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Multisensory Integration and the Regulation of Aversive Behaviors in C.elegans

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Multisensory Integration and the Regulation of Aversive Behaviors in *C.elegans*

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Table of Contents

Abstract 3
Background 3
Sensation and Behavior 3
Chemosensation in *C.elegans* 4
C.elegans as a Model System 4
Sensory system 4
Attenuation of Avoidance in the Presence of *E.coli* Extract 5
Methods 7
Strains 7
Bacterial Extract and Test Compounds 7
Drop Assays 7
Statistics 8
Results 9
Discussion 12
Conclusions 14
Acknowledgements 14
References 15

Abstract

Organisms are constantly taking in stimuli from the environment through their senses. These stimuli provide the organism with information, which is processed in the brain and then used to determine and set into motion certain, appropriate behaviors. Multisensory integration is the process through which stimuli gathered using multiple senses, such as sight and hearing, are integrated within the brain and used to enact complex behaviors. Malfunctions in multisensory integration have been observed to be related to some mental illnesses and neurodevelopmental disorders, such as schizophrenia and autism. The underlying mechanisms of this integration is poorly understood. *Caenorhabitis elegans* uses a highly specialized system of chemosensation that it relies on to survive within its environment, making it suitable for study in order to further understand these underlying mechanisms. Chemosensation is gathering information from the environment specifically through the detection of odorants and small molecules such as ascarosides, which *C.elegans* produces on its own as a method of communication between individuals. Octopamine Succinyl Ascaroside #9 (osas#9) is produced by L1 larvae who have become starved and is released into the environment. Exposure to osas#9 causes other individuals to avoid areas where it is present. Young Adult worms, however, have been found to attenuate this avoidance behavior when starved and upon detecting *E.coli* extract. It is possible that an *E.coli* metabolite is responsible for this attenuation, and avoidance assays were performed on two candidate compounds, L-Proline and Cyclo(Phe-Pro). Avoidance assays were also performed on a number of *C.elegans* mutant strains to determine specificity of the attenuation. Neither L-Proline nor Cyclo(Phe-Pro) in single treatments or synergistic treatments recreated the attenuation seen using *E.coli* extract, and attenuation was observed to be dependent on the presence of serotonin and gpa-2/gpa-3 alpha-subunits, implicating the ADF neuron as part of the neuronal circuit for attenuation.

Background

Sensation and Behavior

Behaviors are actions an organism takes in response to a stimulus or set of stimuli present within their environment. Organisms may interact with or respond to stimuli in the environment for a number of reasons and in a variety of ways: running away from a predator to survive, moving to a new source of food to feed from, and approaching or courting a potential mate to reproduce and pass on its genes. The peripheral nervous system takes in stimuli from the environment through vision, auditory processing, tactile perception, or olfaction and is processed in the brain. The brain cognitively assesses the stimuli and directs the organism to act appropriately through series of signals that are sent to the rest of the body.

Multisensory integration (MSI) refers to neurons processing environmental stimuli gathered through multiple senses—sight, hearing, touch, taste, smell—and assimilating the information gathered from each sense. The combination of environmental cues from multiple senses allows the organism to better understand their environment, and enact specific, complex

behaviors appropriate to the situation (Meredith & Stein, 1986). Malfunctions in MSI have been found to be related to some mental illnesses and neurodevelopmental disorders. For example, Williams, Light, Braff, and Ramachandra found in a study on patients with schizophrenia that their reaction times to simultaneous visionary and auditory cues were much slower than control participants due to their brains inability to integrate stimuli from both senses at once (2010). Similarly, a study on autistic children found that decreased multisensory processing was linked to difficulty in speech processing, which may be related to difficulties in processing social interaction (Stevenson et al. 2014). Further understanding in how MSI works in smaller, simpler systems can allow for progress in understanding how MSI occurs in humans and its relationship to human behaviors.

