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Neural Signaling Dynamics of Conditioning in *C. elegans*

By

Micaela R. Pribic

Accepted in Partial Completion

of the Requirements for the Degree Master of Science

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Master's Thesis

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Date: April 15th, 2020

Neural Signaling Dynamics of Conditioning in *C. elegans*

A Thesis Presented to

The Faculty of

Western Washington University

In Partial Fulfillment

Of the Requirements of the Degree

Master of Science

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Micaela R. Pribic

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ABSTRACT

Retrograde signaling from downstream effectors (i.e., motor neurons) can modulate plasticity. Much research has focused on the learned association of closely timed sensory stimuli. By comparison, there is less research probing the potential influence of how or if activation at downstream neuromuscular junctions (NMJ) could modulate associative conditioning. Using channelrhodopsin activation of body wall muscle and different motor neuron subsets (cholinergic motor neurons that drive contraction and GABAergic motor neurons that drive relaxation of muscle) in the *Caenorhabditis elegans (C. elegans)* model system, we examined if concurrent excitation in these downstream circuits influences associative conditioning.

Conditioning consisted of pairing two distinct sensory stimuli, mechanosensory (vibration) and blue light (~480nm). Each stimulus drives a locomotor response on its own and we have shown that pairing delivery of these two stimuli alters the subsequent locomotor response to vibration. Animals that expressed channelrhodopsin in the body wall muscle (*pmyo*-3::ChR2), excitatory motor neurons (*punc*-17::ChR2) or the inhibitory motor neurons (*punc*-47::ChR2) received associative vibration-light conditioning. Thus, the blue light stimulus simultaneously functioned as both associating sensory stimulus and activator of channelrhodopsin, when the necessary cofactor was present, all-trans-retinol (ATR+).

Results showed wild type *C. elegans* typically pause for a longer duration following associative vibration-light conditioning. Following vibration-light conditioning, *pmyo*-3::ChR2 exhibited a complete disruption of learning. While trained ATR+ *punc*-17::ChR2 and *punc*-47::ChR2 animals showed partially disrupted conditioned locomotor behavior, as compared to controls. Together, this data suggests that co-activation of the downstream body wall muscle and motorneurons interferes with upstream associative conditioning.

Key Words: Acetylcholine - Associative Conditioning – Body Wall Muscle - *Caenorhabditis elegans* – GABA - Learning - Neuromuscular Junction - Optogenetics

Abbreviations:

ACh-Acetylcholine

BWM – Body Wall Muscle

ChR2 – Channelrhodopsin

CR – Conditioned Response

CS – Conditioned Stimulus

NS – Neutral Stimulus

UR – Unconditioned Response

US – Unconditioned Stimulus

WT – Wild Type

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GENERAL INTRODUCTION

From *C. elegans* and *Aplysia* to mice and humans, animals can integrate multiple sensory inputs, such as smell, touch, and sight, into associative memories (Gawronski and Bodenhausen, 2014; Lattal and Delamater, 2014). For instance, people can have distant memories brought to our attention by the simplest of sensory cues. The smell of chocolate might remind you of grandmother's famous chocolate cake even if she isn't by your side or no chocolate cake is within sight. In an experimental context, an association can be formed between a harmless stimulus (ex. flash of light) and a painful stimulus (ex. electric shock), after a few quickly timed presentations (Cheng et al., 2003). This leads to a conditioned startle response at the flash of light in the absence of the shock. Thus, recall of the association is involuntary and reflexive.

Given the involuntary nature of reflexive responses, model organisms are used to study associative learning in greater cellular and molecular detail. For example, the marine mollusk, *Aplysia,* shows a stronger siphon retraction response to touching of the animal's gill after repeated pairings with electric shock (Carew, Walters, and Kandel, 1981). Another example, *Caenorhabditis elegans* (*C. elegans). C. elegans* can associate olfactory cues (smells) with the presence or absence of food and respond by moving towards (or avoiding) the olfactory cue as is contextually appropriate (Saeki, Yamamoto and Lino, 2001). These are examples of animals with simple nervous systems that demonstrate involuntary learning. Using model organisms like *Aplysia* and *C. elegans*, has allowed for studies to bridge the gap between how the nervous system changes in response to conditioning, and confirmed by behavioral phenotypes (Walters and Byrne, 1982).

Changing behavior is a result of underlying neural plasticity. This plasticity is marked by physical changes in local connections (i.e. neuron to neuron, at the synapse), as well as more diverse rewiring in connectivity of larger circuits that are thought to represent the previous experiences, also known as an "engram" or "neural network" (Barron et al., 2017). Simply put, behavioral plasticity is a result of changing the strengths of synaptic connections between the sensory neurons and interneurons, which then

initiate a motor response, resulting in new behavior, that is quantifiable (Malinow and Malenka, 2002; Svoboda and Holtmaat, 2009). Investigation of plasticity in greater molecular detail highlights the abundance of signaling molecules, known as neurotransmitters and neuropeptides, which differentially affect how neurons "speak" to one another, and how they "wire" together in larger circuits. Specifically, the balance between excitatory and inhibitory keep these circuits "in-check" and allows for memories to be brought forth at the appropriate time (Barrows et al., 2017).

Using *C. elegans,* researchers have a unique array of methods and techniques to understand how cellular and molecular changes in the nervous system can modulate learning and memory. These benefits include, but are not limited to, a fully sequenced genome (Hillier et al., 2005), a completely mapped nervous system (Varshney, et al., 2011), and transparent outer cuticle allowing for direct fluorescence imaging and optogenetics (Nagel et al., 2005; Kerr, 2006; Liu, Hollopeter and Jorgensen, 2009). Pairing the in-depth molecular and cellular knowledge of the worm, with well-known behavioral patterns and phenotypes (Yemini, et al., 2013), as mediated by a well-understood locomotor system (Liu, Hollopeter, and Jorgensen, 2009; Haspel, O'Donovan, and Hart, 2013), it is possible to gain a more complete understanding of what a nervous system can do in an intact and moving animal.

In this study, we use a rapid associative conditioning assay to condition worms that vibration will precede the onset of blue light, henceforth known as vibration-light conditioning. After training, we present the vibration alone, one-, five- and ten-minutes after vibration-light conditioning, then measure changes to locomotor behavior, as compared to untrained controls. Since testing occurs within minutes after training, we are measuring locomotor behavior immediately following the period of learning. To quantify changes in locomotor behavior, which are our markers of learning, we measure time and distance ratios of various *C. elegans* motor patterns, such as forward swimming, backward swimming, pausing (no movement) and omega turns (complete change of direction). In this research, our assay successfully conditions the locomotor behavior of wild type animals, as being marked by increased time spent pausing and reduced forward and backward locomotion. To further assess how overstimulation of different neural

circuits could disrupt or enhance learning, we utilized optogenetic strains of *C. elegans* that activate body wall muscle (*myo*-3), cholinergic (*unc*-17) or GABAergic (*unc*-47) sub circuits, at the exact time of vibration-light conditioning. Results showed overstimulation of the body wall muscles during conditioning ablated conditioned locomotor behavior. While overstimulation of GABAergic and cholinergic sub-circuits resulted in modified conditioned locomotor behavior. These experiments allow us to assess how learning is affected by downstream activation of locomotor responses.

INTRODUCTION

PART I: CLASSICAL CONDITIONING

Classical Conditioning

Associative conditioning is what allows an organism to learn a sequential, cause and effect relationship of events. Allowing the organism to generate an appropriate response for any number of situations that may occur. Research into how organisms learn predictable relationships between stimuli to then eventually change their behavior is known as Classical Conditioning (Domjan, 2015).

Classical Conditioning was first reported by the Russian physiologist, Ivan Pavlov. In Pavlov's work, he noticed the dogs he worked with would salivate not only when food was available in their dish, but when the food was within sight or even when just seeing the person who usually fed them. After further investigation, Pavlov laid the framework for classical conditioning. During the conditioning period, a neutral stimulus (NS) is presented with an unconditioned stimulus (US) several times. Eventually, the NS will become associated with the US, and will then be known as the conditioned stimulus (CS). This CS will then elicit a conditioned response (CR) on its own. It is important to note that a US will always elicit an unconditioned response (UR) by itself, as it is an automatic response. For example, food (US) being placed in front of a dog will always elicit salivation (UR). On the other hand, the CS (bell) does not elicit any response before conditioning, but after several US-CS pairings (ie. food + bell), the CS alone will eventually elicit the CR (salvation). Since the change in behavior is due to an association between the CS and US, it requires no higher-level cognition, the CR is due to an involuntary physical process in the animal's nervous system. From the work of Pavlov, many researchers have explored a wide variety of stimulus combinations and measures of several CRs, as discussed below.

Conditioning Procedures

Several options of conditioning procedures are employed in most learning and memory laboratories; delayed conditioning, trace conditioning, simultaneous conditioning, and backward

conditioning. The variation in timing and order in which the CS and US are presented lead to significantly varied results and rates of acquisition (or time it takes to fully learn the new association), as well as activating different behavioral and neuronal mechanisms (Bangasser et al., 2006). The optimal association typically follows delayed conditioning, where the CS is presented first, then with some delay, the US is presented. Delayed conditioning tends to be the most consistent, likely due to the distinct presentation of the CS alone, before the onset of the US. Some examples of delayed conditioning being; a tactile stimulation paired with electric shock (Antonov et al., 2001), odor paired with electric shock (Quinn, Harris and Benzer, 1974) or odor paired with tone and flavored pellet (Chang et al., 2015). All components of the classical conditioning assay are modifiable regarding intensity, salience, and timing. Thus, researchers must identify stimuli and procedural parameters that will be biologically appropriate for the organism, and produce a measurable change in response after conditioning.

Conditioned and Unconditioned Stimulus

The choice of stimuli for a classical conditioning procedure is important for the success of learning an association between the CS and the US. Most notably, the more intense or salient the US or CS, the faster the acquisition of the association (Imada, Yamazaki and Morishita, 1981). Some examples include varying intensity of foot shock and subsequent rate of inducing conditioned fear response (Olshavsky et al., 2013), or how intense the light is as a method of inducing eye blinks (Braggio and Putney, 1975). Another consideration when choosing stimuli is how biologically relevant the CS-US pairing are with one another (Öhman, A., & Mineka, 2001). The idea being that certain cues are important to an animal's survival and thus are more likely to be noticed and associated faster. An example being rats are more likely to associate audiovisual cues with a foot shock, but not as well with taste (Garcia and Koelling, 1966). This is likely due to the audiovisual CS paired with the feeling of electric shock mimics the natural cause and effect relationship of a predator being seen or heard and then the animal being physically attacked. However, when the reinforcer is a sickness (done via radiation), the more appropriate CS-US pairing is with flavored pellets, as this mimics the cause and effect relationship of taste aversion,

where the animal avoids something due to a previous association with physical illness (Garcia and Koelling, 1966).

Measured Conditioned Response

Once the conditioning schedule has been chosen and pairings are presented several times, the animal will reach consistent responsiveness (More and Jensen, 2014). This is known as acquisition and would indicate the organism has learned the association between the CS and the US. However, to truly assess the association between the CS and US, one must test the organism's response to the CS alone. This allows investigators to measure the CR without the influence of the US. Because the CR is due to the CS-US pairing, and thus mimics the UR, the resulting behavioral responses are involuntary and predictable. Some examples of measurable CR in model organisms include anything from a rabbit's eye blink (Weeks, et al., 2007), an odor avoidance in *Drosophila* (Quinn, Harris, and Benzer, 1974), siphon contraction in *Aplysia* (Carew, Walters and Kandel, 1981) or increased freezing behavior in rats (Hobin, Goosens and Maren, 2003). Typically, the magnitude of the response (the robustness of response), probability (the likelihood of response occurring) and latency (time until onset of the conditioned response) is used as a measure of the CR and establishes a method of quantifying learning (Schreurs and Alkon, 1990).

Brief Summary of Classical Conditioning and Associative Learning

The CS-US relationship is dynamic, yet predictable when it comes to generating the CR. Observed differences in intensity and salience of the US, how biologically relevant the chosen CS, the order in which the CS-US is presented will affect the rate of acquisition. Once the CS alone begins to generate the CR, a newly learned behavior is acquired, but not by conscious thought, as associative conditioning and behavioral changes have been observed in many species (e.g., primates, dogs, rats, pigeons, sea slug, and fruit fly). Due to the involuntary nature of associative conditioning, it is the modifications to the nervous system that incorporates relevant information and shapes new responses. Hard evidence of underlying circuit plasticity was first recorded in the sea slug, *Aplysia*, where removal

of the ganglion (central nervous system) resulted in disruption of the conditioned siphon withdrawal reflex. Effectively providing hard evidence of the nervous system mediating the CS-US association (Carew, Walters, and Kandel, 1981). Behavioral observations paired with associative conditioning of model organisms allows investigators to further study the underlying dynamics of neural plasticity of conditioning in a laboratory environment.

Connectivity and Signaling in the Nervous System after Associative Conditioning

Research into the neurobiology of learning and memory focuses on changes in connectivity between neurons and of overall larger changes in the neural circuit. Generally, the pipeline of how a nervous system responds to the environment is as follows: sensory neuron (SN) responds to various environmental stimuli and synapses with downstream interneurons (IN) that summate the various inputs from many SNs and collectively send a signal to further downstream motor neurons (MN) that synapse with body wall muscle (BWM). Upon BWM activation or inhibition, a locomotor response (i.e., the measurable behavior) is generated. Considerable research has gone into uncovering the molecular and cellular details of how individual synapses and larger populations of neurons change in response to modifications in neuronal firing and allow for the learning and subsequent retention of new information (for review: Abel and Lattal, 2001). Physical storage of memories is dependent, in part, on regulation and remodeling of the synapse, which is sensitive to incoming information from the pre-synaptic neuron. Upon repetitive signaling from the same pre-synaptic neuron, increased connectivity and sensitivity occurs, this is known as long-term potentiation (Bliss and Lomo, 1973). For example, the memory of an event induces the strengthening of synapses (where one neuron projects onto another) by increasing glutamate receptor localization (AMPA-type and NMDA-type receptors) at the synapse in response to increased activity by pre-synaptic neurons, this is known as long-term potentiation (Bliss and Collingridge, 1993). Additionally, increased strength of synapses within a circuit, or neural network, allows for the memory to be encoded and recalled at the appropriate moment (Anderson, 1972; Barron, et al., 2017). It is generally well supported that there are physical changes in the nervous system that

underlie learning and memory, specifically with changes at the synapse, and with overall signaling patterns in the cortex (for review: Holtmaat and Svoboda, 2009). However, newer research suggests long-term memory is stored and encoded by epigenetic modification in the DNA of trained cells (Bedecarrats, et al., 2018).

Early research elucidating neural plasticity favored *Aplysia* for its easily accessible nervous system and well-established siphon withdrawal response (Kriegstein, Castellucci and Kandel, 1974). To classically condition *Aplysia*, researchers would pair several intracellular activations of tail SNs (CS), mediated by the electrode implants, with a shock to the skin (the US), to then measure the CR (siphon withdrawal response). One notable benefit of the siphon withdrawal response in *Aplysia,* due to the easily accessible nervous system, it is possible to use electrophysiology to measure real-time changes in amplitude and frequency of neuronal firing in subpopulations of neurons that make up the CS-US pairing. Thus establishing the relationship between cellular changes in the nervous system and the acquisition of associative learning. In *Aplysia,* Walters and Byrne (1982) showed CS-US pairings resulted in enhanced excitatory postsynaptic potentials of the MNs, suggesting that changing neuronal firing patterns in MNs were due to experience; and these changing neuronal firing patterns predicted the CR. Looking deeper, investigation of how specific chemical modulators generate the CR, Ocorr, Walters, and Bryne (1985) used a modified conditioning protocol, by measuring levels of cyclic adenosine-monophosphate concentration (cAMP: a modulator of neuronal connectivity) following CS-US pairings of high potassium seawater (CS) and serotonin exposure (US) in SNs. Serotonin mimics the sensitizing electrical stimulation of the US (tail shock), and its effects were thought to be mediated by cAMP. Using this experimental setup, researchers showed for the first time that a chemical transmitter (serotonin) mediates activity-dependent modulation of cAMP; and that chemical neuromodulators are involved in managing the CR (Ocorr, Walters, and Bryne, 1985). Further dissection of the *Aplysia* central nervous system allowed for direct recording of specific neurons mediating the siphon withdrawal circuit. In Antonov, et al. (2001), the pre-synaptic activity of the SN modulated firing onto the downstream MNs; this change in

presynaptic potential was altered after conditioning with the CS-US pairing of tap to the siphon and shock of the tail. These recordings of presynaptic activity suggest that learning the CS-US association involves modulation of both pre- and postsynaptic sensitivity and connectivity to generate a CR.

Looking to understand morphological and connective changes that mediate associative conditioning in mammals is harder, due in part to the complexity of the nervous system. Recent advancements in computer modeling have helped to shed light on nuanced control of associative conditioning in the rodent. Kim et al. (2015) identified competitive synaptic interactions that underlie a fear memory trace in the lateral amygdala of the rat. The amygdala is a brain region known for encoding fearful experiences (Campeau, Miserendino, and Davis, 1992). After modeling the region, it was found that the plasticity of synapses between different neuronal types (principle neuron or inhibitory interneuron) in the lateral amygdala determines how generalizable a conditioned fear response becomes (Kim et al., 2015). Notably, greater principle neuron plasticity shows less specific conditioned fear response, while greater inhibitory interneuron plasticity shows greater specificity of the conditioned fear response. These findings, both in *Aplysia* and in computer modeling of the rodent brain sub-regions, suggest that diversity in order and excitability, as well as chemical transmitter, makeup, and neuronal type, within the same brain region or neural trace, can lead to the specific acquisition and memory of classically conditioned events.

