Aversive Conditioning through Optogenetic Activation in *C. elegans*

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Abstract

C. elegans are known to undergo associative learning, also termed *conditioning*: a process whereby two stimuli (conditioned stimulus + unconditioned stimulus) are integrated and used to inform future behavior (conditioned response). The neural correlates underlying this process have been subject to recent investigation, although the unconditioned stimuli in question are often systemic stressors, such as heat shock, starvation, pathogen infection, that have broad effects on the organism through multiple signaling pathways (serotonergic and stress response pathways, insulin signaling). We have developed an associative learning paradigm and automated apparatus that make use of optogenetic stimulation to administer an acute aversive stimulus paired with an odor stimulus. The major benefits of this approach are: (a) training can be done on food, (b) an aversive stimulus can be delivered without tissue damage or injury, and (c) animals do not exhibit systemic stress responses as they do with most other unconditioned stimuli administered during *C. elegans* conditioning. The apparatus used to deliver both optogenetic stimulation (unconditioned stimulus) and odorant vapors (conditioned stimulus) was termed the OptoBox: a 3D printed box-like device wherein agar plates containing *C. elegans* are treated for a given length of time prior to various forms of behavioral assessment (chemotaxis) or quantitative imaging. In this way, the increased specificity achieved by using targeted optogenetic activation rather than systemic stimuli (starvation, heat shock) allows for the investigation of associative learning using a microcircuit model involving only two sensory neurons and a small network of interneurons. Following OptoBox treatment, the behavioral testing and imaging (quantitative fluorescent imaging and single-neuron calcium imaging) are used to assess effects on sensory processing. In addition to monitoring the effect of OptoBox training on wildtype *C. elegans* populations, strains carrying mutations in genes known to play a role in associative learning were subjected to treatment and subsequent behavioral testing and neuroimaging.

Résumé

La capacité du nématode *Caenhorabditis elegans* à apprendre de façon associative et être conditionné fut démontré dans la littérature. Dans ces deux cas, afin d'induire un comportement spécifique aux nématodes deux stimuli sont utilisés : l'un permettant le conditionnement et l'autre étant non conditionné (contrôle). L'intégration de ces stimuli est permis par l'activation de circuits neuronaux spécifiques. De nos jours, la compréhension de leur implication dans la réponse au conditionnement est étudiée intensivement en utilisant des conditions (*e.g.* choc de température, le manque de nourriture, l'infection à des pathogènes) menant à un stress systémique ayant de vastes répercussions sur l'organisme. Ces circuits neuronaux utilisent une pléthore de voies de signalisation induite par le stress, la sérotonine ou encore l'insuline. Nous avons donc développé un paradigme d'apprentissage associatif ainsi qu'un dispositif automatisé intégrant la stimulation optogénétique pour associer un stimulus négatif sévère à une odeur. Les bénéfices de cette approche permettent principalement que : *(i)* l'apprentissage peut être mener sur des plaques ensemencées, *(ii)* le stimulus négatif peut être appliqué sans infliger de dommages aux tissus ou créer de blessures aux nématodes et *(iii)* les vers ne développent pas de réponse de stress systémique comme c'est le cas avec la plupart des approches présentement utilisées pour étudier le conditionnement chez le *C. elegans*. Le dispositif développé fut nommé OptoBox et permet donc d'associer la stimulation optogénétique (stimuli non-conditionné) et les vapeurs d'odeur (stimulus conditionné). L'OptoBox est une boîte conçue, modélisée et imprimée à l'aide d'une imprimante 3D permettant de placer des plaques d'agar à l'intérieur de celle-ci pour exposer les souches de *C. elegans* à l'odeur associée ou non au stimulus optogénétique. Les animaux sont alors soumis au traitement pour une durée spécifique déterminée. Leur apprentissage associé aux stimuli est ensuite testé en utilisant des tests comportementaux ou en les imageant. De cette façon, en ciblant l'activation par optogénétique, il fut possible d'avoir une plus grande spécificité que si un stimulus systémique aurait été utilisé. Il est, alors, possible de regarder les effets spécifiques au niveau d'un simple circuit d'intérêt comprenant deux neurones sensoriels et quelques interneurones. Après le traitement avec l'OptoBox, les tests comportementaux et l'imagerie (imagerie quantitative et imagerie

calcique à l'échelle du neurone individuel) furent utilisés pour étudier les effets au niveau de l'intégration sensorielle. Ce processus fut également utilisé dans ce projet sur des souches de nématodes ayant des mutations pour des gènes connus et impliqués dans l'apprentissage associatif. Grâce au dispositif de l'OptoBox développé pour ce projet, il sera donc possible d'étudier plus spécifiquement des circuits neuronaux et l'effet d'activer certains neurones en condition ciblées sans créer un stress systémique chez les vers.

Chapter 1: Introduction

Behavioral Conditioning and Learning: a historical perspective

Studies involving the investigation of learning have been ongoing throughout human history: from ancient Greek epistemology to mid 20th century pedagogical study, and even into contemporary biological research. Indeed, the post-industrial revolution era is widely considered the *age of information*, characterized by a dissemination of knowledge via print and more novel forms of media (radio, internet, etc). Despite this massive expansion in knowledge propagation, several branches of science have opted to focus inwards in order to study the most fundamental bases of information processing. The neurological basis of learning and memory involves an understanding of the physical means by which information from the external world is relayed to an organism, and ways by which the organism in question responds to this information and uses it to guide future decisions. The following section shall provide a brief overview of how learning and memory came to be studied through a biological perspective, and where this perspective is situated relative to similar disciplines.

While the 19th Century saw the emergence of psychology as a field of study, the focus of early psychological research was mainly qualitative, dominated by the use of introspection to gain insight into mental processes. However, at the same time as Freud heralded psychodynamic theory and the use of psychoanalytics, alternative paradigms were investigated in both Europe and America. Indeed, while psychoanalytic theory made use of techniques such as free association and dream analysis to understand what leads unconscious drives into conscious activity, behaviorists began making use of animal models to characterize psychological activity that could be generalized to human experience. Famously, the work of Ivan Pavlov in late 19th-early 20th Century Russia set in motion decades of behaviorist theory. Originally a physiologist concerned with the mechanics of animal digestion, Pavlov is now remembered for his work in reflexology and classical conditioning. Pavlov's most notorious achievement involved conditioning experimental subjects, dogs, to salivate in response to non-food stimuli following repetitive presentation of food and/or food odors in conjunction with the stimulus in

question. A salivatory response to a non-food stimulus was termed a "psychic secretion". Although Pavlov is perhaps best known for using a bell to induce such psychic secretions, his notes document that subjects were capable of discriminating between different metronome speeds, salivating only at specific beats per minute. Pavlov described this phenomenon as being the result of an association formed between the sub cortex, understood at the time as being responsible for drives and reflexes, and the outer cerebral hemispheres. Although not yet well characterized, the cerebral cortex was understood as being a hub of complex cognitive activity in both humans and higher-order mammals. In his 1927 lecture on conditioned reflexes, Pavlov described cerebral cortices as such: "Consider the dog, which has been for so many countless ages the servant of man. Think how he may be trained to perform various duties, watching, hunting, etc. We know that this complex behaviour of the animal, undoubtedly involving the highest nervous activity, is mainly associated with the cerebral hemispheres." (Pavlov, 1927) In this way, physiology provided a readout for the psychological activity of cerebral cortices.

Pavlov was not the only proponent of this perspective- in 1898 the American psychologist Edward Thorndike published his dissertation "*Animal Intelligence: An Experimental Study of the Associative Processes in Animals*". Much like Pavlov, Thorndike championed the use of animal subjects and questioned the divide between psychology and physiology, especially in relation to learning. Likewise, Thorndike also questioned the divide between human and animal learning: "The main purpose of the study of the animal mind is to learn the development of mental life down through the phylum, to trace in particular the origin of human faculty." (Thorndike, 1898) Indeed, Thorndike produced the earliest examples of *time-curves* (now commonly known as learning curves) by observing cats' ability to escape box-like contraptions (Thorndike, 1898).

The path bridging the divide between animal physiology and psychology was certainly paved by researchers such as Pavlov and Thorndike, though it was further solidified by the behaviorists and neurobiologists that followed. Pavlov's work on conditioning was continued in America by John Watson in the early 20th Century through his research concerning the development of phobias and, later, child-rearing (Watson & Rayner, 1920). Thorndike's approaches towards animal learning were influential to B.F. Skinner and his use of complex contingency devices in experiments demonstrating

operant conditioning. Both Watson and Skinner propagated the use of terminology which, originally stemming from Pavlov's work, are still used to this day: animal learning requires both an instinctive, naïve behavior termed an **unconditioned response** (ex. pain, hunger, fear) and an extrinsic factor (ex. an auditory tone, a certain object). If this extrinsic factor is presented along with the unconditioned response with enough frequency, the two become linked resulting in what is known as a **conditioned response**. In this context, the extrinsic factor in question is termed the **conditioned stimulus**. Applied to Pavlov's experiments on dog salivation, the auditory tone (bell, metronome) is the conditioned stimulus, the salivation elicited by meat powder during training is the instinctive unconditioned response, and the "psychic secretions" resulting from repetitive pairing of meat powder with the auditory tone constitute the conditioned response. In this way, the application of quantitative, physiological methods onto the study of psychology was popularized in America and continued as behaviorism gained a foothold in contemporary psychology.

By the mid 20th Century, rapid advances made in the field of molecular biology informed new directions of psychological research. An unprecedented understanding of DNA and biological processes allowed for the possibility of studying psychological phenomena at a cellular resolution. Indeed, the blending of psychological and anatomical theory was exemplified by the work of Eric Kandel, a Viennese expatriate who, after completing a medical degree, opted to intern at a neurophysiology lab. During his medical studies, Kandel developed a keen interest in psychoanalysis and was eager to study and characterize physiological substrates for Freud's structural theory (id, ego, superego). As such, Kandel spent much of his postdoctoral career searching for biological counterparts of psychological phenomena such as psychopathology, learning, and memory. With Brenda Milner's discovery of implicit versus explicit memory, Kandel saw an intriguing opportunity. In his biography, "In Search of memory", Kandel explains: "Implicit memory often has an automatic quality. It is recalled directly through performance, without any conscious effort or even awareness that we are drawing on memory (…) This is that type of reflexive learning studied by behaviorists, notably Pavlol, Thorndike, and Skinner." Kandel went on to design a series of studies investigating the cellular properties of reflexive learning. When deciding which organism to make use of for these studies, Kandel posited: "To make any reasonable progress

toward understanding how memory storage occurs, it would be desirable, at least initially, to study the simplest instance of memory storage and to study it in an animal with the simplest possible nervous system, so I could trace the flow of information from sensory input to motor output." In keeping with his focus on reductionism, Kandel chose to study reflex behavior in the giant marine snail *Aplysia*: an organism with a relatively simple nervous system comprised of 20, 000 neurons, sufficiently large for microelectrode insertion, organized into nine clusters.

Using *Aplysia*, Kandel applied tenants of behaviorist theory while making use of neuroanatomical techniques such as electrophysiology. By applying electrical pulses to one ganglia while recording postsynaptic responses in another ganglia, Kandel studied both nonassociative and associative forms of synaptic plasticity Nonassociative synaptic plasticity includes **habituation**, which is a decreased response to an electrical pulse due to application of repetitive, weak pulses, as well as **sensitization**, which is an increased response elicited by fewer, stronger electrical pulses. Associative synaptic plasticity (**classical conditioning**) was observed by pairing electrical pulses to certain ganglia in a constant temporal sequence. Importantly, each of these forms of synaptic plasticity had been described in terms of salivatory responses by Ivan Pavlov over half a century prior. Kandel explained, "After all, habituation, sensitization and classical conditioning- the three learning protocols described by Pavlov- are essentially a series of instructions on how a sensory stimulus should be presented, alone or in combination with another sensory stimulus, to produce learning." Kandel further studied habituation, sensitization and classical conditioning using a behavioral circuit: the gill withdrawal reflex, which is controlled by just one of the snail's ganglia. Interestingly, the strengthening and weakening of this reflex, which generally lasts up to a day, could be extended to weeks if stimulation of the ganglia was spaced and repetitive. This observation appeared to demonstrate a rudimentary form of long-term synaptic changes that one could argue constitutes memory.

Molecular biology of Conditioning

Less than a century after Pavlov published his work detailing associative learning in canines, studies were undertaken to demonstrate analogous forms of learning, only using

an invertebrate model organism and making use of only a handful of neurons. Indeed, the transition from assaying an entire organism to observing electrophysiological responses in a small network of neurons allowed a higher resolution of investigation. Indeed, several lines of investigation pursued in the latter half of the 20th Century sought to elucidate molecular mechanisms underlying learning and memory, many of which operate on the level of a single synapse. By the 1930s, it was widely accepted that neurons, both within the central and peripheral nervous system, communicate via the secretion of molecules termed neurotransmitters. However, until recently it remained unclear which changes occurred to neuronal communication during learning and memory acquisition/storage.

While pursuing his electrophysiological research in *Aplysia*, Kandel made the observation that the same identifiable neurons in one individual snail were in the same position as in others, and even more intriguingly, stimulation of the same neurons led to the same behavior across all individuals. This phenomenon, **neuronal invariance**, was similarly observed in other invertebrate model organisms, for instance, the soil-dwelling nematode *C. elegans* which boasted even fewer cells than *Aplysia*. Indeed, the 302 neurons of a hermaphroditic *C. elegans* nematode were found to demonstrate neuronal invariance (Kimble and Hirsh 1979). What these discoveries signified, in relation to learning, was a reliance on changes to an already hard-wired system- that is, there is plasticity even between two hard-wired synaptic partners. Synaptic partners are thus susceptible to modulation that can alter the output of a synaptic circuit. An illustration of this concept was provided by the modulation of central pattern generator (CPG) neuronal circuits. CPG circuits consist of a sequence of neurons whose activation results in a stereotyped behavioral output, such as rhythmic contractions and locomotion. Stomach contractions of decapod crustaceans, the result of CPGs within the stomatogastric ganglion, were observed to change in frequency depending on which neurotransmitters and neuropeptides were present in the vicinity of the CPG input fibers (Hooper and Marder, 1984).

With the consensus that invariant neuronal sequences are susceptible to modulation, lines of inquiry sought to identify modulatory factors capable of inducing synaptic plasticity within the context of learning and memory. Using the gill-withdrawal reflex in *Aplysia*, Kandel identified that although the motor neurons mediating this reflex

were glutamatergic, the release of serotonin from a nearby layer of interneurons was sufficient to modulate the strength of gill withdrawal (Frost and Kandel, 1995). Indeed, the application of serotonin induced synaptic facilitation, a molecular process whereby a neuron becomes more responsive to excitatory input (Brunelli, Catellucci and Kandel, 1976). This observation offered a biological substrate for the process of sensitization- a behavioral phenomenon documented close to a century earlier by Ivan Pavlov. In a 1976 paper, Kandel Brunelli and Catellucci posit, "we propose a specific and, it is to be hoped, testable model of the mechanisms underlying habituation and sensitization". Moreover, it appeared quite plausible that any neuron, not necessarily a specialize hippocampal or cortical neuron, contained the potential for memory.

