JULS RESEARCH

Serotonin mediates *Caenorhabditis elegans* associative learning by indicating presence or absence of food

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Abstract

What does it mean to learn? The full molecular mechanisms underlying the formation and storage of a memory are unknown in even the simplest model organisms. The nematode worm, *Caenorhabditis elegans*, despite having only 302 neurons, is able to learn and can undergo classical (Pavlovian) conditioning. For example, when worms are given an attractive odorant (such as benzaldehyde, Bnz) during a period of starvation, they learn to find this stimulus aversive. Previous research indicates that serotonin signaling in worms acts as an endogenous food signal. When given exogenously, serotonin blocks the formation of this Bnz-starvation association.

This study hypothesized that the Bnz-starvation association is negatively regulated by serotonergic signalling. The absence of this satiety signal was considered the unconditioned stimulus in the associative learning paradigm. Since Bnz represents the conditioned stimulus, understanding the nature of the unconditioned stimulus signal will help explain stimuli integration and, consequently, memory formation.

Serotonin synthesis and receptor mutants were screened in the Bnz-starvation associative learning paradigm. Worms were given Bnz during a period of starvation, and then tested for their approach to a point source of Bnz. It was found that worms missing a single serotonin receptor, SER-4, were able to form a starvation-odorant memory even in the presence of exogenous serotonin.

This study implicates SER-4 as the crucial molecular component necessary for receiving the serotonin/satiety signal and, consequently, the regulation of the associative memory. Therefore, the structural simplicity and facile genetics of *C. elegans* was used to understand the nature of the unconditioned stimulus and gain insight into a fundamental question: what exactly is a memory?

Introduction

The struggle to define "learning" and "memory" is a fundamental problem addressed by a multitude of behavioural and neuroanatomy studies. With the relative neural complexity of popular model organisms (135 000 neurons in *Drosophila melanogaster*, 4 000 000 neurons in mice), it remains a challenge to understand learning on anything more than the level of neuronal wiring and regional firing, much less to isolate the biochemical representation of a memory.

The nematode *Caenorhabditis elegans*, despite having only 302 neurons, can undergo classical (Pavlovian) conditioning, and can therefore be tested in an associative learning paradigm. When worms are given an odourant (such as benzaldehyde, Bnz) during a period of starvation, their natural preference for this smell switches from attractive to aversive [1]. In this way, worms are able to integrate internal and external cues in order to behaviourally adapt to their environment. This presents a simple model organism with which to study the molecular mechanisms of learning, something that is neither well understood nor easily characterized in a more complex animal.

Classical conditioning is defined as the association of a conditioned stimulus (CS) and an unconditioned stimulus (US) to produce a conditioned response. In the Bnz-starvation paradigm, the US is the innately aversive physiological state of starvation. The CS is Bnz, which untrained worms find naively attractive. Worms can be trained by exposure to the CS in the presence of the US, represented in the lab by the presence of Bnz in absence of food. Learning occurs when an association between Bnz and starvation is formed such that worms actively avoid a point source of the Bnz CS. Since this learning paradigm involves starvation, it is clear that the presence or absence of food represents an important external signal to worms. This regulation of "satiety-state" in worms has been shown to be mediated by serotonergic signaling [2,3].

Serotonin is a biogenic amine neurotransmitter that mediates multiple food-related processes in worms. The effects of applying exogenous serotonin to worms were first characterized in Horvitz et al. (1982), which described three main behavioural responses: depressed locomotion, stimulated pharyngeal pumping, and increased rate of egg laying [4]. These phenomena correlated with those observed in earlier studies describing food-mediated

behavioural responses in worms [5]. Furthermore, later studies clarified the causal role of endogenous serotonin signaling in these phenomena. The modulation of the satiety state was linked to serotonin by showing that exogenous serotonin reversed starvation-responsive behaviour [6]. Likewise, the "depressed locomotion" was a result of the role of endogenous serotonin signaling in mediating an "enhanced slowing" response of fasting worms [2]. Increased pharyngeal pumping was found to also result from the presence of food in a worm's environment, and multiple serotonin-binding receptors were implicated as necessary for modulating this response [7]. In addition, increased rate of egg-laying was also found to be mediated by serotonin-binding to different serotonin receptors [7].