Brief Summary of Connectivity and Signaling in the Nervous System

This research highlights how structural remodeling mediates neuroplasticity which underlies the changes in connectivity that support CS-US pairings that are known as associative conditioning. Historically, there has been a heavy focus on connections between SN-IN connectivity, with the primary study of unidirectional flow of information in the nervous system, as it changes during and after conditioning. Yet, driving inhibition in specific brain areas of the rat disrupted the acquisition of a conditioned fear response to shock (Assareh et al., 2017) and that overexcitation of specific brain regions during a prediction assay increased subsequent prediction error (Chang et al., 2016). It is in this research

we focus on how learning in an associative conditioning assay is affected following targeted activation of downstream IN-MN and MN-BWM connections. Given the wide variety of experimental tools and the simpler nervous system, we use *Caenorhabditis elegans (C. elegans)* in our study of classical conditioning.

PART II: LEARNING IN *C. ELEGANS*

Caenorhabditis elegans as a Model Organism

C. elegans was first proposed as a model organism by Sydney Brenner in 1963 and is now regularly used in cellular and molecular research. Their use has rapidly expanded due to their small size (~1mm in length), hermaphroditic nature that keeps a genetically consistent stock and growth to adulthood which takes 3-4 days (Stiernagle, 2006). In *C.elegans*, the hermaphrodite has 959 somatic cells, with 302 of those cells being neurons (White, Thomson and Brenner, 1986). Additionally, the animal has the first fully sequenced genome (Hillier et al., 2005). *C. elegans* have a transparent exterior cuticle which allows for easy fluorescence imaging of gene expression and protein localization (Kerr, 2006). Remarkably, *C. elegans* was the first multicellular organism to have green fluorescent protein (GFP), expressed in its nervous system, guided by the *mec*-7 promoter, resulting in specific GFP expression in the six touch receptor neurons (Chalfie et al., 1994). Further development of fluorescent imaging techniques of neuronal signaling, known as calcium imaging, where a fluorophore changes conformation in the presence of calcium, allows for tracking and monitoring of neuronal activity (Suzuki et al., 2003). Lastly, optogenetics is a non-invasive method of depolarizing (channelrhodopsin) or hyperpolarizing (halorhodopsin) specific populations of neurons by shining of blue or green light respectively (Nagel et al., 2005; Liu, Hollopeter and Jorgensen, 2009). These benefits have allowed laboratories to use this small nematode in many dynamic ways, especially in the field of neuroplasticity, as pertains to worm learning and memory (White et al., 1986).

Neurobiology of *C. elegans*

C. elegans have only 300 neurons, of which they have the first and only Connectome. The Connectome is a complete map of all sensory, inter and motor neurons with identified connections between different neurons; these neuronal links consist of ~7,000 chemical and ~3,500 electrical connections (White et al., 1986; Varshney et al., 2011). This significant collection of work has allowed researchers to map specific sub-circuits that respond to distinct stimuli and initiate specific locomotor responses (Haspel, O'Donovan and Hart, 2010; Kato et al., 2015). For instance, the mechanosensory circuit (Wicks and Rankin, 1995), olfactory circuit (Chalasani et al., 2007) and thermosensory circuit (Mori and Ohshima, 1995). To respond to a dynamic environment, single neurons typically receive multiple signals and send out different signals to drive distinct, context-dependent locomotor responses (Li, et al., 2014; Tao, et al., 2019). For example, the sensory neuron ASH has olfactory and mechanosensory receptors, and either receptor activation drives the release of dual transmitters to change behavior appropriately (Maricq, et al., 1995). To drive reversals in response to gentle nose touch, glutamate is released onto downstream command interneurons driving locomotor behavior while olfactory sensation drives another transmitter to modulate behavior accordingly (Maricq, et al., 1995). This early work highlights the functionality of the *C. elegans* nervous system, as it is important to appreciate how basic neuronal function occurs so that the mammalian nervous system will eventually be better understood. Especially since many fundamental genes and proteins required for learning and memory in the *C. elegans* are homologous to many genes and proteins found in vertebrate systems (Stein and Murphy, 2014; Lakhina et al., 2015).

Associative Conditioning in *C. elegans*

Studying learning in *C. elegans* is common, as these animals exhibit context-specific behavioral plasticity. It is important to highlight that nonassociative learning, such as habituation (decreased responsiveness to a repetitive stimulus) and sensitization (increased responsiveness to repetitive stimulus), changes the magnitude of stereotypical response. For example, *C. elegans* will habituate to a

repetitive presentation of a single stimulus and decrease responsiveness after several presentations (Rankin, Beck and Chiba, 1990). Retention of this habituation is demonstrated 24 hours later (Beck and Rankin, 1997). This decrease in responsiveness is not a result of sensory adaptation (i.e., the sensory receptors are overwhelmed and cannot signal) or fatigue (i.e., the changed behavior occurring because the animal itself is too tired to respond). Unlike associative conditioning, changes in responsiveness are not caused by an association with a positive or negative, neutral stimulus, like you would expect with classical conditioning.

C. elegans can associate two distinct stimuli, and change their behavior (usually observed by locomotor changes) depending on the nature of the association. Two common assays used to study associative conditioning in *C. elegans* are chemotaxis (association of a chemical with the US) and thermotaxis (association of temperature with the US). These procedures using this model organism allow researchers to bridge gaps between genes, nervous system circuitry and conditioned behavior. In chemotaxis assays, *C. elegans* are presented with a chemical CS, usually NaCl (due to the animal's natural chemotaxis behavior towards NaCl) and presence or absence of food (US). Depending on how favorable the resulting association is, the animals either swim towards (positive chemotaxis) or avoid (negative chemotaxis) the conditioned chemical, based on if food is associated with that chemical CS. These subsequent behavioral changes can be seen soon after conditioned incubation with the chemical CS (~4 hours) and up to 24 hours later (Saeki, Yamamoto and Iino, 2001; Amano and Maruyama, 2011). To highlight how sensitive *C. elegans* are to chemical cues, by varying the concentration of NaCl used during cultivation will result in the animal seeking the specific NaCl concentration the animal was reared at (Lou, et al., 2014). This further supports the animal's retention of the associative memory up to a day later and reinforces the notion that *C. elegans* can exhibit experience-dependent behavioral plasticity.

C. elegans can also form associations with temperature. In thermotaxis assays, animals will move up, down or show no locomotive pattern, along a thermal gradient, usually 15ºC – 25ºC. In standard thermotaxis experiments, *C. elegans* exhibit associative learning with their cultivation temperature (CS),

as it will indicate food (US), in which the animals will migrate toward that cultivation temperature at testing (Hedgecock and Russell, 1975). Further assessment with learning mutants will show atypical thermotaxis behavior. If the animals are learning defective, they show reduced or no thermotaxis towards the cultivation temperature (Biron, et al., 2006) or otherwise atypical temperature responses - such as cryophilic behavior for colder temperatures or thermophilic behavior for warmer temperatures (Mori and Ohshima, 1995; Land and Rubin, 2017). Furthermore, these animals can "un-learn" associations previously made, if the thermal temperature is no longer presented with food for several hours, known as extinction (Li et al., 2013). Showing extinction supports the idea that *C. elegans* exhibit context-specific behavioral plasticity that is more dynamic than once thought. Research published by Gomez et al. (2001) showed animals that were presented with food in an environment at 25ºC, not the rearing temperature originally associated with food, exhibited thermotaxis behavior towards 25ºC when tested. However, when then introduced to food at 20ºC, the animals showed a lesser thermotaxis index at 25ºC, with greater thermotaxis towards 20ºC again, suggesting the previously learned association was updated with new information (Gomez et al., 2001). These data, as gathered by chemotaxis and thermotaxis experiments, support experience-dependent associative learning in the worm.

It is important to clarify that chemotaxis and thermotaxis protocols require a minimum incubation period of one-hour, yet it is feasibly possible that learning occurs more rapidly. In this research, we demonstrate learning in *C. elegans* using a rapid associative conditioning assay. This assay consists of five delayed US-US pairings, not the usual CS-US pairings, which will be discussed later on. Of the delayed US-US pairings, a five-second mechanosensory vibration (US; 300Hz) is overlapped with a three-second blue light illumination (US; ~460nm) and both stimuli terminate together. Between pairings, there are 60 seconds between intertrial intervals. As established during testing, a single presentation of the five-second vibration, unless otherwise specified, is presented at different retention periods: one-, five- or ten-minutes postconditioning. These retention periods are closer in time to our training assay and thus focus on learning (< 1-hour post-training), as opposed to a short-term association (2+ hours) or long-term

memory (16-40 hours) (Kauffman, et al., 2011; Kauffman, et al., 2010). Changes in locomotive behavior - forward movement, backward movement, pauses and omega turns are quantified and used as our measure of learning. This vibration-light assay demonstrates that *C. elegans* are capable of conditioned locomotor behavior within minutes of learning.

Mechanosensation and Photosensation

Vibration and blue light are two distinct and aversive stimuli that result in competing locomotor, responses (CR). Repetitive non-specific mechanosensory stimulation results in increased reversal frequency and magnitude in wild type *C. elegans (*Wicks and Rankin 1995). The mechanosensory response is mediated by six mechanosensory neurons located at the anterior (ALMR/L, AVM) and posterior (PLMR/L, PVM) of the worm (Chalfie et al., 1985). Regarding photosensation, wild type animals are non-responsive to green light and most responsive to UV and blue light, showing increased forward locomotion under whole-body illumination (Edwards et al., 2008). Early work for this thesis showed wild type *C. elegans* had a similar lack of response to green light, data not shown. Within the Edwards et al. (2008) study, LITE-1 expressing interneurons (AVG and PVT) were identified as driving neurons, mediating the locomotive response. An additional study by Ward et al., (2008) showed an avoidance response that depends on either head or tail specific activation, resulting in reversals or forward locomotion, respectively. More recent work by Bhatla and Horvitz (2015) shows LITE-1 expression in several additional sensory neurons and interneurons, suggesting a complex photosensitive circuit. (See Figure 3, for a schematic of converging mechanosensory and photosensory circuits driving differing locomotor behavior).

We use a vibration-light associative conditioning assay and each stimulus is biologically relevant to *C. elegans*. Evolutionarily, there is a class of predacious fungi that form hyphal rings that upon touch, contract and ensnare the worm (Perri and Alkema, 2012). It is thought that the rapid escape response (e.g., reversal or acceleration) evolved as a method to escape being eaten, as animals with deficits in touch sensation show an increased probability of being trapped (Maguire, et al., 2011). On the other hand,

prolonged exposure (e.g., 20+ minutes) to UV light resulted in paralysis and eventual death of the animal (Ward et al., 2008). Since *C. elegans* are a soil-dwelling nematode photosensation likely evolved as a response to keep the animal safe from UV mutagenesis, by directing locomotor responses based on the anatomical location being exposed (Ward et al., 2008). Because both stimuli always result in a locomotor response (UR), this pairing is technically a US-US association, otherwise known as beta conditioning. Beta conditioning is a form of associative conditioning in which both stimuli will always generate a response (Schreurs and Alkon, 1990; Choi, et al., 2011). In this research, it is important to ensure the animal is detecting both stimuli, hence why conditioning with the traditional CS-US pairing would be impossible. Using our US-US (vibration-light) assay, we were able to verify that both vibration alone and light alone were being detected, as observed by immediate locomotor responses (observational data).

The Motor Circuit and Neuromuscular Junction

Understanding the *C. elegans* motor circuit is key to this research, especially since our measure of learning and conditioned behavior is the change in the proportion of time and distance the worm travels after vibration-light conditioning. To start, the motor circuit is comprised of parallel excitatory and inhibitory motor neurons that synapse with body wall muscles, and thus form the neuromuscular junction (NMJ) (Schuske, Beg, and Jorgensen, 2004). As body bends are initiated to produce forward or backward swimming, each segment initiates body wall muscle contraction by acetylcholine release, while the parallel segment relaxes the opposite body wall muscles by inhibitory GABA release (Richmond and Jorgensen, 1999; Bono and Maricq, 2005). This synchronized release of opposing neurotransmitters, mediated by graded synaptic transmission leads to a sinusoidal like swim pattern (Liu, Hollopeter, and Jorgensen, 2009).

Regarding the identity of the motor neuron sub-circuits that make up the neuromuscular junction, there are A-type, B-type and D-type motor neurons. A-type and B-type are excitatory motor neurons that release acetylcholine into the neuromuscular junction upon activation, and further studies indicate A-type motor neurons mediate reversals and B-type mediate forward locomotion (Haspel, O'Donovan and Hart,

2010). D-type motor neurons cause the parallel body wall muscle to relax (Schuske, Beg, and Jorgensen, 2003). See figure 1A for a schematic of connectivity of the neuromuscular junction and with respective input from upstream command interneurons.

It is the command interneurons (which receive multiple inputs from sensory neurons) that are the key to *C. elegans* generating appropriate locomotor behavior in an ever-changing environment (Xu, et al., 2011). Command interneurons (AVB, AVA, PVC, AVD, and AVE) have been characterized and are shown to initiate specific locomotive responses due to their connectivity with A-, B- or D- type motoneurons (Bono and Maricq, 2005). Liu et al. (2017) demonstrated that the strength of acetylcholine signaling from AVA onto A-type motor neurons is modified by retrograde signaling via gap junctions from A-type motor neurons back onto AVA. Disruption of these gap junctions resulted in defective backward, but not forward, locomotion. Overall, circuit connectivity affects locomotor behavior and in this research, we assess if co-activation of body wall muscle, cholinergic or GABAergic circuits during conditioning effect learning in *C. elegans.* See part III, Optogenetics, for discussion of how to achieve temporally precise sub-circuit activation.

PART III: NEURAL SIGNALING AND DYNAMICS

Neurotransmitters in Learning

Research into the specific roles of neurotransmission in the central nervous system has advanced learning and memory research, particularly with neural plasticity. For example, glutamate is a primary excitatory neurotransmitter that modifies synaptic plasticity throughout the nervous system, thus underling some cue responses (Reiner and Levitz, 2018). Other signaling molecules involved in both mammalian and *C. elegans* nervous systems are GABA, a common inhibitory neurotransmitter that causes hyperpolarization of the neuron, and acetylcholine (ACh), a common neuromodulator in the central nervous system. Defining a neurotransmitter as excitatory or inhibitory simply means the chemical

signal either increases or decreases the target cell's chance of firing, while a neuromodulator can act as both am inhibitory or excitatory signal, depending on the target type (Iversen, et al., 2009).

With long-term potentiation, different kinds of chemical transmission can alter the structure of the target post-synaptic membrane and thus neural plasticity. Cholinergic input from the amygdala into the hippocampus shows activity-dependent pre- and post-synaptic firing that leads to induction of long-term potentiation in rat hippocampal cultures (Adams, Winterer, & Müller, 2004). While GABAergic input from inhibitory interneurons in rat hippocampal neuron culture shows an inhibition of long-term potentiation of excitatory synapses, which is thought to flexibly tailor excitability in the hippocampal circuit (Chapman, Perez, & Lacaille, 1998). Since long-term potentiation and the modulation of synaptic connection is the basis of neural plasticity, understanding the different effects of neurotransmitters, especially glutamate, GABA and ACh in learning and memory is necessary.

Glutamate: in Learning and *C. elegans*

Glutamate is a prevalent neurotransmitter in the mammalian nervous system and glutamatergic signaling is known to promote excitatory signaling by activating ionotropic glutamatergic receptors and/or subsequent activation of metabotropic glutamate receptors on the post-synaptic neuron (Reiner and Levitz, 2018). Mechanistically, long-term potentiation, as mediated by glutamate signaling, is shown to initiate increased AMPA receptor localization on the postsynaptic neuron as mediated by CaMKII (a calcium indicator) (Kristensen, et al., 2011). Both receptor types, AMPA and NMDA, are common and work together to promote neuronal plasticity (Reiner and Levitz, 2018). While glutamatergic-ionotropic NMDA receptors are involved in long term potentiation by inducing increased calcium levels in postsynaptic neurons, leading to higher excitability and synapse strengthening (Boehm and Malinow, 2005). Glutamate signaling has been well characterized in its role of neural plasticity in mammalian nervous systems.

In *C. elegans*, GLR-1 is an AMPA type - ionotropic glutamate receptor (Hart et al., 1995) and is responsible for mediating noxious avoidance responses to a variety of stimuli (Bono and Maricq, 2005). Responses to noxious stimuli can be modified by changes in neuronal activity, primarily by varying localization and abundance of GLR-1 at the synapse. Examples of misregulation of the glutamate response, as shown by the loss-of-function mutant, *unc*-43 (a CaMKII homolog), show reduced localization of GLR-1 at the synapse, and subsequent accumulation of GLR-1 in the cell body. While additional loss-of-function mutants, *unc*-2 (a voltage-gated calcium channel) and *eat*-4 (a vesicular glutamate transporter), both required for plasticity, show overexpression of GLR-1 at the synapse (Grunwald et al., 2004). These changes in glutamatergic signaling processes by AMPA type glutamate receptors influence *C. elegans* behavior, such as reduced nose touch avoidance (Maricq, et al., 1995), while changes in *eat*-4, which medicates presynaptic glutamate release, results in initially rapid habituation, however, it is not retained 24 hours later (Rose, et al., 2002). Further study showed GLR-1 is required for long-term memory of habituation (Rose et al., 2003). NMR-1, another *C. elegans* glutamate receptor subtype is an ionotropic NMDA receptor homolog that is known to mediate locomotor behavior, as shown in Brockie, et al. (2001) *nmr*-1 mutants show reduced switching from forward locomotion to backward locomotion. Glutamate is a common signaling molecule that interacts with well-known learning and memory proteins in *C. elegans*.