Indeed, further study elucidated several other molecules involved in the underlying mechanisms of both habituation and sensitization. The involvement of Cyclic Adenosine Monophosphate (cAMP) as a second messenger was posited by several researchers within the field of invertebrate learning (Davis, Cherry, Dauwalder, Han and Skoulakis, 1995). cAMP was a well-characterized molecule already by this time, having been discovered in the late 1950s- it was well understood that intracellular cAMP levels were increased in response to hormones such as epinephrine, and this transcient cAMP increase led to the activation of protein kinase A (PKA) (Corbin and Krebs, 1969; Brunelli, Catellucci and Kandel). Accordingly, the direct intracellular injection of both cAMP and/or PKA into a sensory neuron was sufficient to induce increased neurotransmitter release from the neuron in question- this effect appeared to be mediated by increased calcium permeability into the sensory neuron, confering a "potentiated" state of increased excitability. Further elucidation of this cAMP-dependent pathway, termed "**activity-dependent facilitation**", revealed the necessity for both increased calcium influx as well as the binding of serotonin, with sufficient temporal overlap (Abrams, Karl and Kandel, 1991). In this sense, cAMP production and subsequent PKA activation serves as a sort of coincidence detector- aptly described as a "*molecular site of stimulus convergence*" (Abrams, Karl and Kandel, 1991). In response to synaptic input, such as serotonin binding or any stimulus resulting in an increase of intracellular calcium, cAMP is produced by the enzyme adenylate cyclase. Indeed, cyclase stimulation % was found to be highest within *Aplysia* ganglia in which the administration of exogenous calcium and serotonin was temporally paired. This dual regulation provided a potential

substrate underlying associative learning: “Because the *Aplysia* enzyme is dually regulated by calmodulin and by the stimulatory G-protein, it provides a potential site of convergence between the conditioned stimulus, which causes activity and Ca2+ influx in the sensory neurons, and the unconditioned stimulus, which causes release of facilitatory transmitter.” (Abrams, Karl and Kandel, 1991) The facilitatory role of adenylate cyclase and cAMP in associative learning was observed in other invertebrate models, such as drosophila (Dudai, Zvi and Segel, 1984) and honeybees (Hellstern, Malaka and Hammer, 1998). The confirmation that the same molecules and enzymes appear involved in rudimentary learning across different species hinted at a shared, evolutionarily conserved mechanism underlying associative learning.

The aforementioned molecular changes (transient calcium increases, cAMP production by increased enzymatic activity of adenylate cyclase) were eventually observed to accompany certain structural changes. Indeed, while one single application of serotonin to an *Aplysia* sensory neuron was sufficient to induce synaptic facilitation, the application of several serotonin pulses to the same neuron could induce long-lasting anatomical changes to the synapse (Mayford *et al*, 1992). This was not completely unexpected, seeing as it had been documented for some time that, even in mammalian neurons, the efficiency of synaptic transmission increased in an activity-dependent manner (Andersen & Lomo, 1973). Indeed, by the mid-1970s it was well established that the application of high-frequency electrical pulses (tetanus stimulation) to hippocampal neurons increased the strength and responsiveness of the synapse in question: this is the notion of **long-term potentiation (LTP)**. As such, activity-dependent molecular changes (increased cAMP) were found to initiate changes in gene expression, which in turn could instigate anatomical changes within the synapse (Dash, Hochner and Kandel, 1990). Genes that were found to be regulated by increased cAMP levels were thus termed “cre-responsive elements”, under the control of the **cre-responsive element-binding (CREB) protein**. In both vertebrate and invertebrate species, there are several CREB isoforms, with different isoforms involved in short-term, long-term, or early phase/late phase long term memory. Unexpectedly, there does appear to be a general correlation between activated CREB and various forms of long-term memory and LTP, in both vertebrate and invertebrate models (Barco, Alarcon & Kandel, 2002; Yin & Tully, 1996).

In short, invertebrate models of learning have been instrumental in identifying both the molecular and structural changes underlying several types of learning. Indeed, the gill withdrawal circuit studied in *Aplysia* provided one of the earliest models for demonstrating the physiological changes underlying rudimentary Pavlovian learning (sensitization, habituation, conditioning). Both *Aplysia* and other invertebrate models were later used further study slightly more complex forms of associative learning, for instance olfactory conditioning is extensively studied using *Drosophila* (Livingstone, Sziber and Quinn, 1984). The same molecules identified in relatively simple invertebrate neural pathways appeared evolutionarily conserved; for instance, 5-HT, cAMP and calcium appear to play a role in the development and maintenance of LTP in the neurons of higher mammals (Barco, Alarcon and Kandel, 2002; Teixera *et al*, 2018). The following sections seek to demonstrate the further use of invertebrate systems, notably the nematode *Caenorhabditis elegans*, in the characterization of molecular substrates for learning and memory.

Conditioning in *C. elegans*

At the same time as Eric Kandel went about the early studies of learning and memory using the marine snail *Aplysia*, a researcher named Sydney Brenner began using another invertebrate model, the roundworm *C. elegans,* to study molecular genetics. A contemporary of Crick and Watson, Brenner was involved with the early characterization of molecules underlying heredity: RNA, codons and eventually stop codons (Brenner *et al*, 1961; Crick *et al 1961*; Brenner *et al*, 1965). However, Brenner soon came to the realization that the extensive study of both heredity and cellular biology could be studied in tandem using the same organism should this organism be sufficiently simple and genetically tractable. In Berkeley, researcher Ellsworth Dougherty had begun publishing accounts of nematode genetics as early as 1948, and it was through these publications that Brenner realized the potential of nematodes in the study of advanced genetics (Dougherty and Calhoun, 1948). By 1967, Brenner began using nematodes to characterize ethyl methanesulfonate (EMS)- induced mutants and the heredity of these mutations, “Getting a a mutant of a complex organism and confirming Gregor Mendel in only 2 weeks was most satisfying” (Brenner, 2009). By 1974, Brenner published *The Genetics of*

Caenorhabditis elegans, an article detailing 300 mutations, and positing the many benefits of using *C. elegans* as a model organism: a transparent body, rapid generation time, its hermaphroditic self-reproduction coupled with the use of males for genetic transfer, as well as the small number of cells comprising the whole organism and nervous system. In the decades to follow, researchers would go on to elucidate a complete *C. elegans* cell lineage (Sulston and Horvitz, 1977), as well a comprehensive wiring diagram for each of the 302 neurons of adult hermaphrodite nematodes (White *et al*, 1986). These 302 neurons, much like those studied by Eric Kandel in *Aplysia,* were found to demonstrate a large degree of invariance in the 7 000 synapses they formed- a major benefit of using invertebrate model organisms.

While early *C. elegans* research focused on forward genetics, that is, understanding the genetic bases and heredity of observed mutant phenotypes, the relative simplicity of *C. elegans* made it an ideal candidate for studying the nervous system. Despite its small nervous system, *C. elegans* is nonetheless capable of several behaviors: for example, the nematode can navigate towards appetitive odors and away from repulsive ones (**chemotaxis**), and it can navigate radial temperature gradients in order to remain at its preferred cultivation temperature (**thermotaxis**) (Hedgecock & Russel 1975; Ward, 1973). It was soon observed that, much like the gill withdrawal reflex of *Aplysia*, there were several stereotyped actions performed by *C. elegans* that required only a small circuit of neurons. By selectively disrupting the function of individual neurons within these characterized circuits, one could infer neuronal functionality by observing the resulting behavior. A common method used to critically disrupt the function of individual neurons is **laser ablation**: a technique developed in 1980 to study *C. elegans'* developmental cell lineages (Sulston & White, 1980). As such, the ablation of certain amphid neurons led to a characterization of the chemosensory circuit and the process of chemotaxis (Bargmann & Horvitz, 1991). Chemotaxis, and an understanding of its underlying neuronal circuits, provided a readout for the interaction between *C. elegans* and its environment. As stated in their 1991 *Cell* paper, Bargmann & Horvitz described, "Chemotaxis represents a complex response of the animal to its environment: the animal must sense continuously changing external conditions and convert sensory information into movement in a particular direction." (Bargmann & Horvitz). As such, chemotaxis became a viable behavioral assay for quantifying both non-associative and associative

learning in *C. elegans*. For instance, the cellular basis of olfactory adaptation was one of the first forms of non-associative learning studied in *C. elegans*: that is, a decreased sensitivity to a given odorant following sustained presentation. It was observed that odorant-specific neurons (AWC and AWA) underwent habituation in a calcium-dependent manner, seeing as treatment with EGTA, a calcium chelator, abolished olfactory habituation (Colbert & Bargmann, 1995).

Forms of non-associative learning in *C. elegans* were also studied in the context of nematodes' responses to mechanosensory stimulation (Rankin, Beck and Chiba, 1990). *C. elegans*' locomotion on agar plates consists of sinusoidal body bends along the dorsal-ventral plane. The application of a sudden mechanosensory stimulus (ex. a tap to the nose using an eyelash pick, or the application of a tap to the petri dish containing the animal) reliably induces a **reversal**, that is, a series of backwards undulations. Using different schedules of taps applied to petri dishes, it was observed that *C. elegans* could undergo either short-term or long-term habituation or sensitization- quantified by either an increased or decreased number of reversals (Rankin, Beck and Chiba, 1990). This finding is in line with previous publications using vertebrate models. For instance, the pattern of stressor presentation (frequency, duration) was found to influence the levels of corticosterone, a proxy for stress response, quantified from rats (Pittman, Ottenweller & Natelson, 1990).

Seeing as forms of non-associative learning (habituation and sensitization) have been described in *C. elegans* chemosensory and mechanosensory pathways, researchers began to question whether *C. elegans*, with its simple nervous circuitry, was capable of undergoing associative learning. Indeed, in a 1997 paper, it was demonstrated that presenting a population of *C. elegans* with an ion in the absence of food modulated that populations' response (quantified by chemotaxis) upon subsequent presentation of the same ion (Wen *et al*, 1997). In this way, food deprivation (**unconditioned stimulus: US**) becomes associated with the ion (**conditioned stimulus: CS**), and the paired presentation led to decreased chemotaxis towards this ion (**conditioned response: CR**). Simply put, *C. elegans* are indeed capable of associative learning, as demonstrated by their ability to learn an aversion to a stimulus encountered during food deprivation. The impact of this learning paradigm, which constitutes an example of Pavlovian classical conditioning, was quantified using the chemotaxis- a popular choice of invertebrate behavioral assessment

Chemotaxis has been proven to be a reliable assay that can be used to quantify a complex behavior, and therefore posits many possible uses: from traditional forward genetics (ex. mutational screens) to assessing the effects of laser ablation. Seeing as chemotaxis is the result of activity in sensory neurons, interneurons and motor neurons, assessing this behavior provides an excellent opportunity to study sensory integration. In food-status dependent conditioning, trained *C. elegans* integrate the past experience of starvation/food deprivation along with the ion or odorant used during such training (Wen *et al*, 1997). Accordingly, further study found interneurons to be required for integrative behavior (Hiroyuki & Mori, 2013), with the requirement for specific interneurons varying by the sensory input involved in the associative learning (Ardiel & Rankin, 2010). There were, however, certain genes that appear to be required for learning across sensory modalities. For instance, the gene encoding the enzyme tryptophane hydroxylase (TPH-1) was found to be required for the integration of feeding status with thermosensory information, as well as olfactory information (ex. response to volatile compounds/odorants), resulting in thermotaxis and chemotaxis, respectively. Similarly, serotonin was found to be necessary for a process known as **pathogen learning**: that is the formation of a long-lived association between a pathogenic bacterial food source (*Pseudomonas aeruginosa*: PA14) and the resulting gut infection (Zhang, Lu & Bargmann, 2005). Standard *C. elegans* maintenance generally involves raising animals on agar-filled petri dishes where they feed from a thin lawn of nonpathogenic *E. coli* bacteria (OP50 or HB101). Naïve animals, that is, animals that have not previously fed on pathogenic PA14 bacteria, show no chemotaxis preference between OP50/HB101 and PA14. However, if *C. elegans* are fed PA14 for long enough to develop a gut infection, they show a long-lived aversion to PA14 when given the choice between this pathogenic strain and a non-pathogenic strain (assayed by chemotaxis or similar olfactory mazes) (Zhang, Lu & Bargmann, 2005). Similar to thermotaxis and other feeding-status dependent forms of associative learning, pathogenic learning was found to require the expression of TPH-1. Moreover, the expression of one specific serotonin receptor, a serotonin-gated chloride channel: MOD-1, is required in certain interneurons (AIY, AIZ) (Hobert, 2003). Much like early studies of synaptic facilitation undergone through the gill withdrawal reflex in *Aplysia*, these forms of associative learning (pathogen learning, chemotaxis) appear to involve modulation by serotonin at the interneuronal level.

In short, the past several decades have seen the emergence of invertebrate models to study forms of both non-associative (habituation, sensitization) and associative (classical conditioning) forms of learning. The benefits of these models involve invariant neuronal architecture, genetic tractability, and relatively simple learning assays (ex. the observation of reflexes in *Aplysia*, chemotaxis in *C. elegans*). Invertebrate learning assays have been instrumental in providing a basis of the molecular underpinnings of learning, for instance the discovery of cAMP/PKA/CREB pathway in synaptic plasticity, and the necessity for adequate serotonergic signaling in associative learning.

Invertebrate learning assays generally fall into two categories: the first involves the study of dissected circuits at the single-cell level, for instance electrophysiological studies done using small *Aplysia* circuits (ex. examining the process of synaptic facilitation between a sensory neuron and downstream interneurons). The second category involves slightly more ecologically relevant study designs that measure the behavior of freely-moving animals, this includes chemotaxis, thermotaxis and olfactory maze tasks undertaken by *C. elegans*. Recent advances in molecular techniques offer the possibility of merging these learning assay types in order to obtain a method that allows for the study of a neuronal circuit at the single-cell level while also obtaining behavioral insight. This type of behavioral assay has been implemented in vertebrate models: for instance researchers have used hippocampal optogenetic stimulation to activate an artificial aversive (fear) memory in mice (Ramirez, Tonegawa & Liu, 2014). The light-sensitive Channelrhodopsin cation channel (ChR2) was selectively expressed in hippocampal neurons mediating the animals' fear response using activity-dependent expression. The subsequent optogenetic stimulation of these neurons results in engram activation, which can then be used as an artificial unconditioned stimulus (US) during learning paradigms. This type of experiment permits more direct study of the link between a specific neuronal circuit and a given behavior- in this case the link between hippocampal neurons active during fear conditioning, and the resulting aversive behavior.

Invertebrate models, especially transparent-bodied organisms such as *C. elegans*, offer considerable potential for this type of experiment. Selective expression of optogenetic constructs allows cell-specific activation, which can be achieved in freely-moving nematodes without the need for intracranial LED insertion (as is required for mice models). Moreover, seeing as individual *C. elegans* sensory neurons perform well-

defined functions, activity-dependent ChR2 expression is not required. Instead, ChR2 expression can be targeted to specific sensory neurons, depending on the type and valence of unconditioned stimulus desired for a given learning paradigm.