These data implicate serotonergic signaling as a key regulator of satiety status in worms. Serotonin signaling has also been implicated in the aforementioned starvation-odourant associative learning paradigm. This was accomplished by demonstrating that the presence of food during the training period could block the Bnz-starvation association, and that this phenomenon could be recapitulated with the application of exogenous serotonin. These two training paradigms described a "food block" and a "serotonin block", respectively [3]. This research also showed that worms deficient in serotonin signaling (mutant for the genes tph-1 or cat-4, involved in serotonin synthesis) were not able to be food blocked, and learned to associate Bnz with starvation in the presence of food. This raises the question of whether serotonin signaling is not just important as a satiety signal, but whether the lack of serotonin signaling is the nature of the starvation US. Therefore, previous research indicates that the presence or absence of serotonin signaling appears to mediate the US arm of the associative learning paradigm by regulating a worm's satiety state.

C. elegans has five canonical serotonin receptors, and four receptors with strong serotonin receptor homology. Of the canonical receptors, four (ser-1, ser-4, ser-5, and ser-7) are G-protein coupled receptors and one (mod-1) is a ligand-gated ion channel [7]. Different receptors are involved in mediating different aspects of the food response, including pharyngeal pumping, locomotion, and increased egg-laying [8-12].

This study shows that worms mutant for the five canonical serotonin receptors ("quintuple mutants") are defective in their ability to be serotonin blocked. The receptors necessary for this phenomenon were investigated, and it was found that the loss of ser-4 alone replicated the minimal serotonin blocking seen in quintuple mutants, implicating the SER-4 receptor in mediating the US arm of the Bnz-starvation classical conditioning paradigm.

The goal of this study is to explore the mechanisms of Bnz-starvation associative learning in *C. elegans* in order to better understand the nature of memory formation and storage on a molecular level. Worms are a good model organism with which to investigate these pathways because of their structural simplicity and facile genetics. In addition, many serotonin receptors in worms are homologues of human serotonin receptors [7]. The mechanisms involved in their associative learning may comprise a simplification of the mechanisms involved in mammalian associative learning. Based on the prior research, this study hypothesizes that serotonin signaling is involved in mediating the starvation/food signal in *C. elegans*, which, upon integration with Bnz, results in associative learning of the Bnz-starvation association.

Materials and methods

Strains: N2 Bristol (wild-type), GR1321 tph-1(mg280) II, UT1310 ser-1(ok345) ser-7(tm1325) X ser-3(ad1774) I, ser-1(ok345) ser-7(tm1325) X ser-4(ok512) III ser-5(tm2654)I mod-1(ok103) V, MT9668 mod-1(ok103) V, RB745 ser-4(ok512) III.

In the standard classical conditioning paradigm, N2 (wild type) worms were trained for one hour by suspension in 1 mL M9 buffer + 0.005% Triton X, with or without 0.006% benzaldehyde [3]. Worms were then transferred to the center of an NGM agar plate, where 1uL of 1% Bnz (diluted in 100% ethanol, EtOH) was spotted on the testing side, and 1uL of 100% EtOH was spotted on the control side. NaN3 (sodium azide) was spotted at either end to preserve the worms' first chemotactic choice. Worms were allowed to move freely for one hour, the duration of the testing period. The chemotactic index (CI) was calculated as number of worms on the test spot minus number of worms on the control spot, divided by the total number of worms on the plate. A negative chemotaxis value indicated movement away from Bnz, or learning, and a positive value indicated chemotaxis towards Bnz. Food blocking was achieved by either training worms on NGM agar plates seeded with OP50 *Escherichia coli*, or on a plate with 100uL of culture diluted to an OD600 of 0.5 [3]. Serotonin blocking was achieved by the addition of 10 or 40 mM serotonin to the training tube, as per Nuttley et al. (2002) [3].

Results

Tph-1 can be food blocked at high concentrations of food.

