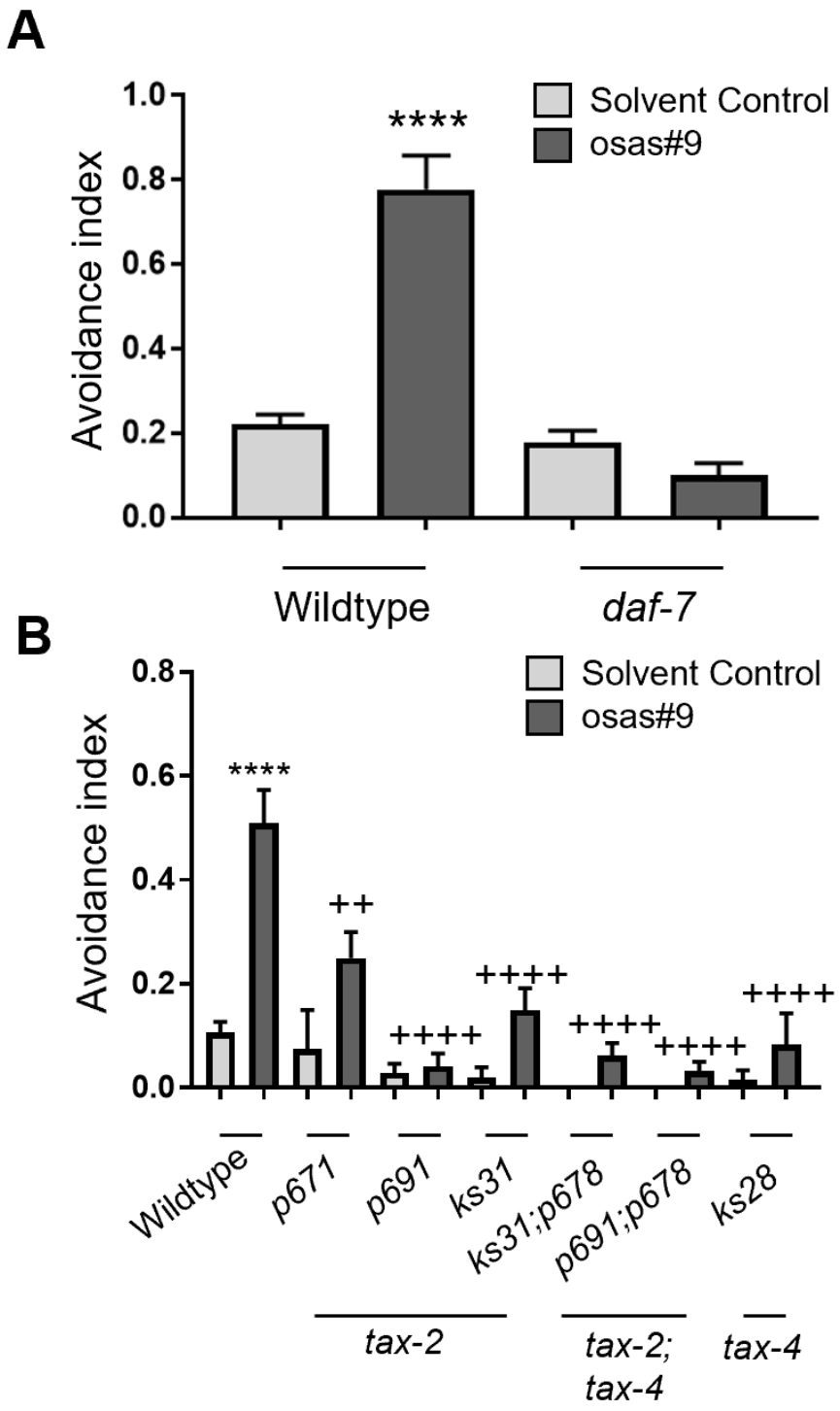

Figure B4. Non- ASH sensory neurons contribute to osas#9 response. **A)** DAF-7 signaling is required for the osas#9 avoidance response. *daf-7 lof* animals do not show aversion to osas#9. n≥3. **B)** The cyclic nucleotide gated channels, TAX-2 and TAX-4 are

required for normal aversion to osas#9. n≥2. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups, "+" symbol denotes comparison to wildtype.

Neuropeptides have known roles in establishing circuits underlying behavioral states, for example, the neuropeptide PDF-1 acts as a switch for roaming and dwelling behavioral circuits (96). We tested several neuropeptides of the FMRFamide related family (FLPs) for their potential role in modulation of the osas#9 response. Of the FLPs tested (3,6,12, and 19) FLP-19 is required for normal response to osas#9 (Fig. B5). Further studies elucidating its site of release and action are needed for further insight.

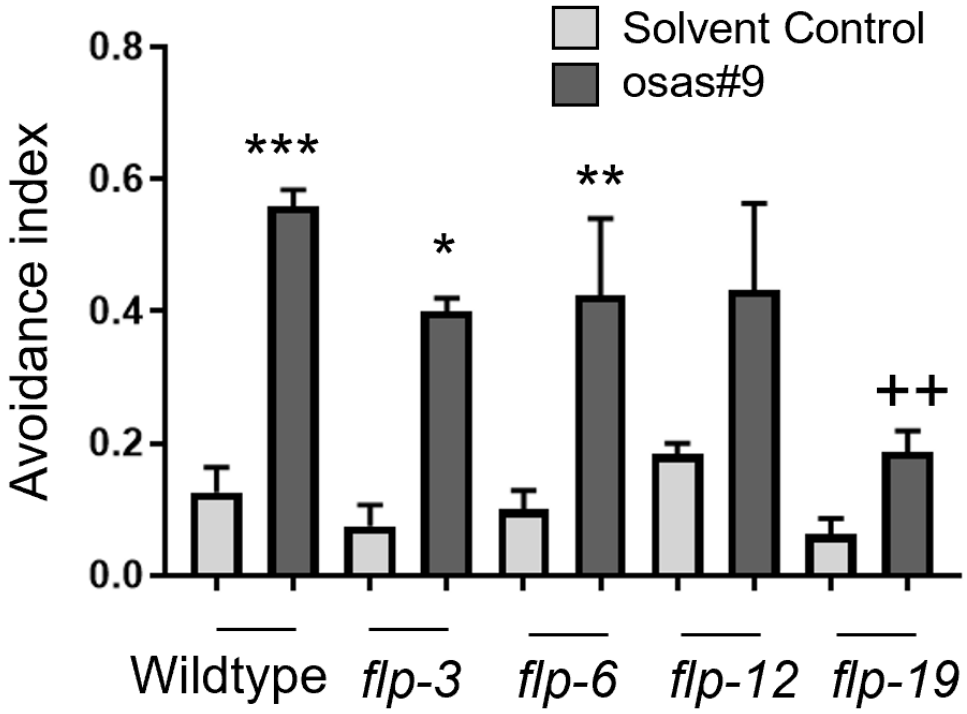

Figure B5. Preliminary screen of neuropeptides involved in osas#9 perception. The neuropeptide, FLP-19, was determined to be necessary for avoidance to osas#9. n≥3. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups, "+" symbol denotes comparison to wildtype.

2 B.3 Further behavioral paradigms

While the focus of the role of osas#9 has been understanding how it drives an avoidance response, the pheromone offers other avenues of interesting questions that can be asked. We investigated two other avenues of osas#9 behavior, choice and developmental memory.

Ascarosides have been shown to have developmental memory effects, namely, early exposure to ascr#3 in larval stage 1 (L1) animals was found to modulate adult behavior (100). We found that exposure to 1 µM osas#9 for 30 minutes as L1 animals abolished aversive responses in adults exposed to osas#9 in the avoidance assay (Fig. B6). Furthermore, it was found that exposing L1 animals to osas#9 also resulted in trans-generational effects, offspring of pre-exposed animals not only showed reduced avoidance as adults, bust subsequent generations do as well (101). This data demonstrated that pre-exposure to osas#9 is capable of altering adult behavior, and future generations.

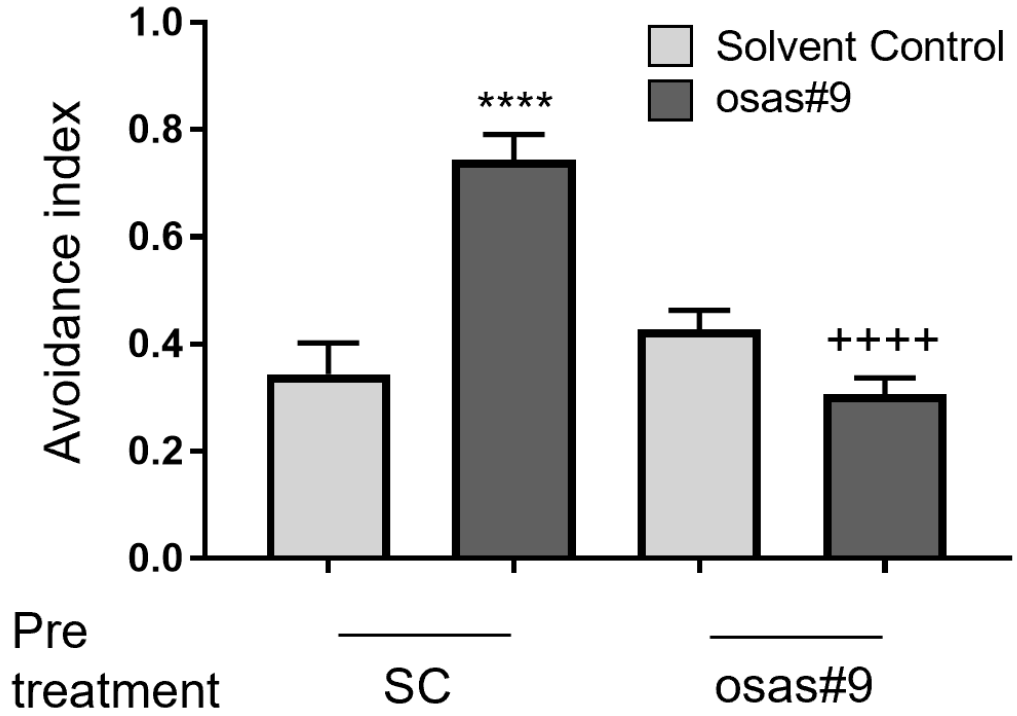

Figure B6. Pre-exposure to osas#9 at the L1 stage affects adult behavior. Animals pre-treated with osas#9 show aberrant response to osas#9 as young adults. n≥11. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups, "+" symbol denotes comparison to wildtype.

Lastly, it was demonstrated that starved *C. elegans* exposed to osas#9 in conjunction with *E. coli* extract show an attenuated response to osas#9 (Chapter Three) (22). However, these tests were limited to acute avoidance behavior. We hypothesized that although the osas#9 response is attenuated by *E. coli*, given a choice, animals would prefer a food patch without osas#9. We placed animals on a plate equidistant from two food patches, one containing osas#9, and observed the animals for twenty minutes. It was observed that animals, given a choice were more apt to feed on the non-osas#9 containing patch (Fig. B7A). Moreover, when animals entered the osas#9 containing patch, they were significantly more likely to exit (Fig. B7B). Taken together, these data demonstrate that although *E. coli* can attenuate the acute avoidance response, animals still show aversive behavior to osas#9 in food patches.

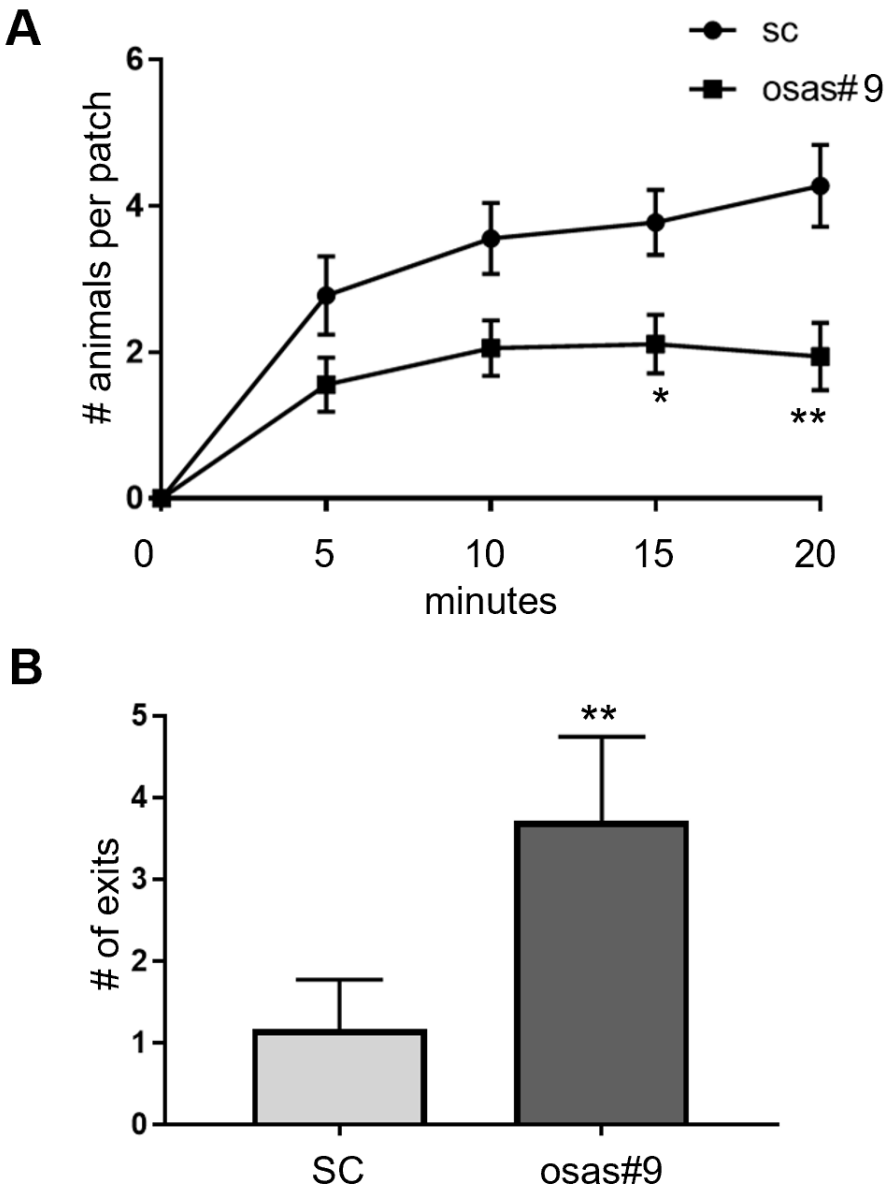

Figure B7. *C. elegans* prefer *E. coli* patches without osas#9. **A-B)** Starved animals were placed on a plate containing two patches of *E. coli*, with either osas#9 or the solvent control. **A)** After 15 minutes, significantly more animals were in the food patch without osas#9. n≥18. **B)** Significantly more animals exited the food patch containing osas#9 than the solvent control during the duration of the assay (20 minutes). n≥18.Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, A) One-Way ANOVA,

followed by Sidak's multiple comparison post-tests. B) Mann-Whitney test. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups.

2 B.4 Discussion

We have provided strong preliminary data for the regulation of the osas#9 response and provided insight beyond that of acute exposure to osas#9. The requirement of OSM-9 indicates that it is downstream of TYRA-2 activation by osas#9 (Fig. B8). It is generally believed that OSM-9 depolarization is achieved through lipid signaling after GPCR activation (99). Our data from Chapter 2 Part A implies that the Gα subunit GPA-6 is coupled with TYRA-2, and thus it may propagate the signal via control of secondary phospholipid messengers (Fig. B8). Further studies would need to be carried out to see if GPA-6 is acting directly to modulate OSM-9 or if Gβγ subunits dissociated upon receptor activation are responsible. Additionally, how the G protein regulators are contributing to the signal remains to be discovered. Generally, RGS and GRK proteins serve to dampen signals, suggesting that their absence may result in hyperactive responses (61, 102). However, we determined that animals lacking these regulators show a defective response to osas#9 (Fig B3,8). Previous studies have also indicated that in *C. elegans* RGS-3 and GRK-2 mutants show abolished responses to water-soluble repellents (91, 92). They hypothesize that overcompensation for the loss may result in more drastic downregulation of the signaling pathways or that Gα saturation may be responsible for the observed defects in chemosensory responses (91, 92). One method in which we could parse out this effect would be to overexpress GPA-6 and see if it mimics the phenotype seen in the regulation mutants.

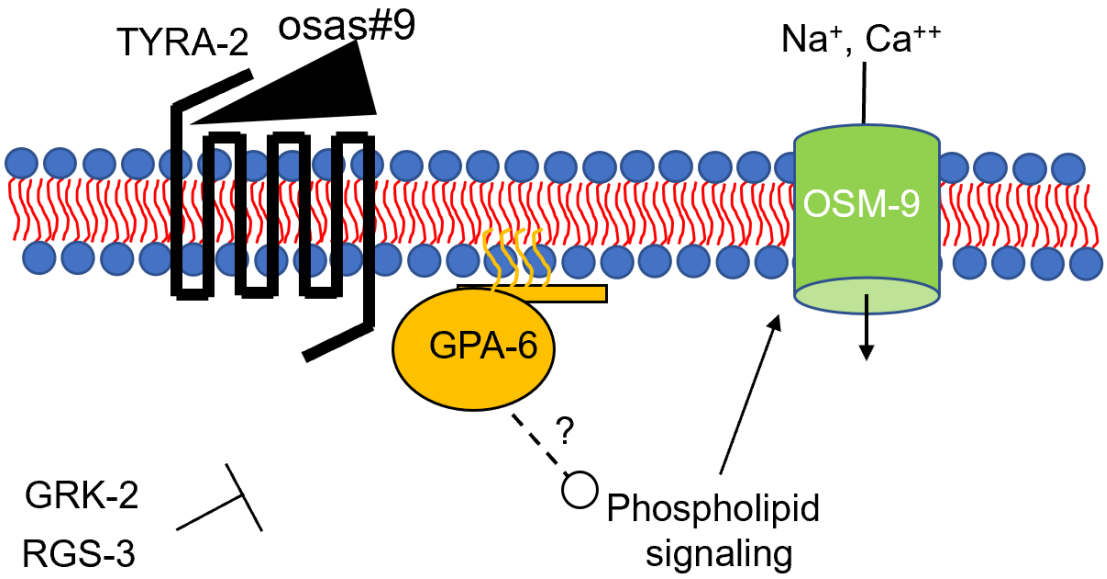

Figure 8. Schematic depicting signaling pathway in ASH sensory neurons in response to osas#9. The aversive pheromone, osas#9, interacts with the GPCR TYRA-2 activating the transduction pathway. GPA-6, also present in the cilia of ASH and required for aversion is hypothesized to partner with TYRA-2 and relay the signal. The target of GPA-6 is unknown, but it may regulate phospholipid production or consumption (dotted line) to modulate the required OSM-9 channel. The regulatory proteins, RGS-3 and GRK-2 are required for the response.

The aggregation pheromone, icas#3, increases reversals upon detection (35). However, when ASK sensory neurons are ablated, the increased reversal rate is abolished, and attraction is no longer observed (35). This demonstrates that detection of an attractant cue can increases reversals. Therefore, our data demonstrating that reprogrammed *C. elegans* show increased reversal frequency when exposed to osas#9 (Fig. B1C) implies that animals turn to remain near the attractive stimulus.

Distinct from testing osas#9 transduction, we are interested in unveiling the role of other sensory neurons. We found that the cyclic nucleotide gated channels TAX-2 and TAX-4 are required for the response (Fig. B4B), despite not being present in ASH sensory neurons. However, *tax-2* and *tax-4* channels are also required for the proper development and formation of sensory neurons (99). Therefore, to decouple the developmental effect from the behavioral abnormality observed, additional studies would need to be carried out. For example, temporal rescue of these channels in adult animals would provide insight into the function of these CNGs in osas#9 behavior. Similarly, DAF-7 behavioral defects (Fig. B4A) may be an effect of reduced DAF-7 signaling dauer formation (66, 99); this reduction may be important for establishing the state dependent response of osas#9, and the null *daf-7* mutant may mask this effect. To provide more insight on the role of DAF-7 in osas#9 avoidance, we could express the ligand in starved animals, which usually have decreased DAF-7 signaling, and assay animals for avoidance. If starved animals no longer avoid osas#9, it would indicate that the concentration of DAF-7 is an important regulator of this aversion response to osas#9.

Lastly, this work provides the framework for two further avenues of study utilizing the aversive compound osas#9: developmental memory and food patch assessment.

Previous work has identified that ascaroside exposure at the L1 stage can affect adult behavior by increasing activity in post-synaptic neurons (100). However, a stark difference from our data is that pre-exposure increased avoidance behavior to ascr#3 (100), whereas osas#9 pre-exposure dampened both adult and future generations response to the aversive cue (Fig. B6)(101). This provides an interesting opportunity to compare the imprinting effects of two different pheromones. It is also intriguing that L1 animals would show a reduced response to osas#9 as adults when pre-exposed to osas#9, as the cue is produced exclusively by starved L1 animals (22). Perhaps this effect would be mitigated if animals were exposed for less time, as natural animals would disperse upon detection of the cue. Another interesting paradigm investigated in this section was the observation that starved animals, when given a choice, choose food without osas#9 (Fig. B7). As acute co-exposure to osas#9 and *E. coli* attenuates the avoidance response, it is interesting that animals are observed to exit a food patch containing the aversive cue (Fig. B7). This suggests that the detection of *E. coli* does not completely override osas#9 sensation as implied by acute exposure, as animals over time migrated and stayed in the food patch without osas#9 (Fig. B7). How this is encoded in the nervous system in contrast to acute exposure is an intriguing avenue for follow up studies on decision making by the animal.