The following chapter seeks to explore the use of targeted optogenetic activation to create a classical conditioning paradigm using *C. elegans*, where chemotaxis is used as a behavioral readout for associative learning.

Chapter 2: Designing a Pavlovian conditioning assay using optogenetic stimulation

Introduction

The observation that non-human mammals are capable of forming learned associations between stimuli dates back to the late 17th Century, when physiologist Ivan Pavlov performed experiments demonstrating appetitive conditioned responses in canines. These responses, most notably salivation, were initially observed only if the canines were presented with food aromas, however, if these aromas were frequently paired with certain non-appetitive stimuli (ex. auditory tones), salivation could be observed in response to non-appetitive stimuli presented alone. This process, described as classical conditioning, was eventually demonstrated to occur even in simple invertebrate organisms, such as fruit flies, honeybees, marine snails and nematodes (Livingstone, Sziber and Quinn, 1984; Bitterman *et al*, 1983; Kandel *et al*, 1983). Indeed, the use of invertebrate organisms to investigate rudimentary forms of learning became widespread in the latter half of the 1900s, resulting in the discovery of several cellular components of learning and memory (Bong-Kiun, Kandel & Grant, 1993; Kandel, 2012). In this way, the study of learning through a reductionist lens led to the understanding that mechanisms underlying plasticity (cAMP increases and CREB activation, synaptic facilitation) are contained within small neuronal networks and even single neurons.

Despite the vast insight available through the study of plasticity within dissected circuits and individual neurons, an integration of whole-organism behavior is nonetheless ideal. *C. elegans*, a soil-dwelling nematode presents an ideal model organism as it is easily genetically and optogenetically modulated, and exhibits well-characterized stereotyped behaviors (Brenner, 1974). With a nervous system consisting of 302 neurons, the self-fertilizing hermaphroditic nematode is capable of recognizing odorants and responding to them using forwards and backwards undulatory locomotion. This behavior, known as **chemotaxis**, can be assayed in order to assess chemosensory function **(**Bargmann & Horvitz, 1991). Through a variety of sensory, motor and interneurons, *C. elegans* are capable of travelling up a gradient of chemotattractant (an appetitive stimulus) by course-correcting turning, wherein the probability of executing a turn while navigating up a chemoattractive gradient is reduced while the probability of turning away from a downwards gradient is increased (Bargmann & Hotvitz, 1991). Over the past two decades, researchers have made use of chemotaxis to study associative learning in *C.*

elegans. As discussed in the previous chapter, *C. elegans* have proven amenable to Pavlovian classical conditioning paradigms involving a "training" regime wherein nematodes are presented with an ion or odorant (the conditioned stimulus) in conjunction with food deprivation (the unconditioned stimulus) (Zhang, Lu & Bargmann, 2005). Following training, nematodes are subjected to a chemotaxis assay used to assess whether the animals have associated the negative valence of food deprivation with the ion or odorant used during training. The degree to which a population avoids the ion/odorant post-training, inferred from the chemotactic index of naïve (untrained) nematodes, is a metric for the degree of associative learning exhibited by this population of *C. elegans* (Zhang, Lu & Bargmann, 2005; Kuhara & Mori, 2006). Mutant screening and laser ablation studies have demonstrated that associative learning in this context requires intact serotonergic signaling as well as the activity of certain interneurons downstream of the sensory neurons that recognize the ion/odorant used during training (Zhang, Lu & Bargmann, 2005; Kuhara & Mori, 2006; Stetak *et al*, 2009). The requirement for serotonergic input and interneuronal modulation is congruent with early synaptic plasticity research done in *Aplysia* marine snails (Brunelli, Catellucci and Kandel, 1976).

However, the exact activity of serotonin in the case of food-status dependent learning in *C. elegans* is difficult to assess seeing as changes in serotonin levels signal changes in feeding status (Ji Ying *et al*, 2000). As such, it is difficult to accurately quantify defects in associative learning using training paradigms that rely on feeding status as an unconditioned stimulus, seeing as both associative learning and food availability are known to require changes in serotonin signaling. Moreover, the assessment of strains containing serotonin mutations (ex. 5-HT receptor mutants, 5-HT biosynthetic enzyme mutants) using this type of training paradigm presents a similar confound: it is difficult to tease apart genuine learning defects from an inability to adequately signal changes in feeding status.

This issue is partly circumvented by studies that make use of pathogen infection, rather than starvation, as the unconditioned stimulus (Zhang, Lu & Bargmann, 2005). In this way, nematodes that have previously fed from a pathogenic bacterial lawn (*Pseudomonas aeruginosa*: PA14), and experienced subsequent gut infection, are less likely to feed from this lawn upon encountering it a second time (compared to naïve worms that have only fed from nonpathogenic bacterial lawns). Seeing as there is no food

deprivation involved in this experimental design, one could assume that any changes in serotonergic signaling that occur during training are due to associative learning and not feeding status. However, the physiological effects of pathogenic bacterial infection in *C. elegans* are diverse and potent: nematodes possess an innate immune system that mediates an acute inflammatory reaction, stimulating the production of pro-inflammatory cytokines, many of which are known to affect neurological processes (DBL-1/TGFβ, INS-7) (Irazoqui *et al*, 2010; Rothwell & Hopkins, 1995). Moreover, there is some evidence to suggest that activation of pathogen-binding immune receptors upregulates serotonergic signaling by increasing the expression of the serotonin biosynthetic enzyme *tph-1* (Xie *et al*, 2013; Rubio-Godoy, Aunger & Curtis, 2007). As such, there is a need for unconditioned stimuli, to be used in associative learning paradigms, that (1) do not involve food deprivation and (2) do not invoke an immune response.

In regards to the cellular basis of learning and plasticity, the logic of a reductionist perspective dictates that the resolution of an experiment increases the fewer cells are involved. In this sense, the activation of a single cell, or a small network of cells capable of conferring a negative valence, would be an ideal choice of unconditioned stimulus. Nematodes present an opportunity for this reductionism, as there are sensory neurons that, individually or in small groups, are responsible for the transduction of sensory stimuli and modalities (ex. odorants, thermal input, oxygen sensation) (White, Southgate, Thomson & Brenner, 1986). Specifically, *C. elegans* possess a relatively simple nociceptive system wherein there is one[1] primary, polymodal nociceptive neuron, ASH, that responds to harmful odorants as well as certain types of mechanical input (Yoshihiro *et al*, 1999). Simply put, selective activation of the ASH nociceptive neuron would subject the nematode to a negative valence stimulus devoid of any physiological damage. Moreover, *C. elegans* are transparent, which lends to logistically simple single-cell activation using targeted expression of light-activated optogenetic constructs (Husson, Gottschalk & Leifer, 2013).

In *C. elegans*, the ASH nociceptive neuron is located within the nerve ring, a cluster of predominantly sensory neuron axons located in the anterior region of the

[1] There are in fact two bilaterally symmetrical ASH neurons. However the pair of ASH neurons are functionally identical and therefore will be referred to as simply the ASH neuron (singular).

nematode (Ware *et al*, 1975). ASH, like several other sensory neurons, possesses rod-like ciliated structures that are partially exposed to the exterior environment through the amphid pore: an open structure located at the anterior of the nematode (Peckol, Zallen, Yarrow & Bargmann, 1999; Hart & Chao, 2010). ASH and other sensory neurons share a high degree of overlap in their networks of downstream interneurons. For instance, ASH and several chemosensory neurons share common interneurons that synapse onto the same command and motor neurons (AVA, RIV, SMD) controlling the forward and backward locomotor behavior involved in both nocifensive and chemotactic responses (Gray, Hill and Bargmann, 2005). One such chemosensory neuron, AWC, detects the volatile odorant isoamyl alcohol (lAA) which, at low-to-medium concentrations, acts as an appetitive stimulus for *C. elegans*, generally instigating a high chemotactic index when assayed alongside a control stimulus (Bargmann, Hartwieg & Horvitz, 1993). Seeing as these individual neurons, ASH and AWC, confers the sensory modalities of nociception and appetitive chemoreception, respectively, these neurons provide a reasobable basis for conditioning at a microcircuit level.

The basis of our Pavlovian conditioning assay involves the optogenetic stimulation of ASH as the unconditioned stimulus, while the paired administration of the odorant IAA acts as the conditioned stimulus. In this way, the dual activation[2] of the nociceptive neuron, ASH, and the chemosensory neuron, AWC, constitutes the neurological basis of this training protocol. The degree of IAA preference, measured by chemotactic index post-training, is used as readout for the degree of associative learning undergone by the animals. In order to permit the dual presentation of optogenetic stimulation (by blue light LED pulses) and IAA odorant administration, a 3D printed device was designed. This device, termed the OptoBox, incorporates arduino-controlled LED drivers and programmable valve-controlled airflow for the delivery of IAA vapors. In this way, a microcontroller (Arduino Uno) can be programmed to direct the synchronous presentation of blue light pulses from LEDs attached to the OptoBox, and

[2] The chemosensory neuron AWC is technically inactivated by the administration of the IAA odorant- that is to say, AWC responds to the removal of an odorant rather than its administration. This form of odorant transduction nonetheless signals activation to downstream interneurons, as AWC is tonically active when not responding to an odorant (O'Halloran *et al*, 2009).

IAA vapor delivery through a valve system. The following section describes the OptoBox assembly and preliminary tests to verify its efficacy at delivering conditioning stimuli.

Materials and Methods

Worm maintenance: all strains were grown at 20°C and raised on OP50 *E. coli* bacteria according to standard methods (Brenner, 1974).

Four-quadrant counterattractant assay:

Procedure: Post-training, 1 mL of NGM buffer was used to wash worms from training plates into a 1.5 mL eppendorf tube. Worms were then washed three times with NGM buffer and left to settle by gravity at the bottom the tube. Each wash cycle was approximately two minutes meaning that worms did not remain in NGM buffer longer than 10 minutes to avoid possible chemotactic confounds due to starvation. After washing, approximately 20μL of buffer containing 50-100 worms were transferred, using a glass capillary pipette, to the center of a four-quadrant counterattractant assay. Excess buffer was blotted away using a folded Kimwipe and worms were left to crawl for 20 minutes (plate lids were sealed with Parafilm to avoid possible odorant contamination).

Design: Chemotaxis agar was prepared, poured into 9cm petri dishes and left at 20°C to dry for two days. Once dry, a marker is used to divide the petri dish into four quadrants. 1μL of each an odorant, either IAA (diluted 1:300 with water) or a second, also appetitive odorant: diacetyl (DA, diluted 1:350 with water), and sodium azide (10%, diluted with water) are pipetted onto each quadrant at a 3cm distance from the center of the assay plate. During scoring of the assay, only worms found within a 2cm diameter of the odor spot region are counted. In order to assess the relative preference for IAA, the following formula was used: [(*worms in IAA spot - worms in DA spot*) / (*total worms in IAA and DA spots*)].

OptoBox design

The general design of the OptoBox machine sought to allow synchronous presentation of both optogenetic and odorant stimuli such as to constitute an aversive form of Pavlovian classical conditioning. In order to achieve synchronous timing of presentation, both the LED drivers and airflow-controlling valves were programmed using an Arduino microcontroller. As such, odorant presentation was consistently timed to occur only when LED light pulses were being administered. A 3D printed box was used to mount four LED disks inside of apertures on the lid, while two small holes in the box acted the intake and outtake of IAA vapors. The intake was connected to tubing from an air outlet ball valve, which ran through the Arduino-controlled valve. Specifically, the airflow intake tube was separated into two valves: one preceding the a glass vial containing IAA diluted with water and the other preceding a glass vial containing only water (control vial). When LED lights are off (the OFF period), the valve connected to the control vial is open while the valve connected to the IAA vial is closed. As such, there are two possible OptoBox stimulation states: (1) **Lights ON, Control Valve OFF, IAA Valve ON**, (2) **Lights OFF, Control Valve ON, IAA Valve OFF**.

The OptoBox interior contains round inlets to allow four plates to be placed underneath blue LEDs. Plates (6 cm petri dishes) were filled with standard NGM agar and seeded with OP50 bacteria, for control plates, and OP50 + 100mM all-trans retinol (ATR) for experimental plates (Nagel *et al*, 2005). Age-synchronized worms were grown in the dark at 20°C (for both ATR and non-ATR plates) and trained as young adult animals. The code used for OptoBox stimulation is provided in Appendix A.

Results and Discussion

A counterattractant assay (**Figure 1B, Figure 2**) was used for testing following OptoBox conditioning due to the fact that the use of IAA as a conditioned stimulus poses a confound seeing as this odorant is not neutral. Indeed, IAA is an appetitive odorant, meaning that screening for IAA preference before and after classical conditioning yields a narrow learning index (assayed via chemotaxis) due to a high baseline attraction to IAA

regardless of training (Saeki, Yamamoto & Iino, 2001). As such, diacetyl (DA) diluted 1:350 with distilled water was used as a counterattractant, allowing a relative preference for IAA to be assayed (**Figure 1B, Figure 3**). Furthermore, IAA is recognized by the AWC chemosensory neuron, while DA is recognized by the AWA chemosensory neuron, meaning that conditioning using the odorant IAA should not affect chemosensation in the AWA neuron (Bargmann, Hartwieg and Horvitz, 1993).

Prior to testing the OptoBox conditioning paradigm, we sought to confirm that the two available unconditioned stimuli (optogenetic stimulation and food deprivation) were in fact non-systemic and systemic stressors, respectively. In order to verify the nature of the stressors, we used confocal imaging to monitor the activity of a GFP-tagged transcription factor known to mediate several forms of systemic stress. This transcription factor, DAF-16, is known to mediate metabolic stress due to its involvement in the insulin signaling pathway, and has since been implicated in other forms of stress resistance such as heat shock and oxidative stress (Warnhoff *et al*, 2014; Ogg *et al*, 1997). To visualize the activity of DAF-16, we monitored a fluorescent reporter strain, TJ356, which expresses GFP tagged DAF-16. TJ356 was crossed into the optogenetic ASH::ChR2 strain (AQ2335). The resulting strain: **MMH171** (TJ356; AQ2335) was observed under a confocal microscope following OptoBox treatment by optogenetic stimulation on ATR (**Figure 2C**), as well as following food deprivation (**Figure 2D**). Images resulting from these conditions were compared to MMH162 animals that were subjected to heat shock, as a positive control, (**Figure 2A**) and untreated animals as a negative control (**Figure 2B**). Fifteen minutes of heat shock at 37°C was sufficient to induce nuclear DAF-16 accumulation (**Figure 2A**), as expected due to the systemic nature of heat stress (Ghazi & Lamitina, 2015). Interestingly, animals treated in the OptoBox on OP50 + ATR seeded agar plates did not show DAF-16 translocation, meaning that repetitive optogenetic ASH stimulation (i.e. repetitive nociceptive input) does not induce a systemic stress response (**Figure 2C**). Images of ATR-supplemented OptoBox treated animals did not deviate significantly from those of untreated animals (**Figure 2B, 2C**). Animals treated inside the OptoBox (without LED stimulation) on unseeded agar plates for 2 hours showed DAF-16 accumulation to the same degree as heat-shocked animals (**Figure 2A, 2D**). As such, both heat shock and food deprivation appear to induce a systemic stress response, as demonstrated by DAF-16 activity, whereas repetitive nociceptive stimulation does not act

through this systemic stress pathway. An additional factor that was investigated through the visualization of DAF-16 activity was whether the LED light pulses caused a significant temperature increase to the agar plates. Blue light LEDs are positioned approximately 2.5 inches above the agar plates: at that distance the intensity was measured to be 2mW/mm^2. Seeing as LED stimulation did not cause DAF-16 nuclear translocation, we concluded that the luminance did not create sufficient heat to trigger a heat shock response in treated worms.