Chemosensation in *C.elegans*

C.elegans as a Model System

Caenorhabditis elegans are microscopic worms that have been thoroughly studied and used as a model system for a variety of studies. Its nervous system is relatively simplistic, and the connectome—the connections between all of the neurons within a brain—has been extensively mapped (Sengupta & Samuel, 2009). However, *C.elegans* still performs complex behaviors in response to specific stimuli. By taking advantage of the simplistic inputs and complex behavioral outputs in accordance with the knowledge of the intricate connections within the brains, it is possible to reveal the underlying mechanisms responsible for specific behaviors.

Chemosensation refers specifically to the sensation of chemicals or odorants, either naturally present in the environment or released by other organisms that trigger specific behaviors (Chute & Srinivasan, 2014). For example, the detection of pheromones can lead organisms to a mate, or the detection of a specific odorant may be used as a cue to denote that a specific environment is lacking food and organisms need to move elsewhere.

Sensory system

A highly complex and versatile system of chemosensation is used by *C.elegans* to gather information about its environment (Bargmann, 2006). Bargmann explains that chemosensory neurons in *C.elegans* have sensory cilia attached that are exposed to the environment in one way or another (2006). In this way, the cilia detect chemicals present in the environment and information from this sensation is sent directly to the cilia's corresponding neuron (Bargmann, 2006). One class of chemicals that *C.elegans* are known to produce for the purposes of chemical communication are ascarosides.

Ascarosides are a type of glycolipid released into the environment by *C.elegans* as a form of social communication between organisms. An ascarylose sugar serves as the base of an ascaroside, and can host a range of constitutive moieties, allowing for extreme variability between similarly structured molecules (Ludewig & Schroeder, 2013). This variability allows for different ascarosides to cause different behavioral responses in organisms that detect them. For example, ascaroside #3 has a saturated fatty acid chain, and causes hermaphroditic repulsion. Ascaroside #10, however, contains an unsaturated fatty acid chain, and causes hermaphrodite attraction (Izrayelit et al. 2012). One such ascaroside is octopamine-succinyl ascaroside #9 (osas

#9), which is produced by worms during their first larval stage (L1) when they become starved (Artyuhkin et al., 2013).

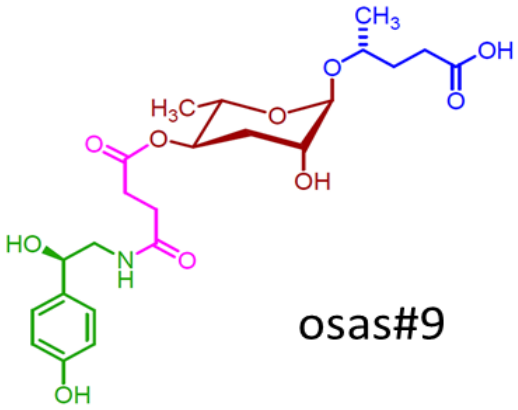

***Figure 1*: Molecular structure of osas#9.** Shown are the ascarylose sugar base (red and blue) and octopamine-succinyl (green and pink) components.

Starved L1 worms release osas #9 into the environment, and exposure causes worms to reverse their body movement (Artyuhkin et al., 2013).

Attenuation of Avoidance in the Presence of *E.coli* Extract

Artyukhin et al. found avoidance behavior triggered by osas #9 was diminished in the presence of *E.coli*, indicating that an *E.coli* metabolite may be responsible for attenuating the response (2013). Turland examined the relationship between nutrition and the subsequent attenuation of avoidance behaviors in the presence of osas #9 in *C.elegans* (2015). He concluded that avoidance attenuation was concentration dependent when he observed *C.elegans* three different thresholds of avoidance behavior that correlated with different ranges of concentration of *E.coli* extract.