This study sought to replicate the results of the food blocking experiment as described by Nuttley et al. (2002) [3]. The gene tph-1 encodes tryptophan hydroxylase, an enzyme responsible for the last step of the serotonin synthesis pathway. Therefore, tph-1-null mutants have no endogenous serotonin signaling. This study was unable to food block tph-1(mg280) mutants at a 6X concentration of *E. coli* OP50 (Figure 1A).

However, when the food block condition was performed on *E. coli* OP50 seeded plates (containing a much higher concentration of food), tph-1 mutants were able to be food blocked (Figure 1B). This may indicate the presence of a serotonin-independent mechanism of satiety signaling at high versus low concentrations of food in the environment. The overall lower chemotaxis observed in tph-1 may be attributed to its known developmental defects hindering the assay, such as slowed development, and small brood size [13].

Learning in quintuple mutants can be blocked at a 40mM, but not a 10mM, concentration of serotonin.

This experiment involved a serotonin blocking experiment as per Nuttley et al. (2002) [3]. As seen before, N2 (wild type) worms

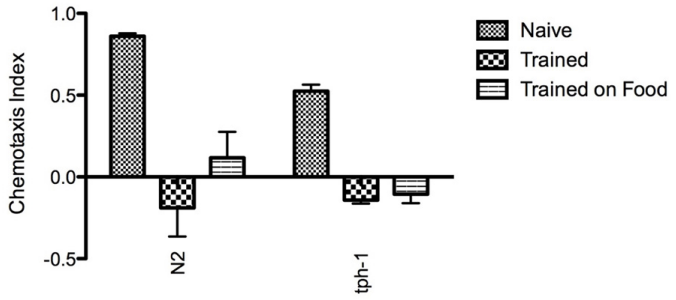

Figure 1A: Food blocking paradigm performed on the tph-1 mutant: Bnz given during training on a 6X concentration of OP50. A partial food block was observed in N2, but no food block was observed in tph-1.

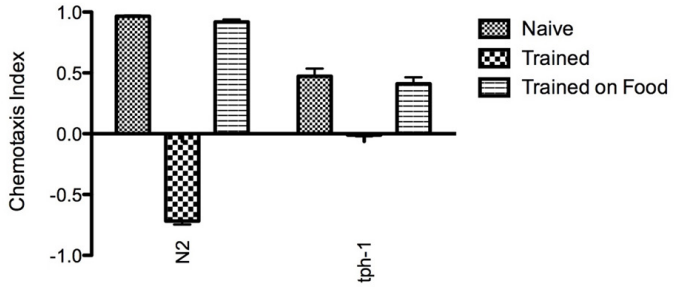

Figure 1B: Food blocking paradigm performed on the tph-1 mutant: Bnz given during training on seeded OP50 plates. A total food block was observed in both N2 and tph-1.

showed a strong serotonin blocking phenotype when trained to Bnz in a 40mM serotonin solution (Figure 2A). A quintuple serotonin receptor mutant, missing the five canonical serotonin receptors (ser-1, ser-4, ser-5, ser-7, and mod-1), was also able to be serotonin blocked at a 40mM concentration of serotonin.

It was then tested whether 40mM was the minimum concentration required to achieve a block of learning. A serotonin blocking dose response curve with N2 and quintuple mutant worms was constructed, testing separately at a 5mM, 10mM, and 40mM concentration of serotonin (Figure 2A, 2B, 2C). N2 worms did not show serotonin blocking at the 5mM concentration (Figure 2C), but were equally unable to form a Bnz-starvation memory in both the 10 and 40mM concentrations. However, relative to N2, quintuple mutant worms showed a greatly attenuated serotonin block at the 10mM concentration (Figure 2B). Therefore, at 40mM, there may be non-specific, low-affinity serotonin binding occurring to