In addition to the role of glutamate in learning and memory, it is responsible for mediating both mechanosensory and phototaxis response. In mechanosensation, glutamatergic signaling mediated by *eat*-4 affects touch cell sensitivity (Lee at al., 1998) and non-NMDA type receptors (GLR-1) are responsible for nose-touch driven locomotor behavior (Brockie et al., 2001). Further *eat*-4 and *glr*-1 mutation showed defective electrotaxis locomotor behavior (Gabel et al., 2007), which is important as electrosensation and photosensation are mediated by a similar set of neurons (Ward et al., 2008). Given these previous findings, we hypothesize the vibration-light pairing is driving by upstream glutamatergic signaling.

GABA: in Learning and *C. elegans*

GABA is an amino acid neurotransmitter that inhibits mammalian neurons from generating an action potential, usually by increasing intracellular Cl- concentration, hyperpolarizing the post-synaptic neuron which results in synaptic inhibition (Werhahn, et al., 2004). Loss of GABAa receptor alpha5 subunit in mice resulted in significantly improved hippocampal learning (i.e., better performance in a water maze task). Collinson et al. (2002) demonstrated this improvement was potentially due to decreased inhibitory postsynaptic currents and inversely increased excitatory postsynaptic potentials in the CA1 region of the hippocampus. In the context of classical conditioning, GABAergic Purkinje cells of the rabbit cerebellum project onto interposed nuclei (i.e., a type of motorneuron) that initiates conditioned eye-blink response (Parker et al., 2009). In the same study, after injection of GABA receptor antagonist (a compound that interferes with receptor activity) into the interposed nuclei, the animals showed a dose-dependent elimination of the conditioned eye-blink response. Suggesting down-stream GABAergic transmission is required to mediate the CR GABAergic signaling is also involved in conditioned olfactory learning in mice, as conditioned odor preference tests result in increased GABA and glutamate release in the olfactory bulb after the presentation of conditioned odor (Brennan, et al., 1998). These results suggest the preferential release of neurotransmitters is required to encode meaningful odor cues, which is useful in directing the changing behavior in a meaningful, context-specific way.

GABAergic signaling is present in the *C. elegans* nervous system and has been shown to have similar functional and sequence homology with mammalian GABAergic signaling (Eastman, Horvitz and Jin, 1999). UNC-30 (transcription factor) binds to promoters for UNC-25 (GAD; synthesizes GABA) and UNC-47 (Vesicular GABA reuptake transporter), thus regulating their transcription in D-type, GABAergic motor neurons. UNC-47 is further expressed in all GABAergic neurons. More recent work (Gendrel et al., 2016) has expanded the map of the GABAergic nervous system. Their work broadens the expression profile of UNC-47 to D-type motor neurons, as well as RME, RIS, AVL, DVB, RIB, SMD, ALA, and some other non-GABA releasing neurons. GABAergic signaling is important for several

behaviors in the animal, including escape behavior (Kagawa-Nagamura, Gengyo-Ando, Ohkura, & Nakai, 2018), foraging deficits and locomotor behavior (Schuske, Beg, & Jorgensen, 2004).

Acetylcholine: in Learning and *C. elegans*

Acetylcholine (ACh) is expressed throughout the mammalian brain and has been established in modulating cognition, attention, and sensory processing (Picciotto, Higley, and Mineur, 2012). Acetylcholine has a modulatory effect on signaling since it can play excitatory or inhibitory roles, likely due to the large variety of receptor subtypes which fall into two classes: ionotropic nicotinic receptors or metabotropic muscarinic receptors (Picciotto, Higley, and Mineur, 2012). In rats, acetylcholine blocking both muscarinic and nicotinic receptors before reward-specific cue presentation modified motivation (e.g., increased lever presses) in response to drug reward (Collins et al., 2016). Also in rats, muscarinic acetylcholine receptors are necessary for long-term potentiation in hippocampal CA1 neurons (Adams, Winterer and Muller, 2004).

Cholinergic transmission in the *C. elegans* nervous system widely expressed (Pereira, et al., 2015). However, it is best studied at the neuromuscular junction (Richmond and Jorgensen, 1999; Francis et al., 2005; Liu, Hollopeter and Jorgensen, 2009). In regards to locomotion. A-type and B-type excitatory motor neurons of the neuromuscular junction release acetylcholine and mediate either backward or forward locomotion, respectively (Haspel, O'Donovan and Hart, 2010). Cholinergic signaling has been further studied in greater locomotive detail. For example, cholinergic signaling mediates behavioral output, such as changing direction (Li et al., 2014), and partially the response to injury signals (i.e., nociception) by harsh touch (Cohen, et al., 2014). Given these prior findings, we hypothesize cholinergic signaling may be necessary for mediating the locomotor response to our vibration-light assay. Especially given its role at the neuromuscular junction.

Both GABAergic and acetylcholine signaling play a role in learning and memory, in other species as well as in *C. elegans*. As GABA and cholinergic signaling at the neuromuscular junction control

locomotor behavior we opted to assess how GABAergic and cholinergic signaling would affect learned locomotor behavior in *C. elegans.* To assess how different neurotransmitter subcircuits and body wall muscles affect learning, we utilized optogenetics to experimentally manipulate each subcircuit during conditioning.

Optogenetics

The field of optogenetics has provided researchers with a noninvasive method to depolarize or hyperpolarize the resting membrane potential of neurons (Fiala, et al., 2006). To depolarize a neuron means making the membrane potential more positive and more likely to signal to downstream neurons or body wall muscle; while hyperpolarizing the neuron makes it less likely to signal and thus decreases the membrane potential. Channelrhodopsin (ChR2) was first isolated from *Chlamydomonas reinhardtii*, a green alga (Nagel et al. 2003) in *Xenopus* oocytes and mammalian cells (HEK293), in which they successfully drove photocurrents. Later, ChR2 was used to successfully drive locomotor behavior in *C. elegans* when expressed in motor neurons and full-body contraction when expressed in body wall muscle, under the *myo*-3 promoter (Nagel et al., 2005). These experiments were the first to express ChR2 in a neuron-specific manner, as well as to control neuronal activity in a fully intact animal due to illumination. Exactly how ChR2 functions mechanistically are as follows: upon blue light illumination (460nm), these channels make a conformational change which allows for Na+ ions to enter the cell, thus depolarizing the membrane potential and eventual exocytosis of neurotransmitter cargo is achieved (Husson, Gottschalk, and Leifer, 2013). When expressing ChR2 in *C. elegans*, these animals are transparent and ChR2 can be activated externally using LED lights to dictate control of neuronal activation (Stirman et al., 2011). In combination with the genetic toolbox of *C. elegans*, ChR2 expression can be localized to specific neuronal types, by using known promoters.

In this research, we used *pmyo*-3::ChR2 (*pmyo*-3 = body wall muscle promoter) and *unc*-47 and *unc*-17 promoters to express ChR2 in GABAergic or cholinergic neurons respectively, to specifically alter the activity of certain muscles and neuronal sub circuits during conditioning. In the dual activation of

endogenous photoreceptors driving the photosensitive response during delayed conditioning, the different ChR2-expressing circuits are simultaneously controlled with the onset/offset of the blue light during the conditioning procedure. The sensory integration thought to occur due to pairing is thought to proceed despite the manipulation of downstream neurons or muscle (See figures 6, 9 and 12 for expression profiles of ChR2 in the different strains of *C. elegans* used, in conjunction with the mechanosensory and photosensitive circuits that drive locomotion).

Research Question

Manipulating signaling in different sub-circuits during conditioning may affect learning. In this research, we describe a rapid associative conditioning assay in *C. elegans* that drives two distinct and competing circuits (mechanosensory and photosensitive) and measured locomotor output at retention periods close to the time of conditioning to assess learning. Our vibration-light conditioning protocol results in increased pausing in wild type animals, suggesting that dual activation of competing circuits prime the locomotor response to a paused state. However, when animals undergo the same conditioning procedure, but with simultaneous optogenetic activation of body wall muscle (*pmyo*-3::ChR2) or different neuronal sub-circuits, GABAergic (*punc*-47::ChR2) or cholinergic (*punc*-17::ChR2), at the same time of conditioning; we see a disruption of conditioned locomotor behavior. This suggests that the sequential activation of downstream neuronal and muscle components in *C. elegans* is important for learning.

METHODS

Strains and strain maintenance

All strains were acquired from the *Caenorhabditis elegans* Genetics Center (CGC). N2 (Wild Type), EG5025, ZX460, and EG5027. Each strain was maintained at 20ºC and fed OP50 *E. coli*, grown on NGM agar plates (Stiernagle, 2006). Before testing, the desired strains were age synchronized using a 2:1 bleach to 1M NaOH solution and tested as four-day-old animals. For optogenetic strains, the cofactor (all-trans-retinol (ATR); Sigma Aldrich, 95%) was present for ChR2 to be in an active conformation. This was achieved by mixing ATR with OP50 *E. coli* (1:100) and allowing strains to grow egg to adult on the OP50+ATR lawns (Liu, Hollopeter, and Jorgensen, 2009). For control (ATR-), the ATR is replaced with, 100% ethanol. Up until testing, *C. elegans* were maintained in light-proof containers with limited vibrational stimulation by placing on foam blocks.

Behavioral assay and apparatus

Behavior collection is conducted on seeded NGM plates, with ~20 *C. elegans* per plate. Testing is conducted in the dark with blue light filters on all computer monitors and the microscope stage, to control for blue light from other light sources. See figure 2 and figure 5 for assay schematics of the training and testing protocols used for data collection. Changes in locomotive behavior measured as forward movement, backward movement, pauses, and omega turns are quantified as our measure of learning, figure 1A example. Each trial consisted of an NGM + OP50 plate with multiple animals, had a minimum of five replicates of each trial and each group has trials that were tested over a minimum of three different days with trained and untrained matched controls for every test day. This randomized any effects due to environmental fluctuations. For conditioning, a five-second vibration (300Hz, X-Vibe speaker) and three-second blue light exposure (470nm @1000mA; Mightex LED) were used. Behavioral data were observed using an Olympus SZ7 Stereoscope (@8X total magnification) with an AmScope MUB2003 CCD camera for recording.

Behavioral analysis - TierPsy

All behavior videos were analyzed using TierPsy Multi-Worm tracker (V1.4.0). This program is a free download, found here: https://github.com/ver228/tierpsy-tracker. Total magnification and software allow for multiple animals to be analyzed at once. Behavioral scoring can be verified via wormbook.org, descriptions of output files for TierPsy are found here: https://github.com/ver228/tierpsy-tracker/blob/master/docs/OUTPUTS.md.

Of our response measures, we use the below criteria to define behavioral responses:

Definition of Behavioral Response:

Forward: The animal is moving in the "head direction" for at least 0.5 seconds and at least 5% of its length

Backward: The animal is moving in the "tail direction" for at least 0.5 seconds and at least 5% of its length

Pause: The animal is moving neither in the head or tail direction for at least 0.5 seconds

Omega Turns: The animal moves forward, the head side then turns back at a sharp angle to become even with the tail and swims off in the direction at which the animal was coming from in a forward motion

Of the various metrics TierPsy generates, we have isolated only a few metrics of choice: time and distance ratio of forward, reverse, pause, and omega turns, as well as motion mode. Using these parameters, responses during the 10 seconds before test tone and 60 seconds post-test tone were calculated accordingly for each worm, in each trial, and a time and/or distance ratio of each response type was calculated by the software. Below descriptions are the TierPsy definitions of the calculated locomotor measures used.

Time Ratio: (no units) ratio between the time spent at the event over the total trajectory time. This is calculated for forward, reversal, pause and omega turn behaviors

Distance Ratio: (no units) ratio between the total distance traveled during an event and the total distance traveled during the whole trajectory. This is calculated for only forward and reversal behaviors

Motion Mode: vector indicating if the worm is moving forward (1), backward (-1) or is paused (0). This measure is unique for every animal, as it assigns a number for the above movements for every frame that animal is in.

Statistical Analysis

Analysis of wild type behavioral data consists of two-way ANOVAs, with post hoc comparisons to determine the effect of training, by comparing the results of vibration-light training, light-only and tone-only to the appropriate untrained group, of the same retention period ($p<0.05$ significance) (Stein and Murphy, 2014). Optogenetic experiments measured differences by two-way student's t-tests between vibration-light trained and untrained/naive controls, of the same cofactor level ($p<0.05$ significance) (Donnelly et al., 2013; Ardiel et al., 2016). Because trained and untrained ATR- animals are superficially wildtype, these data were pooled across strains, to normalize the environmental effects of testing conditions on different days.

All analyses were completed in R, using package "car" for analysis and "ggplot2" for data visualizations. Visualization of behavioral data for trained, light-only or tone-only animals is normalized to untrained data and presented as normalized mean +/- normalized standard error of the mean (SEM) (Hart, 2006; Li et al., 2013). Lucidchart was used to create illustrated diagrams and schematics.

RESULTS AND ANALYSIS

Vibration-light conditioning of wild type *C. elegans*

To establish how baseline locomotor behavior changes after a rapid classical conditioning protocol with vibration-light pairing, we conditioned wild type *C. elegans* (Fig. 2). Since the two stimuli drive opposing locomotor behavior, vibration (mechanosensation) drives reversals (White et al., 1985; Wicks and Rankin, 1995) and light (photosensation) drives forward locomotion (Edwards et al., 2008; Ward et al., 2008), we expected changes in locomotor behavior to reflect successful vibration-light conditioning - see figure 3 for the schematic of mechanosensory-photosensory circuitry in *C. elegans*. In addition to vibration-light pairing, it was important to establish baseline responsiveness to tone-alone, light-alone, as compared to untrained (naïve) responding (Fig. 2). This was to ensure that the changes in locomotor behavior were a response to the vibration-light pairing, not an artifact of the individual stimuli alone, nature of stimulus delivery, or an environmental factor (Schreurs, 1989). The following results (Fig. 4) suggest that vibration-light conditioned *C. elegans* increase their time spent pausing, with a significant decrease in forward and backward locomotion. While controls (untrained, light only and tone only), generally exhibit no response to testing across retention periods, with a couple of exceptions to be discussed later.

Pause time ratio

Pause time ratio is our measure of the proportion of time the worm is moving neither forward or backward, over the whole time it is being recorded, i.e.- the greater the value, the more time the animal is spending in a paused state. In figure 4A, a two-way ANOVA showed a significant interaction of condition and retention period on paused time ratio postconditioning ($F_{6,557}$=2.65, p=0.01), as well as simple main effects of condition ($F_{3,557}$=6.46, p<0.001) and retention period ($F_{2,557}$=6.89, p=0.001). Follow up analysis of the interaction suggest there is no difference of conditioning effect at one-minute postconditioning ($F_{3,179}$=0.15, p=0.92), but are differences in conditioning five-minutes ($F_{3,205}$=6.14, p<0.001) and ten-minutes($F_{3,173}$=4.67, p=0.001) postconditioning. Follow up planned comparisons

suggest the effects of conditioning are due to training and the responsiveness is more robust at the longer retention period. Vibration-light conditioned animals ($p<0.001$) and tone-only animals ($p=0.01$) showed increased pause time at ten-minutes postconditioning compared to one-minute postconditioning, while untrained ($p=0.72$) and light-only *C. elegans* ($p=0.94$) showed no changes. To reiterate, at ten-minutes postconditioning trained and tone-only *C. elegans* increase their time spent pausing, while light-only and untrained (naïve) *C. elegans* showed no change.

Forward time ratio

Forward time ratio is a measure of the time the worm is moving forward, as a proportion of the whole duration the worm is being recorded - i.e., larger forward time ratios mean the animal is spending a greater duration of time moving forward. Assessing forward time ratio (Fig 4b), a two-way ANOVA showed no significant interaction of conditioning and retention period ($F_{6,633}=0.60$, $p=0.72$), but did measure significant simple main effects of condition ($F_{3,633}=45.11$, $p<0.001$) and retention period ($F_{2,633}=11.04$, $p<0.001$). Posthoc analysis reports the following differences of trained and control groups, as compared to untrained *C. elegans*: at one-minute postconditioning, light-only *C. elegans* move forward more frequently ($p=0.03$), tone-only *C. elegans* showed a trending difference ($p=0.06$) and trained *C. elegans* spent significantly less time moving forward ($p<0.001$). Then, at five-minutes postconditioning, light-only *C. elegans* show no difference ($p=0.85$), tone-only *C. elegans* move at a slightly greater forward time ratio than unpaired controls ($p=0.06$), while trained *C. elegans* spend significantly less time moving forward ($p=<0.001$). At ten-minutes postconditioning, paired *C. elegans* spend significantly less time moving forward ($p<0.001$), with tone-only animals still maintaining slightly more forward movement ($p=0.05$) and light-only *C. elegans* show no change in forward time ratio ($p=0.23$) as compared to untrained controls. Taken together, these data suggest that trained wild type *C. elegans* have significantly reduced forward time ratios, at all three retention periods. Meaning vibration-light conditioned animals spent a reduced proportion of time moving forward, across all retention periods. In response to light-only training, wild type *C. elegans* at one-minute postconditioning showed more

forward time ratio at a slightly higher proportion, but five-, and ten-minutes postconditioning forward locomotor behavior resembles that of untrained *C. elegans*. We can then assume blue light sensation does not affect underlying sensitivity to vibration only presentation. For the tone-only condition, the reported values were trending towards increased time spend moving forward, but only at ten-minutes postconditioning did a significant difference emerge. Taken together, untrained, light-only and tone-only wild type *C. elegans* maintain a fairly consistent proportion of forward movement across retention periods, while vibration-light trained *C. elegans* maintained a consistent decrease in forward movement across retention periods.