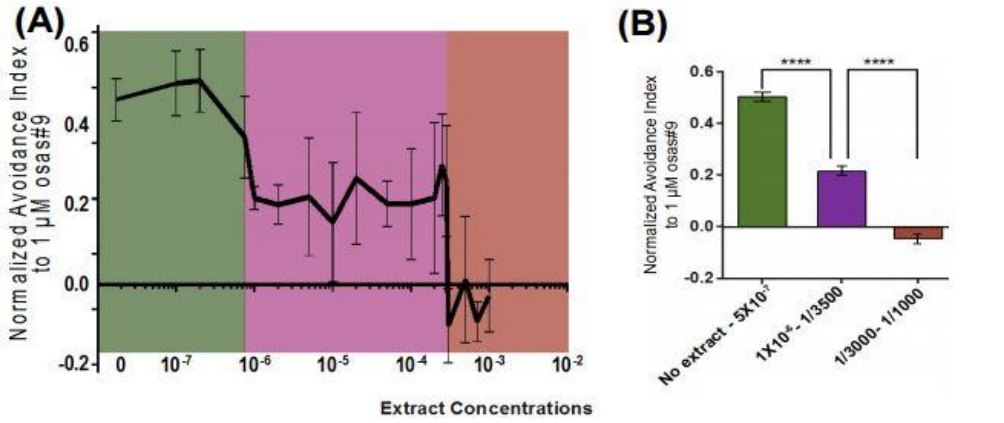

***Figure 2: Normalized avoidance index of C.elegans in response to E.coli concentrations.** Dose response curve **(A)** and graph **(B)** of C.elegans avoidance index in relation to E.coli extract concentrations (adapted from Turland, 2015).*

The presence of these thresholds further supported that an *E.coli* metabolite triggered a mechanism to inhibit avoidance of osas #9 (Turland, 2015). Activity-guided fractionation experiments were performed on the *E.coli* extract in order to separate small molecules within. Fractionations with high attenuation of avoidance compared to controls were sent to a collaborator lab for isolation and identification. Turland examined niacin and nicotinamide as candidate metabolites, but neither alone nor synergistically did either recreate the attenuation observed when using the extract as a whole (2015).

The exact neuronal pathway of avoidance and its attenuation in regards to osas#9 sensation remain largely unknown. Inglis et al. have identified sensory neurons in the head that have roles in chemoattraction: ASE, ASG, ASI, ASK, AWA, and AWA (2007). Most of the cilia of these neurons are exposed through *C.elegans* nose (Chute & Srinivasan, 2014). Additionally, neurons that have been identified with roles in chemorepulsion are ASH, ADL, AWB, PHA, and PHB (Inglis et al., 2007). Further examination of avoidance attenuation could identify the *E.coli* metabolite, the metabolite's receptor, and the neurons involved. Studies from there could characterize integrated neuronal interactions that occur in response to chemosensation of osas#9 and the *E.coli* metabolite to inhibit the avoidance behavior.

Methods

Strains

N2 strain *C.elegans* were used as wild-type animals, and were obtained from the *Caenorhabditis* Genetics Center. Mutant *C.elegans* strains were obtained from Alkema lab (*tdc-1, octr-1, and tyra-3*), Chase lab (*dop-1, dop-2, dop-3*, and *dop-4*), Komuniecki labs (*ser-3*), Kim lab (*gpa-2,gpa-3*), CGC (*cat-2*), Ferkey lab (*egl-4*), and Li lab (*flp-3* and *flp-6*). The genetically ablated strains for ASK (*PS6022*) and AWC (*PY7502*) were obtained from the Wakabayashi lab and Brandeis University lab respectively. All worms were grown at 20ºC incubation on lawns of OP50 *E.coli* (Stiemangle, 2006).

Bacterial Extract and Test Compounds

E.coli extract and potential *E.coli* metabolites L-Proline (Pro) and Cyclo(Phenylalanine-Proline) (Cyclo(Phe-Pro)) were isolated and obtained from Schroeder Lab at Cornell University and were diluted using 100% EtOH. Pro and Cyclo(Phe-Pro) were diluted further using 100% EtOH to create concentrations of 1 nM, 1 µM, and 1 mM. Drop assay solutions were created by combining 98 µL of dH_2O, 11 µL of 10 µM osas#9, and 1 µL of either 10% *E.coli* extract, 1 nM/µM/mM Pro, or 1 nM/µM/mM Cyclo(Phe-Pro). The *E.coli* extract was used at a 10% concentration in order for the drop assay solution to have a final concentration of 1/1000, which was determined to be the concentration at which avoidance is attenuated (Turland, 2015).