Two hours of intermittent training inside the OptoBox (**Figure 1A**) was administered to both control (non-ATR) and experimental (ATR) animals (**Figure 3A**). The presence of all-trans retinol (ATR) is required for channelrhodopsin function, and as such both experimental and control plates are treated simultaneously inside the OptoBox and tested in the same round of counterattractant chemotaxis assays (**Figure 3A**). ATR-treated animals demonstrated significantly lower IAA preference than non-ATR treated animals (**Figure 3B)**. That is to say, ASH activation during IAA exposure appears to reduce the relative appeal of IAA upon subsequent exposure, meaning that aversive conditioning using the OptoBox training appears successful. Starvation in the presence of IAA was done in the OptoBox using vapor exposure: worms in this condition were trained on petri dishes lacking both OP50 bacteria and ATR (**Figure 3B**). This provided the chemotactic index (relative IAA preference) of worms trained with food deprivation as the unconditioned stimulus rather than ASH nociceptive activation. Both ATR-treated and starved worms demonstrated a reduced IAA preference compared to non-ATR treated worms, although starved worms showed a slightly lower preference index (-0.5) compared to that of ATR treated worms (0) (**Figure 3B)**. This discrepancy is likely due to the increased saliency of systemic stressors (ex. starvation) compared to cell-specific nociceptive activation.

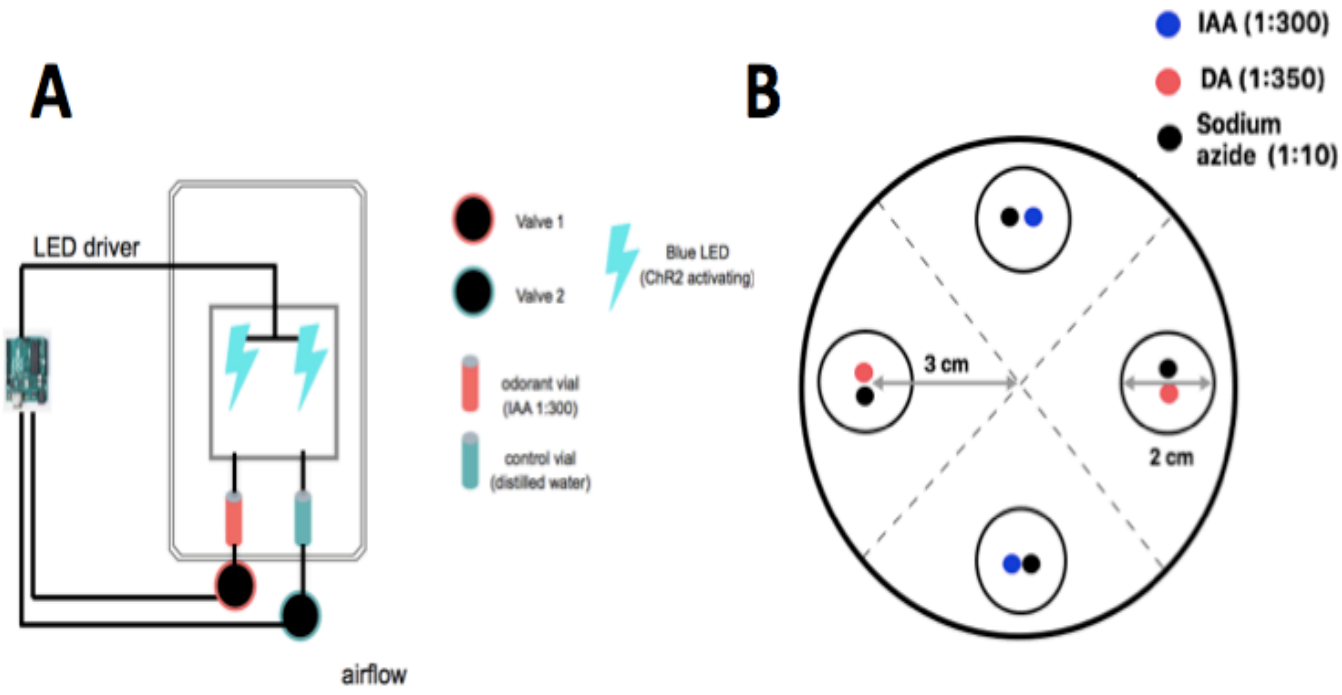

Figure 1

(**A**) Schematic overview of OptoBox design. Programmable Arduino microcontroller is connected to both the blue-light LED driver and two valves: valve 1 is connected to the odorant (isoamyl alcohol: IAA) vial, while valve 2 is connected to the control vial (distilled water only). (**B**) Schematic of the chemotaxis assay plate. Each 9 cm plate is divided into four quadrants, two containing IAA odorant spots and two containing diacetyl (DA) odorant spots. Sodium azide was used to immobilize worms upon reaching a spot. 1 µL spots were pipetted for both odorants and sodium azide. Chemotaxis agar plates made according to standard procedure and allowed to dry on the bench (temperature: 20°C) for two days prior to use.

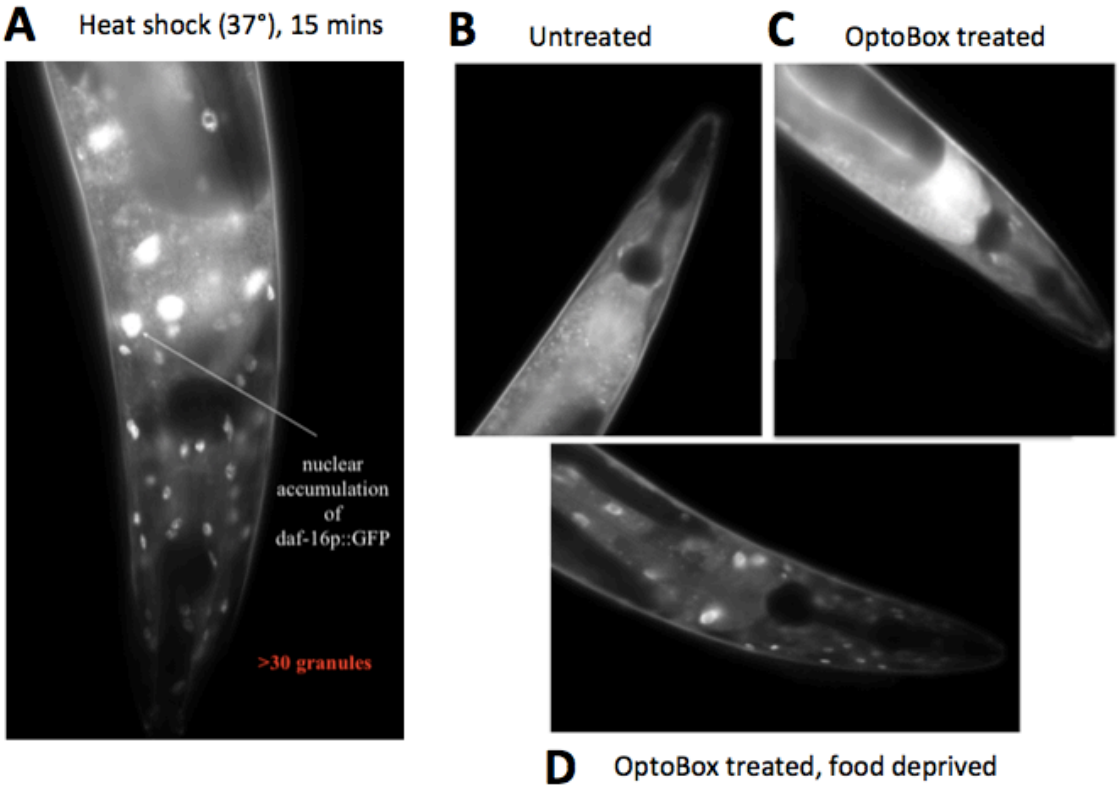

Figure 2

Representational images of TJ356; AQ2335 animals (**A**) heat shock (37°C) for 15 minutes. (**B**) untreated, maintained at 20°C on a seeded OP50 plate (**C**) Treated in the OptoBox with IAA vapors and blue LED pulses, as described in **Figure 1A**, on OP50 + ATR for two hours (**D**) Treated in the OptoBox with IAA vapors on an unseeded agar plate for two hours (food deprived condition).

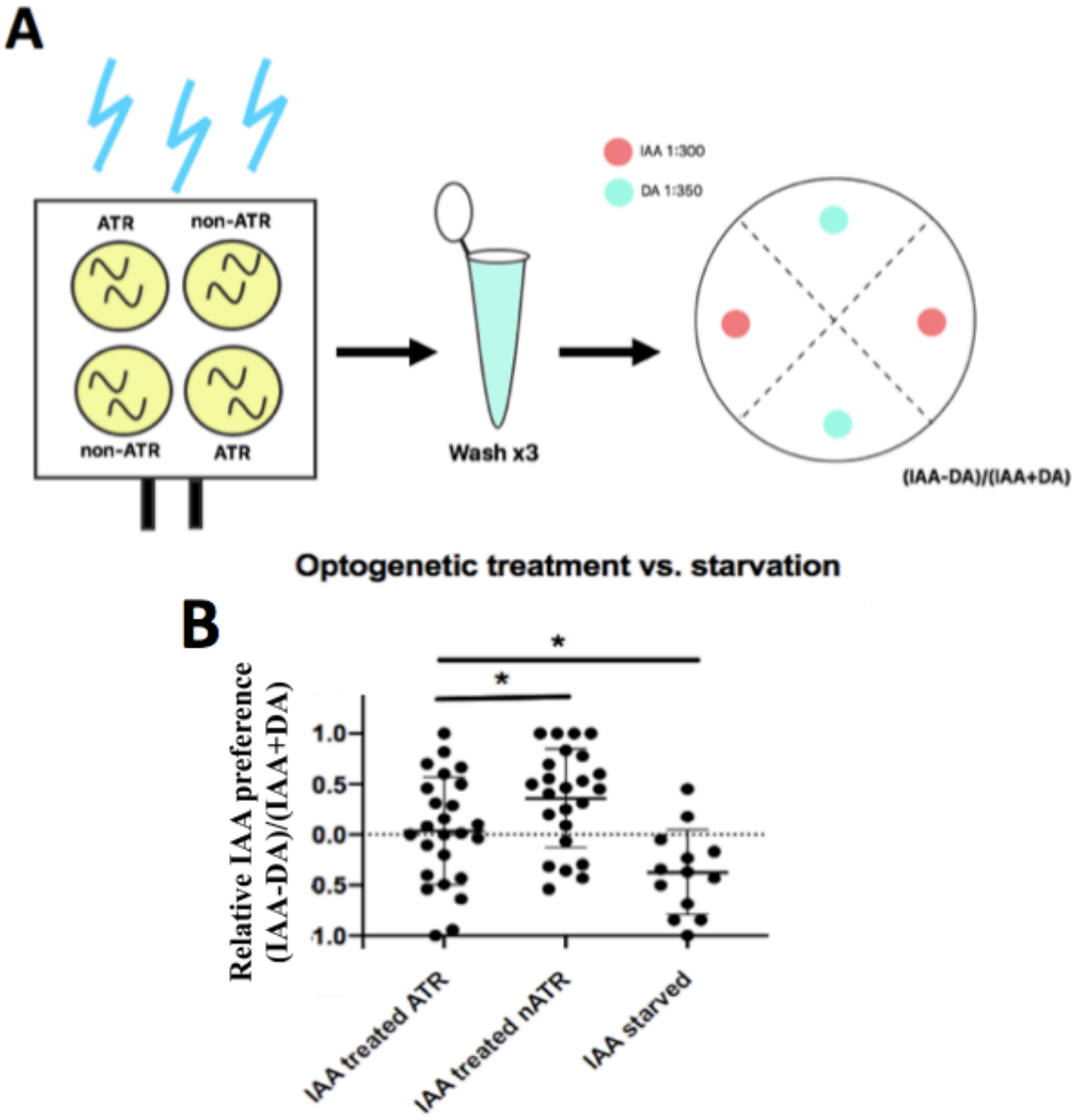

Figure 3:

(**A**) Overview of treatment protocol for OptoBox conditioning. Both ATR-containing OP50 plates and non-ATR OP50 plates (containing young-adult age synchronized worms) are contained within the OptoBox, above each plate is mounted an Arduino-controlled blue LED. Total conditioning time in the OptoBox is two hours, consisting of 10 minute dark cycles (control valve ON, LEDs OFF) punctuated by two minutes of 10 second light pulses (IAA valve ON, LEDs ON). Following conditioning, worms are washed off petri dishes and into eppendorf tubes where they are washed three times with NGM buffer. Worms are then transferred to counterattractant chemotaxis plates containing pre-pipetted odorants & sodium azide. Worms are left to crawl for twenty

minutes before the total number at each spot is scored. Relative IAA preference (chemotactic index) is calculated as follows: [(*worms in IAA – worms in DA*) / (*total number of worms at each spot*)]. (**B**) Relative IAA preference follow three conditioning treatments: IAA treated ATR: worms are treated in the OptoBox on plates containing OP50 + all-trans retinol (ATR), the necessary cofactor required for channelrhodopsin function. IAA treated nATR: worms treated in the OptoBox on plates containing only OP50 without ATR. IAA starved worms have been placed into the OptoBox on unseeded petri dishes to induce starvation. In this case food deprivation acts as the unconditioned stimulus, while IAA vapours are still the conditioned stimulus. Each point is one chemotaxis assay plate, N= 50-100 worms. * = $p \leq 0.05$

Upon confirmation that optogenetic ASH activation functions as a non-systemic stressor capable of inducing a conditioned response (IAA avoidance), we began investigating the processes underlying this form of conditioning. As such, we turned our focus to molecules known to play a role in invertebrate memory. Activity-dependent memory has been demonstrated to occur, even in a single-cell model, through the Cre-responsive element binding (CREB) protein (Leutgeb, Frey and Behnisch, 2005). CREB activity is induced by increased intracellular cAMP production, which is dependent on synaptic input (Dash, Hochner and Kandel, 1990; Barco, Alarcon and Kandel, 2002). In *C. elegans*, the CREB homologue is encoded by the gene CRH-1 (Suo, Kimura & Van Tol, 2006). Prior to assessing the function of CRH-1 using the paired administration of IAA vapors and optogenetic ASH activation, we opted to first test mutants using IAA vapors and starvation, as demonstrated in the **IAA-starved** condition of **Figure 3**, seeing as this yielded a robust chemotactic effect. Worms were treated in the OptoBox as described in **Figure 1B**, using IAA vapors only (without LED blue light pulses since the unconditioned stimulus in this case was acute food deprivation).