Together, this section of Chapter Two extends the data from part A, further demonstrating TYRA-2 as a driver for behavioral and physiological responses to osas#9 and provides additional data regarding the transduction machinery underlying the primary response. It also lays the foundation for further studies on circuit components required for the aversive response to osas#9, namely DAF-7 and FLP-19. Lastly, the development of

new paradigms offers an experimental basis in which to study the effects of imprinting, epigenetics, and decision making.

2 B.5 Methods

Avoidance drop test

The tail end of a forward moving animal is subjected to a small drop (~5 nl) of solution, delivered through a hand-pulled 10 µl glass capillary tube. The solution, upon contact, is wicked up the side of the animal to the amphid sensory organ. Upon sensory cilia exposure the animal is observed for four seconds, where in that time it displays no response or an avoidance response. Aversive responses are characterized as at least one half of a complete head-swing followed by a change in direction of at least 90 degrees from the original direction of locomotion. The total number of avoidance responses is divided by the total number of trials and termed the avoidance index. Methods based on Hilliard et. Al, 2002 (83). Each trial is done concurrently with with osas#9 and the solvent control (osas#9 diluted in DIH_2O). Osas#9 was synthesized by methods outlined in Artyukhin et. Al, 2013 and provided by the Schroeder lab at Cornell University.

Integrated mutant strains and controls are prepared using common M9 buffer to wash and transfer a plate of animals to a microcentrifuge tube where the organisms are allowed to settle. The supernatant is removed and the animals are resuspended and allowed to settle again. The supernatant is again removed and the animals then transferred to an unseeded plate. After 1 hour, young adult animals are subjected to the solvent control and the chemical of interest at random with no animal receiving more than one drop of the same solution. Extrachromosomal transgenic animals were selected for

under a fluorescent scope and gently passed to an unseeded plate and tested after one hour.

Pre-exposure experiments were assays in the same manner. However, L1 animals were placed in 100 µL of 1 µM osas#9 in an epitube for 30 minutes, and then cultured at normal conditions. Young adults were then prepared as detailed for the avoidance assay.

Calcium Imaging

Calcium imaging was performed using a modified microfluidic olfactory chip as outlined in Reilly et. al, 2017 (103). Briefly, a young adult worm was loaded into a microfluidic device with its amphid sensory cilia exposed to a channel that's contents can be temporally controlled. Each animal is exposed to stimulus for 10 seconds and the recorded. The change in fluorescence can then be quantified using imageJ software.

Reversals

Reversals were analyzed and measured using WormLab4.1 (MBF Bioscience, Williston, VT USA) from videos recorded for the holding assay between minute one and two as it was when the divergence was first seen in distance between strains in the holding assay.

Choice assay

Animals were transferred to an unseeded plate and allowed to starve for one hour. Assay plates were prepared by placing two 10 µL drops of *E. coli* OP50 from overnight culture in LB media onto two regions indicated on the template near the edge of 60 mm NGM plate. After one hour, 1 µL of 1 µM osas#9 or solvent control was placed on the OP50 spots. Animals were placed in the center of the plate and recorded for 20 minutes. At 5,

10, 15, and 20 minutes the number of animals in each spot was quantified. Through out the entire duration, it was tracked when animals left a spot.

Statistical analysis

Statistical tests were run using Graphpad Prism. For all figures, when comparing multiple groups, ANOVAs were performed followed by Sidak's multiple comparison test. When only two groups were compared, a Student's t-test was used (Figure B7B). When comparing different strains/conditions, normalized values of osas#9 avoidance index response relative to the respective solvent control were used. This was done to account for any differences in the response to solvent control for the respective groups. When normalizing fold change of osas#9 response to solvent control response for the avoidance assay within a strain/condition, data was first log transformed so a fold change could still be calculated for control plates that had a "0" value. For avoidance assays and the choice assay statistical groups were based on the number of plates assayed, not the number of drops/animals. Calcium imaging statistics were based on pulses and at least 10 animals.

Strains

See Table BS1.

Acknowledgements

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Table BS1: Strains used in 2B

Source	Strain	Gene (allele)
Albrecht	CX14887	N2;kyIs598 [gpa-6::GCaMP2.2b 50 ng/µL]
Bargmann	CX7265	*osm-9*(ky10) IV;yzEx53 [*osm-10::osm-9*, *elt-2*::gfp]
Bargmann	cx2989	*tax-2(p691)* I; *tax-4(p678)*III
Bargmann	cx3085	*tax-2(ks31)* I; *tax-4(p678)*III
Bargmann	CX6750	*tax-4(ks28)*kyEx747
CGC	CB1372	*daf-7*(*e1372*) III.
CGC	PR671	*tax-2(p671)* I.
CGC	PR691	*tax-2(p691)* I.
CGC	FK104	*tax-2(ks31)* I.
Ferkey	FG0001	*grk-2 (gk268)*
Ferkey	LX0242	*rgs-3 (vs19)*
Komuniecki	FX01846	*tyra-2* (tm1846)
Li	N/A	*flp-3(pk361)*
Li	NY183	*flp-6(pk1593)* x7
Li	NY106	*flp-12(n4902)*
Li	NY193	*flp-19(pk1594)*
Srinivasan	JSR45	*tyra-2* (tm1846);worEx15[pLR305_p*odr-10_tyra-2*]
Srinivasan	JSR51	JSR45;kyIs598 [*gpa-6*::GCaMP2.2b 50 ng/µL]
Sternberg	PT839	*osm-9(ky10); him-5(e1490)*

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3 Attenuation of osas#9 response by *E. coli* extract

The work in Chute *et al.* (submitted to eLife, 2018, and also submitted as the previous chapter in this thesis) established the framework of osas#9 sensation, thereby enhancing our understanding of brain function at both the molecular and cellular levels: linking a ligand to a receptor, and ultimately: behavior. Chapter 3 seeks to build on this foundation, and characterize how the primary response is modulated in the presence of multiple stimuli. When starved *C. elegans* encounter osas#9 alongside *E. coli* metabolites, the osas#9 aversive response is abrogated. This chapter aims to determine how this information is integrated; the identity of compound(s) in the *E. coli* extract responsible for the attenuation of the osas#9 response; and the identity of the neurons and modulators are involved. The first half of this work presents the nature of circuit modulation and multisensory integration, in order to provide the reader with necessary background information to inform the logic behind the circuit model of osas#9 perception and state-dependent integration, postulated in the second part of this chapter.

The primary detection information from the previous chapter serves as a launching pad for constructing a microcircuit from which we can begin to glean insights into how multiple conduits of external information are integrated and perceived by the brain. As mentioned in the second half of this chapter, this information will serve to bridge the gap between our knowledge of multisensory integration abnormalities underlying social disorders, and the mechanisms responsible.

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3 A Processing of Sensory Stimuli is Complex and Modular

Understanding how a stimulus is processed by the olfactory system with respect to other stimuli and physiological state is critical in understanding how chemical social cues are routed and integrated in the brain to enact instinctive behaviors. Due to sex-based differences, the complexity of intracellular signaling,, the modulatory nature of a circuit by the physiological state, and the integration of multiple stimuli, this proves to be an extremely challenging undertaking. When considering a response to a stimulus, it is important to consider how the response may be influenced by these factors.

3 A.1 Divergent functions within a neuronal class

It is well documented that within the *C. elegans* nervous system, a single neuronal class is involved in sensation of diverse stimuli to elicit varying behaviors. For example, the polymodal nociceptive neuron, ASH, detects a myriad of different mechano-, osmo-, and chemo- stimuli that all result in aversive behavior (1-7). However, not all stimuli utilize the same pathways and connections, as one might expect – given that detection occurs via a single sensory neuron to serve as a progenitor for those circuits.

The diversity in neuronal circuitries is in part due to the intracellular machinery used within individual neurons. *C. elegans* is equipped with a large set of G protein subunits that exhibit overlapping expression, rendering particular intracellular pathways important in behavioral circuits (8). The nematode's genome codes for 21 Gα protein subunits alone, along with two subunits each of both Gβ and Gγ proteins (8, 9). Of the 21 Gα subunits, 16 are expressed throughout the chemosensory neurons, with many overlapping in their expression profiles (8, 9). For example, on its own, ASH expresses ten different Gα subunits, while ASE expresses only three (8-10).

Surprisingly, ASH studies have revealed differential use of both intra- and inter-cellular signaling molecules upon detection of various stimuli, yet activation of the neuron always results in the same behavioral outcome: avoidance (3, 6, 7, 11-13). For example, nose-touch avoidance, which is assayed by allowing the animal to run into an eyelash positioned perpendicularly to the animal's movement, requires expression of *itr-1* in ASH (Fig. A1) (11). Yet, *itr-1* has not been found to be necessary for osmotic aversive responses mediated the same neuron (Fig. A1) (11). This implies that specific signaling pathways may be utilized by the same neuron in response to individual stimuli, in turn activating unique pathways within the neuron and ultimately deciding which synapses relay the response, establishing the functional connections. Indeed, response to nose touch and benzaldehyde require *itr-1* in ASH neurons (11). However, only nose touch requires IP3 production via the phospholipase C enzymes *egl-8* and *plc-3* (11). It is therefore likely that the upstream Gαq subunit, *egl-30*, is only involved in nose touch, but not benzaldehyde detection (11).

Downstream of the initial sensation of stimuli, differences in functional post-synaptic connections have also been observed. As with *itr-1,* the glutamate receptor, *glr-1,* is utilized primarily in nose touch avoidance, as well as regulating subtle reactions within that of osmosensation (Fig. A1) (7, 12, 13). Conversely, specific genes within ASH, such as *osm-10,* are specific to osmotic detection, and not tactile responses. These genes have been shown to have specific downstream targets, such as *nmr-1* (Fig. A1) (3, 6, 13). Thus, it is possible different stimuli evoke different intra-signaling pathways, which in turn lead to varied synaptic release profiles, enabling activation of specific downstream targets within the functional circuit. Supporting this notion is the clear

presence of both synaptic and dense core vesicles in ASH (14). Furthermore, it has been shown in ASI neurons that, depending on the signaling molecule, different neuropeptides are released from distinct neuronal compartments, asymmetrically, between the neurons of the pair (15).

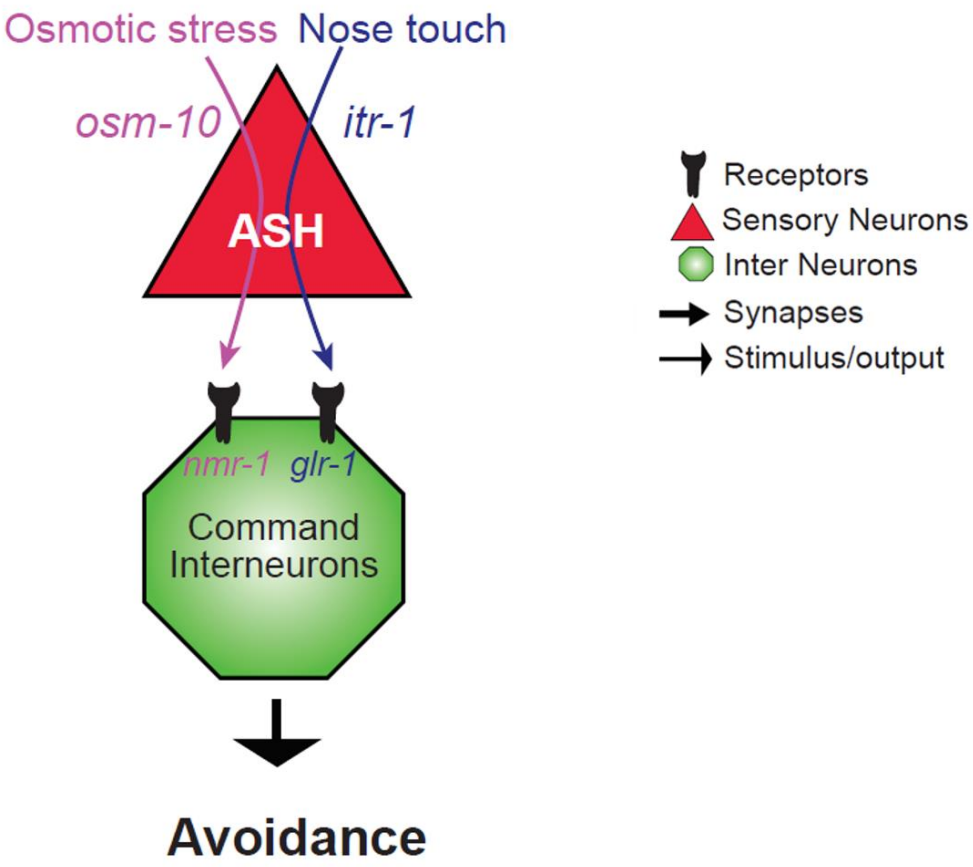


Figure A1. Different pathways can drive the same behavior. The polymodal sensory neuron, ASH, detects a plethora of signals, varying from heavy metals to tactile sensation. Stimulation of ASH sensory neurons results in a characteristic avoidance response, however, the transduction machinery and pathway vary based on stimulus.

While certain intracellular components and synaptic connections are vital in some behaviors, they may be irrelevant in other behavioral circuits which utilize the same neurons. One example of this is the amphid sensory neuron, ADL, and its involvement in the response to ascaroside #3 (ascr#3). Hermaphrodites are observed to avoid ascr#3 through ADL chemical synaptic transmission, presumably, to the backward command interneurons, AVA and AVD (14, 16). Promotion of ADL response to ascr#3 is achieved through the gap junction hub-and-spoke RMG circuit, wherein the interneuron RMG serves as a hub to modulate sensory neuron responses (16, 17). RMG, through the activity level of the neuropeptide receptor *npr-1*, and input from the sensory neuron ASK, can inhibit ADL-triggered avoidance by altering gap junction properties (16, 17). Thus, chemical synapses are involved in the avoidance to ascr#3, whereas gap junctions are necessary for modulating the response, in an *npr-1* dependent manner, to elicit aggregation or attraction (Fig. A2).

Interestingly, the sex of the animal can establish the synaptic connection and function of a neuron. In males, ascr#3 is also sensed by the serotonergic neuron ADF, as well as hermaphrodites which have been masculinized through expression of the transcription factor, *fem-3,* which inhibits the sexual regulator gene, *tra-1* (18, 19). Neuronal activation of ADF by ascr#3 requires *mab*-3, which is naturally inhibited in hermaphroditic animals (18). As ADL is still activated in males, masculinized ADF inhibits the aversive response to ascr#3. This inhibition may be taking place via extrasynaptic connections, or direct serotonin signaling on a downstream neuronal target of ADL (Fig. A2). Biological sex can also determine different physical circuits, wherein synapses between certain neurons are only present in males, and pruned in hermaphrodites (20).

This highlights the necessity of investigating how sex results in specific connections underlying a behavioral circuit, not merely the requisite neuron, in order to generate a more complete functional connectome.

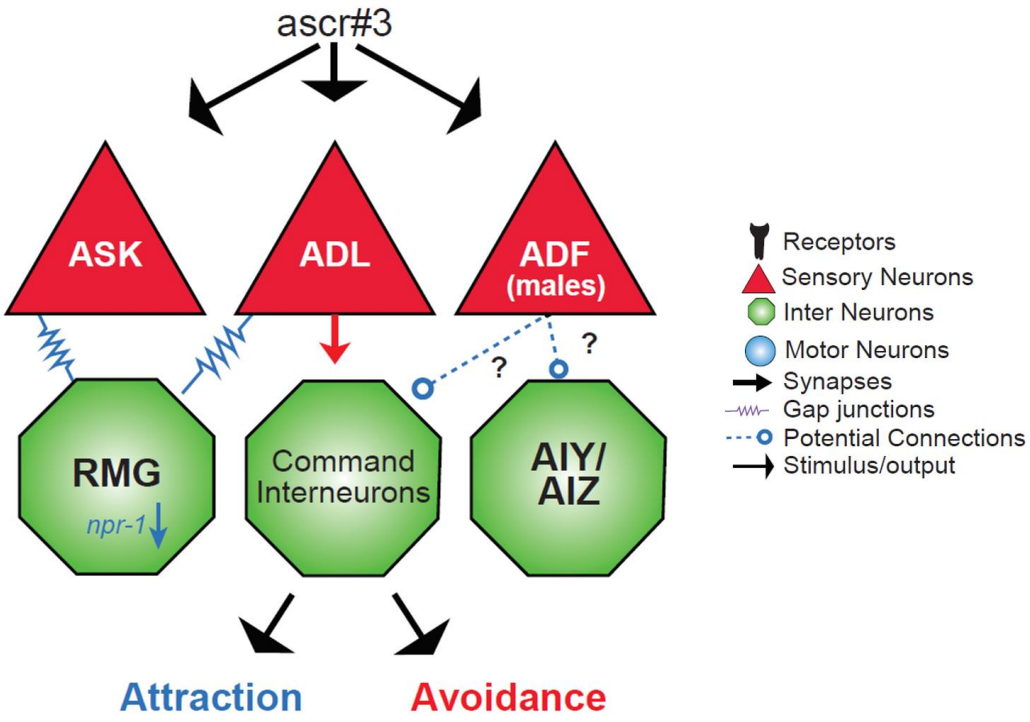


Figure A2. Multiple sensory neurons and outcomes can be modulated by sex and social status. The ascaroside, ascr#3, is sensed by ASK, ADL, and ADF sensory neurons under various circumstances. The ADL neurons detect ascr#3 strongly in hermaphrodites and weakly in males. ASK neurons, detect ascr#3 in social animals with a low *npr-1* levels, and hinders avoidance in hermaphrodites and promotes attraction in males. Internal circuit modulation takes place via a gap junction network centered on the interneuron RMG. Male attraction to ascr#3 is through masculinized ADF, as well as the male-specific CEM neurons (omitted, as they are only present in males). The downstream components remain to be elucidated.

3 A.2 Modulation

Behavioral circuit activity is dependent on the physiological state of the animal. While receptor expression profiles and the sex of the animal are predetermined variables, more flexible states – largely the physiological state of the animal – help to shape and modulate these functional circuits. Sensory networks are altered by neuromodulators (neurotransmitters and neuropeptides) in a context-dependent manner; over varying distances and timescales. The effect of these modulations varies based on site-of-release and local concentration, as governed by release, degradation, and reuptake of neuromodulators.

Serotonin (5-HT) has been shown to play a dominant role in behaviors related to foraging, egg laying, and locomotion, dependent on the presence or absence of food, as expression levels are correlated with being either fed or starved. For example, when food is present, 5-HT acts via GPA-11 to sensitize ASH to 30% 1-octanol aversion and shows a quicker response time then when food is absent (21). Interestingly, when dissecting the role of 5-HT, it was found that the site of release is important, and can result in opposing outcomes. 5-HT released from NSM sensitizes ASH to initiate reversals more rapidly upon exposure to 30% 1-octanol. However, 5-HT released from ADF acts on ASH to shorten the reversal distance, and restarts forward locomotion (22). This highlights how a single neurotransmitter, within the same circuit, can give rise to different synaptic strengths and fine-tuned behavioral outputs, revealing that it is critical to not just consider neurotransmitters on a global scale. Moreover, the same stimulus does not necessarily utilize the same circuit at different concentrations. Different functional circuits are realized when animals are responding to 100% versus 30% 1-octanol (21). At 100% 1-octanol,

ADL and AWB act via electrical synapses, formed by GLR-1 with command interneurons when animals are starved – whereas in 30% 1-octanol aversion, regardless of food presence, is mediated only by ASH (21).

Furthermore, the timescale of stimulus detection appears to be programmed in the response circuit itself. As seen in copper avoidance, a cross-talk inhibition circuit between ASI and ASH fine tunes the behavioral response, with ASH responding quickly and robustly in comparison to a slower, weaker response by ASI which inhibits further ASH activation (23). Whereas this is a short-term reciprocal inhibition state, long-term behavioral states also exist which shape functional circuits. For example, roaming and dwelling states in the presence of food alternate, and last for minutes at a time. This switch is achieved via two opposing neuromodulators, dwelling is promoted by serotonergic neurotransmitter signaling, whereas the roaming state is established by the neuropeptide, PDF-1 (Fig. A3) (24). Strikingly, this functional circuit acts in a seemingly unorthodox manner, defying classical circuit logic of sensory-to-motor organization: motor and interneurons modulate the activity of sensory neurons (24). This largely extrasynaptic, long-term timescale circuit has many potential inputs that can bias signaling of one state over another. Interestingly, the only overlap between these two circuits involves the interneuron AIY (24). Perhaps, the odor of food biases the switch between dwelling and roaming.