Drop Assays

Drop assays were performed to compare the avoidance behaviors of different worm strains. Young adult (YA) *C.elegans* were washed from 5cm plates using M9 solution, transferred to a 1.5 mL Eppendorf tube and washed again with M9, and re-plated onto 5cm plates without any bacteria. The worms were allowed to sit for one hour in order to ensure starvation. Drops of osas#9 and the solvent control (SC) were placed on 20 worms' tails and enveloped the worm to reach the head and nose, and avoidance behavior (avoid or not avoid) was recorded. This process was repeated on 20 worms using the test solution. Avoidance was defined as a two body turns and change of more than 90º in the direction the worm was traveling in at the time the drop was administered. The Avoidance Index (AI) of a worm was calculated by dividing the number of worms that avoided by the total number of worms assayed. An example calculation is shown below:

$$AI_{test} = (\text{Worms Avoided/Total Worms Assayed})$$
$$= 18/20$$
$$= 0.9 \text{ or } 90\%$$

Statistics

Statics were performed using the Data Analysis Tools as part of the FUNCRES.XLAM add-on to Microsoft Excel. Data was analyzed using parametric one-way ANOVAs.

Results

The goals of this research were to identify the *E.coli* metabolite responsible for attenuation, identify the *E.coli* metabolite's receptor, and characterize the avoidance attenuation neuronal circuit. Drop assays were performed on YA *C.elegans* in order to observe the avoidance behavior of wild-type worms two candidate compounds Pro and Cyclo(Phe-Pro) in both in single and synergistic treatment concentrations to determine if either were the metabolite involved in avoidance attenuation. ***Figure 3*** shows the avoidance index (AI) of N2 worms in response to ***(A)*** osas#9, osas#9+1/1000 extract, and single treatments of Pro and Cyclo(Phe-Pro); and ***(B)*** osas#9, osas#9+1/1000 extract, and synergistic treatments of Pro and Cyclo(Phe-Pro).

In wild-type N2 worms, nearly all animals performed avoidance behavior when exposed to osas#9. Exposure to osas#9+1/1000 *E.coli* extract, however, exhibits a steep decline in avoidance behavior. All three concentrations of Pro exhibited avoidance similar to that of