There were four conditions to be assayed using this setup: both wildtype (WT) and *crh-1* mutant worms on food (fed condition) and off food (starved condition). As with ATR and non-ATR plates in **Figure 3**, fed and starved plates were trained in the OptoBox during the same trials (**Figure 3A**).

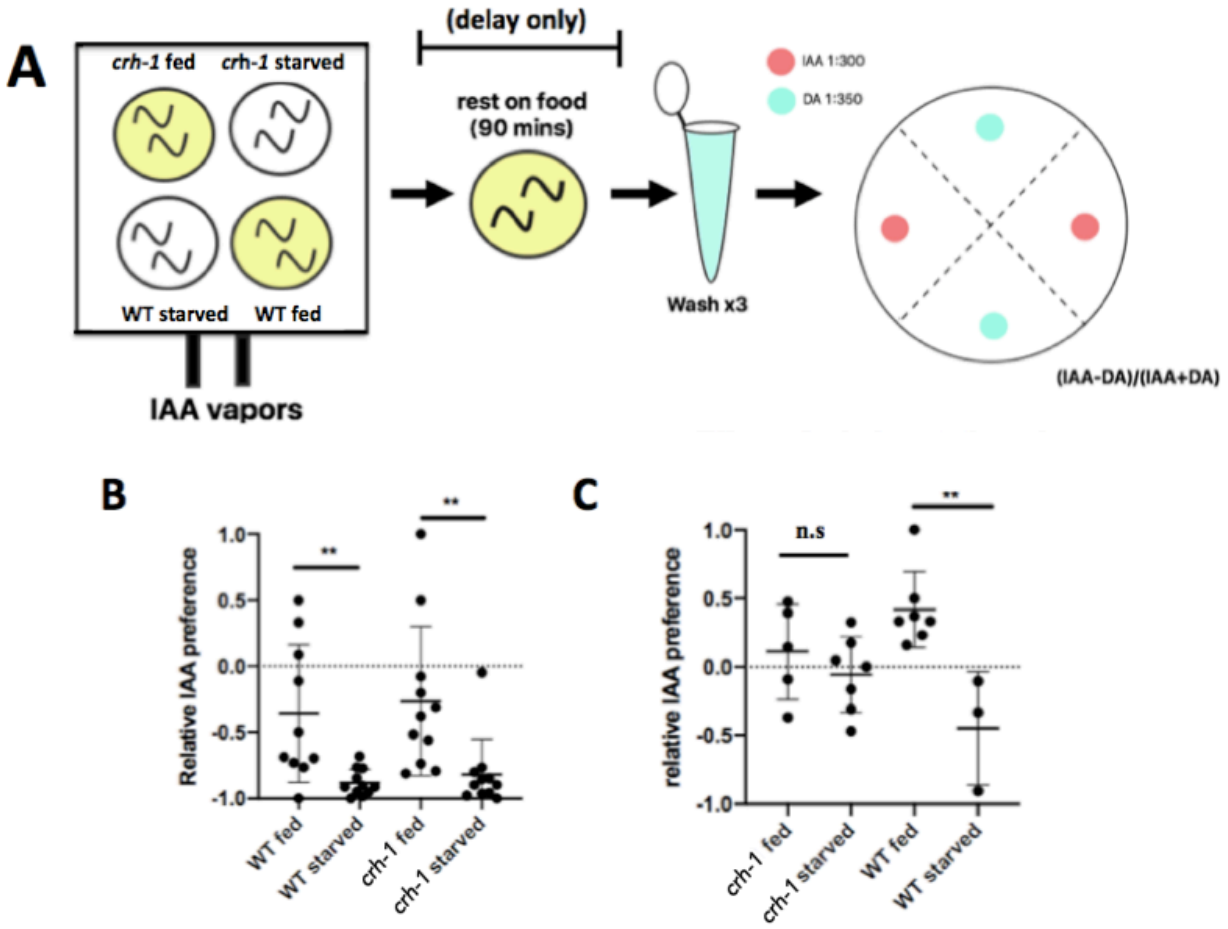

Figure 2

(**A**) Schematic overview of the training process administered to *crh-1* and wildtype (WT) animals. 6 cm NGM agar-filled petri dishes are placed inside plate inlets in the OptoBox, either OP50-seeded (fed condition) or unseeded (starved condition). IAA vapors are administered through the Arduino-programmed valve system described in **Figure 1A** for a duration of 2 hours. LED lights are not used in this setup. After training, worms are either washed immediately with buffer (short-term memory performance) or left to rest on OP50-seeded plates for 90 minutes. Counterattractant chemotaxis assay performed as described in **Figure 1B**. (**B**) Short-term relative IAA preference. Animals assayed by chemotaxis immediately after OptoBox training (administration of IAA vapors on or off food). (**C**) Relative IAA preference assayed after 90 minutes of rest on OP50-seeded plates. After 2 hours of OptoBox treatment, worms in this condition were washed and placed onto seeded plates prior to being washed a second time (three washes) and transferred onto counterattractant chemotaxis plates. ** = $p \leq 0.01$, n.s. = $p > 0.05$

Fed worms, both WT and *crh-1*, were trained on OP50-seeded plates inside the OptoBox, meaning that they were expected to associate the experience of encountering IAA vapors with satiety. Starved worms were trained inside the OptoBox on unseeded agar plates, and therefore associate IAA vapors with the experience of food deprivation. For the assessment of short-term memory, both fed and starved WT and *crh-1* worms were rinsed from training plates, washed with buffer, and transferred immediately onto counterattractant chemotaxis plates (**Figure 4A, 4B)**. We observed that, following starvation, both WT and *crh-1* worms demonstrated a significantly decreased IAA preference compared to on-food trained animals (**Figure 4B**). As such, *crh-1* mutant worms appear capable of forming a short-term association between starvation and IAA vapors. For the assessment of memory following a 90-minute on-food rest period, WT and *crh-1* fed and starved animals were trained as for the assessment of short-term memory (2 hours of IAA vapor administration in the OptoBox). However, after training, fed and starved worms were transferred onto OP50-seeded rest plates for 90 minutes prior to counterattractant chemotaxis (**Figure 4A**). Interestingly, while WT starved worms maintained a significant aversion to IAA after training, *crh-1* starved worms did not demonstrate a significantly lower IAA preference than *crh-1* fed worms (**Figure 4B**). We took this to indicate that *crh-1* worms appear to exhibit memory deficits only when there is a significant time delay between training and testing. Simply put, *crh-1* worms appear capable of forming a short-lived association between the experience of food deprivation and IAA vapors, but this strain demonstrates lower memory retention than WT, as demonstrated by a lack of starvation-induced IAA avoidance after a 90-minute rest period (**Figure 4A, 4C**).

Conclusions

The OptoBox design offers several potential uses in terms of conditioning assays. In brief, classical conditioning assays involve the pairing of a conditioned stimulus (CS) with an unconditioned stimulus (US) in order to achieve a conditioned response (CR) to the aforementioned conditioned stimulus (CS + US = CR). For OptoBox conditioning, Arduino-controlled valves manipulate airflow to allow temporally controlled

administration of odorant vapors, ex. IAA vapors, which constitute the CS in this setup. Unconditioned stimuli (US) that can be used in the OptoBox include non-systemic stressors (optogenetic stimulation) as well as systemic stressors (food deprivation: worms on unseeded agar plates). Simply put, IAA vapors can be paired with either optogenetic stimulation (ASH stimulation in the case of the AQ2335 strain) or food deprivation.

OptoBox conditioning is quantified post-training using counterattractant chemotaxis, an assay that provides a measure for relative IAA preference: this is the conditioned response (CR) resulting from OptoBox training. Although food deprivation induces a more robust avoidance of the IAA odorant after training, repetitive optogenetic stimulation of the nociceptive ASH neuron did induce a significantly reduced preference for IAA. Moreover, ASH stimulation was confirmed to be non-systemic as it did not induce nuclear translocation of the DAF-16 transcription factor, unlike food deprivation, which reliably induces a systemic stress response via the DAD-16 pathway.

As demonstrated by experiments making use of the *crh-1* (*C. elegans* CREB homologue) mutant strain, OptoBox training and subsequent chemotaxis can be used to screen for associative learning deficits. In order to test whether a given gene is involved in conditioning induced by either systemic or non-systemic stressors, training can be done using two different unconditioned stimuli (US): starvation (systemic US) and optogenetic ASH stimulation (non-systemic US). Our testing of CRH-1 mutants using starvation as the US revealed that CRH-1 expression is required for the maintenance, but not initial formation, of conditioned memory. Further study is required in order to verify whether this effect is specific to systemic stressors, or whether CRH-1 plays a role in maintaining memories induced by non-systemic stressors.

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Chapter 3: The role of Serotonin in non-systemic conditioning paradigms

Introduction

Associative learning occurs when two concepts, which could include a stimulus, feeding status or location, become linked through a process of repetitive tandem presentation. Many associative learning paradigms make use of one neutral stimulus, for instance a non-startling tone, and a second stimulus that reliably elicits an instinctive response, such as electric shocks or the presentation of food. In this way, a linkage between these stimuli can be observed when the instinctive response in question is elicited by the previously neutral stimulus. This type of associative learning is commonly known as *classical conditioning*, or *Pavlovian conditioning* and has been observed in several species, from humans to invertebrate model organisms such as nematodes and sea snails. The study of associative learning in organisms with simple nervous systems is beneficial as it provides a reductionist model for neurologically complex phenomena. For instance, the use of semi-dissected *Aplysia* marine snails allowed researchers to study associative learning at the level of individual ganglia and even individual neurons (Kandel, Walters & Carew, 1981). Using this method, which consisted of immobilizing individual snails and exposing neuronal clusters, researchers were able to study instinctive behaviors (ex. gill withdrawal reflex) at a single-neuron level and, more importantly, study how this behavior could be conditioned by the administration of current injection into nearby neurons.[3] This method proved invaluable to the study of long-term potentiation (LTP) and molecules involved in plasticity (CREB, cAMP, PKA) as it provided an easily manipulated *in vivo* model.

One particular insight explored using *Aplysia* neurons is the necessity of serotonergic signaling for cellular plasticity on a network-level as well as within single neurons. It was established that serotonin is required for synaptic facilitation: a process whereby a neuron becomes increasingly responsive to a given repetitive stimulus (Boyle,

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[3] The precise method used for the study of associative learning using semi-dissected *Aplysia* was electrophysiology. The gill-withdrawal circuit was studied by the stimulation of the siphon sensory organ, or further downstream at the level of motor neurons within the abdominal ganglia.

Klein, Smith & Kandel, 1984). This is observed as an increase in the quantity of neurotransmitter released from the neuron in response to a constant stimulus amplitude; for instance, synaptic facilitation in *Aplysia* occurs in the gill withdrawal reflex circuit when sensory neurons increase synaptic input to downstream motor neurons (Jacklet & Rine, 1977). Interestingly, synaptic facilitation was observed to occur in the absence of sensory input when pulses of exogenous serotonin were administered directly to the downstream neurons involved in this circuit (Schacher, Montarolo & Kandel, 1990). Indeed, it has since been confirmed that serotonin is involved in the molecular cascade responsible for the formation of short-term and long-lived synaptic mechanisms that constitute memory, as it acts upstream of the CREB protein (Marinesco, Wickremasinghe & Carew, 2006).

More complex learning paradigms involving freely-moving invertebrate animals have also confirmed a requirement for serotonin (Nuttley, Atkinson-Leadbeater & van der Kooy, 2002). Specifically, studies of associative learning in *C. elegans* usually involve a learned association between feeding status and a given stimulus; the strength of this association can be tested through the use of behavioral assays. To test for an association between food (or the lack thereof) and a volatile odorant, the chemotaxis assay is used; similarly, the association between food and temperature can be tested using a similar behavioral assay: thermotaxis (Lino *et al*, 2013; Nuttley, Atkinson-Leadbeater & van der Kooy, 2002). A drawback to the use of these feeding-based behavioral assays is discussed in Chapter 2: in short, feeding status is signaled to *C. elegans* via organismal serotonin levels, and this presents a confound to the study of how serotonin affects associative learning. As such, the optogenetic stimulation of a nociceptive neuron, ASH, was explored for use as the unconditioned stimulus while vapors of a volatile odorant, IAA, was the conditioned stimulus. The conditioned response resulting from paired ASH activation and IAA administration was quantified using a counterattractant chemotaxis assay, which provided a metric of relative IAA preference (**Chapter 2, Figure 1B**).

Using the optogenetic activation + IAA vapor training paradigm (stimuli presented in conjunction using the programmable OptoBox) (**Chapter 2, Figure 1A**), aversive conditioning was demonstrated to occur after two hours of training (**Chapter 2, Figure 3**). Moreover, the OptoBox is permissive to the application of two different types of unconditioned stimuli: ASH optogenetic activation by LED pulses (non-systemic

stressor), as well as acute food deprivation (systemic stressor) (**Chapter 2, Figure 2**). Food deprivation paired with IAA vapors was used to investigate a gene known to mediate invertebrate memory (CRH-1: invertebrate homologue of CREB) (**Chapter 2, Figure 4**). While the CRH-1 gene was observed to be required for the maintenance, but not acquisition, of aversive conditioning, this was only confirmed for conditioning induced by a systemic stressor (food deprivation) (**Chapter 2, Figure 4**). Further study is required to confirm whether CRH-1 is involved in non-systemic conditioning. As such, the following chapter seeks to elucidate molecular mechanisms involved in non-systemic aversive conditioning. Specifically, the requirement for serotonin in this learning paradigm was investigated, as well as the regulation of serotonin-related genes during learning.

Materials & Methods

Worm maintenance: all strains were grown at 20°C and raised on OP50 *E. coli* bacteria according to standard methods (Brenner, 1974).

Four-quadrant design & procedure + OptoBox design & procedure (as discussed in **Chapter 2)**

Molecular Biology: tryptophan hydroxylase-1 *(tph-1*) mutants (**MT15434** strain:) were crossed into optogenetic strain **AQ2335** (sra-6::Channelrhodopsin(H134R)::mCherry): resulting strain (ASH::ChR2; *tph-1*): **MMH156**. Deletion verified by PCR: forward primer: CTAATAGTGTGTAAGTTTTCATCTTTCAAA, reverse primer: AGCTGGTAGAATTTTAAATTTCTGGA. The fluorescent reporter strain (tph-1p::GFP) used, **SK4013**, was crossed into the optogenetic strain (AQ2335: ASH::ChR2), resulting in the novel strain **MMH172**; worms carrying both transgenes were identified by fluorescent microscopy.
Additional optogenetic strains: **EEG98**: tph-1p::ChR2 in an N2 background, and **EEG107**: tph-1p::ChR2 in a tryptophan hydroxylase mutant (*tph-1*) background. For calcium imaging, the optogenetic AQ2335 strain was crossed into **CX17256** (kyls722

[str-2p::GCaMP5(D38OY) + elt-2::mCherry]). The resulting strain, **MMH173** (CX17256, AQ2335), was compared to CX17256 during calcium imaging analysis.