In fact, an odor detection switch in local search behaviors also intersects onto AIY (25). AWC detects food depletion in a dose-dependent manner: as food is removed, AWC is disinhibited, thereby allowing for inhibition of AIY (25, 26). Thus, it is plausible that short timescale detection by AWC recognizes changing concentrations of food, and relays this

information to AIY, biasing a switch between the long-term roaming and dwelling states when on food (Fig. A3). Together, functional connectomes can vary and take shape in drastically different ways than physical wiring diagrams may suggest, with individual synaptic importance being dictated by physiological states and timescales. Additionally, functional circuits do not work in isolation: the final behavioral output is a readout of the fine tuning of multiple functional circuits creating a functional connectome.

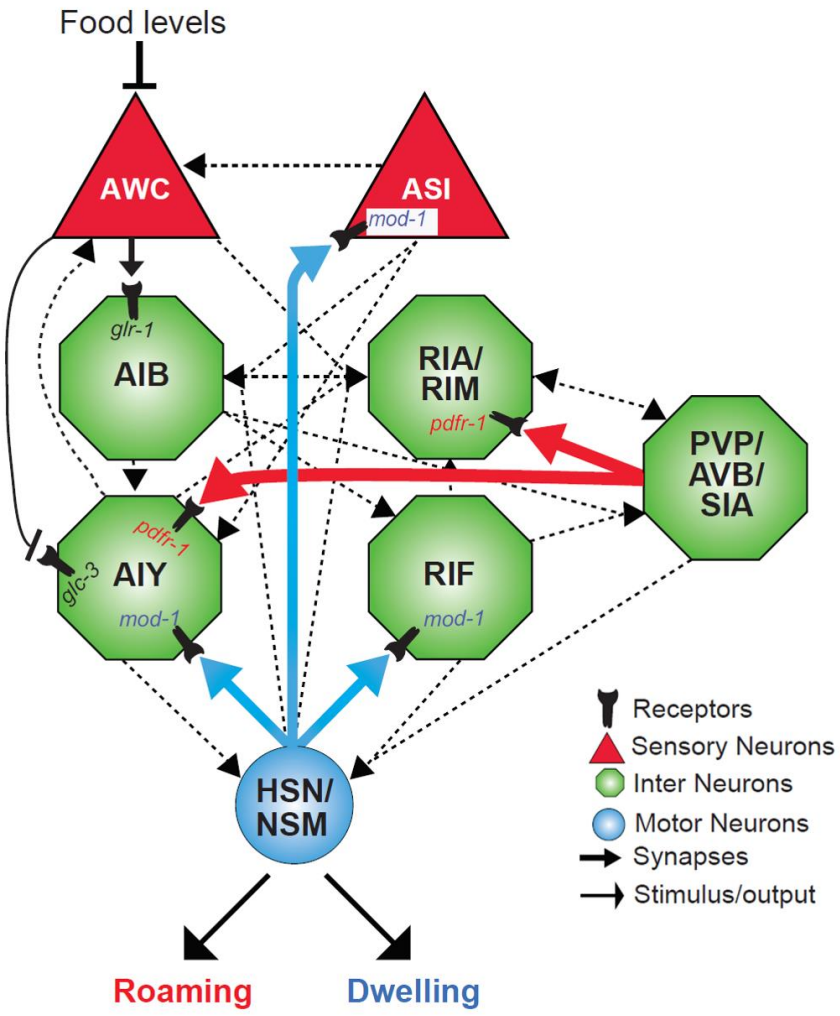


Figure A3. Two behavioral states of *C. elegans* are modulated non-canonically, via both serotonergic and peptidergic signaling. The switch between roaming and dwelling states highlights how functional circuitry may differ from common circuit logic. Dashed lines represent connections based on the physical wiring diagram, whereas the bolded lines (blue = serotonin, red = PDF-1) display the minimal, *functional* connections of circuits driving roaming or dwelling behavior, and are largely extrasynaptic. Furthermore, this circuit defies the canonical organization of relaying information as suggested by the wiring diagram: sensory neuron to interneuron to motor neuron. Note

that first-layer amphid interneuron, AIY, is the only site at which the neuroptidergic and serotoninergic signaling are both integrated directly. Therefore, the seemingly stochastic switch between behavioral states may be biased by input from AWC, which conveys information regarding food level to AIY.

3 A.3 Multisensory Integration

The functional circuit activated by a single stimulus does not act alone. Just as the internal state modulates the response to a particular cue, the presence of multiple stimuli is integrated into larger networks. One example of this is the “flip/flop” model of integration of contradictory “good” and “bad” stimuli. At high levels of repulsive quinine (sensed by ASH), and low levels of the attractant diacetyl (sensed by AWA), animals do not exhibit pharyngeal pumping. However, as the concentration of quinine is decreased, the pumping rate, in a steep sigmoidal fashion, increases, displaying a “flip” to increased pharyngeal pumping (27). Likewise, if quinine levels remain unchanged, a non-linear switch in pharyngeal pumping can be seen as diacetyl concentration increases (27). This flip/flop requires 5-HT and tyramine signaling among the RIM, RIC, and NSM interneurons via the serotonin-gated chloride channel channel (MOD-1) and tyramine receptor (SER-2), respectively (27). Interestingly, animals that lack MOD-1 and SER-2 still show a decrease in pumping as quinine is increased, but in a linear fashion instead of a flip/flop, on/off switch at a particular threshold. Thus, these two sites of action for the neuromodulators are required for fine tuning the response around critical levels, but not for the overall integration of the two stimuli.

The need to understand how stimuli integration allows for the modulation of circuits can further be exemplified by examining threat tolerance. Expectantly, well-fed *C. elegans* are not willing to cross a high osmotic barrier to chemotax towards the attractant, diacetyl: the risk is not worth the reward. However, animals which are deprived of food will cross the same osmotic barrier, suggesting that the risk no longer outweighs the reward (28). This modulation requires tyramine – the neurotransmission of which increases in

extended periods of starvation (29) – thereby desensitizing ASH to the osmotic stressor. It requires a few hours to reach an internal concentration of tyramine which allows for the decision to cross the osmotic barrier (28).

The aforementioned examples showcase the complexity underlying functional circuits, as there seem to be multiple levels of neuronal processing acting in parallel to finely adjust how the animal responds, including: 1) specific intercellular machinery that allows for rapid adjustment of neuronal responses, thereby affecting the output, and 2) the evidence that these modulations can take place over longer time scales - not merely minutes, but instead hours.

However, this characterization has largely omitted the complexities underlying the cross-roads of multisensory integration of social, behavioral communication (pheromones) and food odors. Here, we look at the inter- and intra-cellular components underlying the modification of a starvation-dependent, unisensory pheromone response, in light of multiple stimuli processing and integration.

3 B Multisensory integration of osas#9 and *E. coli*

3 B.1 Introduction

Animals must interact with their environment in a favorable way to overcome challenges and ensure the continuation of their gene pool, and thus the species. In an ever-changing environment, this includes processing of both abiotic and biotic factors, such as temperature, food availability, predators, and mates. Critical to the success of navigating such a complex environment is the nervous system's ability to properly integrate these many stimuli and balance them with the current needs of the animal (30, 31). Optimal foraging theory dictates that behaviors that increase foraging efficiency translate to increased fitness, due to the net gain of energy that can therefore be allocated for survival, growth, and reproduction (32, 33). Underlying this theory is the notion that an animal must have excess energy to expend from foraging before it can escape threats, grow, or allocate resources for reproduction and the care of young (in some life histories). Empirically demonstrating this theory is difficult, as it requires knowing the amount of energy spent per unit energy gained, and an obtainable read out of fitness (33). Therefore, evidence supporting the optimal foraging theory has been largely limited to controlled or modeled net energy intake in short-lived organisms (33-35). A recent study showed the dependency of efficient mother foraging in fur seals to be correlated with pup weight at weening in the wild (33). Together, these studies demonstrate energy balance is crucial for reproductive fitness.

As such, some behaviors or actions outweigh others in importance for efficient foraging, e.g. surviving a threat before continuing to forage. This requires accurate risk-assessment: if the animal is in dire need of food, it may risk foraging despite a nearby

threat and a scarcity of food (28, 36). The nervous system's ability to "understand" the environment through sensation and perception of multiple stimuli, and place them in context of the animal's current physiological needs and enacting appropriate fitness related behaviors is crucial to survival.

The importance of integrating multiple stimuli with the internal state has not been lost in humans. In fact, many neurological disorders, such as, attention deficit hyperactivity disorder (ADHD), autism spectrum condition (ASC), bipolar disorder, schizophrenia, and sensory-processing disorder, exhibit irregular integration of multiple stimuli and neuromodulatory state (30, 31). Particularly well described are the impairments in ADHD, ASC, and schizophrenia, where improper integration is strongly correlated with the disorders (31, 37). A striking commonality to these neuropsychiatric conditions is the social behavioral symptoms; "normal" social interaction requires proper integration(37). Despite the correlation between sensory processing and social functioning, very little information regarding the underlying mechanisms are known (37).

C. elegans presents a unique opportunity to unveil the mechanisms and connections between multisensory integration of social cues and the effective behavioral output. The small nervous system of the nematode is completely mapped, and contains only 302 neurons – 32 of which project sensory cilia into the environment (14, 38). Furthermore, the animals utilize a class of small molecules, termed ascarosides, for social communication (39-43). The production and response to these pheromones is highly regulated by physiological state and sex (Chute *et al.*, submitted to eLife, presented as the previous chapter of this thesis) (18, 39, 40, 44, 45). Lastly, the animal is genetically

tractable and transparent, allowing for characterization of the machinery necessary for molecular and cellular mediation of social behavioral responses.

When considering the ease of *C. elegans* manipulation, their social behaviors, fully mapped connectome, combined with their fully sequenced genome, *C. elegans* serves as a remarkable tool for bridging the gap between multisensory integration and social behaviors. Herein, we show that several sensory neurons, receptors, neuromodulators, and G protein signaling pathways are required for proper behavioral response to an aversive pheromone, as well as *E. coli* metabolites - all in relation to the internal state of the animal. The model network provides insight into how neural circuitries integrate and assess multiple conduits of external sensory information to “decide” upon an appropriate response with respect to internal state.

3 B.2 osas#9 behavioral aversion is attenuated by *E. coli* extract

C. elegans secrete a class of small compounds, ascarosides, that are recognized by conspecifics and result in behavioral and/or physiological changes (39, 41, 42, 44, 46-50) . These chemical cues are modular in nature, with a variable fatty acid derived side chains and moieties attached to a base ascarylose sugar (42, 43, 51, 52). Furthermore, the makeup of the ascaroside “cocktail” secreted into the environment is dependent upon the nematode’s current physiological state, life stage/history, and sex (39, 45). One particular ascaroside, octopamine succinylated ascaroside #9 (osas#9), is released exclusively by starved larval stage one (L1) animals (39). Interestingly, just as the production of the compound is state-dependent, the recipient’s response to osas#9 is dependent on physiological state as well. This is clear as only starved animals respond aversively to the cue when subjected to an avoidance drop test. Additionally, the osas#9

aversive response can be attenuated by the concurrent detection of food odor in the environment (39) (Fig. B1A). We found that *E. coli* OP50 extract attenuates the avoidance to an unchanging concentration of osas#9 in a dose dependent manner (Fig. B1B). The extract is able to attenuate the avoidance response at remarkably dilute concentrations (Fig. B1B), highlighting the ability of the animal to override its innate avoidance behavior in favor of foraging for potential food nearby.

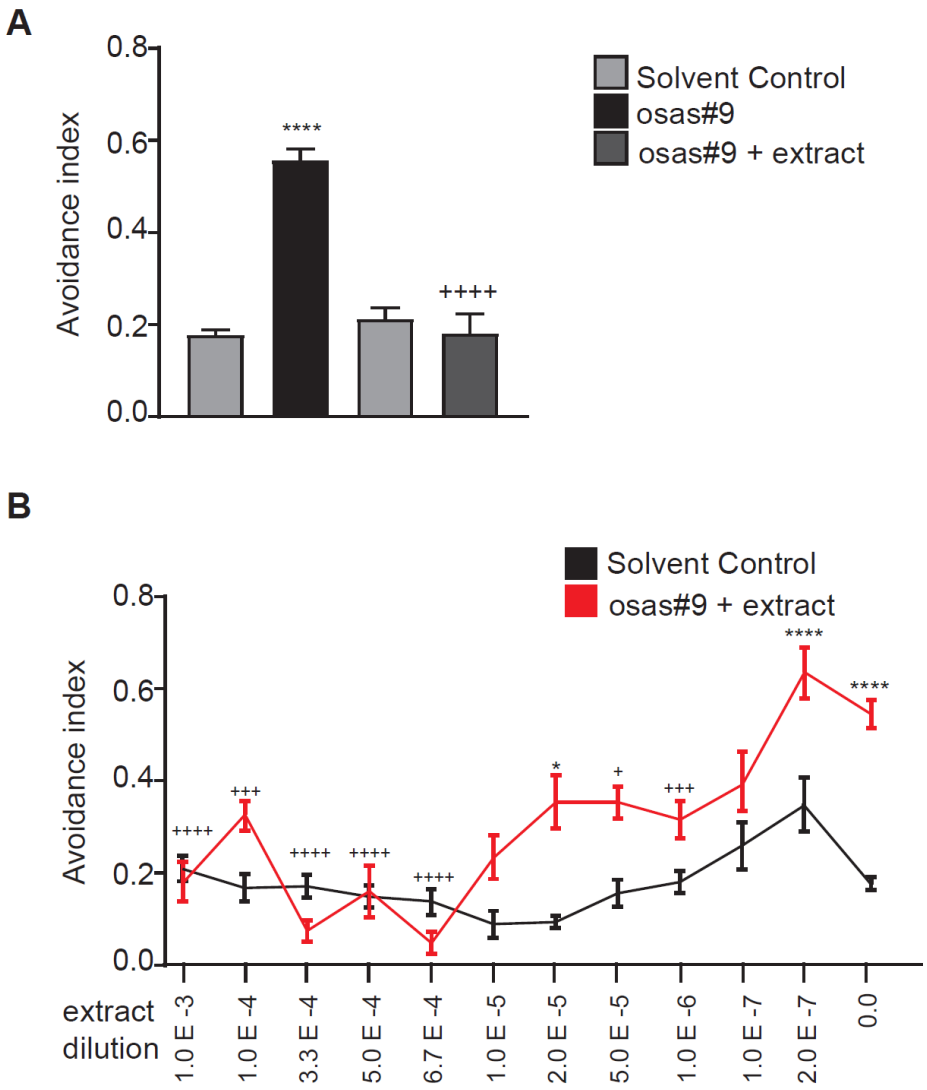
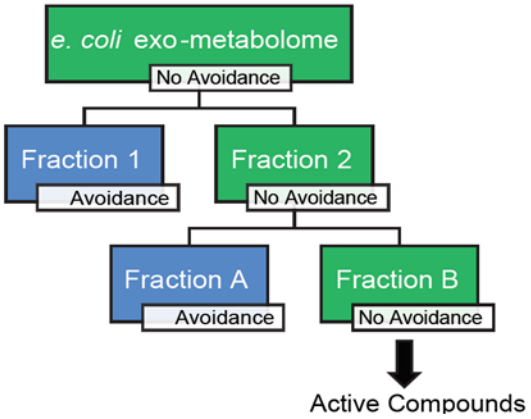


Figure B1. *E. coli* secreted metabolites attenuate the osas#9 response. **A)** When young adult, starved animals are subjected to 1 µM osas#9, they show an aversive response. However, when osas#9 is mixed with *E. coli* extract, starved animals show an attenuated response to osas#9. n≥16. **B)** *E. coli* extract exerts its attenuation effect over a broad range of concentrations. n≥4. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict significance comparing osas#9 avoidance to respective solvent control within groups. '+' signs represent same p value as asterisks,

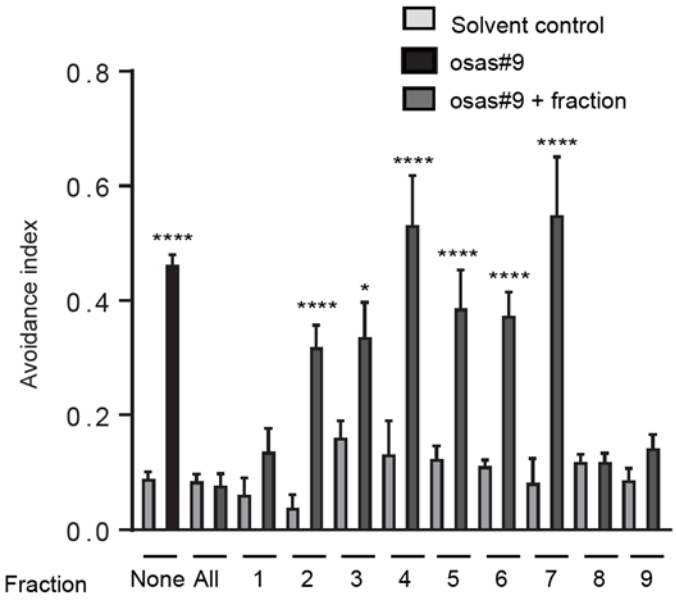
but depict difference between osas#9 avoidance of a strain/conditions in comparison to wild-type.

We sought to determine which compound(s) present in the *E. coli* extract responsible for diminishing the aversive response to osas#9 utilizing activity-guided fractionation (Fig. B2A). Fractionation was performed by our collaborators in the Schroeder Laboratory at Cornell University. Using the drop test, we found that fractions 1, 8, and 9 are all actively contributing to the attenuation of the osas#9 response, indicating that there are redundant compounds responsible (Fig. B2B). From these fractions, it was hypothesized that the compounds nicotinamide, niacin, L-proline, and/or cyclo(phenylalanine-proline) would contribute to the attenuation, due to the abundance of these molecules in the active fractions (Fig B2C). Various concentrations and combinations of nicotinamide and niacin were assayed, revealing attenuation effects at only very high concentrations of niacin (Fig. B3A). No effects were observed by the addition of various concentrations and combinations of L-proline or cyclo(phenylalanine-proline) (Fig. B3B,C). The identity of the active blend of compounds responsible for attenuation at physiologically relevant levels still remains to be identified. A new, more accurate extraction technique is currently being optimized for more abundant and reproducible fractionations by our collaborators.

A


e. coli exo-metabolome
No Avoidance
Fraction 1
Avoidance
Fraction 2
No Avoidance
Fraction A
Avoidance
Fraction B
No Avoidance
Active Compounds

B


Solvent control
osas#9
osas#9 + fraction
Avoidance index
0.0
0.2
0.4
0.6
0.8
****
****
*
****
****
****
****
Fraction
None
All
1
2
3
4
5
6
7
8
9

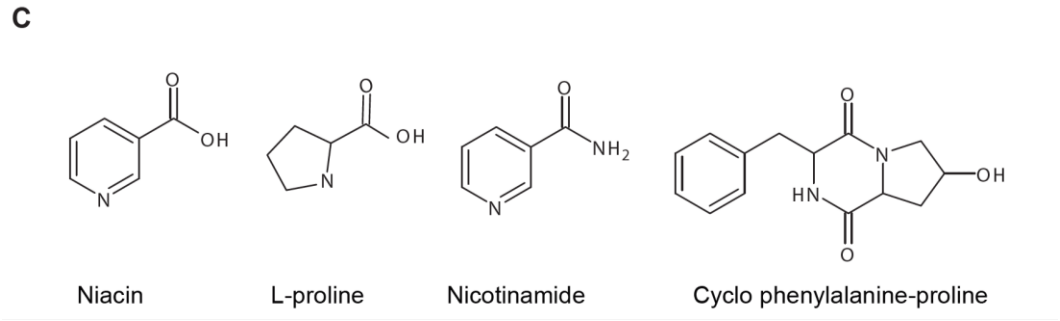
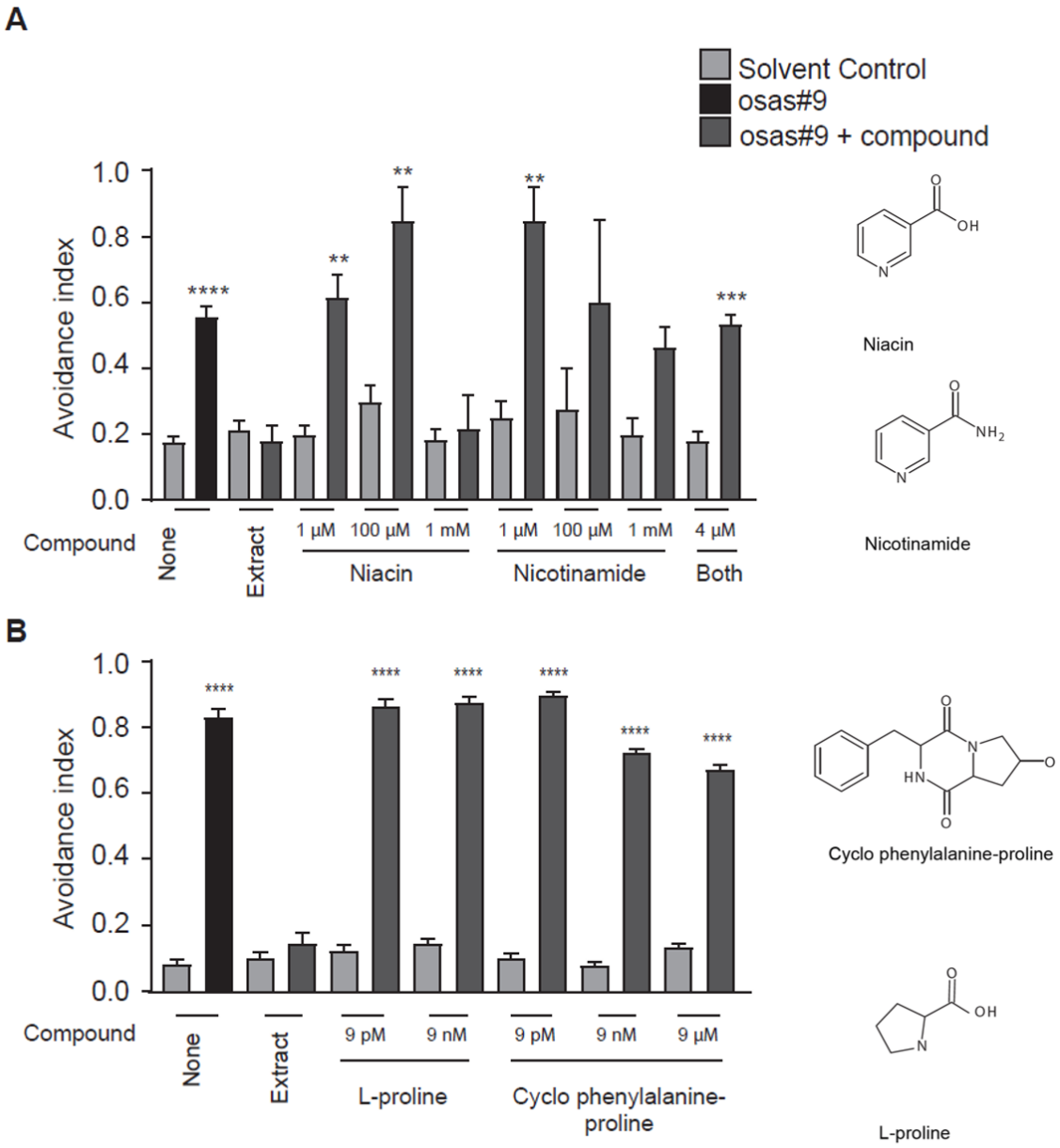


Figure B2. Activity guided fractionation reveals candidate compounds. **A)** Schematic showing activity-guided fractionation. Pools that are still able to attenuate osas#9 response contain active compounds. **B)** Activity-guided fractionation revealed active components are present in fractions 1, 8, and 9. n≥3. **C)** Structured of the candidate compounds niacin, L-proline, nicotinamide, and cyclo(phenylalanine-proline). Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups.