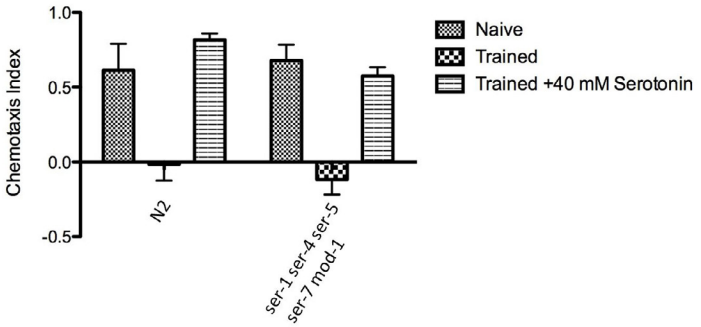

Figure 2A: Serotonin blocking paradigm performed on N2 and the quintuple serotonin receptor mutant (ser-1ser-4ser-5ser-7mod-1), with a 40mM concentration of exogenous serotonin administered during training. Both the N2 and the quintuple mutant exhibited a total serotonin block at 40mM.

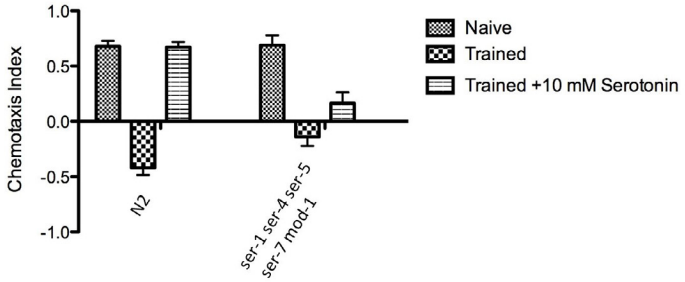

Figure 2B: Serotonin blocking paradigm performed on the quintuple serotonin receptor mutant, with a 10mM concentration of exogenous serotonin administered during training. The quintuple mutant exhibited a greatly attenuated serotonin block, as well as a diminished trained response, in comparison to N2.

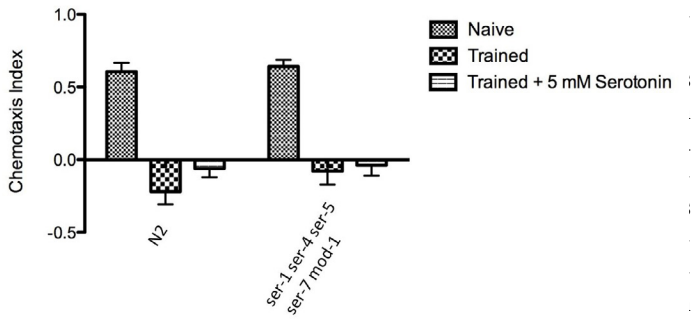

Figure 2C: Serotonin blocking paradigm performed on the quintuple serotonin receptor mutant, with a 5mM concentration of exogenous serotonin administered during training. N2 is unable to be serotonin blocked at a 5mM concentration.

associative memory, too, implicating serotonin signaling in a dual role of satiety state regulation.

Ser-4 recapitulates the attenuated serotonin blocking of the quintuple mutant at a 10 mM concentration of serotonin.

In order to determine which of the five genes in question (ser-1, ser-4, ser-5, ser-7, and mod-1) are necessary for the serotonin block, single or multi-receptor mutants in the serotonin blocking paradigm at a 10mM concentration of serotonin were tested. UT1310, a ser-1ser-3ser-7 triple mutant, was able to be serotonin blocked, indicating that the combination of SER-1 and SER-7 receptors was not the exclusive set necessary for propagating the food- serotonin signal (Figure 3). A mod-1(ok103) single receptor mutant was also serotonin blocked, indicating that MOD-1, too, was not necessary for this satiety stimulus (Figure 4).