Forward distance ratio

Forward distance ratio is a measure of the distance the worm is moving forward, as a proportion of the whole trajectory the worm was recorded- i.e., the greater the value (proportion), the greater the distance is being covered in the forward locomotor state, suggesting the increased magnitude of forward responses. Assessing wild type forward distance ratio (Fig 4C), a two-way ANOVA showed no significant interaction of condition and retention period ($F_{6,633}$=0.35, p=0.90) but did find significant simple main effects of condition ($F_{3,633}$=43.72, p<0.001) and retention period ($F_{2,633}$=19.28, p<0.001). Follow up analysis suggests only vibration-light trained *C. elegans* maintained consistent decrease in forward distance traveled at one-minute (p=<0.001), five-minutes (p=<0.001) and ten-minutes (p=<0.001) postconditioning, as compared to the untrained groups. Tone-only animals showed a trending change in forward distance ratio across retention periods; one-minute (p=0.06), five-minutes (p=0.06) and ten-minutes (0.17) postconditioning. Light only animals showed trending differences as compared to untrained controls at one-minute (p=0.08), but not at five-minutes (p=0.97) or ten-minutes (p=0.20) postconditioning. Taken together, trained *C. elegans* showed a decreased magnitude of forward movement across retention periods. While untrained, light-only, and tone-only controls moved at a fairly consistent proportion of distance forward, and did so across retention periods.

Backward time ratio

Assessing backward locomotion, backward time ratio is a measure of the time the worm is moving backward, as a proportion of the whole duration the worm is being recorded- i.e., greater backward time ratio indicates the worm is generating and performing more backward locomotor responses. Observing differences in backward time ratio (Fig 4D), a two-way ANOVA found no significant interaction between condition and retention period ($F_{6,483}$=0.50, p=0.80) or simple main effect of retention period ($F_{2,483}$=0.08, p=0.91). But did find a significant main effect of condition ($F_{3,633}$=26.30, p<0.001). Follow up analysis showed only trained wild type *C. elegans* had a significant decrease in backward time ratio as compared to untrained controls at one-minute (p=0.001), five-minutes (p<0.001) and ten-minutes (p=0.001) postconditioning. No significant differences between light-only (one-minute p=0.81, five-minute p=0.27, ten-minute p=0.61) or tone-only animals (one-minute p=0.10, five-minute p=0.25, ten-minute p=0.33) were present across retention periods.

Backward distance ratio

Backward distance ratio is a measure of the distance the worm is moving backward, as a proportion of the whole trajectory the worm is being recorded – i.e., greater backward distance ratio indicates more distance moved in the backward locomotor state. As for the backward distance ratio (Fig 4E), there was no significant interaction of condition and retention period ($F_{6,483}$=1.12, p=0.34), nor any significant simple main effects of condition ($F_{3,483}$=2.230, p=0.08) or retention period ($F_{2,483}$=1.09, p=0.33). Thus no further analysis for changes in backward distance was performed. Overall, vibration-light conditioned *C. elegans* spend less time moving backward across all retention periods, but of their overall trajectory, they maintained normal backward locomotion. Meaning, that when a backward motor sequence was initiated, it was performed normally, as we can see in the similar levels of backward distance ratio compared to controls. Regarding controls, untrained, light-only and tone-only *C. elegans*, maintain a consistent time spent moving backward, at a steady trajectory across retention periods.

Omega turn time ratio

Omega turn time ratio is a measure of the time the worm performs an omega turn, as a proportion of the whole duration the worm is being recorded. Another way to state the omega turn time ratio is the greater the value (proportion), the more often the worm generated and performed an omega turn. The omega turn time ratio (Fig 4F) was measured using a two-way ANOVA. There was no significant interaction of condition and retention period ($F_{6,347}$=0.39, p=0.87), nor any significant simple main effects of condition ($F_{3,347}$=1.99, p=0.11) or retention period ($F_{2,347}$=1.01, p=0.36). No follow up analysis was performed, as it appears the vibration-light conditioning and controls have no effect on the proportion of time spent performing omega turns.

It is important to discuss the caveats of wild type delayed pairing of the vibration-light training. Conditioned and control *C. elegans* underwent separate training conditions, yet were still exposed to the test vibration three times; one-, five-, and ten-minutes postconditioning. The repeated presentation of the vibration at testing could potentially prove to be a confound, but there was still an established difference in trained *C. elegans* locomotor behavior, as compared to the other controls. These findings support the need to look at more than just changes in one type of locomotor behavior, but in combination of the different locomotor possibilities.

Additionally, testing at ten-minutes postconditioning resulted in most distinct locomotor responses by the trained group, thus the five-minute retention period was determined to be redundant. With that rationale, we are confident this assay is effective at quickly generating conditioned locomotor behavior in *C. elegans*. However, moving forward with subsequent optogenetic experiments, we ran separate groups of *C. elegans* at each retention period, one-, five- and ten-minutes. However, we only report conditioned behavior one- and ten-minutes postconditioning, the five-minute data is not shown.

Vibration-light conditioning with co-activation of body wall muscle interrupts learning

We sought to discern how activation of body wall muscles during conditioning affected learning in *C. elegans*. Our rationale being that motor learning in primate studies shows activity-dependent use of the limb after spinal lesion, caused rewiring of the cortex (Kambi, et al., 2011). To test the degree to which performing a motor response affects the acquisition of multiple vibration-light pairings, we identified a strain of *C. elegans* that uses the *myo*-3 promoter to drive ChR2 expression in body wall muscle. Exposing this strain of *C. elegans* to the vibration-light pairing with ATR+ activates both the endogenous phototaxis response mediated by LITE-1, as well as the body wall muscles by ChR2 activation (Fig 6). This co-activation and co-termination of both endogenous phototaxis and body wall muscle does not inhibit the sensory integration of upstream sensory neurons and interneurons, but only inhibits the automatic locomotor response the animal would naturally perform during the vibration-light pairings. This allowed us to further investigate the role (if any) of downstream effectors on learning.

In these experiments, the vibration-light training was the same as with wild type *C. elegans* (Fig 5). Except only trained (vibration-light paired) and untrained (naïve) animals were tested at one-, five-, or ten-minutes postconditioning against the five-second mechanosensory vibration (Five-minute data not shown). For blue light to drive ChR2 activation with temporal specificity, *C. elegans* must be fed all-trans-retinol (ATR), this is done so by OP50 *E. coli.* Trained and untrained groups were run with or without cofactor, ATR+ or ATR- respectively. *C. elegans* with the transgene (e.g. - promoter::ChR2) but not fed ATR are superficially wild type and ChR2 is inactive; as non-active ChR2 (100% Ethanol + OP50 *E. coli*) acts as the vehicle condition. Behavioral output from *C. elegans* of the different transgene backgrounds (*pmyo*-3::ChR2 + *punc*-47::ChR2 + *punc*-17::ChR2) that were exposed to ATR- were pooled and the subsequent performance was the baseline of learning. We expect any changes in the pooled trained ATR- *C. elegans* locomotor behavior, compared to untrained ATR- control, to be indicative of successful conditioning. While changes in trained ATR+ *pmyo-3*::ChR2 locomotor behavior, as compared to untrained ATR+ controls, are indicative of learning as well, but due to ChR2 activation of

the body wall muscles, specifically. Again, just as in wild type delayed pairing, the same metrics of forward, backward, pause and omega turn locomotor are assessed. A two-way student's t-test was used to measure the differences between trained and untrained groups of the same cofactor treatments.

Pause time ratio

Pause time ratio is the proportion of time spent pausing over the whole length of recorded video. See figure 7A. At one-minute postconditioning, trained ATR- *C. elegans* showed no change in pause time ratio ($t(239) = 1.34$, $p=0.18$) compared to untrained ATR- controls. At one-minute postconditioning, we see no differences in trained ATR+ *pmyo*-3::ChR2 *C. elegans* compared to untrained controls; pause time ratio ($t(70) = 1.66$, $p=0.24$).

Ten-minutes postconditioning, trained ATR- animals showed an 37% increase in pause time ratio ($t(252) = -2.86$; $p=0.001$), as compared to untrained controls. However, at ten-minutes postconditioning for trained *pmyo*-3::ChR2 ATR+, there are no differences compared to untrained controls; pause time ratio ($t(119)=0.09$, $p=0.92$). Vibration-light conditioned ATR- animals display conditioned paused behavior, while the same animals with co-activation of body wall muscle show no conditioned pause behavior. This suggests the ATR+ *pmyo*-3::ChR2 animals showed disrupted learning when the ability to respond was interrupted.

Forward time and distance ratio

Forward time ratio is the proportion of time spent moving forward over the whole length of time spent moving forward (Fig. 7B). While Forward distance ratio is the proportion of trajectory these animals spent moving in a forward manner (Fig. 7C). At one-minute postconditioning, trained ATR- *C. elegans* showed a 17% increase in forward time ratio ($t(302)= -2.16$, $p=0.03$) but no difference in forward distance ratio($t(302)= -1.52$, $p=0.12$), as compared to ATR- untrained controls. The same is observed for, trained *pmyo*-3::ChR2 ATR+ *C. elegans*, forward time ratio ($t(84)= -0.08$, $p=0.93$), forward distance ratio ($t(84)=0.18$, $p=0.85$), as compared to ATR+ untrained controls, showed no change.

At ten-minutes postconditioning, trained ATR- *C. elegans* showed a 17% decrease in forward time ratio ($t(382)=3.00$, $p=0.01$) and a 15% decrease in forward distance ratio ($t(380)=4.17$, $p<0.001$), as compared to untrained ATR- controls. No differences in trained ATR+ *pmyo*-3::ChR2 *C. elegans* compared to untrained ATR+ controls were observed for forward time ratio ($t(95)=0.14$, $p=0.89$) or forward distance ratio ($t(92)=0.20$, $p=0.83$). These data suggest that conditioned locomotor behavior ten-minutes after vibration-light training affects ATR- *pmyo*-3::ChR2 *C. elegans* forward locomotion, but not forward locomotion for trained ATR+ *pmyo*-3::ChR2 *C. elegans*.

Backward time and distance ratio

Backward time ratio is the proportion of time the animal spends moving backward, over the whole duration of the behavioral video (Fig. 7D), and backward distance ratio is the proportion of distance moved backwards, over the whole trajectory (Fig. 7E). At one-minute postconditioning, trained ATR- *C. elegans* showed no change in backward time ratio ($t(259)=-0.65$, $p=0.51$) or backward distance ratio ($t(259)=-0.82$, $p=0.41$), as compared to untrained ATR- controls. This is also observed in trained ATR+ *pmyo*-3::ChR2 *C. elegans*, backward time ratio ($t(75)=0.67$, $p=0.50$) and backward distance ratio($t(75)=0.61$, $p=0.54$) show no change as compared to ATR+ untrained controls.

At ten-minutes postconditioning, trained *C. elegans* showed a 25% increase in backward time ratio ($t(320)=-2.03$, $p=0.05$), with a 24% increase in backward distance ratio ($t(320)=-3.28$, $p=0.001$). Meaning trained ATR- animals had an increased likelihood of a backward response, and when it does occur, that reversal is likely to be more robust. As for trained ATR+ *pmyo*-3::ChR2 *C. elegans*, there was no measured change for backward time ratio ($t(99)=0.80$, $p=0.42$)) or backward distance ratio ($t(48)=0.81$, $p=0.41$) as compared to untrained ATR+ controls. These data suggest that at ten-minutes post-vibration-light conditioning, ATR- animals had conditioned locomotor behavior that resulted in a more likely and dramatic reversals. While ATR+ animals showed no change in backward locomotion.

Omega turn time ratio

Omega turn time ratio is the proportion of time spent making an omega turn (Fig. 7F). Otherwise known as a "pirouette" motion where the animal is moving forward, stops, initiates a reversal then the animal's head swings back to touch its tail and then moves forward in a drastically different direction. At one-minute postconditioning, trained ATR- *C. elegans* show no change in omega turn time ratio ($t(261)= -0.31$, $p=0.75$) as compared to ATR- untrained controls. The same is seen with trained ATR+ *pmyo*-3::ChR2 *C. elegans,* no change in omega turn time is observed ($t(54)=0.99$, $p=0.32$).

At ten-minutes postconditioning, trained ATR- *C. elegans* show no change in omega turn time ratio ($t(240)=0.08$, $p=0.92$) and trained ATR+ *pmyo*-3::ChR2 *C. elegans* show no change in omega turn time ratio ($t(93)=1.23$, $p=0.22$). Generation of the omega turn time locomotor pattern was unaffected by the vibration-light conditioning protocol, regardless of the presence or absence of cofactor or level of training.

The main takeaway of *pmyo*-3::ChR2 vibration-light conditioning

These data suggest that body wall muscle overactivation during conditioning effects learning in *C. elegans*. More specifically, overactivation of body wall muscle seems to inhibit learning as indicated by the lack of conditioned locomotor response seen at both one- and ten-minutes postconditioning, as compared to untrained ATR+ controls. This lack of conditioned response is not due to the ATR+ *pmyo*-3::ChR2 animals being completely immobilized by the blue light in the vibration-light protocol, as we saw that the animals maintain responsiveness as measured by motion mode results (Fig. 8). Verifying that the cofactor presence does not immobilize the animals in the absence of blue light.

Driving the GABAergic circuit during conditioning modulates learning

GABA is a common neurotransmitter in the *C. elegans* nervous system (Schuske, Beg, and Jorgensen, 2004), as well as a key driver of locomotion due to its complementary activity at the neuromuscular junction (Richmond and Jorgensen, 1999; Fig. 1A). Thus we sought to assess how

overactivation of the inhibitory GABAergic circuit by driving ChR2 expression under the *unc*-47 promoter (Fig. 9) could affect learning. Behavioral experiments were conducted as previously described (Fig. 5). The presence of cofactor (ATR+) allows for active ChR2 to drive activation of inhibitory GABAergic neurons. Statistical comparisons between trained and untrained animals of the same cofactor level and retention period were completed using a student's t-test.

Pause Time Ratio

Pause time ratio is the proportion of time spent pausing over the entire length of recorded video, as an average for each group (Fig. 10A). At one-minute postconditioning, trained ATR- *C. elegans* showed no change in pause time ratio ($t(239) = 1.34$, $p=0.18$) compared to untrained ATR- controls. Again, at one-minute postconditioning, we saw no differences in trained ATR+ *punc-47*::ChR2 *C. elegans* pausing ($t(115)=-1.26$, $p=0.20$), as compared to untrained ATR+ controls.

Ten-minutes postconditioning, trained ATR- animals showed an 37% increase in pause time ratio ($t(252) = -2.86$; $p=0.001$), as compared to untrained controls. At ten-minutes postconditioning, trained ATR+ *punc-47*::ChR2 animals showed no change in paused time ratio ($t(79)=-0.47$, $p=0.63$) as compared to untrained ATR+ controls. Vibration-light conditioned ATR- animals showed conditioned pause behavior at ten-minutes postconditioning. While ATR+ *punc-47*::ChR2 animals display no conditioned paused behavior, regardless of the retention period.

Forward Time and Distance Ratio

Forward time ratio is the proportion of time spent moving forward over the whole duration of time spent moving forward, as averaged for each experimental group (Fig. 10B). While forward distance ratio is the proportion of total trajectory these animals spent moving in a forward manner, as an average for each experimental group (Fig. 10C). Taken together, the forward time ratio is an indicator of the likelihood of generating forward locomotion, while forward distance ratio reflects the magnitude of that forward response when initiated.

At one-minute postconditioning, trained ATR- *C. elegans* showed a 17% increase in forward time ratio ($t(302)= -2.16$, $p=0.03$) but no difference in forward distance ratio($t(302)= -1.52$, $p=0.12$), as compared to ATR- untrained controls. While trained ATR+ *punc-47*::ChR2 *C. elegans* showed no change in forward time ratio ($t(121)=0.35$, $p=0.72$), but showed a trending decrease (-16%) in forward distance ratio ($t(121)=1.72$, $p=0.08$), as compared to ATR+ untrained controls. This suggests that trained ATR+ animals had no change in the likelihood of initiating a forward motion, but when it did occur, it was potentially a slightly smaller magnitude.

At ten-minutes postconditioning, trained ATR- *C. elegans* showed a 17% decrease in forward time ratio ($t(382)=3.00$, $p=0.01$) and a 15% decrease in forward distance ratio ($t(380)=4.17$, $p<0.001$), as compared to untrained ATR- controls. Trained ATR+ *punc-47*::ChR2 *C. elegans* exhibited a trending 24% increase in forward time ratio ($t(135)=-1.84$, $p=0.06$) with a significant 24% increase in forward distance ratio ($t(135)=-2.45$, $p=0.01$) as compared to untrained ATR+ controls.

These data suggest that conditioned locomotor behavior at both one- and ten-minutes after vibration-light training affected forward locomotion for ATR- and ATR+ *punc-47*::ChR2 *C. elegans*. This change in forward pattern generation reflecting differences based on cofactor absence or presence.