A
Solvent Control
osas#9
osas#9 + compound
Avoidance index
1.0
0.8
0.6
0.4
0.2
0.0
****
**
**
**
***
Compound
None
Extract
1 µM
100 µM
1 mM
1 µM
100 µM
1 mM
4 µM
Niacin
Nicotinamide
Both
Niacin
Nicotinamide
B
Avoidance index
1.0
0.8
0.6
0.4
0.2
0.0
****
****
****
****
****
****
Compound
None
Extract
9 pM
9 nM
9 pM
9 nM
9 µM
L-proline
Cyclo phenylalanine-proline
Cyclo phenylalanine-proline
L-proline

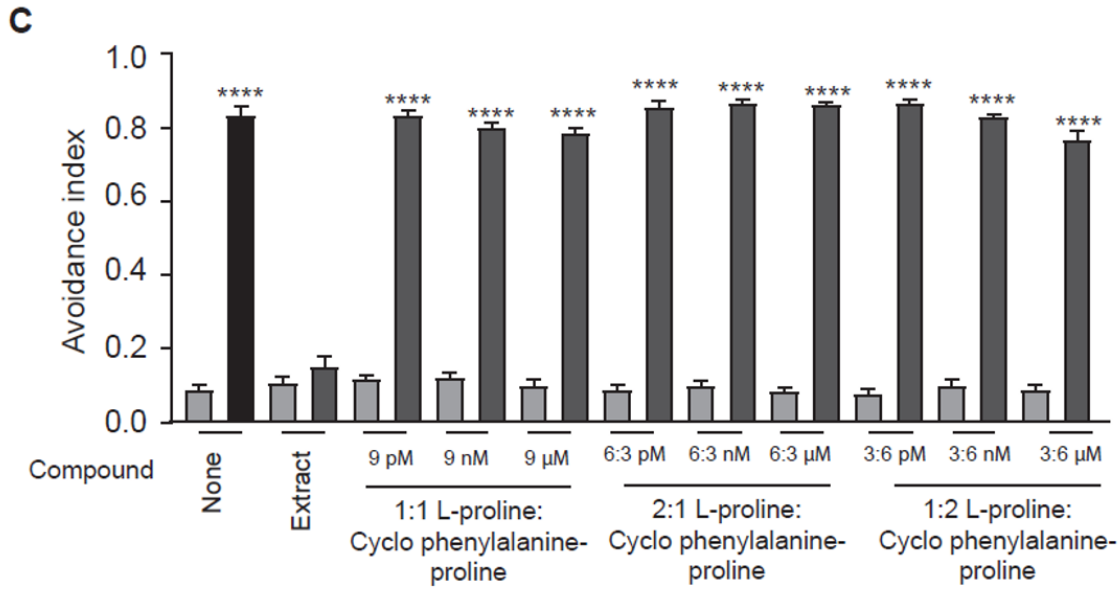


Figure B3. Candidate compounds for osas#9 attenuation. **A)** Niacin and nicotinamdide did not attenuate the response at relevant concentrations. However, 1 mM niacin did abrogate the osas#9 response. n≥2. **B-C)** L-proline and cyclo(phenylalanine-proline) do not attenuate the osas#9 response **B)** individually or **C)** synergistically. n≥9. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA, followed by Sidak's multiple comparison post-tests. Asterisks displayed depict compared osas#9 avoidance to respective solvent control within groups.

3 B.3 Deciphering the *E. coli* Attenuation of osas#9 Circuit

The modulation of the osas#9 response by simultaneous exposure to *E. coli* extract offers an opportunity to better understand the molecular and cellular mechanisms underpinning multisensory integration of social and foraging cues. We first sought to identify the sensory neurons contributing to the attenuation effect. Utilizing the avoidance drop test on mutant animals, we found that genetic ablation of ASK, via cell-specific expression of caspase, resulted in abnormal multisensory integration to osas#9 and *E. coli* extract: the animals continued to avoid osas#9, despite the presence of *E. coli* extract (Fig. B4A). As ASK was necessary for the sensation of the *E. coli* extract, we hypothesized that an ASK released neuromodulator would therefore be required for the attenuation of the osas#9 response.

ASK releases both neuropeptides and neurotransmitters, including *pdf-1*, *flp-21, nlp-8, nlp-10, nlp-14,* and glutamate (17, 53-55). As such, we screened mutant strains available using the drop test with both osas#9 and osas#9 mixed with *E. coli* extract (Fig. B4B). We included animals lacking: the glutamatergic transporter *eat-4*; the NPR-10 receptor, which has been shown to sense the neuropeptide, NLP-14; and the neuropeptide receptor-ligand partners FLP-21, and NPR-1 (53, 56, 57). As expected, *eat-4* loss of function (lof) mutants showed no response to osas#9 alone, likely due to the fact that the primary sensory neuron underlying osas#9 avoidance, ASH, is also glutamatergic (53) (Fig. B4B). To reveal if ASK neurotransmission is important a cell-specific knockdown of *eat-4* would need to be performed. As for peptidergic signaling, we found that *flp-21* and its receptor, *npr-1*, are necessary for the attenuation of the osas#9 response by OP50 extract (Fig. B4B). Interestingly, *flp-21* and *npr-1* have previously been found to play a

role in multisensory integration fine tuning responses to salts and heat under stress conditions of hypoxia or temperature, respectively (58, 59).

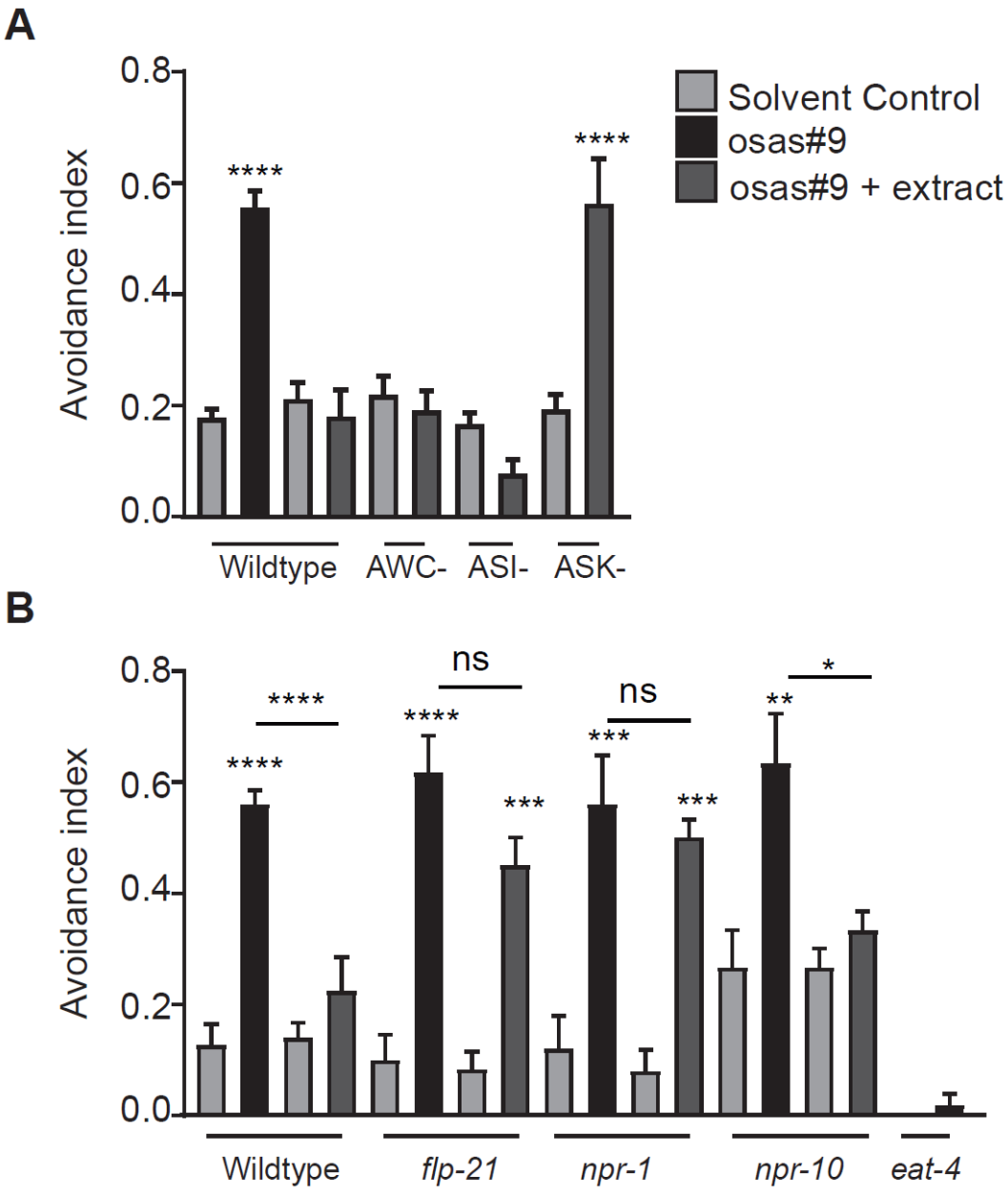
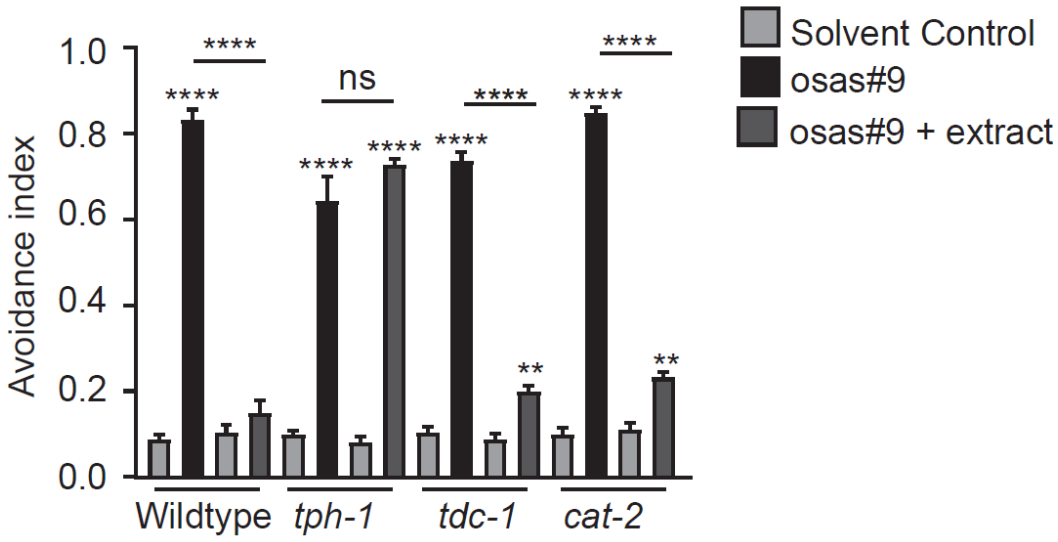


Figure B4. The ASK sensory neuron pair and related modulators are necessary for attenuation of the osas#9 response. **A)** Genetic ablations of AWC, ASI, and ASK sensory neurons revealed the necessity for ASK in *E. coli* mediated attenuation of osas#9 aversion. n≥10. **B)** Directed screen of ASK released modulators (*flp-21, eat-4*) and receptors (*npr-1, npr-10*). *eat-4 lof* animals did not respond to osas#9, whereas FLP-21

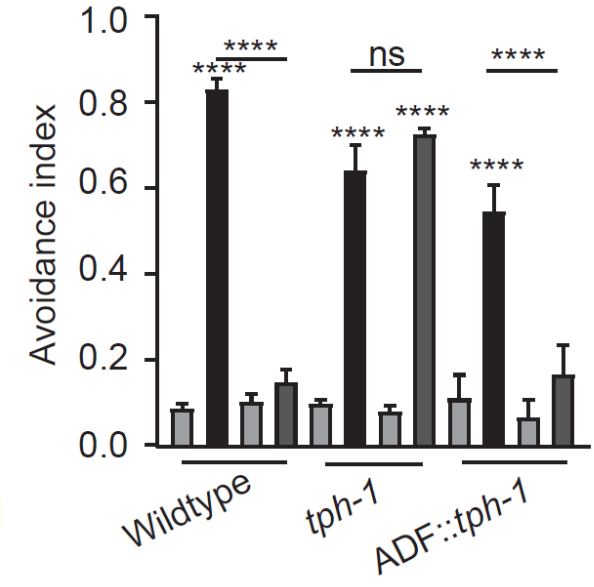
and its cognate receptor NPR-1 were observed to be normal for osas#9 avoidance, but necessary for the attenuation effect. n≥3. Data presented as mean ± S.E.M; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, One-Way ANOVA followed by Sidak's multiple comparison post-tests. Asterisks displayed without bar depict comparisons between osas#9 avoidance and respective solvent control within groups. Asterisks with bar depict comparison between osas#9 alone and osas#9 with *E. coli* extract.

In addition to testing the necessity for glutamate, we assayed loss of function mutants for enzyme involved in the biosynthesis of serotonin, dopamine, tyramine (*tph-1, cat-2, tdc-1,* and *tbh-1,* respectively) (Fig. B5A). Serotonin deficient animals displayed an abnormal phenotype in response to osas#9 and *E. coli* extract (Fig. B5A). As such, we hypothesized the primary serotonergic sensory neuron, ADF, may be involved. Rescuing *tph-1* in the serotonergic ADF neurons of *tph-1* mutant animals reconstituted wild-type behavior, supporting the hypothesis that ADF's release of serotonin is involved in the attenuation of osas#9 avoidance (Fig. B5B). We next sought to find the target of the neurotransmitter within the attenuation circuitry. Therefore, we screened proteins known to be involved in serotonin signaling, including: serotonin receptors (*ser-1, ser-4,* and *ser-7*); the serotonin-gated chloride channel, *mod-1*; and the serotonin reuptake transporter, *mod-5* (60, 61) (Fig. B5C). Of the various serotonin signaling components, the inhibitory channel *mod-1* was found to be required (Fig. B5C). MOD-1 localization includes the first layer amphid interneurons (AIA, AIB, AIY, and AIZ) – which integrate and process information from the amphid sensory neurons – and the interneurons, RIM and RIC (27, 62, 63). AIB and AIZ promote turns, whereas AIA and AIY inhibit them (63, 64). As MOD-1 is inhibitory, and is expressed in the reversal promoting AIB and AIZ interneurons, we asked if a cell-specific rescue in these neurons would restore normal dampening of osas#9 avoidance in the presence of *E. coli* extract. Indeed, expressing *mod-1* in AIB and AIZ resulted in wild-type behavior (Fig. B5D).

A


Solvent Control
osas#9
osas#9 + extract
Avoidance index
1.0
0.8
0.6
0.4
0.2
0.0
****
ns
**
Wildtype
tph-1
tdc-1
cat-2

B


Avoidance index
1.0
0.8
0.6
0.4
0.2
0.0
****
ns
Wildtype
tph-1
ADF::tph-1

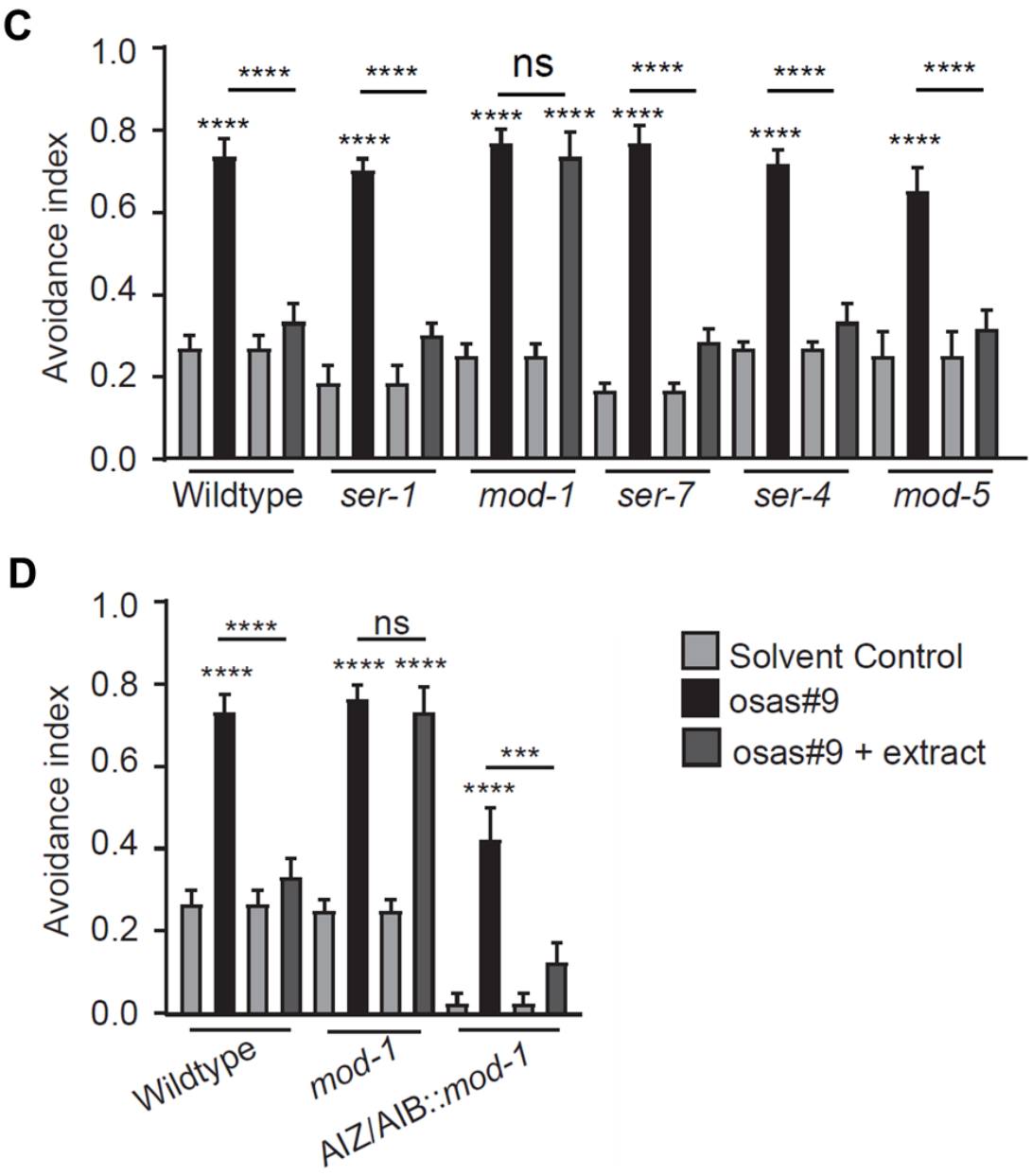
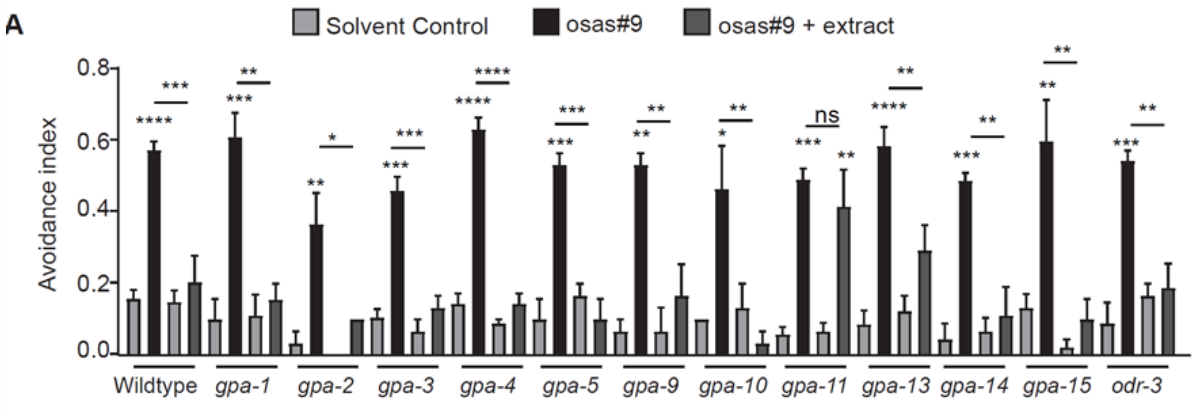