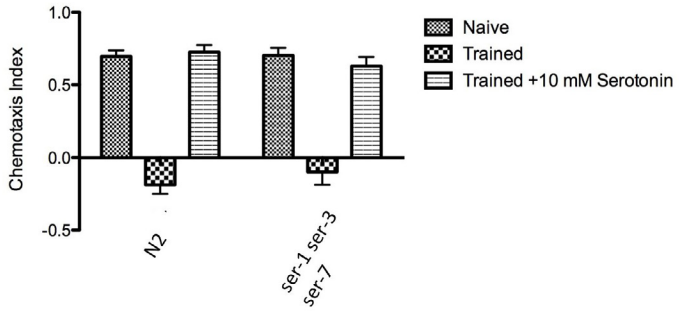

Figure 3: Serotonin blocking paradigm performed on the UT1310 (ser-1ser-3ser-7) triple serotonin receptor mutant, with a 10mM concentration of exogenous serotonin administered during training. Both N2 and UT1310 exhibited a full block of learning.

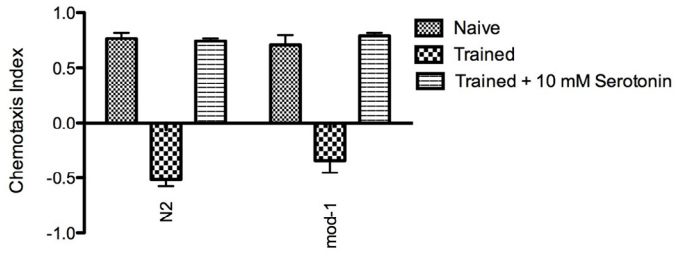

Figure 4: Serotonin blocking paradigm performed on the mod-1 mutant, with a 10mM concentration of exogenous serotonin administered during training. Both N2 and mod-1 exhibited a full block of learning.

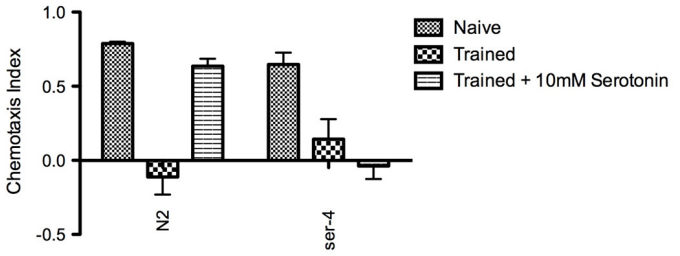

Figure 5: Serotonin blocking paradigm performed on the ser-4 mutant, with a 10mM concentration of exogenous serotonin administered during training. Ser-4 exhibited a greatly attenuated food block, as well as a diminished trained response, in comparison to N2.

However, a ser-4(ok512) single receptor mutant was unable to be serotonin blocked at a 10mM concentration (Figure 5). This data indicates that SER-4 is a necessary receptor for propagating the serotonin-satiety signal representing the absence of the starvation US, as without SER-4 worms are able to form an associative memory between Bnz and starvation in the presence of serotonin. Ser-4 mutant worms also recapitulated the weak Bnz-starvation learning phenotype, first observed in quintuple mutants. Therefore, SER-4 also appears to regulate the association of Bnz with starvation in the absence of serotonin, demonstrating the dual role of serotonin in regulating the starvation signal and mediating associative learning.

Discussion

The full molecular mechanisms underlying the formation and storage of a memory are unknown in even the simplest model organisms. The ability of *C. elegans* to be classically conditioned not only raises questions about the evolution of learning and memory, but also presents a solution to the difficulty of studying the molecular components of learning in more complex animals. With only 302 neurons in the adult hermaphrodite, worms are ideal model organisms in which to study the properties of associative learning, and to understand the nature of memory formation. This paper presented two key findings. First, the serotonin-dependent satiety signal only regulates learning at low concentrations of food; at high concentrations of food, worms have a serotonin-independent satiety signal. Second, the SER-4 receptor is responsible for propagating this serotonin-satiety signal, which negatively regulates the Bnz-starvation associative memory by blocking the formation of the starvation US.

Evidence that serotonin is the primary endogenous food signal in worms came from the observation that the serotonin-synthesis deficient mutant tph-1 is able to associate Bnz and starvation even in the presence of food [3]. However, this study's finding that tph-1 can be food blocked at higher concentrations of food demonstrates the presence of a serotonin-independent pathway mediating satiety state. In fact, while tph-1 mutants require exogenous serotonin to initiate pharyngeal pumping (a serotonin-dependent response to food), a general bacterial extract is sufficient to induce mouth-opening in tph-1, suggesting an unknown serotonin-independent mechanism for feeding stimulation [14].