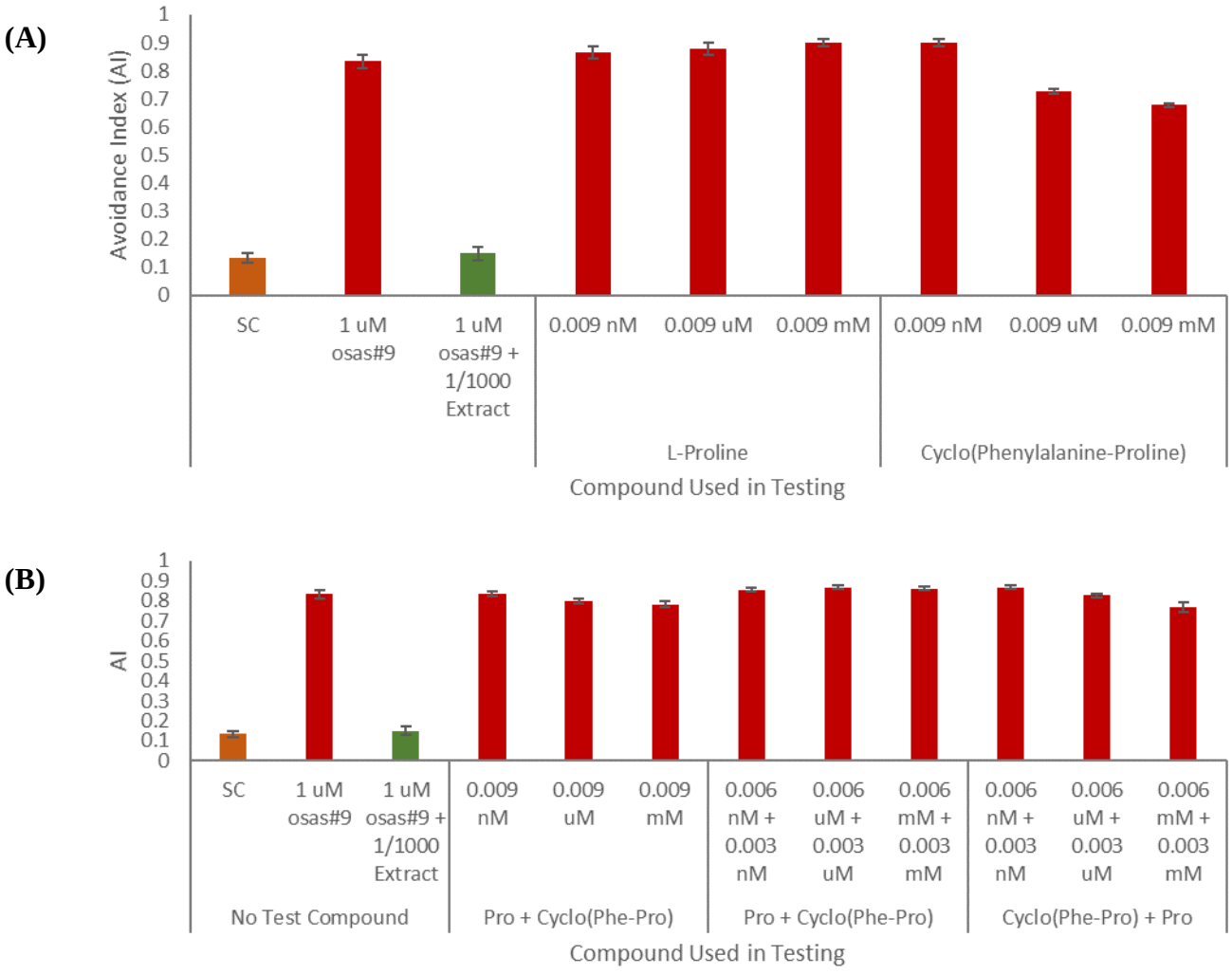

Figure 3: Avoidance Index (AI) of N2 worms during Pro and Cyclo(Phe-Pro) avoidance drop assays. *AI of N2 worms in response to solvent control (SC) of 1% EtOH (orange), 1µM osas#9 (red), 1µM osas#9+1/1000 Extract (green),* ***(A)*** *single treatment concentrations and* ***(B)*** *synergistic treatment concentrations.*

osas#9. 0.009 nM of Cyclo(Phe-Pro) also exhibited a similar AI, but AI then began to decrease as the concentration of Cyclo(Phe-Pro) increased. Signaling in *C.elegans* has proven in previous studies to occur due to synergistic effects of multiple small molecules (Ludewig & Schroeder, 2013). For example, ascarosides # 2, 3, 4 together act as a stronger male attractant than the ascarosides would on their own (Srinivasan et al., 2008). Mixtures made of equal parts Pro and Cyclo(Phe-Pro) exhibited a similar step-wise decrease in AI as only Cyclo(Phe-Pro), however, AI overall remained similar to that of osas#9. Mixtures made of unequal parts where Pro made up a majority of the mixture (i.e. 0.006 nM Pro + 0.003 nM Cyclo(Phe-Pro)) had an AI similar to that of just osas#9. Mixtures made of unequal parts where Cyclo(Phe-Pro) made up a majority of the mixture had an AI similar to that of just osas#9, but also exhibited the step-wise decrease in avoidance as concentration increased.

To identify the *E.coli* metabolite receptor, drop assays were performed on loss of function mutant strains of *C.elegans.* Lin et al. followed a procedure wherein they identified and isolated specific proteins based on activity, and then tested proteins for functionality within different cell types (2008). Similarly, Turland had already identified a concentration of *E.coli* extract necessary for the behavior we were searching for. Instead of testing within different cell types, drop assays on loss of function mutant strains were done in order to determine necessary circuitry for the behavior to occur. ***Figure 4*** shows the AI of each worm strain tested using osas#9 and osas#9+1/1000 extract.