Figure B5. The neurotransmitter serotonin and an associated channel are required for osas#9 attenuation. **A)** *tdc-1, tph-1, and cat-2 lof* animals are deficient in the biosynthesis of tyramine (and subsequently octopamine), serotonin, and dopamine, respectively. *E. coli* extract-mediated attenuation requires the biogenic monoamine, serotonin. n≥9. **B)** ADF sensory neurons are the site of serotonin production via *tph-1* in the attenuation pathway. Expression of *tph-1* in the ADF sensory neurons in a *tph-1 lof* background

reconstituted wild-type behavior to co-exposure of osas#9 and *E. coli* extract. n≥7. **C)** Screen of serotonin related receptors (*ser-1, ser-4,* and *ser-7*), the serotonin gated chloride channel, MOD-1, and the serotonin reuptake transporter, MOD-5. Animals lacking the functional channel, MOD-1, avoided osas#9 in the presence of *E. coli* extract. n≥3. **D)** *mod-1* expressed in the first layer amphid neurons, AIB and AIZ, in *mod-1 lof* animals rescued the attenuation effect of *E. coli* extract. n≥3. Data presented as mean ± S.E.M; * p < <0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, One-Way ANOVA followed by Sidak's multiple comparison post-tests. Asterisks displayed without bar depict comparisons between osas#9 avoidance and respective solvent control within groups. Asterisks with bar depict comparison between osas#9 alone and osas#9 with *E. coli* extract.

Internal transduction machinery plays an important role in sensory perception. We examined G protein signaling components underlying the attenuation effect of *E. coli* extract. Of the 21 Gα subunits in *C. elegans*, 16 are expressed in sensory neurons, and one (*gsa-1*) is required for normal development and behavior (8, 9). In screening the Gα subunits expressed in the sensory neurons, *gpa-11* was revealed to be necessary for extract attenuation of the osas#9 behavioral response (Fig. B6A). It is also worth noting that *goa-1*, which is extensively found throughout all tissues, responded hyperactively to the solvent control, although no difference was observed in comparison to osas#9 with *E. coli* extract (Fig. B6B). Of the four Gβ and Gγ subunits (two each), only one from each family is viable, *gpb-2* and *gpc-1*, respectively. Neither of these subunits were required for complete osas#9 attenuation (Fig. B6C).


A
Solvent Control
osas#9
osas#9 + extract
Avoidance index
0.8
0.6
0.4
0.2
0.0
Wildtype
gpa-1
gpa-2
gpa-3
gpa-4
gpa-5
gpa-9
gpa-10
gpa-11
gpa-13
gpa-14
gpa-15
odr-3
ns

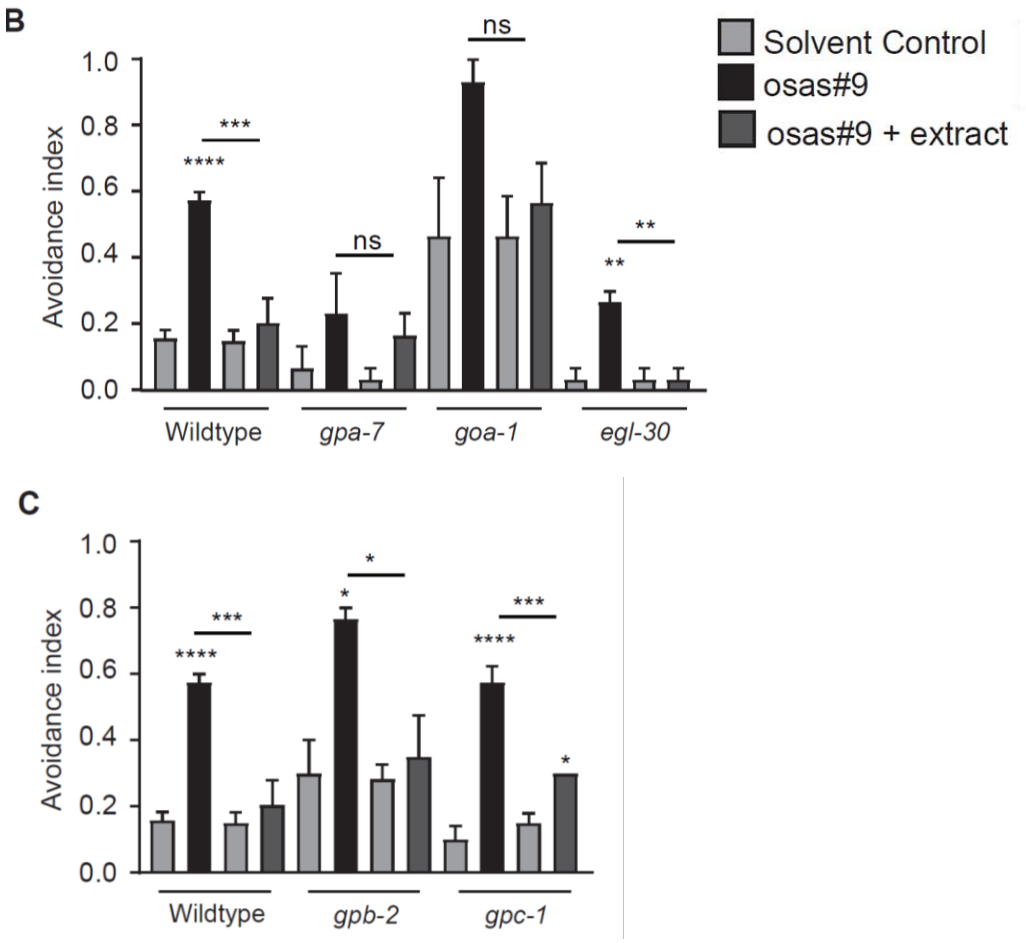


Figure B6. The Gα subunit GPA-11 is required for osas#9 attenuation. **A)** Screen of Gα subunits expressed primarily in neurons without known defects in osas#9 avoidance. The Gα subunit GPA-11 is defective in osas#9 attenuation behavior in the presence of *E. coli* extract. n≥3. **B)** Animals with mutations in Gα subunits expressed in many tissues displayed abnormal responses to osas#9 and solvent controls. This is hypothesized to be due to the defective locomotion and development of the animals, and not a role in the osas#9 behavioral pathway. n≥3. **C)** The Gβ and Gγ subunits, GPB-2 and GPC-1, respectively, did not show a strong phenotype different than wild-type animals. n≥3. Data presented as mean ± S.E.M; * p < <0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001, One-

Way ANOVA followed by Sidak's multiple comparison post-tests. Asterisks displayed without bar depict comparisons between osas#9 avoidance and respective solvent control within groups. Asterisks with bar depict comparison between osas#9 alone and osas#9 with *E. coli* extract.

3 B.4 Discussion and Model

These data highlight cellular and molecular mechanisms necessary for the effect of *E. coli* extract on attenuating the osas#9 aversive response. We have demonstrated key players in the circuit, although certain sites of action and roles in the pathway remain to be determined. Taken together, these data and our hypotheses allow us to begin to construct a tentative, minimal circuit required for *E. coli* attenuation of osas#9 avoidance (Fig. B7).

Sensory Neurons

The osas#9 aversive response requires the polymodal ASH nociceptive neurons (Chute *et al.*, submitted to eLife, presented as the previous chapter in this thesis). Behavioral and physiological responses to osas#9 depend on the G protein-coupled receptor (GPCR), TYRA-2 (Chute *et al.*, submitted to eLife, presented as the previous chapter in this thesis). Ectopic expression of TYRA-2 is capable of driving osas#9-mediated behavioral responses, demonstrating that TYRA-2 is required and sufficient for relaying osas#9 information (Chute *et al.*, submitted to eLife, presented as the previous chapter in this thesis). Lastly, the Gα subunit, GPA-6, is required for osas#9 avoidance, and located in the cilia of ASH sensory neurons, suggesting that GPA-6 may interact with TYRA-2 to drive osas#9 behavior (Chute *et al.*, submitted to eLife, presented as the previous chapter in this thesis). Our working model builds around the primary sensation

of osas#9, and explains how it is inhibited by the presence of *E. coli* metabolite(s). The model is constructed on hypothesized connections of importance, based on the *C. elegans* wiring diagram (wormweb.org) (14), and components of the attenuation response elucidated here (Fig. B7).

Genetic ablation of ASK sensory neurons revealed that they are required for the attenuation effect (Fig. B4A). ASK sensory neurons are known to have roles in local food search and lysine chemoattraction (63, 65). Furthermore, ASK sensory neurons are part of a redundant, parallel dauer controlling pathway that likely integrates food cues with pheromonal sensation (66). This information, coupled with our behavioral data, led us to hypothesize that ASK sensory neurons are sensing the bacterial metabolite(s) (Fig. B7).

The serotonergic sensory neuron, ADF, was demonstrated to be required for the attenuation of osas#9 behavior through cell-specific rescue of the serotonin biosynthetic enzyme, *tph-1* (Fig. B5B). Moreover, we discovered that the inhibitory serotonin gated chloride channel, MOD-1, expressed in AIB and AIZ, and a serotonin receptor-related Gα subunit expressed in ASH (GPA-11) are necessary for attenuation of the osas#9 response (Fig. B5D, B6A). Interestingly, GPA-11 has been implicated in altering ASH sensitivity via serotonergic signaling based on feeding state (21). Furthermore, GPA-11 and MOD-1 are not expressed in the same neurons. *mod-1* is expressed in interneurons, whereas GPA-11 is localized to ASH and ADL (8, 9, 27, 62). Together, these results highlight the importance of serotoninergic signaling in the attenuation pathway, and the possibility of inhibiting the response at multiple hierarchal levels of organization.

The role of ASK sensory neurons must be further investigated before a definitive claim could be made as to the sensory pair being required for sensing *E. coli*

metabolite(s). An essential experiment would be to examine calcium transients in ASK for any physiological response to *E. coli* extract. However, due to the likelihood of multiple metabolites being responsible for the attenuation effect, a comprehensive examination of other potential sensory neurons involved in the pathway would greatly improve our understanding of this circuit. The roles of sensory neurons that have a defect in the attenuation response would best be characterized as primary sensory neurons of the metabolite(s) by performing calcium imaging experiments in each of those neurons in animals with synaptic and/or gap junction deficiencies.

Interneurons

Next, we are able to include interneurons in our model, based on their known functional roles and data presented here, including: the wiring connectivity between them and the sensory neurons shown to be required in the osas#9 modulatory circuit and the known neuromodulators and their cognate receptors. *C. elegans* have a set of interneurons, known as the first layer amphid interneurons (AIA, AIB, AIY, and AIZ), which integrate and process information from half of the total synaptic output of the amphid sensory neurons (63). AIB and AIZ promot turning behaviors, whereas AIA and AIY prevent them (63, 64). Another important set of interneurons are the command interneurons (AVA, AVB, AVD, AVE, and PVC), which communicate with six motor neurons innervating the ventral and dorsal muscles (14). AVB and PVC drive forward locomotion, whereas the remaining command interneurons drive backward movement (63, 67, 68). Furthermore, ASH sensory neurons synapses directly onto AVA, AVB, AVD, and AVE (14).

In response to water soluble repellents, ASH stimulates both AVA and AIB, which in turn inhibits RIM, and leads to aversion (68). Furthermore, RIM is required for inhibition of reversals, potentially via suppressing AVA activity (63). Our data demonstrates the necessity for AIB and AIZ, through the rescue of MOD-1 in these neurons, which restores the attenuation effect of *E. coli* extract on osas#9 avoidance (Fig. B5D). The avoidance inhibiting neuron, AIA, is post-synaptic to the ASK sensory neurons (Fig. B4A), and is electrically coupled with the serotonergic ADF sensory neurons (Fig. B5B) (14). Thus, our model integrates these connections of importance inferred from prior literature (63, 68), the wiring diagram (14), genetic ablations, and our cell-specific rescues of *tph-1* and *mod-1* (Fig. B7).

To confirm the role of both AIB and AIZ amphid interneurons, calcium imaging should be employed. For example, calcium transients in wild-type animals expressing GCaMP in AIZ should be compared to animals with ablated ADF neurons. This data would decipher if AIZ is a site of action for MOD-1 and ADF serotonergic signaling. Likewise, the same experiment can be done with AIB and AIA.

Neuropeptides and neurotransmitters modulate neuronal excitability and synaptic activity, giving rise to a behavioral state that shapes functional circuitries and thus perception and behavioral responses (24, 69, 70). One of the earliest receptor-ligand pairs for neuropeptide signaling elucidated in *C. elegans* was that of NPR-1 and FLP-21, respectively (57, 71). The pair have been found to modulate sensory information, including increasing chemoattraction to salt (58), and setting heat tolerance levels (59). Furthermore, *npr-1* expression specifically in the interneuron RMG has been demonstrated to modify sensation, and change behavioral outputs, acting as a hub which

integrates information (16, 17, 59). We have now demonstrated that the neuropeptide FLP-21 and its receptor, NPR-1, are both required for attenuation of the osas#9 response by *E. coli* extract (Fig. B4B). Due to RMG expression of NPR-1, and its role in integrating pheromonal and context dependent behaviors, we hypothesize that in the osas#9 attenuation response, the site of action of *npr-1* is the RMG interneurons (Fig. B7). Although we investigated *flp-21* due to the necessity of ASK sensory neurons, which release FLP-21, it is also secreted by several other neurons (17), and there is not sufficient evidence to place *flp-21* site of production/release into our model. Cell-specific rescues of *flp-21* and *npr-1* would offer ample evidence for the active site of FLP-21 release and its target neuron in the attenuation pathway.

Taken together, our working model reveals that serotonergic signaling downstream of ASK stimulation attenuates the osas#9 response at multiple levels of organization. We hypothesize ASH itself is being modulated, as well as its downstream targets, AIZ and AIA. Our model is that FLP-21 (from a currently unknown source) inhibits RMG via the neuropeptide receptor NPR-1. RMG, acting as a hub for modulating sensory neuron activity, alters ASK properties through gap junction connections. This, in turn, allows for ASK to trigger serotonergic release, potentially via synaptic connection to AIA, altering the interneuron's properties, relayed through gap junctions with ADF. Additionally, AIA inhibits turning via inhibitory connections with AIB. Serotonin inhibits AIZ via MOD-1 channels, and modifies ASH via GPA-11 signaling (Fig. B7).

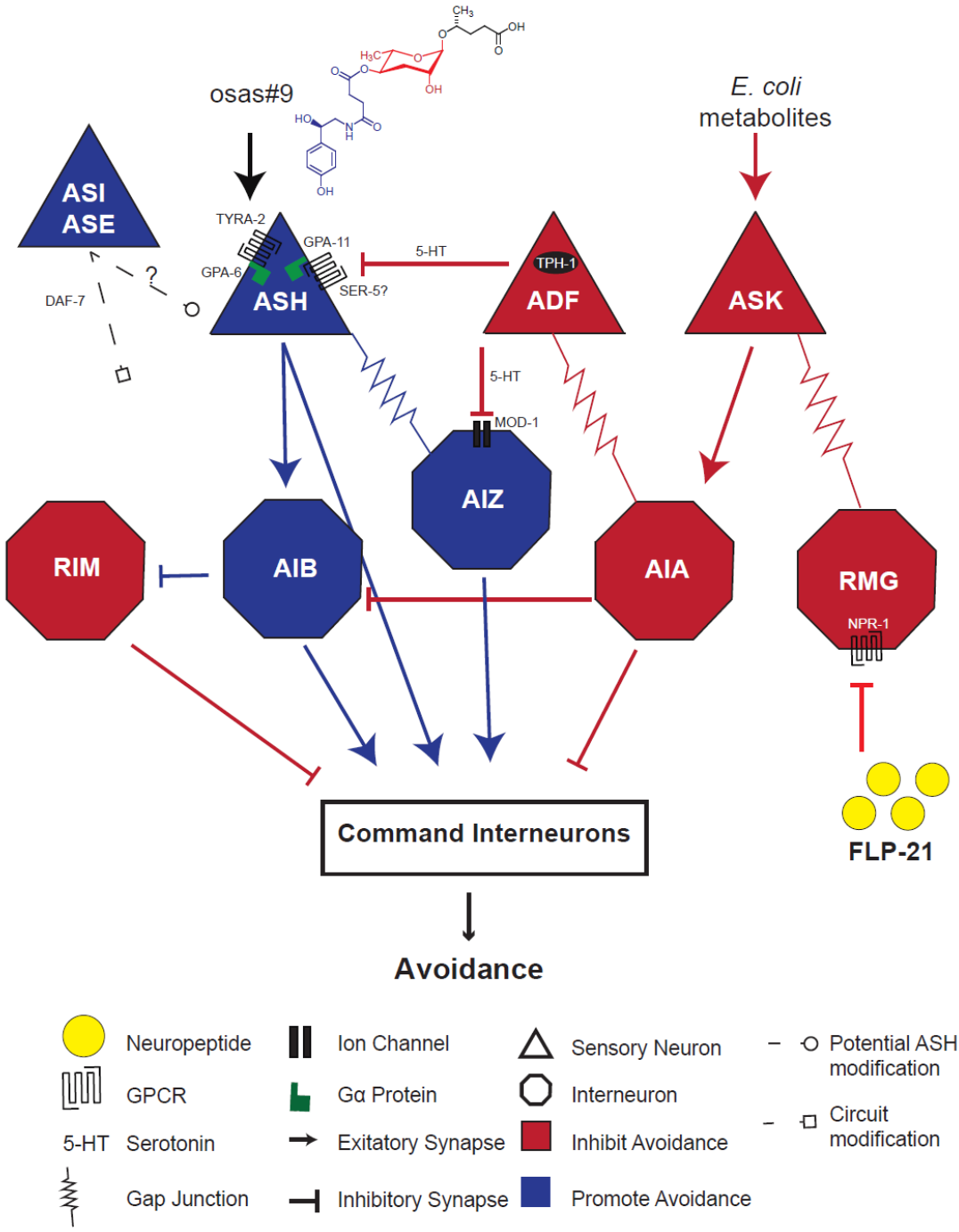


Figure B7. Working model of the circuitry governing the osas#9 response and modulation due to sensation of *E. coli*. Blue represents neurons and connections promoting avoidance to osas#9, whereas red represents those inhibiting aversion in response to *E. coli* extract. Model based on data from this dissertation and the wiring diagram.

3 B.5 Conclusion

Multisensory integration serves to regulate appropriate behavioral responses. Here, we construct a minimal circuit at the cellular and molecular level depicting the integration of social and food cues with respect to physiological needs. This integration results in the animal making a “decision” that bests promote survival. We found that the decision to ignore a threat signal was in part based on risk assessment; when high concentrations of *E. coli* metabolite were present, the animal ignored the aversive cue - the reward outweighs the risk. Contrarily, when low levels of *E. coli* metabolite are detected, the animals still show an aversive response to the repellent osas#9, indicating the risk outweighs the reward. This is in agreement with the optical foraging theory, which implies that seeking a poor food patch is not worth the energy requirement (32). Similar risk assessment studies in *C. elegans* have shown that the level of starvation effects the response to threat cues (Chute *et al.*, submitted to eLife, presented as the previous chapter in this thesis) (28). For example, fed animals are not willing to cross an osmotic barrier when able to sense the potent attractant on the other side. However, as animals starve, they begin to cross the barrier to forage (28). That study demonstrated the use of tyraminergic signaling as a requirement for multisensory integration and decision making, whereas ours found the necessity for serotonergic signaling (28). Interestingly, these two neurotransmitters are known to be regulated by starvation levels antagonistically: tyramine generally is thought to signal starvation, and serotonin the presence of food (21, 29). This demonstrates the importance of balancing hunger/food signals when accessing the risk to pursue food odors when faced with two very different threat cues: biotic versus abiotic.