Backward Time and Distance Ratio

The backward time ratio is the proportion of time the animal spends moving backward, over the whole duration of the behavioral video, represented as an average for each group (Fig. 10D). While backward distance ratio is the proportion of distance moved backward, over the whole trajectory, represented as an average for each group (Fig. 10E). Relating the two, backward time ratio is an indicator of the likelihood of initiating a backward response, while the backward distance ratio indicates the magnitude of that generated backward response.

At one-minute postconditioning, trained ATR- *C. elegans* showed no change in backward time ratio ($t(259)=-0.65$, $p=0.51$) or backward distance ratio ($t(259)=-0.82$, $p=0.41$), as compared to untrained

ATR- controls. In trained ATR+ *punc-47*::ChR2 *C. elegans*, there was a trending 20% increase in backward time ratio (t(112)=-1.75, p=0.08), while no significant change in backward distance ratio(t(108)=-1.32, p=0.18) was observed. This suggests that trained ATR+ *punc*-47 *C.elegans* showed a trend toward increased initiation of reversals, but when initiated, the reversals were of typical magnitude, as compared to ATR+ untrained controls.

At ten-minutes postconditioning, trained *C. elegans* showed a 25% increase in backward time ratio (t(320)=-2.03, p=0.05), with a 24% increase in backward distance ratio (t(320)=-3.28, p=0.001). As for trained ATR+ *punc-47*::ChR2 *C. elegans*, there was no measured change for backward time ratio (t(118)=0.68, p=0.49) or backward distance ratio (t(118)=1.31, p=0.19), as compared to ATR+ untrained controls.

These data suggest that initially, at one-minutes post-vibration-light conditioning, ATR+ animals displayed conditioned locomotor behavior that resulted in the animals being more likely to initiate a reversal response, but this trend was not maintained ten-minutes later.

Omega Turn Time Ratio

Omega turn time ratio is the proportion of time spent making an omega turn, as an average measure for each group (Fig. 10F). At one-minute postconditioning, trained ATR- *C. elegans* show no change in omega turn time ratio (t(261)= -0.31, p=0.75) as compared to ATR- untrained controls. The same is seen with trained ATR+ *punc-47*::ChR2 *C. elegans*, no change in omega turn time is observed (t(108)=0.27, p=0.78).

At ten-minutes postconditioning, trained ATR- *C. elegans* show no change in omega turn time ratio (t(240)=0.08, p=0.92). While trained ATR+ *punc-47*::ChR2 *C. elegans* showed no change in omega turn time ratio (t(71)=-0.18, p=0.85). Omega turns generation appears to be unaffected by the vibration-light protocol.

Main take away of *punc*-47::ChR2 results

Taken as a whole, trained pooled ATR- *C. elegans* and ATR+ *punc*-47::ChR2 *C. elegans* showed differential conditioned locomotor responsiveness based on cofactor level. The conditioned responses appear to differentially affect pausing and the forward circuit. At one-minute postconditioning, trained ATR- animals showed an increased likelihood of making a forward locomotor pattern, while there was a slight decrease in magnitude of forward locomotion for trained ATR+ animals. Then at ten minutes, pooled trained ATR- *C. elegans* showed an increase in pausing with a decreased likelihood and magnitude of forward movement, while trained ATR+ *C. elegans* showed no change in pausing and an increased likelihood and magnitude of forward movement. In other words, because the observed changes in conditioned locomotor behavior were dependent on cofactor level, these results suggest that trace of the vibration-light conditioning was present, except in an altered state, appearing to be due to the asynchronous top-down signaling of the GABAergic subcircuit.

Co-activation of the cholinergic circuit during conditioning

Because endogenous body wall muscle over activation and asynchronous GABAergic signaling showed to influence conditioning, we thought to further assess if the temporally specific stimulation of acetylcholine, a neurotransmitter that feeds into the neuromuscular junction (Richmond and Jorgensen, 1999), is also required for learning (Fig. 1A). Acetylcholine is also known to direct behavior (Dittman and Kaplan, 2008), hence acetylcholine was identified to be of potential importance. Applying the same experimental concept as previously discussed (Fig. 5A), co-activation and co-termination of the endogenous phototaxis responsive via LITE-1 (Edwards et al., 2008; Ward et al., 2008) co-activated the downstream excitatory cholinergic interneurons and motor neurons during the vibration-light pairings. Analysis of the locomotor behavior of trained versus untrained controls, of the same cofactor level, was measured by a student's t-test.

Pause time ratio

Pause time ratio is the average proportion of time spent pausing over the whole length of the recorded video, for each experimental group (Fig. 13A). At one-minute postconditioning, trained ATR- *C. elegans* showed no change in pause time ratio ($t(239) = 1.34$, $p=0.18$) compared to untrained ATR- controls. The same was observed for trained ATR+ *punc-17*::ChR2 *C. elegans,* no differences in pause time ratio ($t(26)=-0.76$, $p=0.45$) were measured, as compared to untrained ATR+ controls.

Ten-minutes postconditioning, trained ATR- animals showed an 37% increase in pause time ratio ($t(252) = -2.86$; $p=0.001$), as compared to untrained controls. While ten-minutes postconditioning trained ATR+ *punc-17*::ChR2 animals showed no change in paused time ratio ($t(75)=1.41$, $p=0.16$) as compared to untrained ATR+ controls. All together, vibration-light conditioned ATR- animals display changes in paused behavior. While the same animals with co-activation of the cholinergic subcircuit exhibited no conditioned pause behavior.

Forward Time and Distance Ratio

Forward time ratio is the average proportion of time spent moving forward over the whole duration of time spent moving forward, as measured for each experimental group (Fig. 13B). While forward distance ratio is the average proportion of total trajectory these animals spent moving in a forward manner, for each group (Fig. 13C). In other words, the ratio of time spent moving forward indicates the likelihood of initiating a forward response, while the ratio of distance spent moving forward is the resulting magnitude of that forward locomotion.

At one-minute postconditioning, trained ATR- *C. elegans* showed a 17% increase in forward time ratio ($t(302)= -2.16$, $p=0.03$) but no difference in forward distance ratio($t(302)= -1.52$, $p=0.12$), as compared to ATR- untrained controls. While trained ATR+ *punc-17*::ChR2 *C. elegans,* showed a 25% decrease in forward time ratio ($t(60)=1.94$, $p=0.05$) and a 25% decrease in forward distance ratio

(t(63)=2.71, p=0.008) as compared to ATR+ untrained controls. Suggesting that forward pattern generation, initiation, and magnitude were reduced after vibration-light conditioning.

At ten-minutes postconditioning, trained ATR- *C. elegans* showed a 17% decrease in forward time ratio (t(382)=3.00, p=0.01) and a 15% decrease in forward distance ratio (t(380)=4.17, p<0.001), as compared to untrained ATR- controls. Trained ATR+ *punc-17*::ChR2 *C. elegans* showed no change in forward time ratio (t(127)=-1.10, p=0.27), or changes in forward distance ratio (t(127)=-1.47, p=0.14), as compared to untrained ATR+ controls.

These data suggest that conditioned forward locomotor behavior for trained ATR- *C. elegans* and trained ATR+ *punc-17*::ChR2 *C. elegans* occurs, but in a manner that is specific to cofactor levels, and only immediately after conditioning. Further interpretation in the discussion.

Backward Time and Distance Ratio

The backward time ratio is the average proportion of time the animal spends moving backward, over the whole duration of the recorded video for each experimental group (Fig. 13D). Backward distance ratio is the proportion of distance moved backward, over the entire pathway trajectory, this is an average measure for each experimental group (Fig. 13E). In other words, the backward time ratio is indicative of the likelihood of performing a backward response, while the backward distance ratio indicates the magnitude of that backward locomotor pattern.

At one-minute postconditioning, trained ATR- *C. elegans* showed no change in backward time ratio (t(259)=-0.65, p=0.51) or backward distance ratio (t(259)=-0.82, p=0.41), as compared to untrained ATR- controls. However, for trained ATR+ *punc-17*::ChR2 *C. elegans*, backward time ratio (t(59)=-0.73, p=0.67) was not significant, yet there a significant increase in backward distance ratio (t(50)=-2.70, p=0.01) as compared to ATR+ untrained controls. These results suggest that trained ATR+ *punc*-17 *C.elegans* showed no change in the likelihood of initiating a reversal, but when a reversal was initiated it was much more dramatic.

At ten-minutes postconditioning, trained *C. elegans* showed a 25% increase in backward time ratio ($t(320)=-2.03$, $p=0.05$), with a 24% increase in backward distance ratio ($t(320)=-3.28$, $p=0.001$). As for trained ATR+ *punc-17*::ChR2 *C. elegans*, there were no changes in backward time ratio ($t(96)=-0.46$, $p=0.64$) or backward distance ratio ($t(102)=-0.39$, $p=0.69$) as compared to ATR+ untrained controls.

Trained ATR- animals showed conditioned changes in reversal behavior. While immediately postconditioning, trained ATR+ animals had a more robust reversal response that was not maintained ten-minutes later.

Omega Turn Time Ratio

Omega turn time ratio is the average proportion of time spent making an omega turn, as measured for each experimental group (Fig. 13F). At one-minute postconditioning, trained ATR- *C. elegans* show no change in omega turn time ratio ($t(261)= -0.31$, $p=0.75$) as compared to ATR- untrained controls. The same is seen with trained ATR+ *punc-17*::ChR2 *C. elegans*, where no change in omega turn time was observed ($t(63)=1.34$, $p=0.18$).

At ten-minutes postconditioning, trained ATR- *C. elegans* show no change in omega turn time ratio ($t(240)=0.08$, $p=0.92$). While trained ATR+ *punc-17*::ChR2 *C. elegans* showed a significant 86% increase in omega turn time ratio ($t(52)=-2.74$, $p=0.01$). The vibration-light conditioning assay affected the omega turn time ratio for only the trained ATR+ *C. elegans* at ten-minutes, we hypothesize on this observation later in the discussion.

Main takeaway of *punc*-17::ChR2

At one-minute postconditioning, trained ATR+ *punc*-17::ChR2 *C. elegans* showed interesting conditioned behavior that was reflected in the forward circuit - e.g., a decrease in forward time- and distance ratio that was the opposite of what was seen for trained pooled ATR- *C. elegans.* Additionally, at ten-minutes postconditioning, trained ATR+ p*unc*-17::ChR2 *C. elegans* were the only group with changes in the proportion of omega turns, while having no other conditioned locomotor behavior at this retention

period. There is some prior evidence suggests acetylcholine signaling is partially involved in the regulation of omega turn frequency (Dittman and Kaplan, 2008). Further analysis in the discussion.

Because we do see some locomotion for these groups at each retention period, results cannot be attributed to the animal's immobilization during the conditioning protocol, as motion mode data show differential responding pre-, during-, and post- conditioning, for all levels of training and cofactor (Fig. 14). Taken together, this data suggests that trained ATR+ *punc*-17::ChR2 *C. elegans* exhibited differential conditioned locomotor behavior compared to trained ATR- animals. Specifically, the forward conditioned behaviors were of opposing outputs, dependent on cofactor absence or presence, suggesting that some trace of the memory for the vibration-light conditioning was maintained, but in an altered state.

DISCUSSION

Wild type (N2) Conditioned Locomotor Behavior

It is generally well accepted that *C. elegans* are capable of associative learning (Kauffman, et al., 2011; Kauffman, et al., 2010; Stein and Murphy, 2014). Yet, most associative conditioning assays using *C. elegans* involve 1 hour or longer conditioning periods (1-4 hours) (Hedgecock and Russell, 1975; Saeki, Yamamoto, and Iino, 2001). In the current study, we were interested in assessing the acquisition of learning, by measuring conditioned locomotor behavior immediately after training. Thus, the first series of experiments aimed to establish a baseline responding to the rapid associative conditioning assay used. We argued that pairing two US's (vibration + light) was sufficient to alter the later locomotor response to a single US (vibration alone; Fig. 4). This is because we see a clear locomotor pattern exhibited by the vibration-light conditioned animals that are different than the locomotor patterns of the controls - e.g., untrained, light-only, and tone-only (Fig. 4). In earlier trials, it was determined that green light alone (~560nm), as well as green light paired with head touch, did not generate a conditioned response (data not shown). Additionally, 100hz vibration and 200hz vibration had subtle locomotor responses, while the 300Hz vibration response was robust (data not shown). Finally, when we paired a 300Hz vibration with blue light pairing (Fig. 2), where we observed conditioned locomotor responses (Fig. 4).

Measuring several metrics of wild type locomotor behavior, one-, five-, and ten-minutes postconditioning, the main take away is that vibration-light trained animals had distinct locomotor behaviors, as compared to controls. Vibration-light conditioned animals had a steady increase in paused time and consistent decreases in forward and backward locomotion as compared to untrained controls, indicating successful conditioning. Because this stimulus combination drives opposing locomotor behavior; mechanosensation drives reversals (Chalfie et al., 1985; Wicks and Rankin, 1995) and blue light drives forward locomotion (Edwards et al., 2008; Ward et al., 2008), thus the US-US pairing results in a CR of pausing behavior. Suggesting that the overall circuit is biased towards a reduction in locomotion, with neither vibration or light outweighing its influence, and pause behavior being the result.

These findings suggest after a few pairings, wild type *C. elegans* could rapidly change behavior that reflects the dual convergence of two directionally competitive stimuli.

Looking at wild type controls, both light-only and tone-only animals show comparable locomotor trends that are similar to untrained animals. With one notable exception, at five- and ten-minutes postconditioning, tone-only animals show an increase in their pause time ratio that mirrors vibration-light conditioned animals, but showed a greater proportion of time spent moving forward. In previous reports, repeated mechanosensory stimulation resulted in an eventual decrease in reversal duration and magnitude due to habituation (Wicks and Rankin, 1995). In our interpretation, results of tone-only animals seem to be reflective of the multiple vibration-only presentations, both during training and testing, as repetitive stimulation occurs, reversal locomotion is stagnant, while forward and paused locomotion increases. Since tone-only animals showed locomotor behavior that is dissimilar to untrained animals, this could be a problem, potentially suggesting our assay has background effects on associative locomotion. However, tone-only animals have a locomotor pattern that is dissimilar to vibration-light conditioned animals, suggesting that the effect of repetitive mechanosensory stimulation is modulated when paired closely in time with another competitive and distinct sensory stimulus. Given this distinction, we argued that to assess conditioned locomotor behavior, all directions of locomotion should be measured, not just a single locomotor pattern (i.e., reversals only). Looking to light-only animals, responding was consistent and steady across retention periods as compared to untrained controls, suggesting that non-associative influences or unexpected environmental confounds are responsible for changes in locomotor behavior. Given these findings, we moved forward with assessing how optogenetic stimulation of downstream effectors influence learned behavior.

Body wall muscle overactivation during conditioning affects learning

Our goal was to assess how activation downstream effectors, such as the body wall muscle, could influence learning a new association. When driving ChR2 under the *myo*-3 promoter, ChR2 is expressed in the body wall muscle. Referring to the training schematic and circuit illustration (Fig. 5 and Fig. 6

respectively), the blue light from the vibration-light pairing drives both the endogenous photosensitive circuit and activation of cells expressing ChR2 in the body wall muscle at the same time. In short, as the vibration-light pairing occurs, it simultaneously activates the body wall muscles of *pmyo*-3::ChR2 animals at the exact time of conditioning. We see that *pmyo*-3::ChR2 animals in the trained ATR+ conditions show no change in locomotor behavior compared to their untrained ATR+ counterparts (Fig. 7). Regardless of locomotor measure or retention period. Since the trained animals respond as the untrained animals would, activation of the body wall muscles during the vibration-light pairing likely disrupted learning. As the recall of the association at testing is comparable to naïve, untrained ATR+ animals. Trained pooled ATR- animals showed conditioned locomotor behavior at both one- and ten-minutes postconditioning, suggesting that conditioning with the vibration-light pairing was successful in these optogenetic animals when cofactor was not present. Thus overactivation of body wall muscle responsible for locomotion at the time of conditioning appears to impair learning in an associative conditioning assay.

Because disruption of the endogenous locomotor response seemed to affect learning in *C. elegans*, we further reviewed previous research involving manipulation of motor pattern generation and subsequent effects on neuroplasticity and learning in other species. Using primates, researchers have shown higher level cortex modification due to motor pattern generation or loss of limb - e.g., error prediction of continuous motor movements is recorded in the cerebellum of Rhesus monkeys and maintained over some time to update long-range connections with the cortex, maintaining and predicting upcoming locomotor behavior (Popa, Streng, and Ebner, 2017). Macaque's with spinal cord lesions that resulted in the loss of both touch and body awareness input from the limb showed reorganization of the motor and somatosensory cortex (Kambi, et al., 2011). This phenomenon in primates suggests that active use of the limb to generate motor responses are important for maintaining higher-level cortical organization. In humans, concurrent cognitive and motor tasks are inversely related, that is, if lower-level motor tasks such as walking are not involved in higher-level processes (e.g., choice and decision making)

then there would not be an inverse relationship, yet there is one (Oliveira, et al., 2018). This could be due to a strain on attention processing, however, walking is an involuntary neurological process that is mediated by the lower, peripheral nervous system. These previously published data from other species suggest that motor pattern generation has a retrograde influence on higher-level cortical structures that are usage dependent and in this study we applied this observation in the context of learning.