A dose-response food block with tph-1 would provide more insight into the relative concentrations of food that stimulate each pathway. Due to the metabolic defects of tph-1 affecting its growth and reproductive rates [13] and, subsequently, its performance in the Bnz-starvation assay, it would be interesting to perform the same experiments on cat-4 mutants, which are also serotonin-synthesis deficient but perform better in our associative learning paradigm [3].

This study found that the loss of ser-4 alone recapitulated the attenuated serotonin block and the weak learning phenotype of the quintuple mutant. This is indicative of a dual role of SER-4, in both propagating the serotonin-based satiety signal and in forming the Bnz-starvation associative memory. Exogenous serotonin, or food that stimulates a certain neuron to release endogenous serotonin, signals though SER-4 to inhibit the starvation signal, which subsequently inhibits the association of Bnz with starvation. Therefore, the loss of SER-4 represents the loss of this inhibitory satiety signal, resulting in ser-4 (and quintuple mutant) worms that can associate Bnz with starvation even in the presence of exogenous serotonin.

The loss of SER-4 also results in the formation of a weaker Bnz-starvation association in the absence of serotonin, indicating SER-4 may play a role in memory formation. However, since SER-4 also appears necessary for a satiety signal, it is possible that ser-4 mutants exist in a semi-starved state, resulting in worms exhibiting a weaker aversion to Bnz when paired with starvation. In addition, ser-4 shows a more attenuated serotonin block than the quintuple mutant. This study speculates that the loss of additional serotonin receptors in the quintuple mutant that might inhibit the satiety signal may be responsible for this observation.

Finally, the sole receptor of the quintuple knockout that has not yet been tested is a ser-5 single mutant. Therefore, it is necessary to also test ser-5 in the serotonin blocking paradigm to understand if SER-5 performs alongside SER-4 in regulating the starvation signal.

As of yet, the full molecular mechanisms for the stimulation, secretion, and propagation of the serotonin-satiety signal are unknown. Several neurons are known to secrete serotonin, most of which have been implicated in the regulation of satiety status, including the ADFs, NSMs, and HSNs. While the enhanced slowing response appears to be mediated by mechanosensory stimulation of NSM, in general the upstream mechanisms of food-induced serotonin synthesis are still unknown [2]. To understand which neurons secrete the serotonin involved in the associative learning pathway, the food-blocking paradigm will be used to test single-neuron knockouts of tph-1. Worms unable to synthesize serotonin from a neuron involved in mediating the US-food signal should be resistant to food blocking.

Bnz sensation in *C. elegans* occurs in the AWC neuron, and odourant-starvation integration must therefore occur in either AWC or a downstream neuron [15]. In order to understand where the starvation-US is integrated with the Bnz-CS, ser-4 will be rescued in individual neurons downstream of AWC in a ser-4 mutant background. A restoration of the serotonin blocking phenotype in any of these rescues would implicate the neuron in question as a downstream recipient of the serotonin-satiety signal, specifically though the SER-4 receptor. The consideration of how this neuron connects to AWC would provide insight into where the two stimuli are integrated, and where the associative memory is formed.

Conclusion

This study used the Bnz-starvation classical conditioning paradigm to explore the molecular components of this CS-US association. The data outlined a pathway in which the presence of food

activates a neuron to synthesize and release serotonin onto a SER-4 receptor, blocking the starvation signal, and thereby inhibiting the Bnz-starvation association. By delineating the role of serotonin and serotonin receptors in regulating the starvation signal, as well as understanding which neurons are necessary for this pathway, insight can be gained into the nature of stimuli integration and memory storage. This may be applicable to higher-level organisms on a broader scale to investigate a fundamental question: what is a memory?

Acknowledgements

Thank you to the members of the van der Kooy lab for their advice and helpful discussion. Thank you to Cornelia Bargmann and Richard Komuniecki, and to the CGC for the strains.

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