We found that the attenuation circuit requires complex interactions utilizing both serotonergic and peptidergic signaling. A key feature of our model is that FLP-21 and NPR-1 are “upstream” of serotonin in the regulation of the attenuation response, which is determined by the physiological state of the animal. This is consistent with mammals, in which top-down input from higher order neurons/processing centers modulate sensation (72-74). This strategy is thought to have evolved to focus the sensory system on relevant cues in a noisy environment to best meet the needs and goals of the organism’s current state (73) (30). Our model provides insights at the molecular and cellular levels of top-down risk assessment behaviors underlying multisensory integration decisions involving social cues. Completion of the proposed studies will enhance our understanding of how multisensory integration abnormalities contribute to neuropsychiatric disorders with characteristic social defect symptoms.

3 B.6 Methods

Drop avoidance test

A forward moving animal is subjected to a small drop (~5 nl) of solution delivered through 10 µl glass capillary tube that has been hand-pulled to a fine hollow point. The solution is dropped at the tail end of the animal and upon contact, through capillary action, surrounds the animal and exposes the amphid sensory neurons to the solution. The animal is observed for four seconds. In that time the animal either continues its forward motion, displaying no response, or is observed to have an avoidance response (75). An avoidance response is scored as such by a reversal consisting of at least one half of a complete “head swing” followed by a change in direction of at least 90 degrees from the original vector. The avoidance index can then be calculated by dividing the number of

avoidance responses by the total number of trials. Each trial is done concurrently with osas#9 + *E. coli* extract, osas#9, and a solvent control. Osas#9 was synthesized by methods in Artyukhin et al. 2013 (39) and *E. coli extracts* and fractions were provided by Maro Kariya of the Schroeder lab.

Integrated mutant strains and controls are prepared by washing animals in M9 buffer. The animals are suspended in M9 buffer and transferred from the culture plate into a microcentrifuge tube. After being allowed to settle for ten minutes, the supernatant is removed, and the animals are resuspended with fresh M9 buffer. The supernatant is again removed, and the animals then transferred to an unseeded plate using a micropipette. After one-hour, young adult animals are randomly subjected to drop of the solvent control or compound of interest. No animal receives more than one drop of the same solution. ‘

Extrachromosomal transgenic animals and controls carefully transferred to an unseeded plate and allowed to crawl around. After one hour of starvation, animals are subjected no more than once for each the solvent control and compound of interest at random.

E. coli extract and compounds.

E. coli OP50 was grown in 4 L of LB media for 18 hr at 37^{o}C. The culture was then pelleted into four portions, each stemming from 1 L. The exo-metabolome from one portion of the pellet was extracted in 95% ethanol: 5% water for 1.5 hr. The extract supernatant had its solvent removed by rotary evaporation. The dried extract was resuspended in 500 uL of methanol and was fractionated using HPLC.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. When comparing multiple groups, ANOVAs were performed and Sidak's multiple comparison test. For B1B, normalized values of osas#9 avoidance index response relative to the respective solvent control was used. This was done to account for differences in baseline response to solvent control for the respective group.

Strains

See supplementary table SB1.

Undergraduate Contributions

Under the guidance of C. Chute:

A. Turland performed the *E. coli* extract dilution, niacin, and nicotinamide experiments; J. Yabut performed the neurotransmitter screen, L-proline, and cyclo(phenylalanine-proline) experiments; M. Andresano performed the serotonin component screen experiments; V. Coyle performed the fractionation assays; and M. Savoie assisted with the serotonin rescue experiments.

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Table BS1. Strains used in chapter.

Source	Strain	Gene (allele)
Albrecht Lab	cx13503	eat-4 (ky5)
Alkema	QW284	tdc-1 (n3420)
Bargmann	CX6968	mod-1(ok103); kyEx985= Podr-2(2b)::mod-1::GFP; ceolomocite GFP]
Bargmann	CX13571	tph-1 (mg280); kySi56; kyEx4077= srh-142::nCre (95 ng/uL); myo-3::mCherry (5ng/uL)
CGC	MT1434	egl-30(n686)I.
CGC	DG1856	goa-1(sa734)I.
CGC	NL332	gpa-1(pk15)V.
CGC	NL1147	gpa-10(pk362)V.
CGC	NL787	gpa-11(pk349)II.
CGC	NL2330	gpa-13(pk1270)V.
CGC	NL788	gpa-14(pk347)I.
CGC	NL797	gpa-15(pk477)I.
CGC	NL334	gpa-2(pk16)V.
CGC	NL335	gpa-3(pk35)V.
CGC	NL790	gpa-4(pk381)IV.
CGC	NL1137	gpa-5(pk376)X.
CGC	NL795	gpa-7(pk610)IV.
CGC	NL793	gpa-9(pk436)V.
CGC	NL361	gpb-1(pk44)II: pkEx170
CGC	DA541	gpb-2(ad541)I.
CGC	NL792	gpc-1(pk298)X.
CGC	CX2205	odr-3(n2150)V.
CGC	CB1112	cat-2(e1112) II.
CGC	RB982	flp-21(ok889) V
CGC	CX4148	npr-1(ky13)
CGC	RB1325	npr-10[C53C7.1(ok1442)]
CGC	DA1814	ser-1(ok345) X
CGC	MT9668	mod-1(ok103) V
CGC	RB1585	ser-7(ok1944) X
CGC	AQ866	ser-4(ok512) III
CGC	MT9772	mod-5(n3314 I
CGC	MT15434	tph-1 (mg280)II.
Sternberg	PS6022	qrIs1[sra-9::mCasp1], (ASK-)
Sternberg	PY7502	oyIs85 [ceh-36p::TU#813 + ceh-36p::TU#814 + srtx-1p::GFP + unc-122p::DsRed], (AWC-)
Sternberg	PY7505	oyIs84 [gpa-4p::TU#813 + gcy-27p::TU#814 + gcy-27p::GFP + unc-122p::DsRed], (ASI-)

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4 *C. elegans* contains promiscuous, redundant sensory neurons for detection of biotic and abiotic cues.

This chapter is a rewrite of the Nature Communication article “Predator-secreted sulfolipids induce defensive responses in *C. elegans*” (1). The published version can be seen as an addendum. The manuscript resulted from a collaboration between the Chalasani Lab (Salk Institute), the Srinivasan Lab (Worcester Polytechnic Institute) and the Schroeder Lab (Cornell University). First authorship is shared equally between Zheng Liu (Chalasani), Maro J. Kariya (Schroeder), and myself. This rewrite is not an exhaustive description of the manuscript and its findings, but rather focuses primarily on my contribution and interpretation of those results independently from the entire data set. This includes data derived from others when necessary to paint a complete picture, but limits presentation of the findings of others to frame the story from my data and perspective for the purpose of non-overlapping dissertation chapters. Primary omissions include: 1) detailed compound identification schemes performed by the Schroeder Lab, 2) characterization of necessary transduction components and physiological responses underlying the redundant chemosensory neurons detecting the predator cue by the Chalasani Lab, and 3) the effects of the selective serotonin re-uptake inhibitor Sertraline on attenuating the behavioral response to predator cue by the Chalasani Lab.

4.1 Introduction

Animals must interact constantly with their environment for survival at the individual and species level. This includes instinctual behaviors such as foraging, thermoregulation, mate acquisition, etc. To appropriately navigate the environment, the nervous system must sense, transduce, and process a plethora of sensory information, ranging from abiotic to biotic factors. A large portion of an individual's interaction with biotic factors is comprised of chemical social signals.

Social signals can be communicated via each primary sense: visual, auditory, tactile, gustatory, and olfactory. The signals can range from auditory alarm calls, to visual dances that communicate foraging locations, to olfactory cues mediating development (2-5). Within a sensory modality, a broad range of specific information can be communicated. For example, the black-fronted titi monkey (*C. nigrifrons*) utilizes alarm calls that vary in duration and rate to encode the type and location of a predatory threat (2). Furthermore, in both birds and mammals, auditory alarm calls can convey information about the relative danger and urgency of a predatory threat shaping the behavioral response in conspecifics (6-8). While auditory alarm calls convey information about a predator to conspecifics, evolution has also selected for social communication between predator-prey relationships (9, 10). For example, aposematic coloring to visually deter predators and ultrasound detection for predatory evasion conveys information between predators and prey (9, 10).

However, the most ancient, and ubiquitous form of social communication among life is chemical communication, present both within and between species. Therefore, it is not surprising that detection of predator odors plays a major role in prey behaviors across phyla (11-14). The detection of chemical signals released by an organism that benefit a

receiver of a different species are known as kairomones. Upon detection of a predator odor, animals can exhibit a primer effect, such as reproductive modulation and physiological changes, or an immediate behavioral response known as a releaser effect (11-14). The importance of kairomone detection is exemplified by the myriad of species that can detect the social cues and the range of elicited effects detection results in. Exemplifying the evolutionary significance of kairomones is that of rodents inbred in laboratory conditions, who have not been exposed to predators in hundreds of generations, still respond aversively to predator scents (15). This indicates that the cellular machinery underlying the innate response is maintained, as it is evolutionarily crucial for survival, and may be both broadly tuned and redundant.

Indeed, much progress has been made recently in unravelling the sensory organization of kairomone detection, and it appears that several different mammalian sensory channels exist for detection of predator olfactory cues. For example, chemosensory detection of predatory excretions has been observed in the vomeronasal organ (VNO), the Gruenberg ganglion (GG), the main olfactory epithelium (MOE), and the necklace subsystem's role in innate avoidance implies it may be as well (15-19). Interestingly, the kairomones trimethylthiazoline (TMT) and 2-propylthietane (2-PT) are detected by the GG, VNO, and MOE olfactory systems (19, 20). This suggests redundancy in neural coding for the detection of predatory aversive cues, highlighting the key selective pressures predator-prey dynamics have on encoding innate behaviors. Although much work has revealed regions of activation by kairomones, our understanding of promiscuous and redundant circuits underlying predator cue responses at the cellular

and molecular level is lacking. Elucidating these mechanisms will provide key insights into the neural coding strategies underlying predator-prey interactions.

To investigate these phenomena at the microscopic level, we investigated the classically studied nematode, *Caenorhabditis elegans*, and its interaction with the predatory satellite model, *Pristionchus pacificus* (21-23). We leveraged the relatively simple, eutelic nervous system of *C. elegans*, which affords cellular and molecular analysis due to a completely mapped wiring diagram of the animals' 302 neurons, and a fully annotated genome (24). This knowledge – coupled with the animals' genetic tractability, robust behavioral responses, and resiliency to neuronal ablations – offers a powerful tool for understanding sensory channels mediating social responses (25-28). Herein, we demonstrate that *C. elegans* detect and avoid sulfolipid compounds secreted by *P. pacificus*, using broadly tuned, redundant chemosensory pathways.

4.2 Results

P. pacificus is a sexually dimorphic, facultative predator that feeds on smaller nematodes, including *C. elegans*, in times of stress (29). Furthermore, like *C. elegans, P. pacificus* is known to secrete small molecules derived from primary metabolic pathways into the environment, which act as social signals (30-32). *C. elegans* detect diverse social, chemosensory information while navigating its natural milieu, ranging from pheromones from conspecifics to kairomones from noxious bacteria (33, 34). Thus, we hypothesized that secreted compounds from the predatory *P. pacificus* may be detected and perceived as kairomones. To test this, we subjected *C. elegans* to the exo-metabolome of two different *P. pacificus* isolates: the canonical strain, PS312 (22, 23) and a more recent isolate, RS5275B (35). Neither strain's secretions resulted in *C. elegans* avoidance upon

detection (Fig. 1A). However, *P. pacificus* is a dimorphic facultative predator, and the better suited form for predation develops in part as a result of starvation (29, 36). Therefore, we subjected *C. elegans* to secretions derived from starved *P. pacificus* and found that 21 hours of starvation elicited the most robust aversive response (Fig. 1A). The secretions from RS5275B resulted in more robust avoidance and will henceforth be referred to as “predator cue” (Fig. 1A). The observed difference in behavioral responses to the secretions may indicate that the invariant *E. coli* diet of the PS312 has resulted in preference for the non-predatory form, relative to animals living in an unpredictable environment – as on a bacterial diet, the nematodes develop and mature faster (29). Interestingly, the *C. elegans* wild type strain, N2, which has not encountered *P. pacificus* for thousands of generations, still maintains its ability detect – and respond to – a predator cue. These results indicate the importance of encoding predatory threats, and suggests that redundant chemosensory channels may exist.

To characterize the sensory pathways initiating the behavioral response to the predatory cue, we analyzed chemosensory neurons of the amphid organ. The amphid organ in *C. elegans* consists of twelve neuronal pairs, with cilia extending to the tip of the animals’ nose. However, one pair of these neurons (AFD) is thermosensory (37, 38). Of the eleven remaining pairs, three (AWA, AWB, and AWC) are believed to primarily detect volatile cues. This left the eight single- and double- ciliated sensory neurons as primary candidates for the detection of the *P. pacificus* cue (38). The transparent nature of *C. elegans*, and the invariant nature of their neuronal location allows for laser microsurgery to ablate specific sensory neurons, and assess their roles in behavioral responses (27, 39). Thus, we performed laser ablation studies of seven of the eight candidate ciliated

neurons; ASE was omitted from these studies, as it has been shown to be extensively involved in salt detection (40-42). Laser ablation of the remaining neurons revealed that four chemosensory neurons – ADL, ASH, ASI and ASJ – are required for wild type response to the predator cue (Fig. 1B).

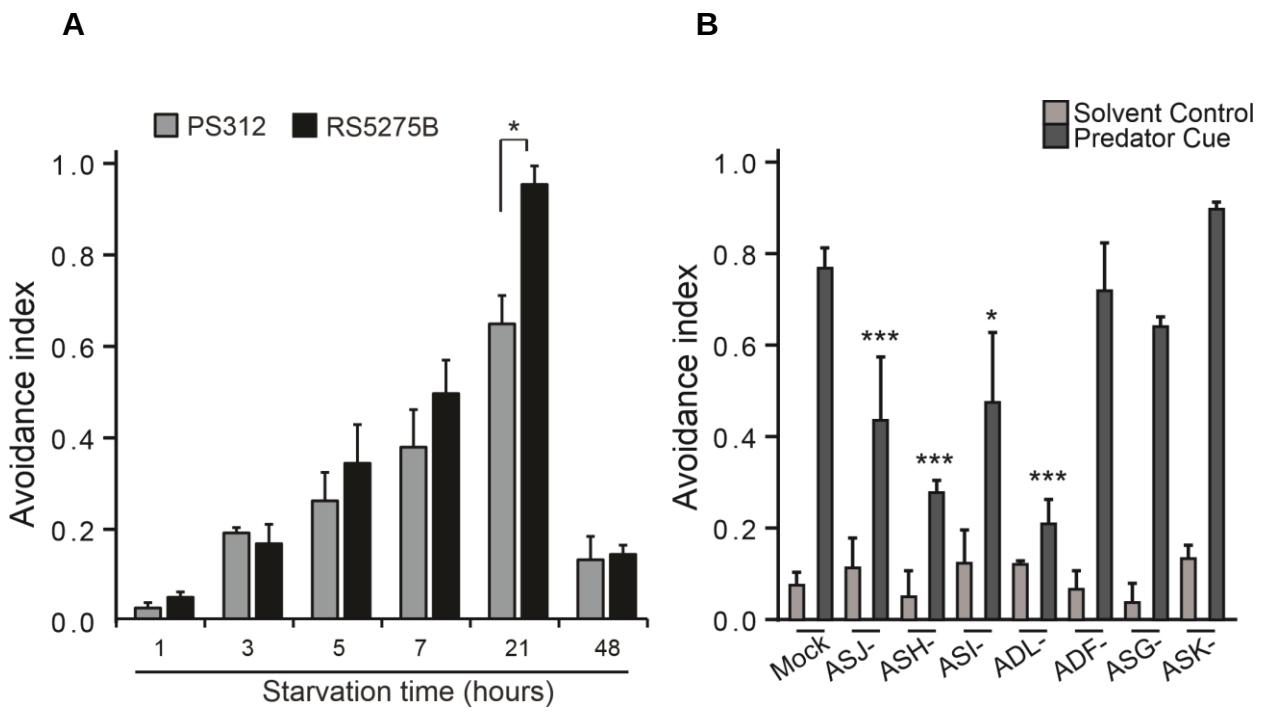


Figure 1. *Pristionchus pacificus* secretions are redundantly detected by *C. elegans* as kairomones. A) *P. pacificus* lab isolate (PS312) and recent wild isolate (RS5275B) secretions when starved elicit aversive behavior in *C. elegans*. Both strains elicited the strongest behavioral response at 21 hours of starvation. The most recent isolate, RS5275B, displayed a significantly stronger effect. Secretions from RS5275B that were starved for 21 hours were used for other assays and referred to as "predator cue". $n \geq 30$ animals. **B)** Laser ablation of amphid sensory neurons revealed that the predator cue is sensed redundantly by four chemosensory neurons: ADL, ASH, aSI, and ASJ. $n \geq 7$ animals. Data represented as mean ± SEM. One-Way ANOVA followed by Sidak's multiple comparison test, * $p < 0.05$, *** $p < 0.001$.

Given that exo-metabolomes consist of many compounds, we asked if the requirement of multiple chemosensory channels was due to a wide array of active compounds in the secretions, or if it instead implied a redundancy for sensation of specific compounds. To isolate any *P. pacificus* compounds responsible for *C. elegans'* aversive response, we utilized activity-guided fractionation to establish pools of compounds from differential 2D NMR spectroscopy comparison between active and inactive metabolite fractions (43). In tandem, UHPLC-HRMS was used to identify compounds present in the active fractions. This analysis led to the discovery of several unique sulfolipids; the terminal alcohols sufal#1and sufal#2, and the carboxylic acid containing sufac#1 and sufac#2 (Fig. 2A). It was found that the sulfolipids terminating with hydroxyl functional groups, rather than a carboxylic acid, were responsible for much of the behavioral response observed in *C. elegans*. Thus, *C. elegans* perceives the *P. pacificus* secreted compounds, sufal#1 and sufal#2, as predatory kairomones.

We next laser ablated amphid neurons to determine if the terminal alcohols can activate the previously observed redundant chemosensory neurons underlying the *C. elegans* response to the predator cue (Fig. 1B). We found that that the sulfolipids at (1:112 dilution) recruited the same set of sensory neurons underlying chemosensation of the predator cue (ADL, ASH, ASI, and ASJ) (Fig. 2B). This demonstrated that the pathways are indeed redundant for detection of the sulfolipids, and that multiple channels were not being recruited by a plethora of secretory compounds. Next, we asked if the different chemosensory neurons were tuned to different concentrations of these terminal alcohols. As such, we repeated the screen of amphid sensory neurons using a four-fold further dilution of the sulfal#1 and sulfal#2. We found that at lower concentrations, only ASJ and

ASH contribute to the avoidance behavior (Fig. 2C). Therefore, the number of recruited pathways in *C. elegans* avoidance to the predatory compounds is dictated by their concentration.