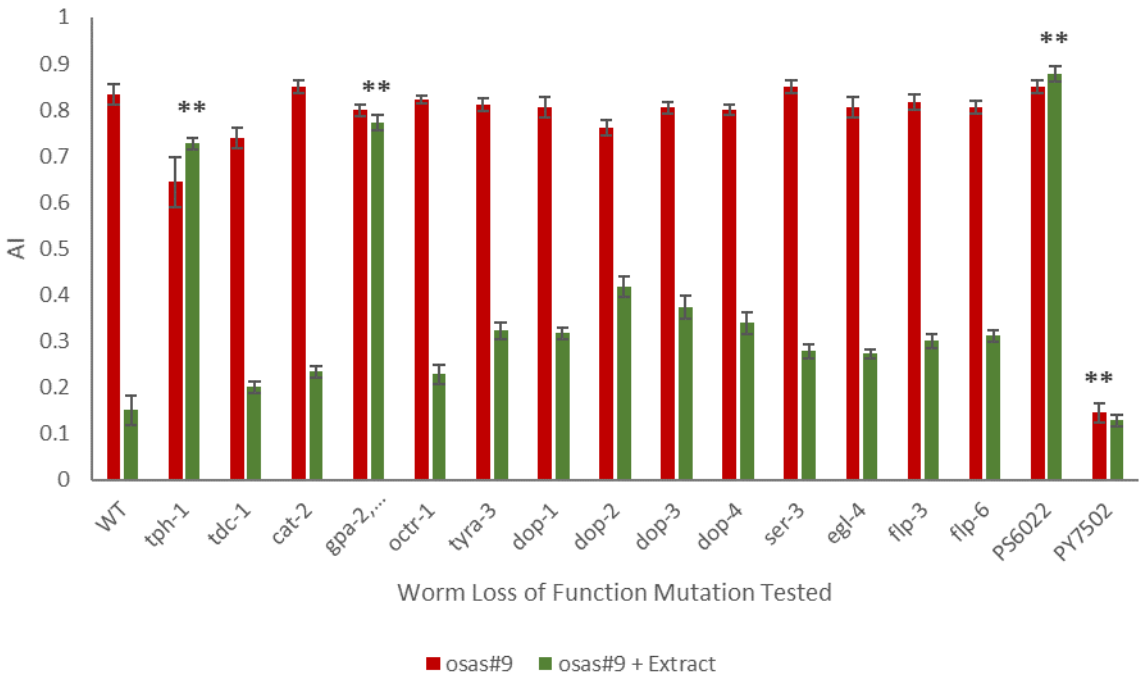

Figure 4: AI of Worm Loss of Function Mutation Strains in Response to osas#9 (red) and osas#9+1/1000 extract (green). *N2 worms were used as WT comparisons. AI in response to 1µM osas#9 (red) and 1µM osas#9+1/1000 extract (green). Noticeable lack of attenuation to osas#9 occurred in tph-1 (p = 9.05E-12) and gpa-2/gpa-3 strains (p=7.13E-12). Lack of attenuation was expected in PS6022, a strain genetically ablated for ASK. PY7502 worms are genetically ablated for AWC, and these worms appear to be unable to detect osas#9.*

We examined loss of function mutations in order to isolate and identify whether particular signaling mechanisms were necessary for attenuation to occur. Neurotransmitter involvement was investigated using strains *tph-1* (serotonin), *tdc-1* (octopamine), and *cat-2* (dopamine) (Bargmann, 1993). Loss of specific receptors was investigated using strains *gpa-2, gpa-3* (loss of alpha-subunits), *octr-1* (loss of receptor in ASH and ASI), *tyra-3* (loss of receptor in ADE and CEP), *dop-1, dop-2, dop-3,* and *dop-4* (loss of dopaminergic receptors), *ser-3* (loss of octopamine receptor), and *egl-4* (inhibition of chemosensation). Neuropeptide involvement was investigated by testing strains *flp-3* and *flp-6.* Involvement of specific neurons was investigated using strains *PS6022* (genetically ablated ASK) and *PY7502* (genetically ablated AWC). *Tph-1* (p = 9.05E-12), *gpa-2, gpa-3* (p = 7.12E-1) and *PS6022* worms, however, avoided when in the presence of extract. *PY7502* (p = 2.61E-13) worms showed no avoidance in response to osas#9 exposure.