Looking further into motor feedback and learning, data gathered from squirrel monkeys that learned novel locomotor tasks showed distinct changes in motor cortex representation during different stages of learning – e.g., training, extinction, and reacquisition (Nudo, et al., 1996), suggesting motor pattern generation influences neuronal firing. Additionally, learned motor movement has been shown to up and downregulate many genes associated with neuronal plasticity and learning, within 1-24 hours after training (Hertler et al., 2016). Reflection on these studies of learned motor memory suggests that generation of locomotion and feedback from movement is important for physiological structuring of the nervous system, and with learning newly conditioned motor patterns. While motor processes do have a retrograde influence on higher-level cortical structures that is likely mediated by up and downregulation of learning and memory genes. In the current study, we showed that an endogenous locomotor response during an associative conditioning assay is important for successful acquisition in *C. elegans*. This falls in line with prior studies regarding motor learning and cortical plasticity.

Acquisition of the vibration-light conditioning occurs between sensory neurons-interneurons and interneurons-interneurons, with information culminating downstream onto command interneurons; which modulate neuromuscular junction activity and initiate conditioned locomotor response via body wall muscle (Fig. 3). In this study, the body wall muscles were activated for the exact duration of the blue light. It is possible that while at the upper circuit level, the vibration-light convergence is uninterrupted, the inability to perform the endogenous locomotor response had disrupted the animal's learning of the vibration-light association, seen in Assareh et al. (2017) where the conditioned fear response is disrupted with optogenetic inhibition of key brain regions during fear conditioning. In our study, upon testing ten-

minutes postconditioning, we see these animals demonstrate a locomotor pattern that reflects that of a naïve/untrained animal.

Given previous studies of learned motor memory, it would appear that the generation of locomotion and feedback from motor movement is important for physiological structuring of the nervous system, and with learning newly conditioned motor patterns. Relating this to the current study, we found evidence to suggest that while *C. elegans* undergo associative conditioning, the generation of the animal's endogenous locomotor response may be important for the successful acquisition of the vibration-light pairing in *C. elegans*.

Influence of the GABAergic circuit during conditioning in *C. elegans*

GABA is a key neurotransmitter in the *C. elegans* neuromuscular junction and drives locomotion (Eastman, Horvitz, and Jin, 1999; Fig 1A-C), and in this study, we sought to investigate how signaling by GABAergic motor neurons influences associative conditioning. To add to this argument, GABA is a prominent neurotransmitter in both the *C. elegans* and the mammalian nervous system (Werhahn, et al., 2004; Schuske, Beg, and Jorgensen, 2004; Lu, Hollopeter, and Jorgensen, 2009) and changes in inhibitory signaling contribute to long term potentiation (Chapman, Perez, and Lacaille, 1998). In the current study, we used *punc*-47 to drive ChR2 expression in interneurons that partially drive the endogenous light response, as well as in command interneurons and the neuromuscular junction (Eastman, Horvitz, and Jin, 1999). In these experiments, under the *unc*-47 promoter, ChR2 was driven in additional neurons that are outside of the illustrated schematic (Fig. 15; Gendrel, Atlas, and Hobert, 2016), this is a potential conflict. Yet, the main question we wanted to address is how overstimulation of downstream neuronal components (interneurons, motor neurons, and body wall muscle) during conditioning, affect learning. We believe that future work expressing ChR2 under a neuromuscular junction specific promoter could distinguish how different inputs of GABA neurotransmission contribute to learning.

Addressing the results, trained ATR+ *punc*-47 *C. elegans* showed no conditioned pause behavior but did show conditioned forward locomotion at one- and ten-minutes postconditioning. For trained ATR-

pooled *C. elegans*, the opposite was observed - i.e., conditioned increase in paused behavior and reversed conditioned forward locomotor patterns. These results suggest that the disruption in linear, top-down, activation of the GABAergic circuit during conditioning resulted in a biasing of the forward response circuit for trained ATR+ animals. Suggesting that when downstream effectors that mediate the locomotor response are activated at temporally inappropriate time points learning is partially disrupted.

It appears that vibration-light conditioning relies on sequential signaling of the GABAergic circuit. In humans, a reduction in GABA concentration in the sensory-motor cortex is necessary for the acquisition of a learned motor task (Floyer-Lea et al., 2006). While using a low-intensity motor task, decreased GABA concentration was correlated with mediating connectivity in the motor cortex (Sampaio-Baptista, et al., 2015). Pharmacological blocking of GABA reuptake in the motor cortex inhibited motor learning (Werhahn et al., 2004), yet pharmacological increases in GABA concentration increased long-term performance on cognitive tasks in aged mice (Tong et al., 2016). In other words, for normal learning of a motor task, appropriate levels of GABA concentrations appear necessary for acquisition and recall of motor tasks. These previously reported findings align with the current finding that short-term increased GABAergic signaling disrupted vibration-light conditioning in *C. elegans*.

Influence of cholinergic circuit during conditioning in *C. elegans*

Acetylcholine is an important neuromodulator that has been linked to learning (Picciotto, Higley, and Mineur, 2012) and is important for a functioning neuromuscular junction and in generating locomotion (Richmond and Jorgensen, 1999; Liu, Hollopeter, and Jorgensen, 2009; Fig 1A-C). In rats, an imbalance in excitatory (cholinergic) input results in the inappropriate recall of neural engrams (Barron et al., 2017), suggesting further that sequential and temporal activation of the cholinergic circuit is important for appropriate recall of memory. Given previous research, we investigated cholinergic motor neurons and their potential role in modulating the downstream components of our learning assay.

Reviewing how overactivation of cholinergic motor neurons during conditioning affected learning (Fig 13), at one-minute postconditioning trained ATR+ *punc*-17::ChR2 animals showed

decreased forward time and forward distance ratios, with a subsequent increase in magnitude traveled moving backward. Suggesting that at least one-minute postconditioning, trained ATR+ *punc*-17::ChR2 *C. elegans* showed a more robust reversal response, perhaps reflecting a mildly increased escape response (Maguire et al., 2011) immediately postconditioning. While pooled ATR- trained *C.elegans,* showed a conditioned increase in paused behavior and increased conditioned forward locomotor patterns. ATR+ *punc*-17::ChR2 *C. elegans* only exhibited a sharp increase in omega turn time ratio.

Interestingly, trained ATR+ *punc*-17::ChR2 *C. elegans* was the only group in the current study to show any change in omega turns. Omega turns are associated with the escape response in *C. elegans*, as they are part of the reorientation of locomotor direction (Broekmans, et al., 2016). But when on food, *C. elegans* exhibit a low proportion of omega turns (Gray, Hill and Bargmann, 2005) and in the current study, all animals were tested on food. Previous research suggests that omega turn completion in response to touch is mediated by GABAergic motor neurons, as directed by upstream command interneurons that release acetylcholine (Donnelly et al., 2013). Because *punc*-17::ChR2 is expressed in command interneurons, it is possible that overstimulation of the cholinergic circuit briefly biased this increase in omega turns. Further evidence suggests hypersensitivity to acetylcholine, as mediated by sensitized muscarinic acetylcholine receptors, significantly increased baseline omega-turn frequency (Dittman and Kaplan, 2008). So far, since this is the only measured increase in omega turns recorded, it is impossible to draw any decisive conclusion as to causation, however, it is possible that excessive cholinergic signaling increased the likelihood of producing omega turns in the ATR+ *punc*-17::ChR2 animals, while disrupting acquisition of conditioned locomotor response.

As a whole, because trained ATR+ *punc*-17::ChR2 animals have differential conditioned responding compared to trained ATR- animals, this change in conditioned locomotor behavior suggests that top-down, linear cholinergic circuit activation is important for normal learning in the worm. When this sequential process is disrupted, as done in this study, normal learning is disrupted. How this occurs requires further investigation, yet, previous research supports retrograde signaling between command

interneurons and A-type (cholinergic) motor neurons by gap junctions in *C. elegans* as a mechanism to modulate spontaneous locomotor behavior (Liu et al., 2017). Gap junctions have also been identified in coordinating switching between forward and backward locomotion by coordination of command interneuron activity with cholinergic motor neurons (Kawano et al., 2011). Of the 302 neurons in the *C. elegans* neural connectome, innexins make this simple circuit considerably more complicated (Hall, 2016; Kunert-Graf, Sakhanenko, and Galas 2017), making gap junctions a plausible mediator of retrograde signaling from downstream effectors.

Limits of the current study

In the current study, some of the following compromises occurred; first, due to the nature of our software, TierPsy, all behavior videos must be 30 seconds or longer, given our frame rate (Javer et al., 2018). This is why we could not assess temporally specific time and distance ratios immediately pre- or post-vibration presentation. We did attempt to splice whole videos into pre- and post- segments and analyze them individually. This was unsuccessful, as TierPsy needs a much larger number of frames to correctly orient the head and tail end of the worm (Javer et al., 2018). With that, we chose to use the temporally more specific, but less directionally specific, measure of motion mode. These data are to be assessed in the context of time and distance ratios. Another aspect of TierPsy data output is the sheer number of metrics the program puts out for each video processed – i.e., there are 700+ data points for each animal recorded, in each video. In this research, we used only six metrics to assess locomotor behavior - forward, backward, paused and omega turns. While we chose to not use some other locomotor metrics (e.g., upsilon turns and coils) as these locomotor patterns account for only a small percentage of worm locomotion. This is why some of the time- and distance-ratios do not add up to exactly 100%.

Another aspect of this research to account for is the degree of natural variation in locomotor response across some of the treatments. It is possible that components of the data that were affected by the experimenter, temperature and humidity of the testing room, NGM plate composition, degree of crowding on the plate, time spent incubating, etc. However, by following protocols for controls we ran a

matched untrained (naïve) control with every trained replicate over a minimum of three days per condition, then used the untrained behavioral data as the baseline measure of behavior (Hart, et al., 2006). It is believed that most of the uncontrolled environmental variation is minimized by this method. In this presented data, 2,983 wild type and optogenetic *C. elegans* were analyzed, further diluting extraneous influence.

Lastly, light- and tone-only control groups for the optogenetic animals were not initially run. At the time, we deemed it important to first ensure that conditioning differences in ATR+ groups were even occurring, before adding in the additional control groups. Currently, these trials are being taken over and run to assess how ATR+ animals respond to vibration after several presentations of the light-alone. Again, it is still possible to draw tentative conclusions given this completed data set.

Future directions

After light-only and tone-only trials for the optogenetic strains are complete, there are several ways to further the current findings. First, drive halorhodopsin (Halo) under the same promoters, *myo*-3, *unc*-17, and *unc*-47 to assess how the inactivation of body wall muscle and the different circuits affects learning. Hypothetically, this should show results where learning is disrupted in *pmyo*-3::Halo animals, while the memory trace should be rescued in *punc*-17::Halo and *punc*-47::Halo. Once, complete, it would be interesting to then drive ChR2 expression in only cholinergic and GABAergic motor neurons in an attempt to further deduce how, if any, effect signaling at the neuromuscular junction has on learning. Another future step would be to express GCaMP – a genetically encoded calcium indicator used to fluorescently detect active neurons – in downstream interneurons and motor neurons. This would allow for measurement of changed downstream neuronal activity during and postconditioning in trained verse untrained animals, thus further investigating the sequential effects of not only conditioned behavior, but how neuronal signaling changes as a result.

CONCLUSION

Imaging of the human brain during the acquisition of a newly learned motor skill shows dynamic neuronal activation between the cerebellum and the higher cortex (Doyon et al., 2002). Studies using non-human primates find even more compelling evidence of cortical structural reorganization post-lesioning or motor task training (Nudo et al., 1996; Kambi et al., 2011); suggesting feedback from lower limbs are imperative in motor learning. Not surprisingly, some of the same cellular and molecular components involved in learning and memory are involved in motor task learning as well (Hertler et al., 2016).

Yet lesioning studies are extremely invasive, so to further study circuitry during conditioning in a temporally precise, and but in a non-invasive manner, optogenetic experiments are useful. As highlighted by optogenetic inhibition of key brain regions during various classical conditioning procedures in rats either induced inappropriate increases in behavioral response (Chang, et al., 2016) or inhibition of conditioned response all together (Assareh et al., 2017). Still, there are limitations to the use of model organisms with complex cortices, such as primates and rodents. This is why optogenetic control of different neural sub circuits during associative conditioning in *C. elegans* is ideal. In this research, we observed similar behavioral observations in *C. elegans,* as previous research noted in mammalian test subjects, where the inability to execute a motor response during conditioning or a task, affected the learned locomotor response. With mammalian studies, imaging methods are used to assess changing structural morphology at the stages of learning, while in this study, we manipulated endogenous behavior in an unrestrained and freely moving animal.

Results of this study suggest four key findings: 1.) Wild type *C. elegans* can rapidly learn an association, given five repetitions of pairings during training, show consistent conditioned locomotor behavior within ten-minutes of training. 2.) Body wall muscle overstimulation during conditioning inhibits the acquisition of a conditioned locomotor response. 3.) Simultaneous stimulation of GABAergic and cholinergic circuits during conditioning disrupted learning but did not completely ablate the memory

trace, suggesting sequential signaling is important for conditioning. 4.) locomotor movement is important for conditioning, across taxa; from *C. elegans* to rats, to primates and humans.

We offer *C. elegans* as a viable model organism to further decipher how sequential activation of different neural networks affect learned behavior. This has huge potential due in part to optogenetic methods to noninvasively turning on or off specific neural circuits, many experience-dependent associative (or nonassociative) conditioning protocols, and a fully sequenced genome with homologous learning and memory genes.

FIGURES

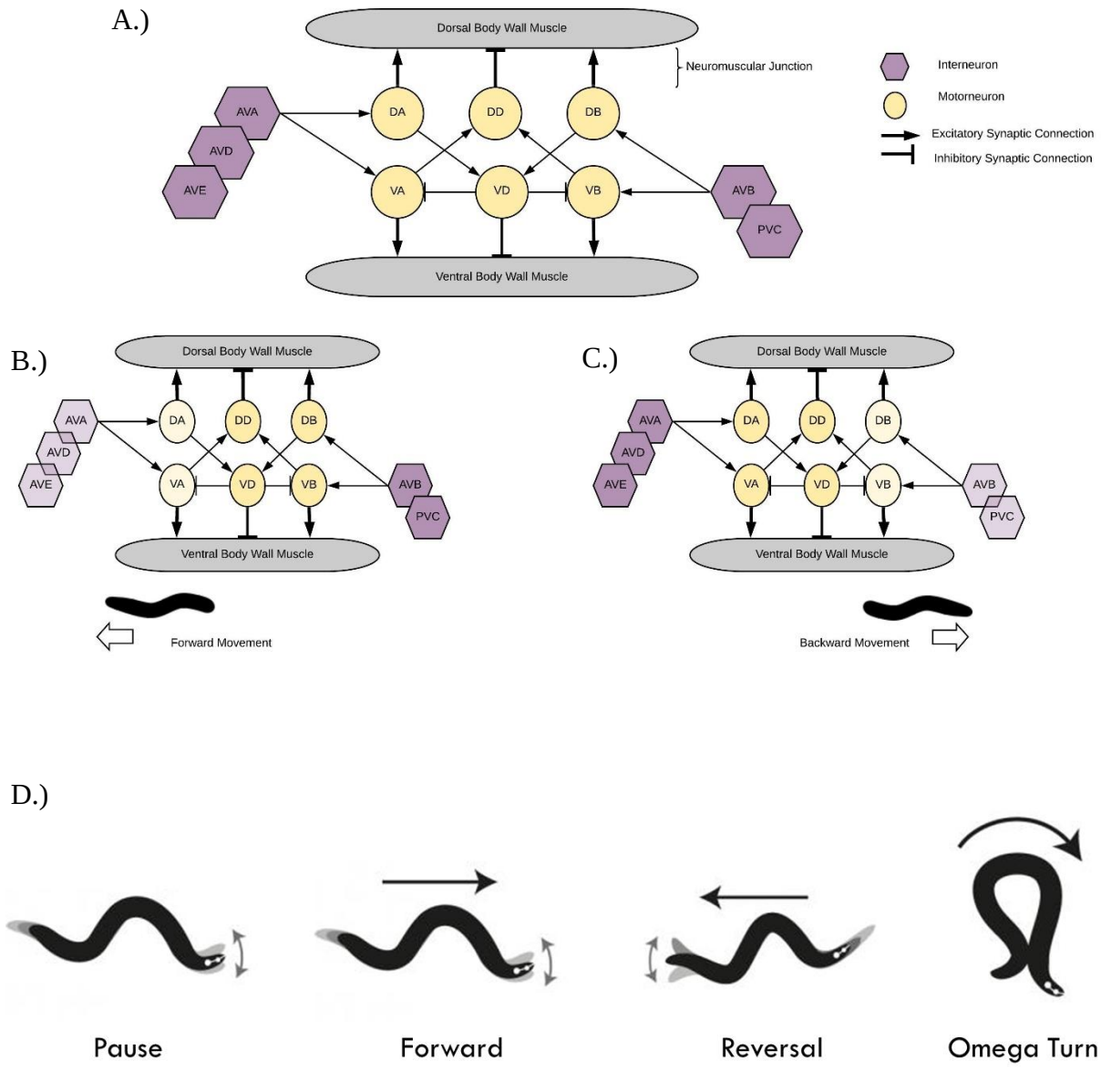


Figure 1. *C. elegans* neuromuscular junction and locomotor example. (A) Circuitry schematic of how command interneurons connect with different subtypes of motorneurons and the subsequent projection onto body wall muscle. Excitatory projections are mediated by acetylcholine and inhibitory projections are mediated by GABA. Differential activation of these subcircuits within the NMJ, as indicated by dark purple, lead**s** to either forward (B) or backward (C) locomotion. Connections are modeled after Haspel, O'Donovan**,** and Hart (2010) and Zhen and Samuel (2015). (D) Example of *C. elegans* locomotor patterns - illustration was adapted from António Carlos da Costa, Vrije Universiteit Amsterdam - https://www.oist.jp/news-center/photos/nuances-c-elegans-crawling-behavior