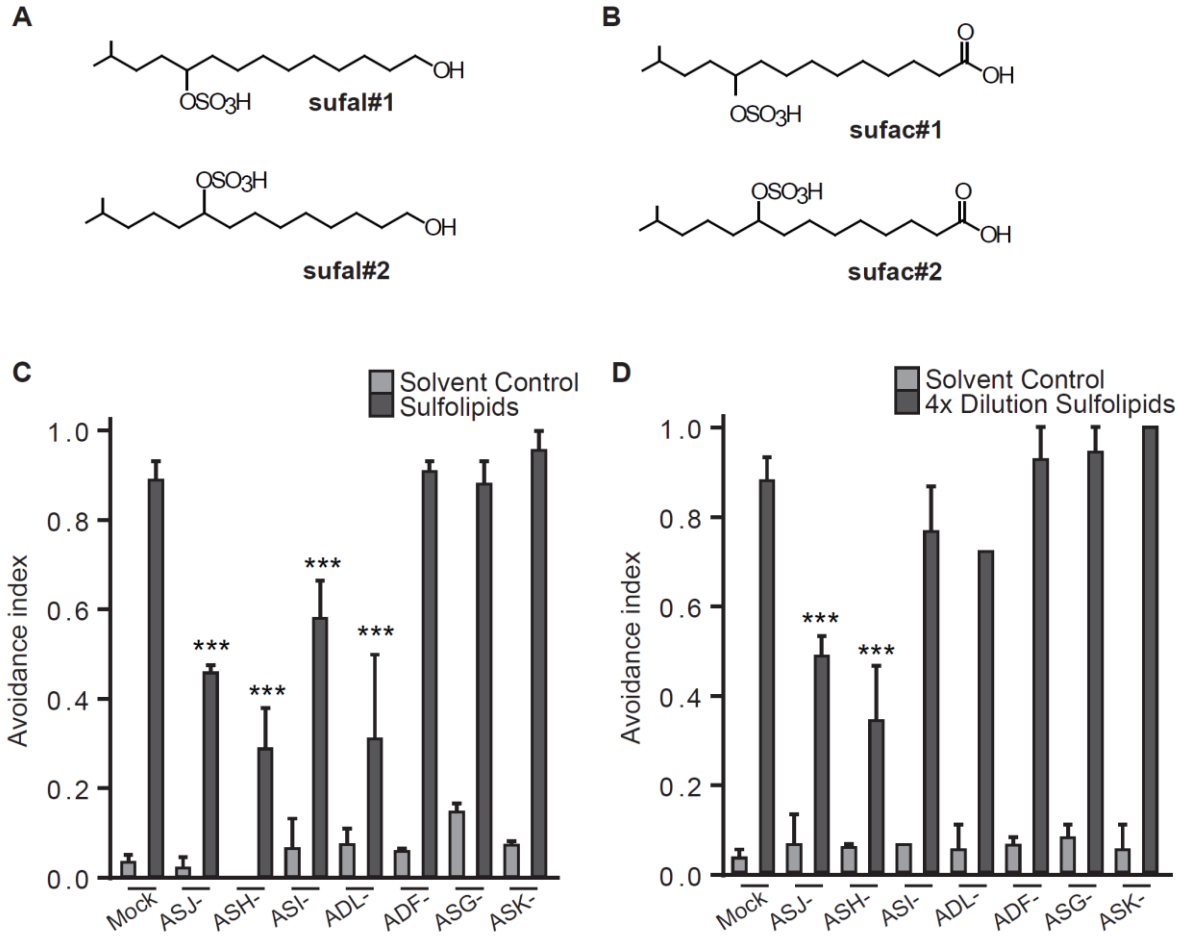
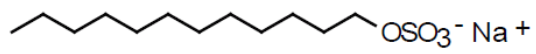


Figure 2. Isolated sulfolipids are active components of the Predator Cue. A-B) Sulfolipids consisting of a 14-carbon chain and sulfate group were found to be unique compounds present in the active fractions of the predator cue. **A)** Sufal#1 and sufal#2 have terminal alcohols, whereas **B)** sufac#1 and sufac#2 terminate with carboxylic acid groups. **C-D)** The most abundant sulfolipids, sufac#1 and sufal#2 were tested **C)** at a 1:112 dilution, and **D)** a further 4-fold dilution. $n \geq 9$ animals. While all four sensory neurons – ADL, ASH, ASI, and ASJ – are recruited for avoidance, a 4-fold further dilution reveals that ASJ and ASH are more sensitive to the sulfolipids than ASI and ADL. Data represented as mean ± SEM; One-Way ANOVA followed by Sidak's multiple comparison test, *** $p < 0.001$.

We noted a striking similarity of the structures of sufal#1 and sufal#2 to the synthetic detergent, sodium dodecyl sulfate (SDS) (Fig. 3A). Moreover, this non-biotic compound is a known repellent of *C. elegans* (44). Although SDS has been used to understand head-tail maps in *C. elegans* navigational strategy (44), the ecological significance of the response to this synthetic compound has not been investigated. As such, we hypothesized that the redundant chemosensory pathways underlying *P. pacificus* kairomone detection system may be promiscuous, and thus responsible for sensation of the aversive detergent, SDS.

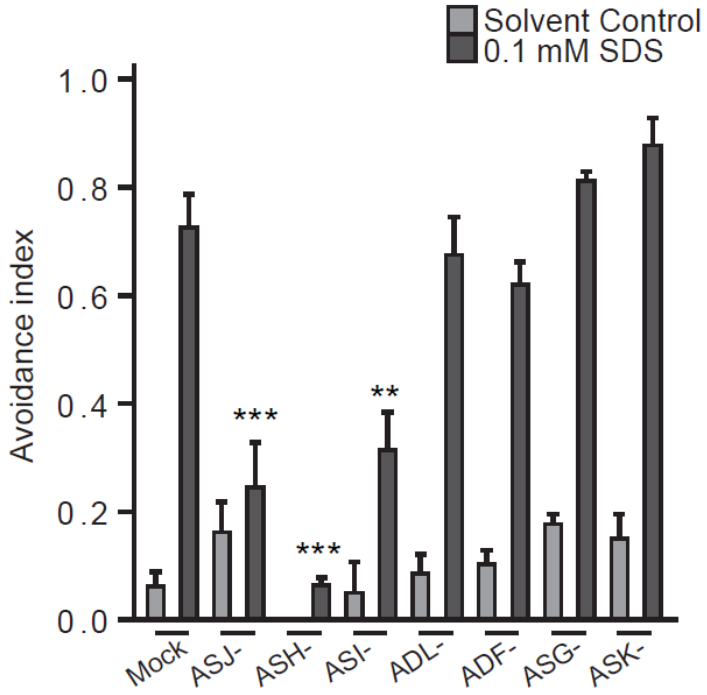
Through laser ablations of the amphid sensory neurons we found that two of the sensory neurons, ASH and ASJ, are required for response to 1 mM SDS (Fig. 3B). This overlaps with the pathways required for wild type perception of sulfal#1 and sulfal#2 (Fig. 2C). Similar to how the concentration of the terminal alcohols effected sensory neuron recruitment (Fig. 2B,C), we observed that a lower concentration of SDS (0.1 mM), recruited ASI, as well as the two encoding *C. elegans* perception of 1 mM SDS (ASH and ASJ) (Fig. 3B,C). Therefore, the avoidance of SDS, a synthetic compound, reflects promiscuous detection by the sensory pathways underlying response to the structurally related predatory kairomones secreted by *P. pacificus.*

A



Sodium Dodecyl Sulfate (SDS)

B



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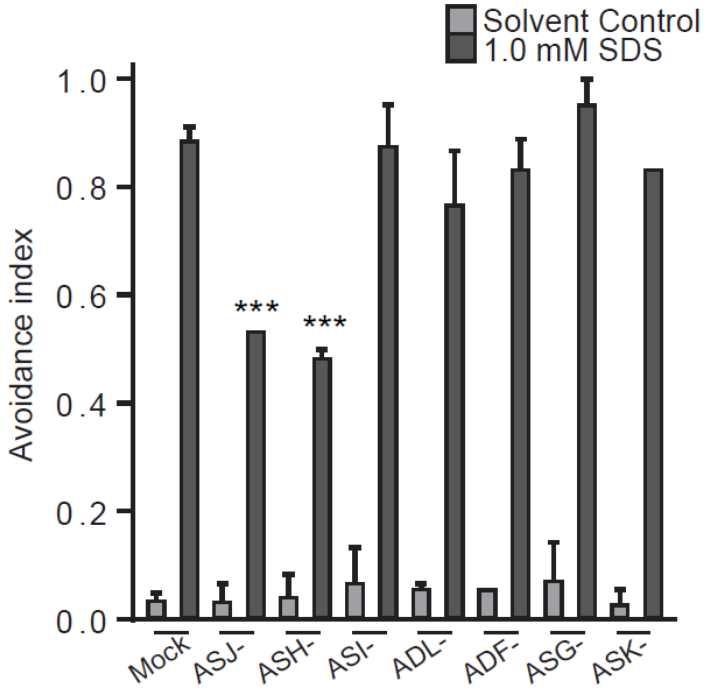

Figure 3. *C. elegans* recruit the same neurons for detection of SDS. A) The chemical structure of sodium dodecyl sulfate (SDS) resembles the isolates sulfolipids isolated from *P. pacificus.* **B-C)** Animals with chemosensory pairs laser ablated were subjected to **A)** 0.1 mM and **B)** 1.0 mM SDS. $n \geq 9$ animals. *C. elegans* ASJ and ASH sensory neurons are recruited for both concentrations of SDS. ASI is required for detection at lower concentrations only. Data represented as mean ± SEM; One-way ANOVA followed by Sidak's multiple comparison test, ** $p < 0.01$, *** $p < 0.001$.

Kairomones often elicit both primer (long lasting, physiological effects) and releaser (short term behaviors) responses. We have demonstrated the effect of the predator cue as a releaser kairomone through behavioral aversion by *C. elegans* upon detection (Fig. 2, 3). However, the question remained whether or not *P. pacificus* secretion elicits primer effects as well. Indeed, it was found that after short term exposure to the predator cue, adult animals laid significantly less eggs in the subsequent hour, in comparison to non-exposed, similarly staged animals (Fig. 4). Moreover, the following hour (60-120 minutes) resulted in exposed animals laying more eggs than non-exposed animals (Fig. 4). This demonstrates that the primer effect of perceived *P. pacificus* kairomones results in a fleeting cessation of egg laying and not a reduction in overall brood size.

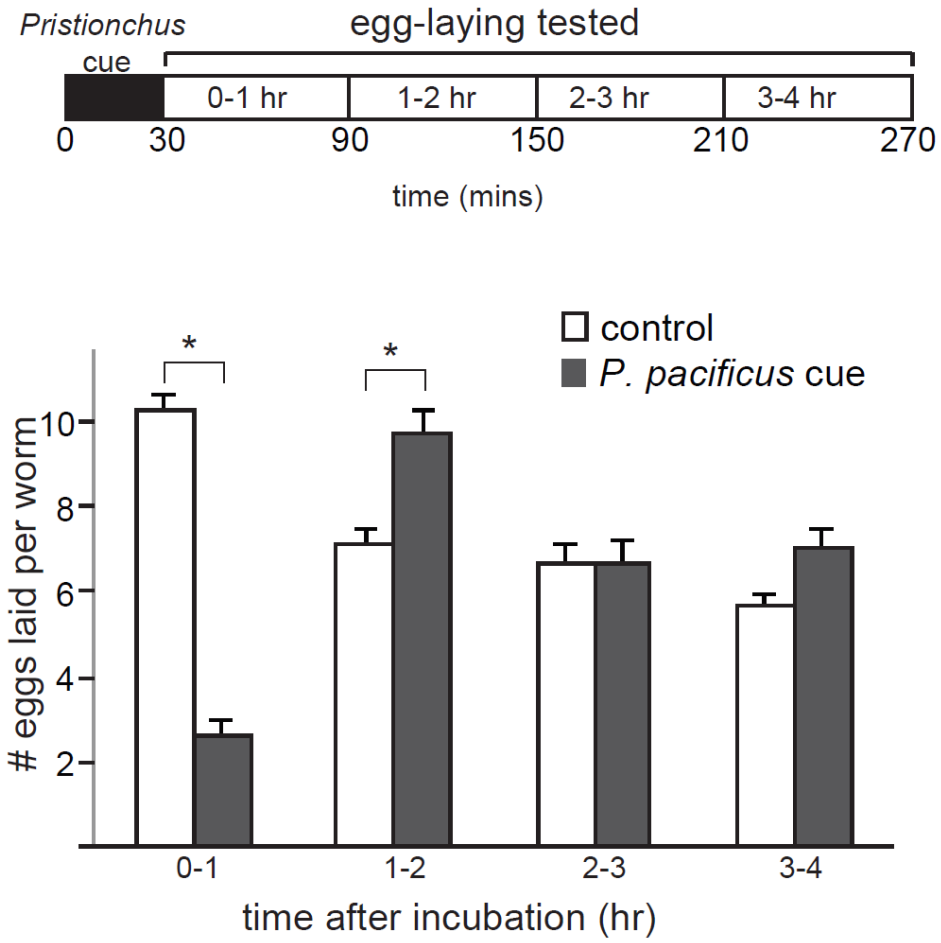

Figure 4. Predator Cue has Kairomone Primer Effects on *C. elegans*. *C. elegans* day 1 adults were exposed to solvent control or predator cue for 30 minutes before being transferred to normal culture conditions (NGM and OP50 lawn). The number of eggs laid was tallied at the end of the first, second, third, and fourth hours post exposure. *n* ≥83 animals; data represented as mean ± SEM; Unpaired t-test, Bonferroni correction, * p <0.05.

4.3 Discussion

We show that the facultative predator *P. pacificus* secretes compounds serve as a molecular signature which *C. elegans* perceives as a kairomone. However, only the exo-metabalome of starved *P. pacificus* induces aversive behavior, suggesting that the production of the active components is physiologically state dependent. Similarly, *C. elegans* secretory profiles are dependent on developmental stage, diet, environment, and sex (45-47). One pheromone secreted by *C. elegans*, osas#9, is exclusively produced by larval stage one starved animals, and results in aversion in conspecifics in a state dependent manner (48). It will be interesting to test if the sulfolipid terminal alcohols are indeed produced only when *P. pacificus* is starved, and analyze if these compounds communicate information to conspecifics like osas#9.

Further implication that sulfolipid production and secretion is physiologically state dependent, is the life history of *P. pacificus*. *P.pacificus* is observed to have one of two distinct mouth forms; the stenostomatous, or the eurystomatous (36). The eurystomatous results in a broader mouth, with an additional denticle that enables for more efficient feeding on prey than the narrower mouth form (29, 36). While both forms occur naturally in the population, starvation increases the percent of animals with the eurystomatic fate (36). Interestingly, coupled with that fate, is an increase in sulfatase activity (49). Thus, it may be that cleaved sulfate groups are incorporated into lipid synthetic pathways, and secreted as a waste elimination pathway in starved predators. *C. elegans* that eavesdrop on these cues would therefore have increased evolutionary fitness, by encoding and perceiving compounds representative of the *P. pacificus* predatory state as kairomones.

Indeed, this follows the typical trajectory for kairomone evolution, as the signal from the emitter is not purposed for eavesdropping (50).

We show here that *C. elegans* responds to the predator cue robustly in two distinct timescales: by rapidly avoiding the detected signals, and delaying egg-laying. Primer and releaser effects on receivers is characteristic of both pheromone and kairomone communication across phyla (11, 50). The adaptive value of both primer and releaser effects in response to predator cues is apparent for the persistence of an animals' gene pool: predatory escape to remain alive, and reproductive modulation for increasing the survival chances of offspring. Predator odors affect reproductive behavior ranging from reduced ovulation to altered oviposition in mammals and insects (11, 12). For example, *D. melanogaster* will avoid depositing eggs when olfactory cues from the parasitoid wasp *L. boulardi* are detected, while rodents show decreased litter production upon sensing predator cues (11, 12). Studies of this nature have given credence to the predator-induced breeding suppression hypothesis, which states that predator presence modulates reproduction to favor survival (11). Our findings that *C. elegans* avoid a predator cue and delay egg-laying for an hour after exposure demonstrate that this hypothesis extends beyond mammals and insects.

Remarkably, *C. elegans* has maintained the ability to detect this predatory cue after decades of being cultured in a laboratory setting. This exemplifies the importance of encoding predatory information, and the immense selective pressures for defensive behaviors. Similarly, lab mice have been shown to detect predatory chemical cues from several predators, such as weasels and foxes (15, 51). Both predatory compounds were shown to be detected redundantly by several olfactory subsystems (15, 51). We found,

at the cellular level, that *C. elegans* utilizes the redundant coding strategies for chemosensation of predator olfactory cues (Table 1), implying that evolution selects for multiple avenues of threat detection in order to ensure that the proper behavioral responses are triggered. Although ADL was not observed to play a role in SDS detection, it may be masked by the high sensitivity of the ASH and ASJ to the repellent. In several cases, a chemosensory neuron pair has masked the role of other sensory neurons in both attractive and aversive behaviors (44, 52, 53). ADF, ASG, and ASI have roles in chemotaxis that is only observable when the ASE sensory neurons are non-functional (52). Our data demonstrating that *C. elegans* uses the same pathways to avoid the isolated predator cues and the structurally related synthetic compound, SDS, indicates that these redundant pathways are also promiscuous.

The recruitment of the same set of sensory neurons to perceive both the predator cue and SDS indicates that the olfactory receptors underlying detection may be broadly tuned. The vast majority of chemoreceptors in vertebrates and invertebrates are G Protein-Coupled Receptors (GPCRs) (17, 54). GPCRs can be narrowly tuned to one ligand, or broadly tuned and capable of detecting a myriad of ligands (54, 55). The implication of having broadly tuned olfactory receptors is an evolved strategy for being able to rapidly and effectively adapt to an ever-changing range and composition of chemosensory information in the environment (50, 54). Thus, it is highly likely that a promiscuous receptor is responsible for detecting both of the terminal alcohols, as well as SDS. However, it is possible that the neurons express receptors that detect a similar set of chemical compounds, or are tuned to different concentrations. Such has been the case with diacetyl receptors in AWA and ASH, where the receptor SRI-14 in ASH detects

only high concentrations of the odorant, while ODR-10 in AWA detects a broader concentration range (56). Similarly, the differential chemosensory neurons observed to be required at different concentrations of the isolated compound and SDS, may be due to differential expression of receptors with various affinities for the compounds (Table 1). Future studies characterizing the receptor(s) would unveil the promiscuous nature of the underlying circuitry involved in detection of the predator cue and SDS.

In summary, our results demonstrate the importance of innate, neural coding of predatory molecular signatures at the single chemosensory neuron level. This data corroborates the notion that predatory olfactory cues are detected by multiple avenues (51) and supports a general evolutionary strategy for selecting redundant pathways to ensure proper execution of defensive behavior crucial for survival. Indeed, this is also seen in foraging behavior (52), further indicating that expending extra energy to encode multiple pathways for sensing critical survival cues is an important allocation strategy. Moreover, the genetic cost of maintaining redundancy may be balanced by promiscuous detection of compounds sharing structural similarity (Table 1). Our data demonstrates this through the observation that *C. elegans'* response to an exotic, synthetic molecule, recruits the same sensory neurons as an evolved kairomone pathway. This strategy allows for rapid detection and suitable fitness behaviors to newly introduced cues, and represents receptor bias as a mechanism for the evolution of olfactory driven aversive behavior.

Table 1. Summary of Chemosensory Neurons Required for Aversive Responses. Four chemosensory neurons show redundant function in sensing the predator cue, the sulfolipids, and SDS. Check mark denotes that cell ablations of that neuron impaired *C. elegans* ability to respond to the chemical of interest. The predator cue is a solution of M9 buffer containing *P. pacificus* secretions from 21 hours of starvation. The sulfolipids were synthesized by the Schroeder Lab and diluted in M9. SDS was diluted in M9.

Cue	Concentration	ASH	ASJ	ADL	ASI
Predator Cue	Undiluted	✓	✓	✓	✓
Sulfolipids	Undiluted	✓	✓	✓	✓
	4x dilution	✓	✓		
SDS	1 mM	✓	✓		
	0.1 mM	✓	✓		✓

4.4 Methods

Predator Cue: P. pacificus (PS312 and RS5275B) secretions were collected from approximately 100 µL of nematode culture after being washed with M9 buffer five times. At the indicated time points, the M9 media, containing the secreted compounds, was removed.

Laser-ablations: Laser ablations were performed as reported in literature (25, 27). In brief, larval stage one (L1) animals were placed on an agar pad (M9 buffer containing 2% agar), mounted on a glass slide, and were then anesthetized by inhibition of oxidative phosphorylation using sodium azide. Individual sensory neuron nuclei were identified at 100x on a compound microscope using Nomarski imaging. The MicroPoint laser system was used to trigger the firing of a laser through the objective of the mircroscope at the nucleus of a neuron. Successful ablation was observed by the loss of definition of the nucleus, which typically has a punctate texture. Both neurons of the sensory pair of interest were ablated in each animal. Post-ablation, animals were gently transferred to an NGM plate with an OP50 lawn, and allowed to recover for three days before performing the avoidance assay. Ablated animals and mock controls (which experience the same procedure minus the laser) were assayed on the same day.

Avoidance Assay: A small volume (~0.5 µL) was placed in front of a forward moving animal. When the sensory cilia (anterior tip of animal) passed into the drop region, animals exhibited either no response, or initiated an avoidance response, consisting of a reversal and an omega turn. Animals that initiated an avoidance response within 4 seconds of exposure were scored to allow for the calculation of the avoidance index. Each animal was tested six times (three drops of the solvent control, and three drops containing the

chemical of interest) with two minutes between stimuli delivery. The avoidance index was calculated by dividing the number of aversive responses by the total number of trials. Data is presented as mean +/- standard error, and were analyzed using GraphPad Prism 7 software. One-way ANOVA, followed by multiple comparisons using Sidak corrections were used for statistical analysis.

Isolated Compounds: For identification of the active compounds in the predator cue, *P. pacificus* eggs were isolated from gravid adults using sodium hypochlorite, and placed in M9 buffer for 24 hours. The supernatant was then lyophilized and fractionated using a water-methanol gradient. Active fractions were then compared to adjacent fractions using UHPLC-HRMS and comparative 2D NMR spectroscopy. The detailed methods of the identification of the compounds and their synthesis for use in the avoidance assay can be found in the published manuscript (1).

Egg-laying assay: Synchronized day one *C. elegans* adults were treated with M9 buffer or concentrated predator cue for 30 minutes, and then transferred to NGM plates with a dried 100 µL OP50 lawn. At the indicated time points the number of eggs were counted. Mean egg number and standard error were plotted, and statistically compared using an unpaired t-test with Bonferonni corrections.