GFP Imaging: Worms were picked from treatment plates onto unseeded agar plates and left to crawl for two minutes to remove any residual bacteria. Agar pads for imaging consisted of 2% agarose dissolved in M9 buffer, pipetted onto glass slides, in 2µL spots, and flattened using a second glass slide. Agar pads were left at 20°C to dry for one hour before use. 1µL of M9 buffer was pipetted onto dry agar pads, and approximately 5 worms were transferred from agar plates into the buffer droplet. Worms were then immobilized with a second glass cover slide and imaged by confocal microscopy within 10 minutes of immobilization. Images were analyzed using Fiji distribution of ImageJ (Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012).)

Calcium imaging: microfluidic chips made according to designs by Chronis et al, 2007 using Polydimethylsiloxane (PDMS). MMH173 (CX17256; AQ2335) worms were treated in the OptoBox for two hours before imaging. Immediately prior to imaging, worms were transferred from treatment plates (containing OP50 +/- ATR) onto unseeded NGM agar plates and allowed to crawl for 2-3 minutes to remove residual bacteria. All worms were imaged within one hour of OptoBox treatment completion. Imaging consisted of 1 minute videos involving alternating exposure (10 second oscillations) to NGM buffer and NGM buffer + 1: 10 000 IAA. All worms imaged once. Videos consisted of TIFF stacks obtained by Olympus IX83 inverted microscope (Olympus, Richmond Hill, Ontario). TIFF stacks analyzed using Fiji by ImageJ to obtain GCaMP5 fluorescence values, data analyzed using JMP 14 (SAS, Toronto, Ontario).

Results & Discussion

Because serotonin has been established as necessary for several forms of associative learning, we crossed a strain containing a mutation in the tryptophan hydroxylase-1 gene (MT15434: *tph-1*) into the optogenetic strain (AQ2335: ASH::ChR2) used in Chapter 2. Both the strain resulting from this cross, **MMH156** (AQ2335; MT15434), and AQ2335 (ASH::ChR2 strain) were treated using OptoBox training (described in Chapter 2). The

crossed strain, MMH156 (AQ2335; MT15434), is nonetheless capable of responding to nociceptive input via the ASH neurons seeing as ASH is a glutamatergic neuron (Hart, Sims & Kaplan, 1995), however, without the functional TPH-1 enzyme this strain is unable to hydroxylate tryptophan to produce the serotonin precursor 5-hydroxytryptophan (5-TPH), meaning this strain is essentially devoid of serotonin (Sze *et al*, 2000). Despite a lack of organismal serotonin, the MT15434 strain appears superficially wild type and is capable of normal behaviors such as olfaction and chemotaxis (Waggoner, Zhou, Schafer & Schafer, 1998).[4]

During OptoBox treatment, both AQ2335 and MMH156 (AQ2335; MT15434) strains were placed on OP50 + ATR seeded agar plates, meaning that all treated worms experienced both ASH stimulation and IAA vapors (**Figure 1A**). Following two hours of OptoBox treatment, worms were washed in buffer and immediately transferred onto counterattractant chemotaxis plates (**Figure 1A**). Post-conditioning, worms devoid of serotonin (MMH156: AQ2335; MT15434) show a significantly higher relative IAA preference than worms with intact serotonergic signaling (**Figure 1B**). Because both strains experience wildtype nociception, olfaction and chemotaxis, the difference in relative IAA preference is likely a product of differential associative learning. The observation that a learning deficit appears to correlate with reduced/disrupted serotonergic signaling is in line with existing literature (Nuttley, Atkinson-Leadbeater & van der Kooy, 2002; Tsui & van der Kooy, 2008; Zhang, Lu & Bargmann, 2005).

Next, in order test the impact of serotonergic signaling on associative learning in a more direct manner, we chose to monitor the expression of an enzyme required for serotonin biosynthesis during conditioning. A fluorescent reporter strain, SK4013, which expresses GFP under the same promoter as the TPH-1 enzyme (tph-1p::GFP) was crossed into the ASH::ChR2 optogenetic strain in order to visualize the regulatory control of TPH-1 during OptoBox conditioning. In this sense, GFP fluorescence intensity can be taken as a proxy for TPH-1 gene expression and, therefore, serotonin biosynthesis (Lee, Crane, Zhang & Lu, 2013). Fluorescence from two pairs of serotonin-producing neurons,

[4] Although *tph-1* mutant worms were viable and superficially wildtype, they do exhibit changes in reproduction and increased fat storage. However, *tph-1* mutants performed wildtype chemosensation and nociception and for that reason we disregarded the aforementioned metabolic changes.

the ADFs and NSMs,[5] was quantified following various OptoBox treatment protocols (**Figure 2A, 2B**). All imaging was done in worms expressing both ASH::ChR2 and tph-1p::GFP (**Figure 2A**). One cohort of worms was treated in the OptoBox (with IAA vapors as well as blue LED pulses) for four hours on OP50 + ATR, while a second was treated for four hours on OP50 devoid of ATR (**Figure 2C**). Worms treated on OP50 + ATR demonstrated a significantly higher degree of fluorescence intensity from the ADF and NSM neurons, indicating an upregulation in serotonin biosynthesis for worms administered both IAA vapors and ASH stimulation (**Figure 2C**).

A third cohort was treated in the OptoBox for two hours, on OP50 + ATR, with only blue LED pulses (no IAA vapors) (**Figure 2C**). The purpose of testing worms treated with ATR and no IAA vapors was to verify whether TPH-1 upregulation was solely dependent on repetitive ASH stimulation. ASH neurons express G-protein coupled receptor subunit GPA-11, which is known to be modulated by serotonin (Chao *et al*, 2004), and in this way changes in serotonin biosynthesis could be due, in theory, to increased nociceptive input. However, treatment on OP50 + ATR without IAA vapors did not result in any significant increase in fluorescence, meaning that both repetitive ASH stimulation and olfactory stimulation (IAA) are required for TPH-1 upregulation (**Figure 2C**). This observation implies that TPH-1 expression is upregulated during learning that requires the formation of an association between two distinct, novel stimuli presented together with sufficient regularity. This is in line with previous studies that have found a correlation between increased serotonergic signaling and the cellular plasticity required for associative learning (Zhang, Lu & Bargmann, 2005; Achee & Zoran, 1997). The reliance on serotonin is likely due to the many intracellular signaling cascades initiated by serotonin receptor activation, for instance ion channel modulation and second messenger (ex. cAMP) production (Achee & Zoran, 1997; Bailey *et al*, 2000). Moreover, interneurons receiving input from both ASH and AWC (AWC: IAA chemosensation) neurons are known to express serotonin receptors, for instance the AIA and AIB interneuron (Harris *et al*, 2009).

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[5] Both ADF and NSM neurons are bilaterally symmetrical pairs. A third pair, the HSNs, is located adjacent to the vulva and involved in the egg laying circuit. Due to their lack of proximity to the nerve ring, the HSN neurons were excluded from analysis.

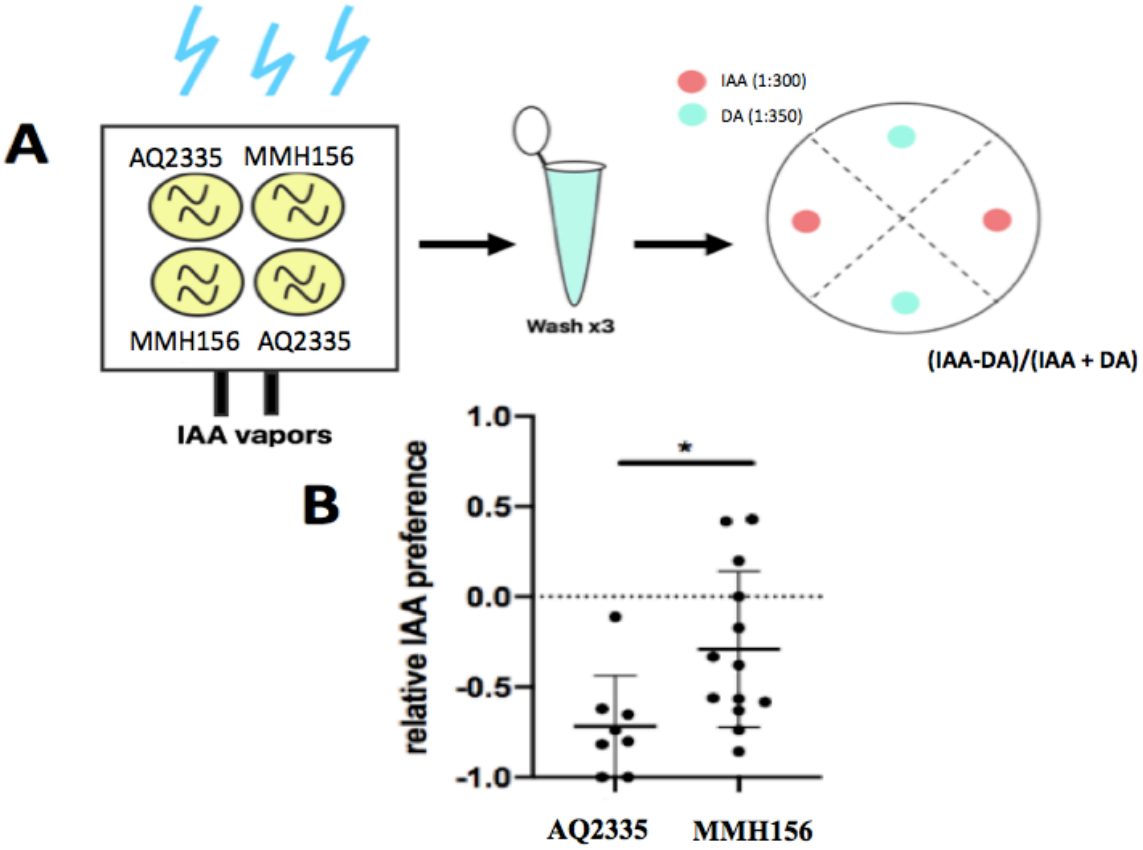

Figure 1

(**A**) Treatment protocol for OptoBox aversive conditioning using the ASH::ChR2 strain (AQ2335) and the ASH::ChR2; *tph-1* strain MMH156 (AQ2335; MT15434). As discussed in Chapter 2, ASH::ChR2 worms treated on plates seeded with OP50 + all-trans retinol (ATR) show a significantly higher aversion to IAA vapors post-treatment than worms treated in the absence of ATR (**Chapter 2, Figure 3B**). As such, conditioning for both AQ2335 and AQ2335; MT15434 strains was done on OP50 + ATR. Worms were treated for two hours, washed in NGM buffer three times and placed on the counterattractant chemotaxis assay plate as discussed in Chapter 2 (**Chapter 2, Figure 1**). (**B**) Quantification of relative IAA preference (worms at IAA spot – worms at DA spot/ total worms at both spots) was done using the optogenetic strain (AQ2335) as well as the optogenetic strain containing a TPH-1 mutation MMH162 (AQ2335, MT15434). * = $p \leq 0.05$

In order to further investigate the influence of serotonergic signaling on the OptoBox associative learning paradigm, we tested a different optogenetic *C. elegans*

strain that expresses the channelrhodopsin construct under the promoter region of the TPH-1 gene (tph-1p::ChR2: EEG98). In this way, rather than blue light LED pulses eliciting nociceptive input (as for the AQ2335 strain), LED pulses induce selective neuronal activation in TPH-1-expressing cells: NSM, ADF and HSN (Pokala & Glater, 2018). Seeing as our previous experiments had demonstrated that conditioned aversion to IAA requires both a functional TPH-1 enzyme and TPH-1 up-regulation, we sought to assess the effect of directly stimulating TPH-1-expressing neurons, so as to elicit serotonergic activity, during training. When placed in the OptoBox and treated with paired blue light pulses and IAA, the EEG98 (tph-1p::ChR2) strain experiences surges of serotonin release only during periods of IAA odorant administration (**Figure 3A**). EEG98 was treated in the OptoBox on both OP50 and OP50 + ATR in order to compare the experience of IAA vapors + serotonergic activation (OP50 + ATR) versus only exposure to IAA vapors (OP50 only) (**Figure 3A**). A similar optogenetic strain, EEG107, expressed the same channelrhodopsin construct under the TPH-1 promoter region, only the background of this strain contained a 1306 base-pair deletion in the TPH-1 enzyme, causing significantly disrupted serotonin biosynthesis. The EEG107 (tph-1p::ChR2; *tph-1*) strain was used as a negative control seeing as blue light pulses during treatment could not elicit serotonin release from NSM, ADF or HSN neurons.

Interestingly, EEG98 worms showed a greater affinity for IAA when treated with ATR, indicating that increased serotonin levels correlate with positive associative learning when timed to correspond with IAA presentation (**Figure 3B**). Accordingly, the EEG107 strain (tph-1p::ChR2; *tph-1*) did not demonstrate any difference in IAA preference +/- ATR, indicating that the change in preference observed in EEG98 worms is dependent on serotonin production (**Figure 3B**). The effect of serotonin release in the context of IAA vapor exposure could be related to serotonin acting to increase cellular excitability, which might in turn increase plasticity and therefore associative learning (Daoudal & Debanne, 2003; Burrell & Sahley, 2005). In short, blue LED pulses activate serotonergic neurons, causing increased serotonin release, which might in turn increase the excitability of nearby serotonin-responsive sensory neurons and interneurons via the cAMP/PKA pathway downstream of serotonin receptors (Burrell & Sahley, 2005; Barbas *et al*, 2003). For instance, interneurons known to receive synaptic input directly from

AWC[6], the neuron that recognizes the IAA odorant, express serotonin receptors and therefore could potentially act as sites of associative learning (ex. AIY, AIA) (Hapiak *et al*, 2009).

Alternatively, increased serotonin levels signal the presence of food & satiety, and therefore the activation of TPH-1-expressing cells might signal food presence (Nuttley, Atkinson-Leadbeater & van der Kooy, 2002). By this logic, a post-training increase in IAA affinity might be due to an association between IAA vapours and perceived satiety. However, this explanation is somewhat problematic seeing as all strains were treated on agar plates seeded with OP50 and therefore all worms experienced satiety to a similar degree- it is unclear whether further serotonergic stimulation would result in increased satiety.