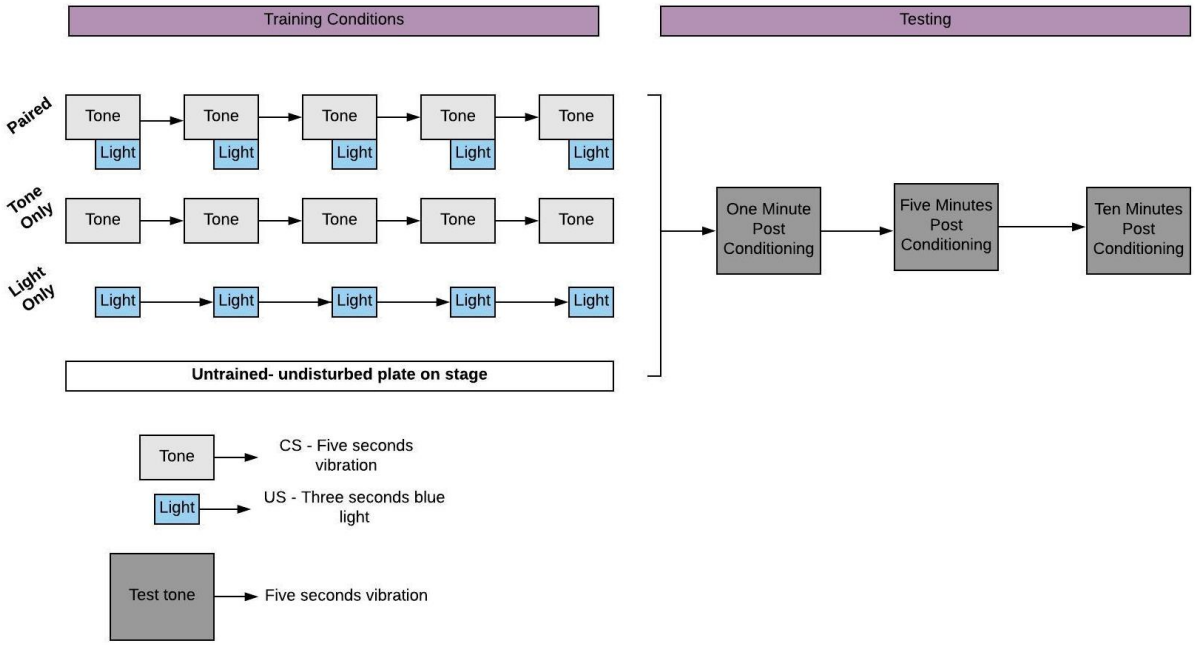


Figure 2. Rapid associative conditioning assay and associated behavioral controls used to assess wild type conditioned locomotor behavior. Pairing consists of a 2 second ISI between **the** onset of the vibration and onset of the blue light. Other training controls (e.g., tone-only, light-only, and naïve/untrained) are as indicated and completed with independent groups of *C.elegans*. At testing, each plate of multiple wild type *C. elegans* were recorded for 75 seconds at each retention period

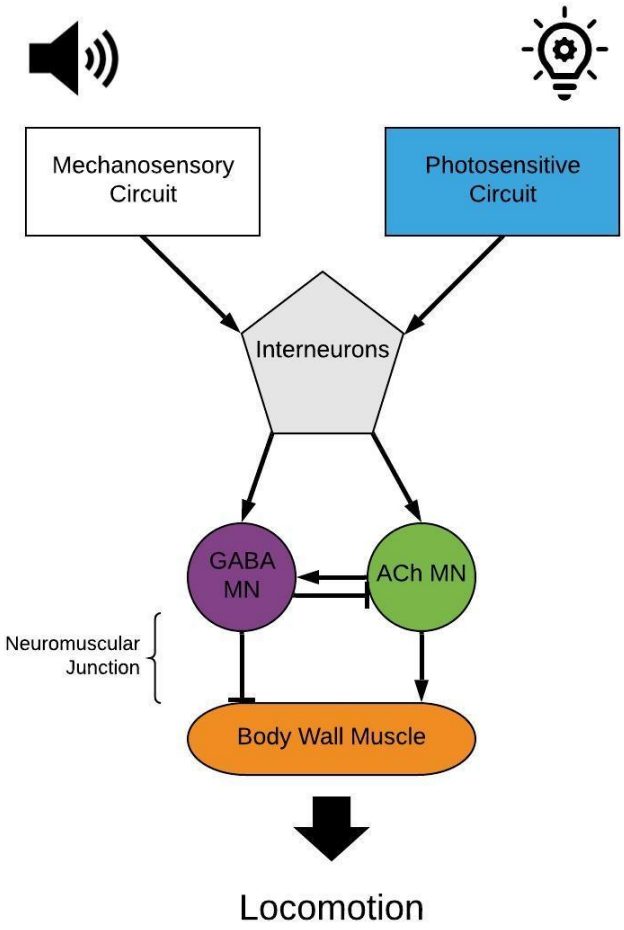


Figure 3. Illustrated diagram of the mechanosensory and photosensitive circuits with connections to downstream effectors controlling locomotor response. The white box represents sensory neurons driving mechanosensation, Chalfie et al. (1985), Wicks and Rankin (1995). The blue box represents neurons that express the LITE-1 photoreceptor that drives photosensation, Edwards, et al. (2008) and Ward et al. (2008). Downstream interneurons (gray hexagon) and motor neurons (purple/green circles) controlling directionality of locomotive movement: Haspel et al. (2010).

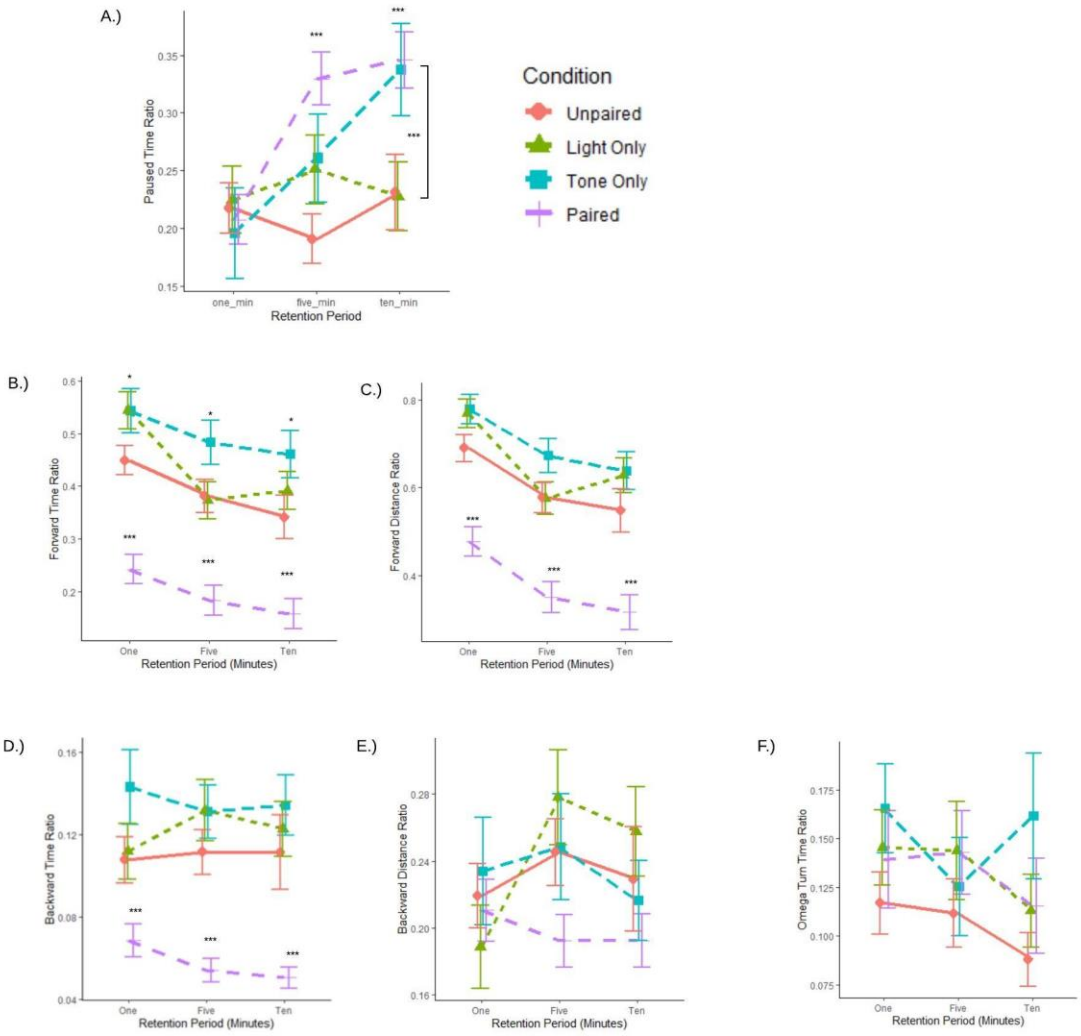
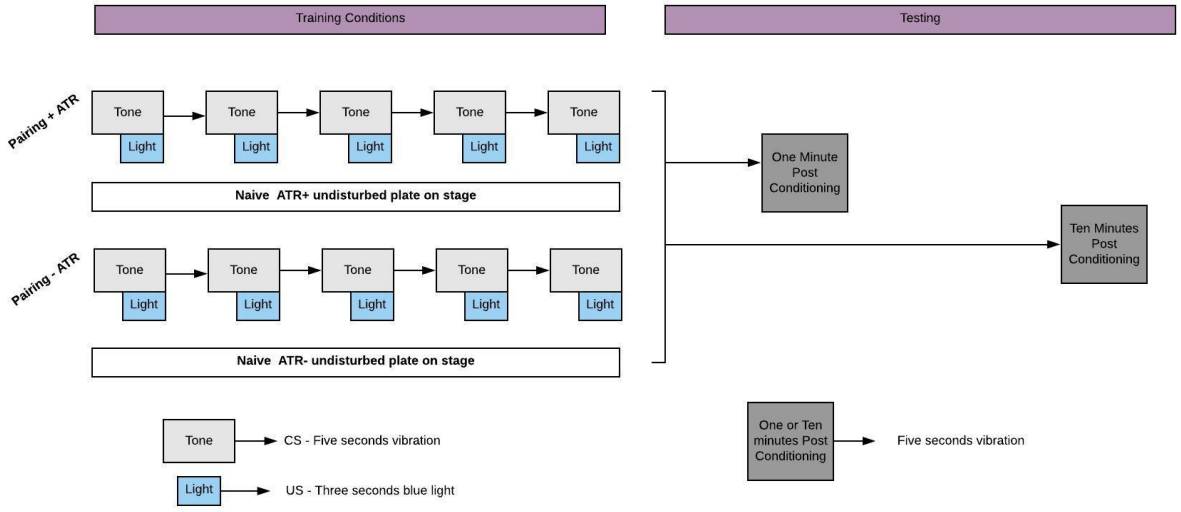


Figure 4. Wild type locomotor behavior after vibration-light conditioning or controls (e.g., tone-only, light-only, and untrained). Responses to a five-second vibration are measured one-, five- and ten-minutes post-training. Charted responses are the means of each group, and error bars are +/-SEM. Analysis for significance was done by two-way ANOVA with posthoc analysis at each retention period to compare differences between condition and controls with the untrained group. (A, B, D, F) Time ratios are the average proportion of time animals of each group spent moving in each possible locomotor direction, pausing, moving forward or backward, or performing an omega turn. (C, E) Distance ratios are the average proportion of the total trajectory that each group of animals moved in either the forward or backward direction. At ten-minutes following vibration-light conditioning, wild type *C. elegans* increased time spent pausing (A), with a subsequent decrease in time (B) and distance (C) spent moving forward, as well as decreased time (D) and distance (E) moving backward. No changes in omega turn time were measured (F). Controls - light-only and tone-only have similar locomotor patterns to the untrained group, but dissimilar to vibration-light conditioned animals.

A.)



B.)

C.)

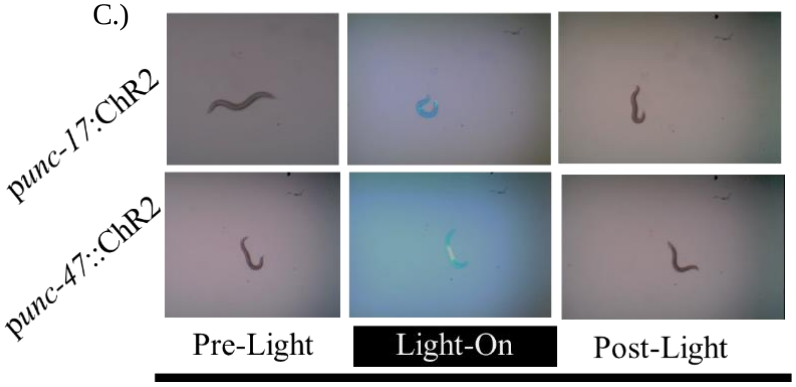


Figure 5. Vibration-light conditioning assay used with optogenetic *C. elegans*. (A) Schematic of vibration-light conditioning and testing protocol. Vibration-light pairing consists of a 2 second ISI between **the** onset of the vibration and onset of blue light. Other training conditions are as indicated and completed with independent sets of animals. At testing, each plate of was video recorded for 75 seconds at one of the following retention periods, one- or ten- minutes postconditioning (B) Example set up of all experiments. (C) Example locomotor changes when ChR2 is activated by blue light under the different neuronal promoters. *punc*-17::ChR2 show a whole body bending movement, while *punc*-47::ChR2 shows temporary paralysis while the blue light is on.

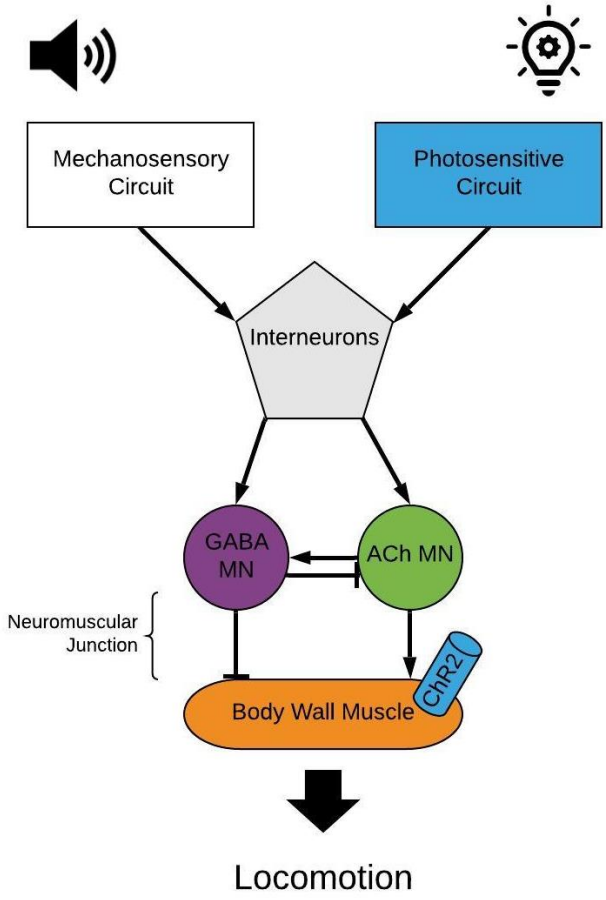


Figure 6. Schematic of the mechanosensory and phototaxis circuit, with indicated expression profile of *pmyo-3*::ChR2. During vibration-light conditioning, blue light drives both the photosensitive circuit and channelrhodopsin (ChR2) overactivation of body wall muscles, thus experimentally disrupting the sequential, top-down signaling process, but not disrupting integration of both mechanosensory and photosensitive circuits that underlie the association. Expression profile of *pmyo*-3 in body wall muscles as previously reported by Nagel, et al. (2005). As before, the white box represents sensory neurons driving mechanosensation, Chalfie et al. (1985), Wicks and Rankin (1995). The blue box represents neurons that express the LITE-1 photoreceptor that drives photosensation, Edwards, et al. (2008) and Ward et al. (2008). Downstream interneurons (gray hexagon) and motor neurons (purple/green circles) controlling directionality of locomotive movement: Haspel et al. (2010).