Discussion

The data suggests that neither Pro nor Cyclo(Phe-Pro) are the *E.coli* metabolite responsible for *C.elegans* sensing the presence of food and attenuating the avoidance response to osas#9. The AI of single treatment concentrations of Pro and Cyclo(Phe-Pro) in ***Figure 3*** do not mimic the AI of worms treated with osas#9+1/1000 extract, which would be expected if either molecule was responsible for attenuation. The synergistic treatment results do, however, further support that attenuation may be triggered via multiple small molecules. Combining Pro and Cyclo(Phe-Pro) in mixtures where Cyclo(Phe-Pro) was the major component resulted in a slight decrease in avoidance, whereas only Pro and even mixtures of Pro and Cyclo(Phe-Pro) has no decrease in avoidance behaviors. Further studies towards the identification of the *E.coli* metabolite responsible could be done via continuing activity-guided fractionation and testing future compounds from a collaborator lab, or by testing molecules/metabolites known to be responsible for chemosensory avoidance and detection by ASK.

Data further suggests that attenuation is serotonin dependent. *Tph-1* mutant *C.elegans* lack the enzyme tryptophan hydroxylase, which is responsible for the synthesis of the neurotransmitter serotonin (Bargmann, 1993). *Tph-1* mutant worms, as shown in ***Figure 4***, exhibited a statistically significant (p = 9.05E-12) increase in avoidance when exposed to osas#9+1/1000 extract, implying that these mutants are unable to detect the metabolite in the *E.coli* extract the triggers the attenuation of avoidance and, thus, continue to avoid the osas#9 signal. Future studies could be completed via serotonin rescue assays, where the recovery of attenuation through the administration of serotonin to *tph-1* mutants would confirm serotonin dependence. Of the lines tested with defects in signal transduction, *gpa-2, gpa-3* also exhibited a statistically significant (p = 7.12E-12) increase in avoidance to osas#9+1/1000 extract. *Gpa-2, gpa-3* mutants lack two alpha-subunits of G-protein receptors and interrupts signal transduction. A ligand will still bind to its receptor, but the signal cannot be carried downstream. Thus, it seems likely that the *E.coli* metabolite(s) responsible for attenuation utilizes a pathway that incorporates the *gpa-2, gpa-3* alpha-subunits. Future studies into the *E.coli* metabolite receptor could be done using screens for G-protein coupled receptors that utilize the *gpa-2, gpa-3* alpha-subunits cross referenced with small molecules known to interact with ASK and/or ADF.

The exact neuronal pathway of avoidance attenuation is unknown. ASK has been identified previously as necessary for food detection, which is supported by drop assay data in ***Figure 4*** (Turland, 2015). PS6022, a strain in which ASK is genetically ablated, had increased levels of avoidance with osas#9+1/1000 extract. The data for *tph-1* and *gpa-2, gpa-3* implicate the involvement of the ADF neuron in the attenuation of avoidance to osas#9. ADF is the only sensory neuron that utilizes serotonin (Jafari et al. 2011). Additionally, ADF is also known to utilize a signaling pathway that incorporates a *gpa-3* alpha-subunit (Bargmann, 2006). Upon examination of the connectome, ADF, ASH (required for osas#9 sensation), and ASK form a circuit. ADF synapses onto and is synapsed onto by ASH, and ASH is connected via gap junction to ASK (Bhatla). Neither *tph-1* nor *gpa-2, gpa-3* mutants exhibited drops in sensation to osas#9, implying that ADF is only involved in the sensation of the *E.coli* metabolite in the extract, and both ADF and ASK are required for attenuation to occur. ***Figure 5*** shows a working

model of the possible neuronal pathway of avoidance attenuation that builds upon what was concluded in Turland's work.