It is important to note that OptoBox training using the AQ2335 strain (ASH stimulation) resulted in IAA aversion (**Chapter 2, Figure 3B**), whereas training using the EEG98 strain (serotonin-producing neuron activation) resulted in increased IAA affinity (**Figure 3B**), despite a requirement for serotonin and *tph-1* upregulation in each respective learning paradigm. This disparity in valence is potentially due to a difference in the quantity of serotonin release and, accordingly, differential activation of downstream serotonin receptors exhibiting different affinities (Hapiak *et al*, 2009). Differential serotonin receptor activation has been reported to result in disparate behavioural patterns: for instance, the excitatory receptors SER-1 and SER-7 receptors are necessary for egg-laying, while an inhibitory serotonin-gated chloride channel, MOD-1, appears to inhibit egg-laying but is required for aversive learning (Hapiak *et al*, 2009; Zhang, Lu & Bargmann, 2005). As such, the serotonin release instigated by the optogenetic activation of tph-1-expressing cells might differ from serotonin release experienced during nociceptive (ASH) stimulation, in such a way that different serotonin receptors are activated, resulting in different behavioural patterns (i.e. different valence allocated to the experience of IAA chemosensation). Further study is required to

[6] Like other *C. elegans* amphid sensory neurons, there are two bilateral (one left and right) AWC neurons. While there is a considerable degree of functional asymmetry in AWC neurons due to disparate olfactory gene expression, both the left and right AWC neurons respond to IAA odorant removal (Huang *et al*, 2007). As such, for the purposes of this discussion we refer to the AWC neurons as one singular neuron due to overlapping IAA responsiveness.

investigate the effect of how specific serotonin receptors impact the encoding of valence during learning paradigm administration. For instance, OptoBox training could be administered to *C. elegans* strains carrying both the ASH::ChR2 optogenetic construct and mutations in specific serotonin receptors (MOD-1, SER-1, SER-7), in order to compare the chemotactic index of these mutant strains against that of wildtype strains.

Seeing as this explanation hinged upon the activation of serotonin receptors downstream of sensory stimulation, i.e. within the interneuronal network, we sought to verify whether any changes were occurring at the sensory level. In the Optobox conditioning paradigm explored in Figure 1, sensory input consists of nociceptive stimulation conferred by repetitive optogenetic activation of ASH neurons, as well as IAA vapours, which are predominantly recognized by AWC chemosensory neurons. We chose to characterize the response of a single AWC neuron to IAA vapours using animals treated without ATR, which therefore do not experience any nociceptive input, and animals treated in the presence of ATR who experience AWC activation in conjunction with ASH-mediated nociceptive input. In this manner, we can monitor single-cell olfactory activity in worms that do or do not experience ASH stimulation (and therefore do or do not experience aversive associative learning). In order to monitor AWC activity[7], we used the strain CX17256 (kyls722 [str-2p::GCaMP5(D38OY) + elt-2::mCherry]), in which the expression of a bright green calcium indicator (GCaMP5) is driven by the gene str-2, known to drive expression in AWC^{ON}. AWC^{ON} mediates the sensory response to IAA removal, meaning that upon removal of IAA, AWC^{ON} experiences a strong transient increase in intracellular calcium levels. CX17256 worms were crossed into the optogenetic AQ2335 (ASH::ChR2) strain and GCaMP5+/mCherry+ worms (MMH163: CX17256, AQ2335) were isolated for treatment. In order to visualize AWC GCaMP5 activity, trained worms (+/- ATR) were

[7] As mentioned previously in this section, the AWC chemosensory neurons are bilateral and functionally asymmetrical although they both respond to IAA in a similar manner (Huang *et al*, 2007). We arbitrarily chose to monitor calcium activity in the AWC^{ON} neuron rather than the AWC^{OFF} neuron. However, additional study should examine whether there are changes to IAA responses post-training in AWC^{OFF}, seeing as new research has found differences in IAA responsiveness between AWC^{ON} and AWC^{OFF} following aversive training (Eliezer *et al*, 2019). Further examination is required to address whether the AWC neurons undergo disparate cell-specific changes during olfactory training.

restrained and imaged in microfluidic devices that allowed precisely timed odorant delivery (calcium imaging chip) (Chronis, Zimmer & Bargmann, 2007) (**Figure 4A**). Videos (TIFF stacks) were acquired using the 40X silicone-immersion objective on an Olympus IX83 inverted microscope (Olympus, Richmond Hill, Ontario). Activity in the AWC neuron was quantified in 10-second increments during which alternating streams of NGM buffer and NGM buffer + IAA (diluted 1: 10 000) were presented sequentially for a total of 1 minute (**Figure 4B**). In this way, the relative fluorescence of GCaMP5 in animals treated on OP50 +/- ATR was quantified in order to ascertain whether there are differences in IAA-evoked sensory activity following OptoBox training. Interestingly, ATR-treated worms demonstrated an overall lower amplitude of AWC calcium activity following IAA removal, in comparison to worms treated without ATR which did not experience ASH stimulation during OptoBox training (**Figure 4C**). This relative reduction in calcium fluorescence appears to increase further with each subsequent IAA exposure, indicating that ATR-treated worms might experience changes in sensory adaptation (i.e. habituation) (**Figure 4C**). The mechanisms underlying these sensory changes are unclear, however they are unlikely to involve serotonin, at least not directly, seeing as the AWC chemosensory neuron does not express serotonin receptors. It is possible, however, that serotonin acts distally on AWC through intermediate neurons- for instance, AWC receives synaptic input form the AIA interneuron which expresses MOD-1, a serotonin-gated chloride channel (Gray, Hill & Bargmann, 2005; Harris *et al*, 2009). AIA receives synaptic input from ASH as well as AWC, making this interneuron a candidate for sensory integration during OptoBox training. Further AWC calcium imaging and behavioural assessment is required to further investigate this possibility. For instance, worms with ablated AIA interneurons or MOD-1 receptor loss-of-function mutations can be tested for their ability to undergo associative learning (OptoBox optogenetic training + subsequent counterattractant chemotaxis assessment).

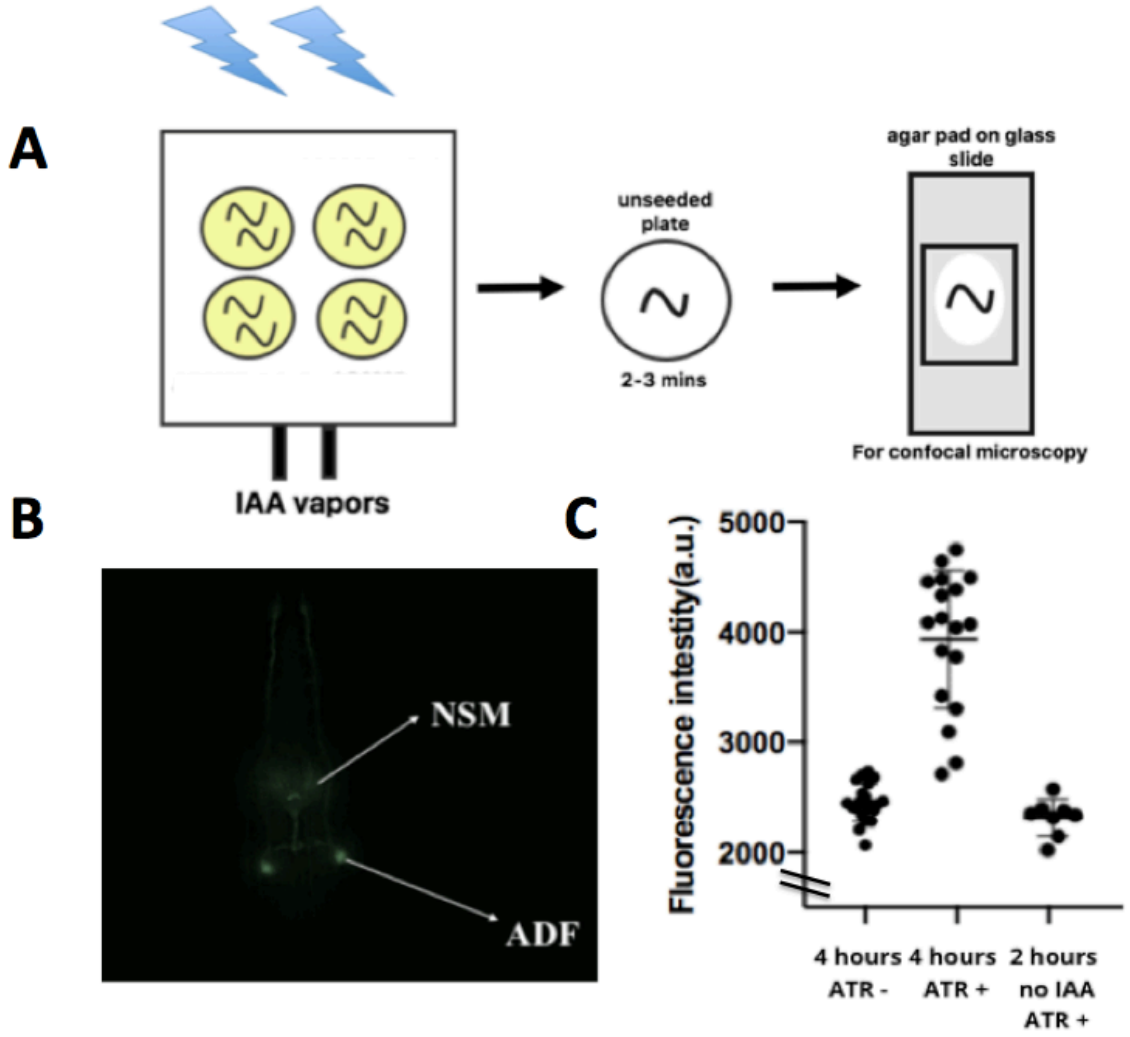

Figure 2

(**A**) Schematic overview of OptoBox treatment. AQ2335; SK4013 strains are treated on OP50 +/- ATR seeded agar plates. Immediately following training (2 hours of IAA vapour + blue LED light pulses), individual worms are picked from treatment plates onto unseeded agar plates to remove residual bacteria. Worms are then transferred onto agar pads and immobilized between glass slides for confocal microscopy. (**B**) Confocal image of SK4013 (tph-1p::GFP) strain showing the two pairs of serotonergic neurons (NSM, ADF) used for GFP fluorescence intensity quantification. (**C**) Fluorescence values of AQ2335; SK4013 worms given one of three OptoBox treatment protocols: (1) 4 hours of treatment on OP50 without ATR, (2) 4 hours of treatment on OP50 with ATR and (3) two hours of treatment on ATR without IAA vapors.

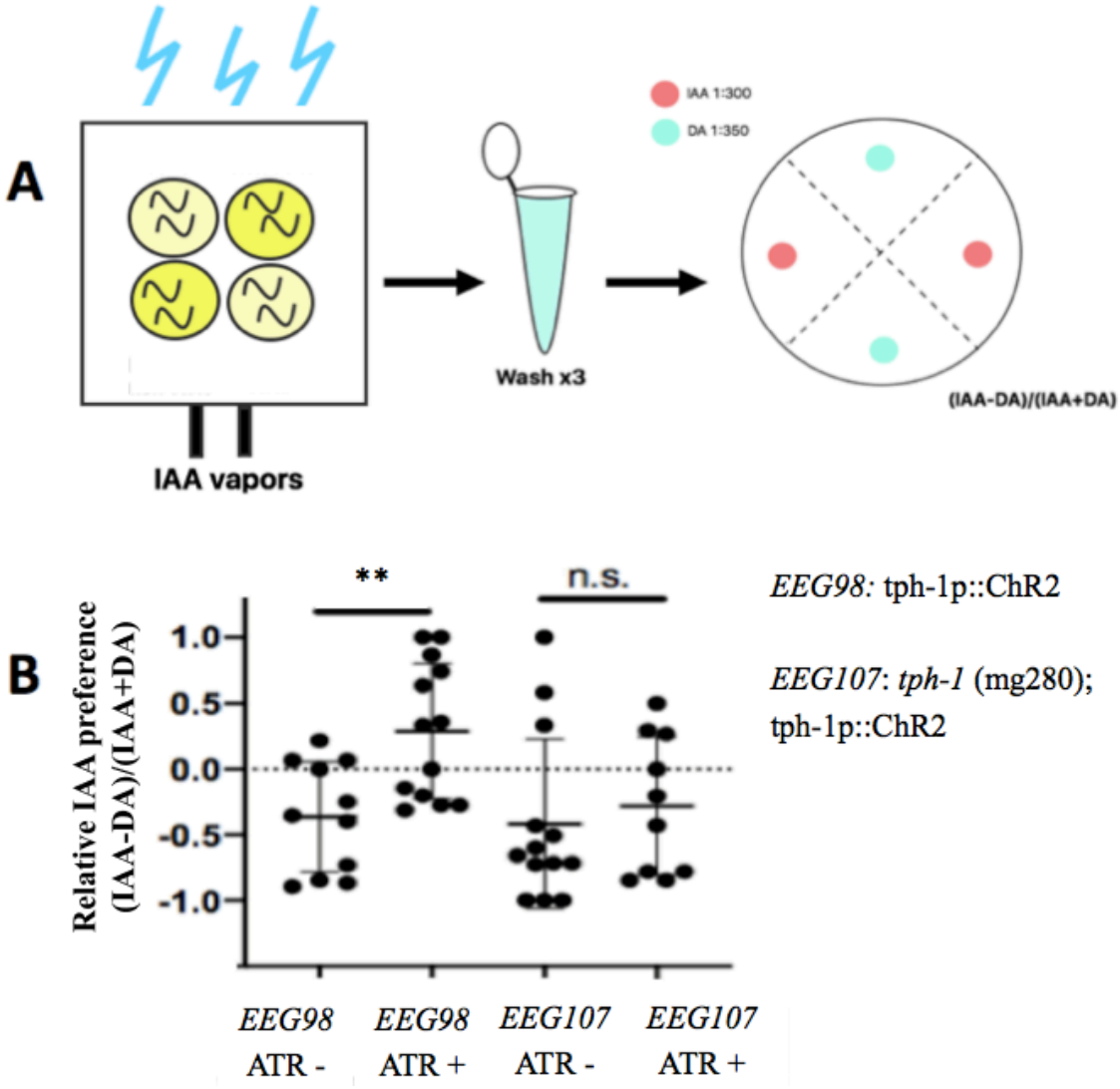

Figure 3

(**A**) OptoBox treatment protocol for paired IAA vapour administration and serotonin release. Two strains, both containing a tph-1p::ChR2 optogenetic construct were treated in the OptoBox for two hours: **EEG98**: tph-1p::ChR2 in an N2 background, **EEG107**: tph-1p::ChR2 in a tryptophan hydroxylase mutant (*tph-1*) background. Each strain was treated on both OP50 + ATR and OP50 – ATR. (**B**) relative IAA preference of each strain, +/- ATR. EEG98 worms demonstrated a higher affinity for IAA when treated on ATR, meaning that serotonin release during IAA administration increases worms' likelihood to prefer IAA upon subsequent presentation. EEG107, which is devoid of serotonin biosynthesis, does not show any significant difference between worms treated on OP50 + ATR and OP50 – ATR. ** = $p \leq 0.01$, n.s. = $p > 0.05$