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Author Contributions: This version of the manuscript was written by C.D.C., and utilizes the predator cue isolated by the Chalasani Lab, and the identified and synthesized sulfolipids by the Schroder Lab. Predator cue secretion of isolates and Egg-laying assay

were performed and analyzed by the Chalasani lab; these figures are from the manuscript (1). All other figures were remade and data analyzed by C.D.C.

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Ch 5 Discussion

The data presented in the previous chapters serves to strengthen our understanding of how social cues are sensed and processed to result in behavioral responses. The social cues studied in chapters two, three, and four – as well as those reviewed in chapter one – vary in nature, from attractive and aversive pheromones, to a predation kairomone, and the responses have been studied in the perspective of the animals' physiological state, and how sensation of multiple stimuli on a molecular and cellular level affects these responses. Deconstruction of these various circuitries which encode social behaviors will enhance our understanding of general brain function and specifically how the brain integrates, perceives, and acts on sensory information. Specifically, it will allow for comparative studies between the sensory strategies of evolutionary distinct species, and how the brain codes different social behaviors – ranging from reproduction to predatory aversion. Lastly, these data and considerations provide insights into the evolution of neural circuitries, signaling pathways, and detection of novel stimuli.

Different coding strategies for different behaviors.

Physiological state and concentration mediate responses

The data obtained and reviewed in this dissertation point to several distinct strategies in regards to coding sensory information. When considering attractive pheromones, we see that mate attraction is elicited across a broad concentration range of both ascr#3 and ascr#8 (1, 2). On the other hand, hermaphrodite aggregation (a behavior distinct from attraction) is signaled via icas#3, which displays a very narrow range of activity (3). This implies that broader range of detection may indicate behaviors

that have more weight of importance for the animal. For example, reproductive success and exchanging of genetic material to maintain diversity in a primarily hermaphroditic population is certainly more important for the survival of the species than aggregation amongst hermaphrodites. Data from all three of the data driven chapters in this dissertation support the notion that crucial chemical cues elicit an effect over a broad range of concentrations. The work performed in Chapter Two revealed that a very broad concentration range of osas#9 results in an aversive effect, highlighting the importance of dispersing from region flagged as depleted of resources when the animal receiving the cue is already starved. In Chapter Three, it was observed that extract of *E. coli* cultures –at dilutions orders of magnitudes apart – abolishes the aversive effect of osas#9. Just as avoidance of regions of depleted food is crucial for survival when foraging, so is attraction to food when starved. In fact, our data supports previous literature which indicates that both starvation state and food signals trump non-life threatening cues. For example, starved males will prefer to remain on food rather than search for a mate, and increasingly starved animals will cross an osmotic barrier towards the volatile attractant, diacetyl (4, 5). We show in Chapter Three that starved animals generally repelled by osas#9, which serves to signal unfavorable food conditions, overrule the avoidance response upon sensation of food odors.

Lastly, in Chapter 4, it was demonstrated that predatory, life threatening cues and structurally related compounds, also elicit robust levels of avoidance across a broad concentration range. Unlike the aversion to osas#9 studied in the previous chapters, the avoidance of predator cues did not appear to be dependent on the animal's feeding state, as *C. elegans* were not starved before conducting the avoidance assay. However, one

would expect starved animals to avoid predatory cues as well, as mammals are known to avoid predatory cues and cease foraging, and optimal foraging theory dictates that excess energy be allocated to survival mechanisms (6, 7). Due to the redundancy of the predator circuit, I would hypothesize it is less flexible in modulation than osas#9 by food odor. Moreover, mammals are known to alter foraging activity in the presence of predators; wild rats, typically nocturnal feeders, will switch to daytime feeding when preyed upon by foxes at night (8). Furthermore, one could vary the concentrations of predator cue and food odor to characterize neural circuitries underlying the multisensory integration of opposing signals. This would provide insight into neural coding of behavioral strategies underlying foraging risk assessment in the presence of predatory threat.

A previous study elegantly showed that hermaphrodites will not aggregate in response to the aggregate pheromone icas#3 in the presence of high concentrations of ascr#3, which has been shown to signal overcrowding (3, 9). A similarly intriguing future experiment would be to see if competing pheromone signals, osas#9 and an attractive pheromone, such as ascr#10, show concentration-dependent alteration of behavior. These studies would show if a dispersal cue has the ability to override attractive social behavior non-critical for survival.

As attraction to hermaphroditic cues is abrogated by starvation, and the osas#9 response is dependent on it, it would be interesting to observe if a well-fed male worm would be attracted to a mating cue in the presence of an odor that signals animals are dispersing. If males exhibit aversion when co-exposed to both, it would be a remarkable tool to decipher decision making circuitries, and demonstrate even further, the remarkable complexity of social chemical communication.

Multiple pathways ensure robust and reliable responses

The data in this dissertation serves to show that, based on the type of social signal, different strategies for detection and behavioral response are employed by the nematode, *C. elegans*. When considering work in this dissertation alongside previously published literature, it becomes abundantly clear that behaviors crucial for survival are robust, and that the consistency of response is likely due to redundant signaling – which serves to ensure appropriate behavior.

When looking at chemically driven social behavior in *C.elegans*, we observe a striking difference between the pheromone osas#9 (Chute *et al.*, eLife, presented as Chapter Two), and the *P. pacificus* secreted sulfolipid kairomones (Chapter Four). While osas#9 aversion resulted in an avoidance index of roughly 0.55, the predator cue sulfolipids resulted in nearly 100 percent aversion. This can be interpreted as an indication of the threat level of the cue. While osas#9 signals an unfavorable environment, it offers the flexibility through the integration of foraging information, and being overruled to alter the animals' behavior (Chapter Three). This suggests that osas#9 may act more as a warning signal that can be integrated in assessment to foraging decisions, than as a danger cue. The same can be observed in male *C. elegans*, and their attraction to hermaphroditic cues: the behavior is ruled by the presence of food (Chapter One) (5). This indicates that pheromones may be encoded with more flexibility for the integration of further environment information to allow for rapid and accurate behavioral adaptation, and allocation of resources in a given scenario, rather than 100% committed response – which may be detrimental to the individual in the long run. In an opposite neural coding strategy, the predator cue elicits a robust response that is likely unaltered by the presence

of mates or food, as it signals an immediate threat. Indeed, mammals have been observed to halt their current behavior – including altering their foraging strategies – in the presence of predators (6). A follow up study would need to be conducted which empirically shows that starved animals still respond to the cue. Anecdotally, it can been seen that starved animals respond to SDS, a structurally related synthetic compound that utilizes the same redundant pathway (Chapter Four), suggesting this to be the case.

Moreover, the redundant recruitment of neurons in predator cue aversion implies it is robustly encoded and hardwired to ensure survivability (Chapter Four). Supporting this notion is the revelation that predatory olfactory cues detected by rodents activate redundant regions of the olfactory system. The predatory kairomones, trimethylthiazoline (TMT) and 2-propylthietane (2-PT), found in fox feces and weasel urine, respectively, are detected by the GG, VNO, and MOE in mice olfactory systems (10, 11). Other critical behaviors have been elucidated to employ redundant circuitries as well, such as sexual behaviors and feeding in fruit flies and mice (12-14). Thus, it is likely that critical, innate behaviors have been selected for in parallel manners to ensure redundancy. This implies that redundancy is an important evolutionary strategy for ensuring robust and reliable responses to key fitness signals. Our findings of the redundant pathway for predatory cue detection provides a platform in a powerful model organism for which further studies can be launched to decipher the cellular and molecular mechanisms underlying redundant coding of predatory social communication.

Co-option represents the opportunistic nature of evolution

Evolution is an opportunistic: often times a particular trait, whether it be macro- or micro-scopic in scale, may acquire an ability to perform something other than its original

purpose (15, 16). In 1982, the term “exaptation” was introduced to describe such co-option, and originally focused on physical structures and functions (15). For example, feathers, originally purposed for thermoregulation and present in non-flying organisms, would later become co-opted, or exapted, for flight (15). Since its coining, the use of the term exaptation has expanded to include co-option of behavioral and molecular elements (16-19).

In regards to behavior, there is an intriguing case highlighting the co-option of feeding and climbing behavior in the Hawaiian goby (17). These fish utilize a scraping movement of their mouth against rocks when feeding on algae – that same motion allows the fish to use their mouths to climb rocks against the current in waterfalls to locate upstream habitats (17). Although it is not clear which behavior would have arisen first, it is clear that regardless of which is the “original” behavior, it had a purpose.

Intriguingly, some examples of exaptation arise from traits that have no apparent use or function (15). For example, at the molecular level, transposable elements are related to retroviruses, and thus have no original function in the human genome: however, repetitions and duplications could give rise to function, in which case it would be a co-option event (15, 20). Also, at the molecular level, it has been implied that the metabolic system may be more promiscuous than originally thought, designed in such a way that multiple resources could be utilized, even though a single carbon source is the primary metabolic input (19). Taken together, these studies highlight that co-option may be a significant manner in which new interactions and behaviors arise, especially with respect to fitness behaviors, such as foraging and reproduction. Therefore, one would expect to uncover similar exaptation events in aversive chemical communication.

The data presented in Chapters Two and Four show strong evidence for exapation as a means for detecting aversive cues. Exemplifying this is the repackaging of a neurotransmitter and its cognate receptor, both of which have been shown to be typically utilized in internal signaling, and not communication between individuals (Chapter Two). Interestingly, this exemplifies co-option at multiple levels. First: it is likely that the signaling pheromone itself, octopamine succinylated ascaroside#9 (osas#9), would have originally evolved as a secreted waste product, likely as a result of amine deactivation and disposal (21). Secondly, the receptor – elucidated in Chapter Two – is a known binding partner of the monoamine, tyramine, and served an endogenous role in multisensory integration (4, 22). Thus, the use of a compound originally serving as a waste product as a cue transmitting information about the environment is in and of itself exaptation. Likewise, the co-option of the receptor, TYRA-2, to be utilized independently of its original function in endogenous signaling. This lends credence to hypothesis that chemoreceptors are broadly tuned and flexible, allowing the animal to quickly detect and process new odors in their environment (23, 24).

Moreover, in Chapter Four we see more evidence of the adaptive value of promiscuous chemoreception pathways. The synthetic molecule, sodium dodecyl sulfate (SDS), is a potent repellent to *C. elegans* (25). However, it is unclear why a nematode would avoid a compound never present in its natural life history. We found the answer (shown in Chapter Four) to be rooted in exaptation. SDS is structurally related to the predator-secreted sulfolipids (sulfal#1, sulfal#2, sufac#1, and sufac#2), which are perceived as aversive kairomones by *C. elegans*. The finding that *C. elegans* recruit the same neurons to respond to both the molecular signature of a predator, as well as the

synthetic compound, suggests that these chemosensory neurons likely express broadly tuned receptors that prime the animal to avoid structures similar in nature redundantly. Although SDS is synthetic, when considering the evolutionary arms race between predators and prey, it becomes evident that detecting variations in the base structure of compounds perceived as threats, and eliciting the same behavioral responses, would greatly advantage the prey. This strategy of similar responses to similar compounds has been seen in mice, wherein alarm pheromones and predatory cues which share a common thiazole group that is detected via the same sensory subsystems (26). However, the pathways and molecular machinery which give rise to this promiscuity remain unknown. Our data provides a platform at the cellular level in which receptors underlying the response to similar structures can be studied. Such studies would reveal if the same receptor is utilized, or if paralogous receptors are expressed in the same neurons. Future studies would be needed to determine which strategy has been employed in *C. elegans*, and will provide much insight as to how chemoreceptors function to detect similar cues and drive similar behaviors.

Taken together, this dissertation provides additional molecular and cellular examples of co-option, and suggests that this phenomena may be an important mechanism by which olfactory detection of new stimuli and signals evolves.

Future work

While many future experiments have been articulated throughout the previous chapters and this discussion, there are several critical experiments that have yet to be discussed that would add to the specific data sets embodied in this dissertation.

It was shown in Chapter Two that TYRA-2 is necessary and sufficient for driving osas#9 behavioral responses, and that the Gα subunit, GPA-6, is required for aversion to osas#9. However, it remains to be shown if these two proteins are coupled. It also has yet to be elucidated whether or not TYRA-2 is acting as part of a heterodimer in detection of osas#9. Biochemical approaches would help unveil these questions. For example, co-immunopreciptation experiments could be performed to uncover any interactions between TYRA-2 and other receptor proteins by engineering an epitope, such as FLAG, onto the N-terminus of the GPCR. The resulting immunoprecipitation could then be analyzed to reveal potential heterodimers, as well TYRA-2-associated G proteins.

We also found that *tyra-2* transcript levels are higher in starved animals than in well fed animals. This correlates with the starvation-dependent response, but we have not determined if this increase in *tyra-2* expression is required for the proper behavioral response. One way to investigate this is would be to overexpress *tyra-2* in well-fed animals, and assay for response to osas#9. However, it seems probable, due to the data shown in Chapter Three, that other modulations of the primary sensory neuron, ASH, may be necessary. One could perform comparative single-cell transcriptomics on the ASH sensory neurons from both well-fed and starved animals. This would likely result in a plethora of potential genes to analyze for a role in the state-dependent osas#9 behavior.

In Chapter Three, a model was constructed to explain the modulation of the osas#9 behavioral response. Calcium imaging of target cells would greatly add to our understanding of the working model for osas#9 attenuation. Using olfactory chip microfluidic devices, coupled with targeted single-cell calcium dynamic analyses, like those employed in Chapter Two, we can reveal how the neurons in our hypothesized

model are functioning. For example, a decrease in calcium transients in ASH animals exposed to both osas#9 and the *E. coli* extract would support the sensory level modification of the model. If this is not seen, we can the hypothesize that the modulation is only occurring downstream of the primary sensation of osas#9, and update the model accordingly. New technologies allowing for simultaneous whole brain imaging would be essential for elucidating a comprehensive circuit underlying osas#9 sensation and modulation. It would allow for a cohesive analysis of all the required neurons, rather than targeted bias approaches. This would also allow for seeing how the global brain state activity changes in a starved animal versus a fed animal, and allow for rapid, informed testing at the molecular level.

We found in Chapter Four that the predator cue had both primer and releaser effects on the recipient. Pheromones are also known to have primer effects, and in *C. elegans* the effect of pheromones on dauer formation is well known (Chapter 1). Given the that *C. elegans* are susceptible to primer effects, and the ascaroside osas#9 is derived from a catecholamine, which are known to modulate egg laying behavior (27, 28), I hypothesize that osas#9, like the sulfolipids, may inhibit egg laying.

Conclusion

Taken together, this dissertation provides molecular and cellular network information underlying social chemical communication, allowed for a framework in which to compare and understand coding strategies, and provided farther insight into the role of co-option as an evolutionary strategy for fast adaptation. Now that the foundation has been built for the primary sensation of osas#9, future work can study how the physiological state is priming the animal for response, and how the nervous system

integrates that information with the external milieu to make the appropriate decision in a given context.

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Addendum

i. Complete strain list

Source	Strain	Gene (allele)
Albrecht	CX14887	N2;kyIs598 [gpa-6::GCaMP2.2b 50 ng/µL]
Albrecht	CX13503	*eat-4 (ky5)*
Alkema	QW569	*octr-1* (ok371)
Alkema	MT13113	*tdc-1 (n3419)*
Alkema	QW284	*tdc-1 (n3420)*
Alkema	QW42	*tyra-2* (tm1815)
Alkema	QW1853	*tyra-2* (tm1846);worEx14[p*tyra-2*::*tyra-2*::GFP @ 1ng/µL]
Alkema	CX11839	*tyra-3* (ok325)
Ambros	NL3321	*sid-1 (pk3321)*
Bargmann	CX6968	mod-1(ok103); kyEx985= Podr-2(2b)::mod-1::GFP; ceolomocite GFP]
Bargmann	CX10979	N2;KyEx2865 [p*sra-6*::GCAMP3 @ 100 ng/µL])
Bargmann	CX7265	*osm-9* (ky10) IV;yzEx53 [osm-10::osm-9, elt-2::gfp]
Bargmann	CX3085	*tax-2(ks31*) I; *tax-4(p678*)III
Bargmann	CX2989	*tax-2(p691*) I; *tax-4(p678*)III
Bargmann	CX6750	*tax-4(ks28)* kyEx747
Bargmann	CX13571	tph-1 (mg280); kySi56; kyEx4077= srh-142::nCre (95 ng/uL); myo-3::mCherry (5ng/uL)

CGC	CB1112	*cat-2(e1112) II.*
CGC	PR672	*che-1* (p672) I.
CGC	CB1372	*daf-7* (*e1372*) III.
CGC	MT1434	*egl-30(n686)I.*
CGC	RB982	*flp-21(ok889) V*
CGC	DG1856	*goa-1(sa734)I.*
CGC	NL332	*gpa-1* (pk15)V.
CGC	NL1147	*gpa-10(pk362)V.*
CGC	NL787	*gpa-11(pk349)II.*
CGC	NL2330	*gpa-13(pk1270)V.*
CGC	NL788	*gpa-14(pk347)I.*
CGC	NL797	*gpa-15(pk477)I.*
CGC	NL334	*gpa-2(pk16)V.*
CGC	NL335	*gpa-3(pk35)V.*
CGC	NL790	*gpa-4(pk381)IV.*
CGC	NL1137	*gpa-5(pk376)X.*
CGC	NL1146	*gpa-6* (pk480)X.

CGC	NL795	*gpa-7(pk610)IV.*
CGC	NL793	*gpa-9(pk436)V.*
CGC	NL361	*gpb-1(pk44)II: pkEx170*
CGC	DA541	*gpb-2(ad541)I.*
CGC	NL792	*gpc-1(pk298)X.*
CGC	CB1489	*him-8* (e1489)
CGC	MT9668	*mod-1(ok103) V*
CGC	MT9772	*mod-5(n3314 I*
CGC	CX4148	*npr-1(ky13)*
CGC	RB1325	*npr-10[C53C7.1(ok1442)]*
CGC	CX2205	*odr-3(n2150)V.*
CGC	DA1814	*ser-1(ok345) X*
CGC	AQ866	*ser-4(ok512) III*
CGC	RB1585	*ser-7(ok1944) X*
CGC	FK104	*tax-2(ks31)* I.
CGC	PR671	*tax-2(p671*) I.
CGC	PR691	*tax-2(p691*) I.

CGC	MT15434	*tph-1 (mg280)II.*
Ferkey	FG0001	*grk-2 (gk268)*
Ferkey	LX0242	*rgs-3 (vs19)*
Iino	JN1713	Is[*sra6* p::mCaspI]
Komuniecki	OH313	*ser-2* (pk1357)
Komuniecki	DA1774	*ser-3* (ad1774)
Komuniecki	FX01846	*tyra-2* (tm1846)
Li	NY106	*flp-12(n4902)*
Li	NY193	*flp-19(pk1594)*
Li	N/A	*flp-3(pk361)*
Li	NY183	*flp-6(pk1593)* x7
Schwarz	VH624	rhIs13 [unc-119::GFP + dpy-20(+)] V; nre-1(hd20) lin-15B(hd126) X.
Srinivasan*	JSR72	*gpa-6* (pk480)X.; WorEx19 (p*nhr-79*::*gpa-6*::RFP @30ng/ul; punc-122::RFP)
Srinivasan*	JSR86	*gpa-6* (pk480)X.; WorEx19 (p*nhr-79*::*gpa-6*::RFP @30ng/ul; p*unc-122*::RFP)
Srinivasan*	JSR51	JSR45;kyIs598 [*gpa-6*::GCaMP2.2b 50 ng/µL]
Srinivasan*	JSR88	N2; WorEx20 [p*gpa-6*::*gpa-6*::RFP::*unc-54* @ 5ng/ul]
Srinivasan*	JSR23	N2;worEx13[p*tyra-2*::*tyra-2*::GFP @ 30ng/µL]
Srinivasan*	JSR89	QW1853; WorEx20 [p*gpa-6*::*gpa-6*::RFP::*unc-54* @ 5ng/ul]

Srinivasan*	JSR50	*tyra-2* (tm1846) ;KyEx2865 [p*sra-6*::GCAMP3 @ 100 ng/µL])
Srinivasan*	JSR19	*tyra-2* (tm1846);worEx12[pLR306_p*nhr-79 _tyra-2*]
Srinivasan*	JSR45	*tyra-2* (tm1846);worEx15[pLR305_p*odr-10 _tyra-2*]
Srinivasan*	JSR47	*tyra-2* (tm1846);worEx15[pLR305_p*odr-10 _tyra-2*]
Sternberg	PT839	*osm-9(ky10); him-5(e1490)*
Sternberg	PY7505	oyIs84 [gpa-4p::TU#813 + gcy-27p::TU#814 + gcy-27p::GFP + unc-122p::DsRed], (ASI-)
Sternberg	PY7502	oyIs85 [ceh-36p::TU#813 + ceh-36p::TU#814 + srtx-1p::GFP + unc-122p::DsRed], (AWC-)
Sternberg	PS6022	qrIs1[sra-9::mCasp1], (ASK-)
Suo	VN280	*ser-6* (2146)

* denotes strain created by Christopher Chute

ii. Chemical Mating Cues in *C. elegans*

iii. Predator-secreted sulfolipids induce defensive responses in *C. elegans*