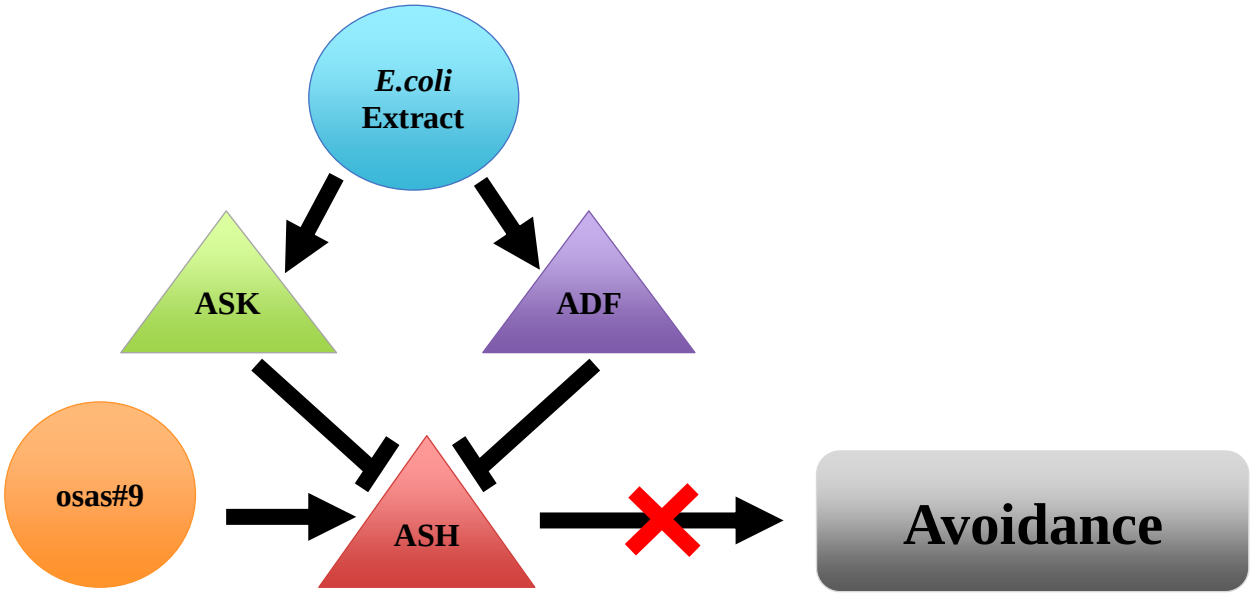

Figure 5: Working model of the neuronal pathway of the attenuation of avoidance to osas#9. *Updated from Turland's work to include the ADF neuron and its possible role in the neuronal circuit of avoidance inhibition in the presence of E.coli extract.*

Future studies could examine and confirm what role, if any, ADF plays in avoidance attenuation by performing avoidance drop assays on worms laser or genetically ablated for the ADF neuron. If ablated worms lose attenuation in ablated worms, it may confirm that ADF and ASK are both required for attenuation to occur. Ablate worms that retain some or all attenuation may indicate instead that ADF is not directly involved in attenuation.

Conclusions

Organisms gather information using their senses, and the integration of sensory information taken in by multiple senses can enact certain, complex behaviors. The underlying mechanisms of this multisensory integration, however, remains largely unknown. Using a highly specialized, but comparatively simplistic model system to study the neurobiological mechanisms that allow for the assimilation of different kinds of sensory data can allow for a better understanding of these behaviors, and how these mechanisms may be used in multisensory integration in more complex nervous systems, such as humans. Specifically, gaining a greater understanding of the process by which multiple kinds of sensory data are processed and the information synthesized to inform behaviors can lead to a better understanding of the behaviors exhibited by those with mental illnesses tied to malfunctions of multisensory integration.

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