A

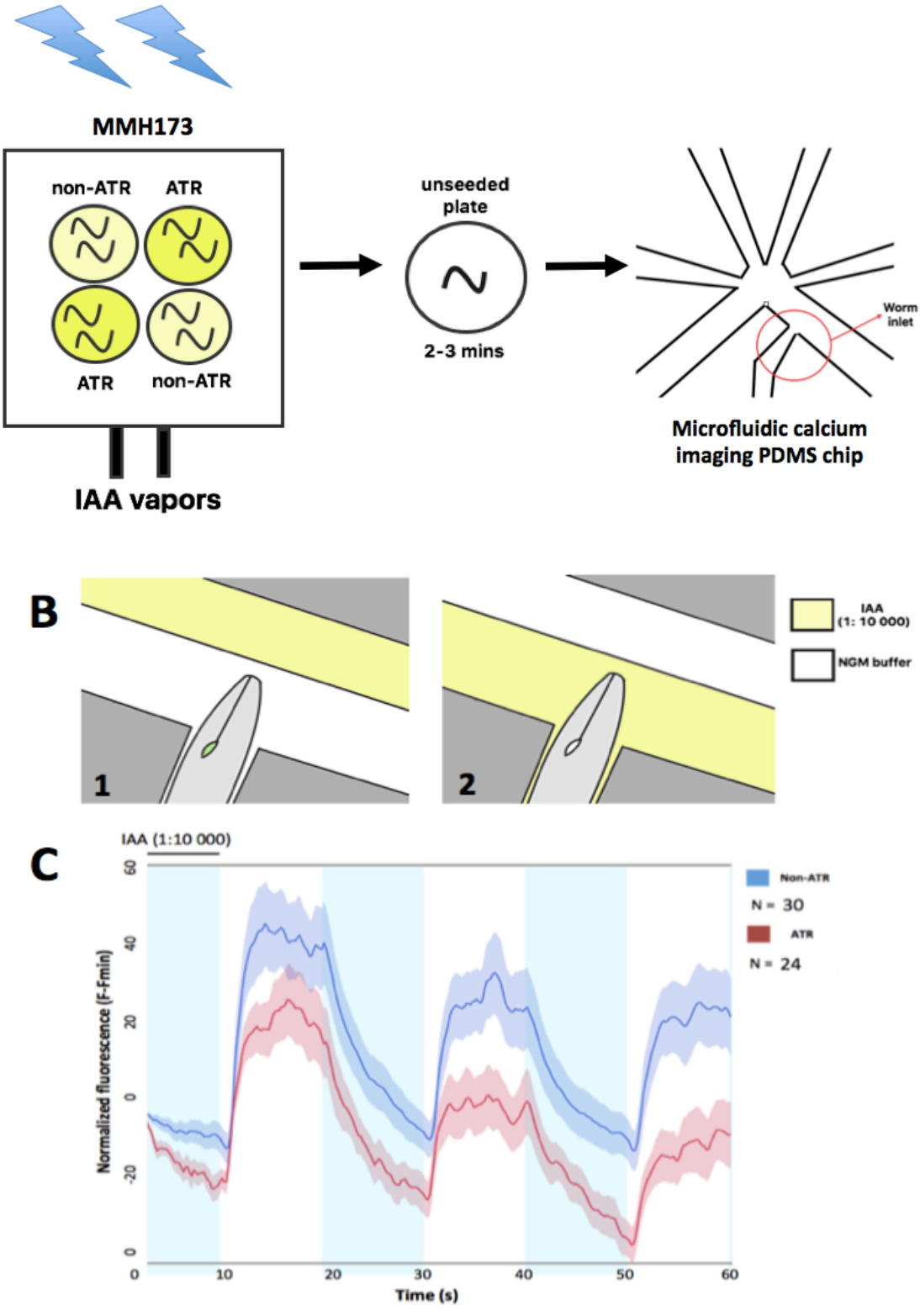
MMH173
non-ATR
ATR
ATR
non-ATR
IAA vapors
unseeded plate
2-3 mins
Worm inlet
Microfluidic calcium imaging PDMS chip
B
1
2
IAA (1: 10 000)
NGM buffer
C
IAA (1:10 000)
Normalized fluorescence (F-Fmin)
Time (s)
Non-ATR
N = 30
ATR
N = 24

Figure 4

(**A**) Overview of OptoBox training + calcium imaging protocol. MMH173 worms (expressing both AWC::GCaMP5 (CX17256) and ASH::ChR2 (AQ2335)) were treated, standard two hour treatment as in Figure 3, with IAA vapours and LED blue light pulses on regular and ATR-supplemented OP50. Next, worms were transferred onto unseeded agar plates and left to crawl for 2-3 minutes in order to remove residual bacteria from the worms' cuticles. Finally, worms are transferred into Polydimethylsiloxane (PDMS) microfluidic calcium imaging chips, where they enter the worm inlet (**B**) Schematic of the microfluidic platform used for restrained single-worm calcium imaging, design from Chronis, Zimmer & Bargmann (2007). Schematic demonstrates the worm held inside the worm inlet with its head exposed to a channel containing alternating streams of NGM buffer and IAA (1: 10 000) diluted with buffer (10 seconds of exposure per stimulus for one minute). Configuration 1 (left) shows the worm exposed to buffer, and AWC activated (green: GCaMP5) by the removal of IAA. Configuration 2 (right) shows the worm exposed to diluted IAA with AWC activity suppressed (white: low GCaMP5 fluorescence). Position of IAA/buffer determined by a programmable valve system. (**C**) Normalized fluorescence of AWC::GCaMP5 in ATR versus non-ATR treated worms (both cohorts treated with IAA vapours inside the OptoBox).

Conclusions

Research in the field of learning and memory over the past couple decades has demonstrated the involvement of serotonin in cellular plasticity as well as associative learning (Mauelshagen, Sheff & Carew, 1998; Marinesco, Wickremasinghe & Carew, 2006). In other words, serotonin can act cell-autonomously as well as on a network level to facilitate several forms of learning. As such, we chose to investigate the role of serotonin in an aversive olfactory conditioning paradigm. While previous studies have addressed the involvement of serotonin in forms of learning that require systemic stressors (i.e. starvation, heat stress), we used the OptoBox training paradigm in order to administer a non-systemic stressor: repetitive optogenetic stimulation of the polymodal ASH nociceptive neuron. Interestingly, worms with a defective tryptophan hydroxylase-1 (tph-1) enzyme (MT15434 strain), which show impaired associative learning when trained using systemic stressors (Nuttley, Atkinson-Leadbeater & van der Kooy, 2002),

also demonstrate reduced conditioned aversion to IAA when treated using the OptoBox. We took this to mean that serotonergic signaling is required for aversive conditioning in paradigms using both systemic (i.e. starvation) and non-systemic (ASH stimulation) unconditioned stimuli. We further investigated the involvement of serotonin by administering OptoBox training to a tph-1p::GFP reporter strain. In this way, GFP fluorescence was observed as a proxy to monitor serotonin production, seeing as tph-1 is the rate-limiting enzyme in serotonin biosynthesis. We observed a dose-dependent increase in GFP fluorescence intensity following training, but only when both ASH stimulation and IAA vapors were jointly present during training. This could indicate that the observed increase in serotonin production signals a sort of sensory input convergence, which is in line with previous reports that posit the involvement of serotonin in regulating cell excitability during multisensory integration (Tang & Trussell, 2017). Next, we chose to directly manipulate levels of serotonergic signaling during OptoBox training by administering OptoBox training (IAA vapors + blue LED pulses) to the optogenetic EEG98 (tph-1p::ChR2) and EEG107 (*tph-1*, tph-1p::ChR2) strains. The purpose of this experiment was to demonstrate whether pulses of serotonergic cell stimulation, presented in conjunction with IAA vapors, was sufficient to instill a behavioral change upon subsequent IAA exposure (assessed by counterattractant chemotaxis following training). Interestingly, EEG98 worms treated on OP50 + ATR show a higher relative affinity for IAA than EEG98 worms treated without ATR. Notably, the EEG107 strain, which cannot synthesize serotonin, does not show any differences in IAA preference whether worms were treated with or without ATR-supplemented OP50.

In short, three separate experiments were undertaken using the OptoBox training protocol to investigate the role of serotonin: (1) assessment of *tph-1* mutant worms' performance using post-training counterattractant chemotaxis assay (2) quantitative imaging of tph-1p::GFP fluorescence intensity following training and (3) treatment of two tph-1::ChR2 optogenetic strains, and subsequent behavioral assessment using counterattractant chemotaxis. Each line of investigation demonstrated a necessity for serotonin during training, and a correlation between increased serotonin production and increased olfactory associative learning.

Seeing as serotonin acts primarily on interneurons (as well as the ASH sensory neurons)[8], we chose to monitor activity in the AWC chemosensory neuron to ascertain whether serotonin-independent sensory changes occur during OptoBox training. Using a microfluidic chip to administer timed streams of either buffer or IAA + buffer, a genetically-encoded calcium indicator (GCaMP5) was imaged to infer AWC activity after OptoBox training with and without ASH optogenetic stimulation. While both ATR and non-ATR treated worms show generally similar response dynamics to IAA removal, ATR-treated worms demonstrated a lower response amplitude- this difference becomes progressively more pronounced upon subsequent odor switches. In this way, repetitive ASH stimulation appears to increase the cellular adaptation of AWC in response to periodic IAA removal. There is no direct synaptic communication between ASH and AWC neurons, and as such the observed differences in calcium activity are likely due to diffuse modulation caused by persistent ASH activity. Seeing as ATR-treated worms show increased tph-1p::GFP fluorescence following training, AWC activity could be affected by serotonin through the AIA interneurons- as AIA expresses the MOD-1 serotonin receptor and sends synaptic feedback to AWC neurons.

In summation, the preceding chapter demonstrates the necessity of serotonergic signaling in associative learning, even when the unconditioned stimulus used in a given learning paradigm is non-systemic (i.e. does not instigate a systemic stress response). However, we observed that increased serotonergic signaling can induce either learned aversion (**Figure 1, 2**) or learned affinity (**Figure 3**) following OptoBox treatment to AQ2335 and EEG98 strains, respectively. As such, further study is required to tease apart the possible involvement of different serotonin receptors in different forms of associative learning (aversive versus appetitive). Moreover, there is evidence to suggest that changes in glutamate levels can interact with serotonin signaling to result in modulated serotonin-associated behavior (Hukema, Rademakers & Jansen, 2008; Cunningham *et al*, 2012). As such, further study is required to characterize whether glutamate released during

[8] Several interneurons downstream of amphid sensory neurons (including AWC and ASH) express at least one type of serotonin receptor (Harris *et al*, 2009). Furthermore, many sensory neurons have also been found to respond to serotonergic feedback from interneurons (Harris *et al*, 2011).

repetitive ASH stimulation (of the AQ2335 strain) interacts with serotonin during associative learning.

In order to test hypotheses of either serotonergic or glutamatergic involvement in non-systemic associative learning, strains containing relevant mutations could be crossed into the AQ2335 optogenetic strain (ASH::ChR2) and screened using OptoBox training and subsequent behavioral or fluorescent imaging assessment. Suggestions of mutations related to altered serotonergic signaling include: (1) homozygote tryptophane hydroxylase-1 mutants (*tph-1*$^{-/-}$) were screened in Figure 1, although heterozygotes (*tph-1*$^{+/-}$*)* could be screened in order to assess the impact of reduced, but not ablated, serotonin biosynthesis. (2) strains that have been chemogenetically altered to restrict AIA synaptic input (ex. tetanus toxin or cell death-inducing protease CED-3[9] expressed specifically by AIA interneurons) (3) loss-of-function mutations in serotonin receptors expressed by ASH or downstream interneurons (ex. MOD-1, SER-4). In addition, candidate mutations related to glutamate signaling include: GLR-1 (ionotropic glutamate receptor expressed by AIB, an interneuron receiving convergent input from both ASH and AWC sensory neurons), GLR-2 (ionotropic glutamate receptor expressed by AIA and AIB interneurons) (Brockie *et al*, 2001). Testing strains with altered glutamate signaling should indicate whether the glutamate released during ASH stimulation modulates downstream interneurons in such a way that affects associative learning.

Additionally, the programmable nature of the OptoBox offers multiple applications besides mutational screening. For instance, the OptoBox offers an ideal platform for long-term, repetitive stimulation of targeted neurons, which could be paired with olfactory stimulation (as was the case in Figure 3, using tph-1p::ChR2 strains). The administration of LED light pulses for two hours does not initiate a heat shock response (Chapter 1, Figure 2) seeing as OptoBox training does not induce DAF-16 nuclear translocation. As such, the OptoBox provides a method for repetitive neuronal activation without causing physiological harm to the worms. Moreover, the frequency and duration of stimulus delivery is easily modulated using the programmable Arduino microcontroller, which synchronously controls both the LED drivers (blue light for

[9] CED-3: a caspase encoding gene. Driving the expression of CED-3 using cell-specific promoters has been demonstrated to lead to targeted apoptosis (Xue, Shaham & Horvitz, 1996).

activating optogenetic constructs) and gas valves (olfactory stimulation). Due to the precise temporal regulation of stimulus delivery, the OptoBox method is amenable to the study of how conditioning acts at varying timeframes, or the effect of different inter-stimulus intervals (ISIs) on conditioning.

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Appendix A

Arduino Microcontroller code for simultaneous blue light pulse and isoamyl alcohol (IAA) delivery

```
                                                    // assigns the number of the PWM output to
cons tint LED1 = 10;                                the control pin of the LED
cons tint LED2 = 11;
cons tint CSvalve = 5;                              // CSvalve: stimulus valve (IAA)
cons tint CONvalve = 6;                             // CONvalve: control valve (distilled water)


//---------- PARAMETERS----------//

const int lightONtime = 1;                        // ON duration each stimulation cycle
const int lightOFFtime = 1;                         // OFF duration each stimulation cycle
const int lightCycles = 20;                       // number of cucles per stimulus presentation
const int ControlStimTime = 1;                      // inter-trial interval
cons tint LEDbrightness = 255;                    //  0 = OFF, 255 = max brightness


// ----------//

void setup ( ) {                                                  // Defines these as outputs
        pinMode (LED1, OUTPUT);                                   //
        pinMode (LED2, OUTPUT);
        pinMode (CSvalve, OUTPUT);
        pinMode (CONvalve, OUTPUT);
        }


void loop ( ) {
                                                                    // close the control valve
        analogWrite (CONvalve, 0);                                  // open the CS valve with 12V
        analogWrite (CSvalve, 255);

                                                                    // cycle the LEDs with PWM output
      for (int i= 0; i < lightCycles; i = i+ 1) {                   to buckpuck timer
        analogWrite (LED1, 255 – LEDbrightness) ;                   // PWM signal to dimmer to turn
        analogWrite (LED2, 255 – LEDbrightness) ;                   LEDs on
        delay (lightONtime * 10000) ;
        analogWrite (LED1, 255) ;                                   // PWM signal to dimmer to turn
        analogWrite (LED2, 255) ;                                   LEDs off
        delay (lightOFFtime * 10000);
       } ;

        analogWrite (LED1, 255) ;


                                                                    // closes the CS valve
                                                                    // open the CON valve with 12V
```

```
analogWrite (LED2, 255) ;

analogWrite (CSvalve, 0) ;
analogWrite (CONvalve, 255) ;
delay (ControlStimTime * 600 000);
}
```