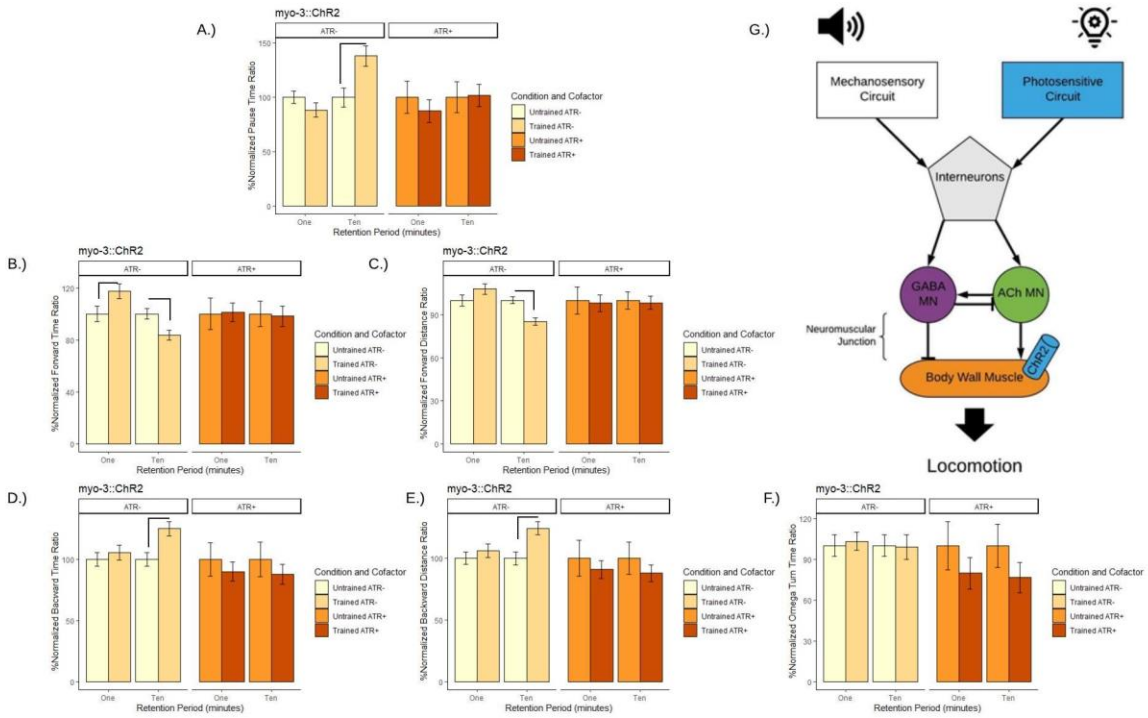
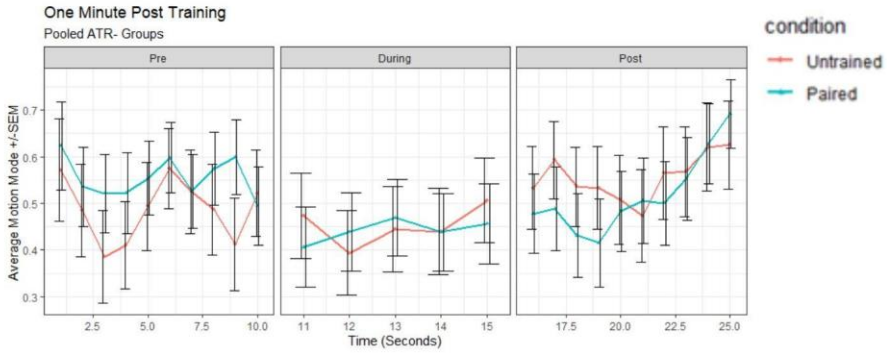
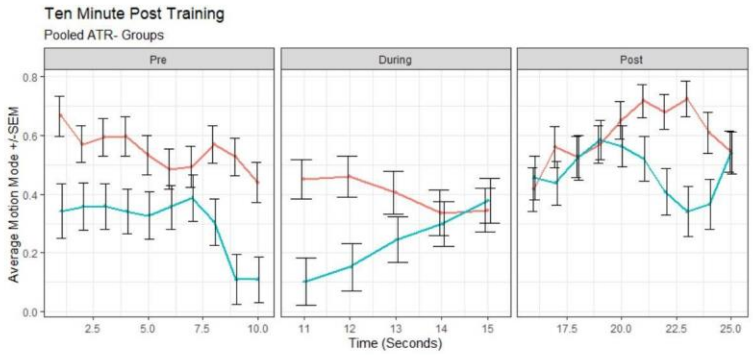


Figure 7. Conditioned locomotive behavior one- and ten- minutes after vibration-light training for *pmyo*-3::ChR2 *C. elegans*, with or without cofactor. All trained output was normalized to untrained output and presented as percentage points. Error bars are normalized SEM. Two-way t-tests were used to assess differences between trained and untrained groups of the same cofactor level. (A, B, D, F) Time ratios are the average proportion of time animals of each group spent moving in each possible locomotor direction, pausing, moving forward or backward, or performing an omega turn. (C, E) Distance ratios are the average proportion of the total trajectory that each group of animals moved in either the forward or backward direction. (G) Circuit diagram from fig 6. Trained ATR+ *pmyo*-3::ChR2 animals exhibit locomotor behavior that is equivalent to their untrained ATR+ controls, suggesting that learning was completely disrupted when overactivation of the body wall muscle during conditioning occurred.

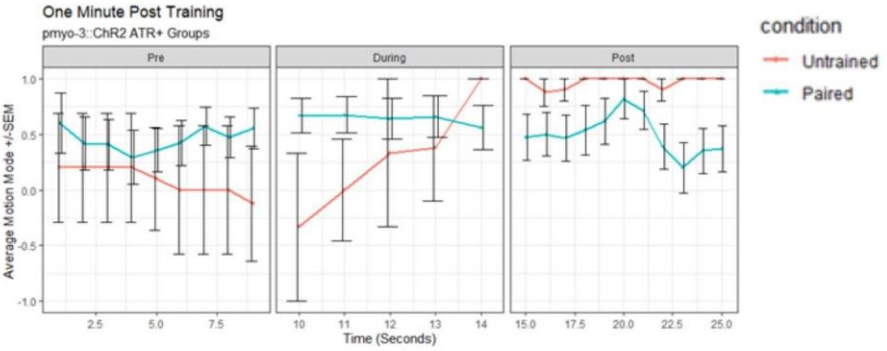
A.)


One Minute Post Training
Pooled ATR- Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)
condition
Untrained
Paired

B.)


Ten Minute Post Training
Pooled ATR- Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)

C.)


One Minute Post Training
pmyo-3::ChR2 ATR+ Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)
condition
Untrained
Paired

D.)

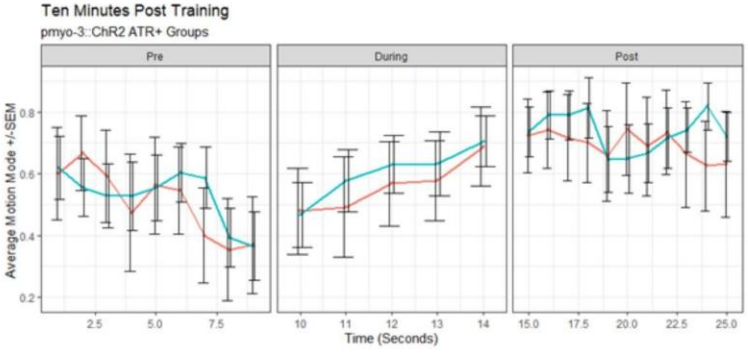

Ten Minutes Post Training
pmyo-3::ChR2 ATR+ Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)

Figure 8. *pmyo*-3::ChR2 motion mode measurement of worm locomotion pre-, during- and post-CS presentation at one- and ten-minutes postconditioning. Line graphs depict variability in locomotion across the pre-, during-, and post- bins (+1 is forward, 0 is paused, -1 is reversal). (A) The average motion mode of pooled ATR- untrained and trained *C. elegans* at one-minute postconditioning and (B) ten-minutes postconditioning. (C) Average motion mode for ATR+ *pmyo*-3::ChR2 trained and untrained animals one-minute postconditioning, and (D) ten-minutes postconditioning. Relative locomotor changes pre- vs. post-CS presentation support that the *pmyo*-3::ChR2 ATR+ animals are still capable of movement, and that the cofactor and transgene do not render the animal immobile.

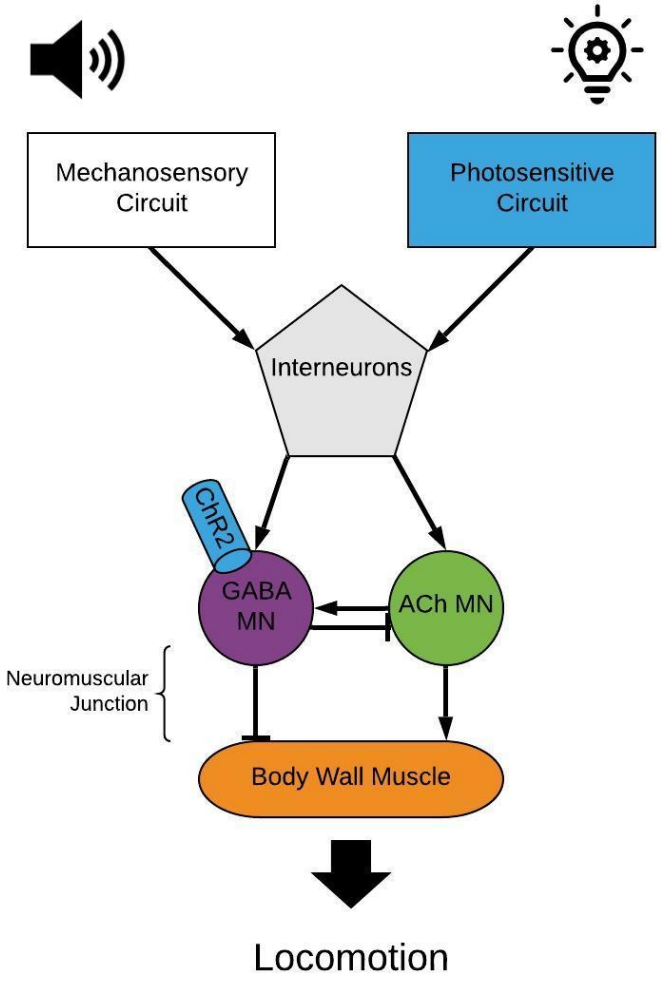


Figure 9. Schematic of the mechanosensory and phototaxis circuit, with the indicated expression profile of *punc-47*::ChR2. During vibration-light conditioning, blue light drives both the photosensitive circuit and channelrhodopsin (ChR2) overactivation of the GABAergic circuit, thus experimentally disrupting the sequential, top-down signaling process, but not disrupting integration of both mechanosensory and photosensitive circuits that underlie the association. Expression profile of *unc-47* in GABAergic motor neurons as previously reported by Eastman, Horvitz**,** and Jin, 1999; Schuske, Beg**,** and Jorgensen, 2004. As before, the white box represents sensory neurons driving mechanosensation, Chalfie et al. (1985), Wicks and Rankin (1995). The blue box represents neurons that express the LITE-1 photoreceptor that drives photosensation, Edwards, et al. (2008) and Ward et al. (2008). Downstream interneurons (gray hexagon) and motor neurons (purple/green circles) controlling directionality of locomotive movement: Haspel et al. (2010).

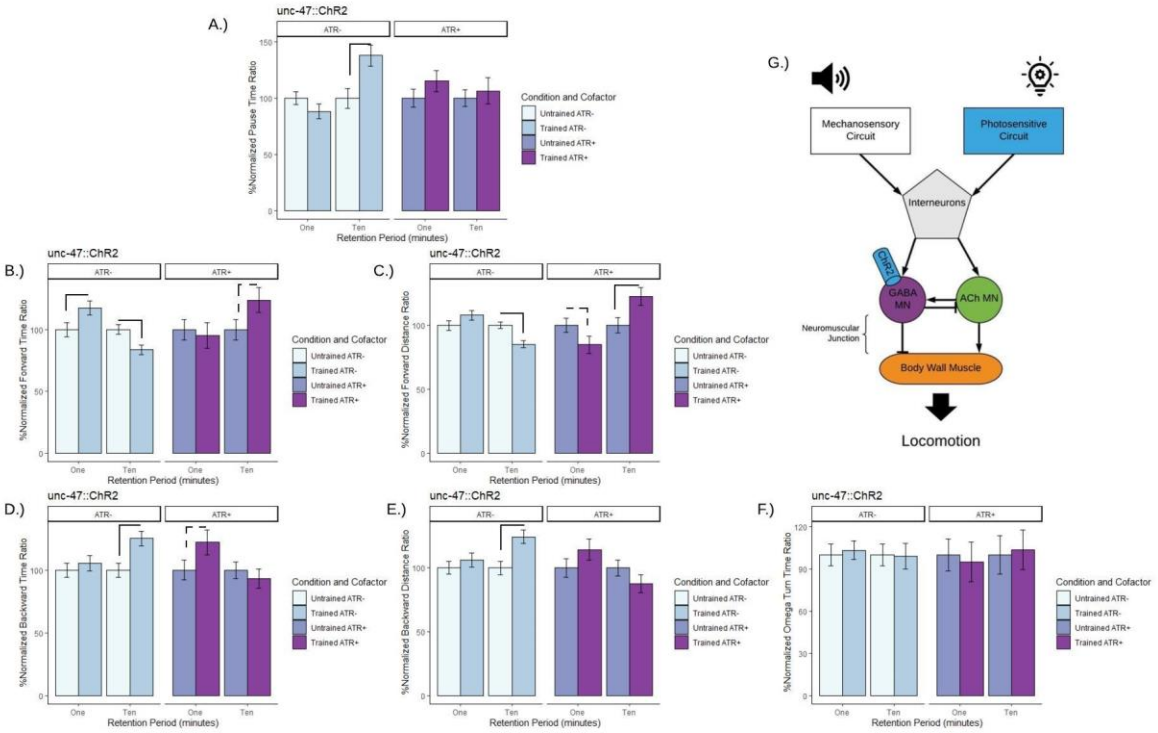
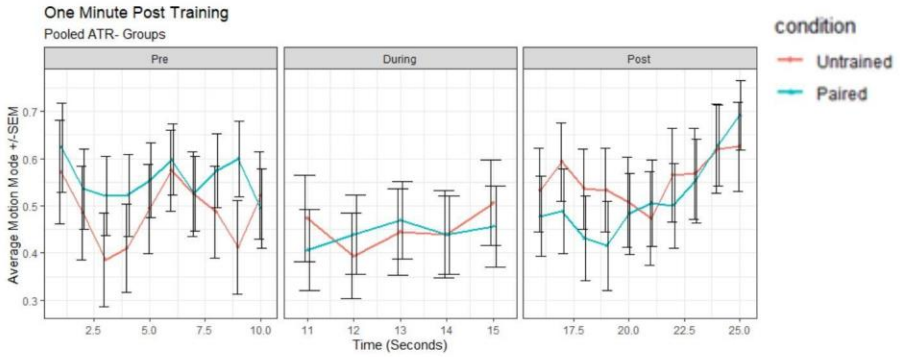
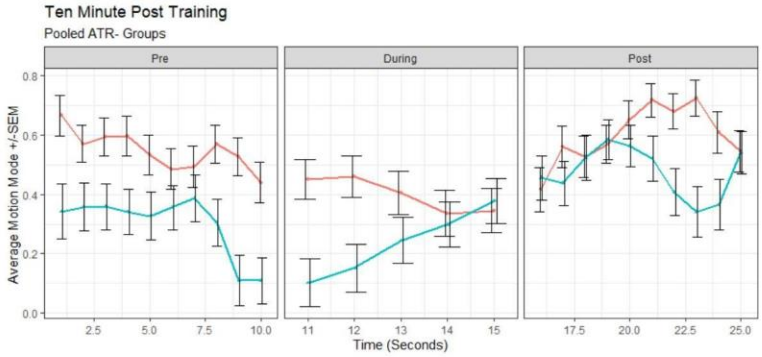


Figure 10. Locomotive behavior one- and ten-minutes after conditioning for *punc*-47::ChR2 *C. elegans*. All trained output were normalized to untrained output and presented as percentage points. Error bars are normalized +/-SEM. Two-way student's t-tests were used to assess differences between trained and untrained groups of the same cofactor level. A solid line indicates a significant difference ($p<0.05$), while the dashed line indicates trending towards significance ($p<0.09$). (A, B, D, F) Time ratios are the average proportion of time animals of each group spent moving in each possible locomotor direction, pausing, moving forward or backward, or performing an omega turn. (C, E) Distance ratios are the average proportion of the total trajectory that each group of animals moved in either the forward or backward direction. (G) Illustrated diagram from fig 9. Trained ATR+ *punc*-47::ChR2 animals show differential conditioned paused and forward locomotor behaviors, suggesting that learning was not completely disrupted during conditioning, but was altered due to the overactivation of the GABAergic circuit.

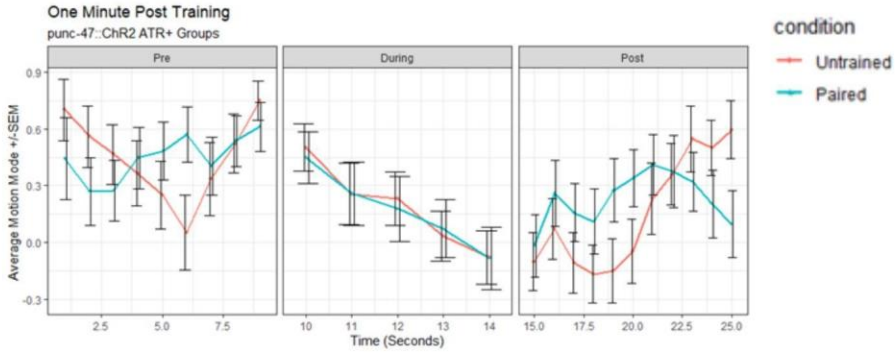
A.)


One Minute Post Training
Pooled ATR- Groups
Pre
During
Post
condition
Untrained
Paired
Average Motion Mode +/-SEM
Time (Seconds)

B.)


Ten Minute Post Training
Pooled ATR- Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)

C.)


One Minute Post Training
punc-47::ChR2 ATR+ Groups
Pre
During
Post
condition
Untrained
Paired
Average Motion Mode +/-SEM
Time (Seconds)

D.)

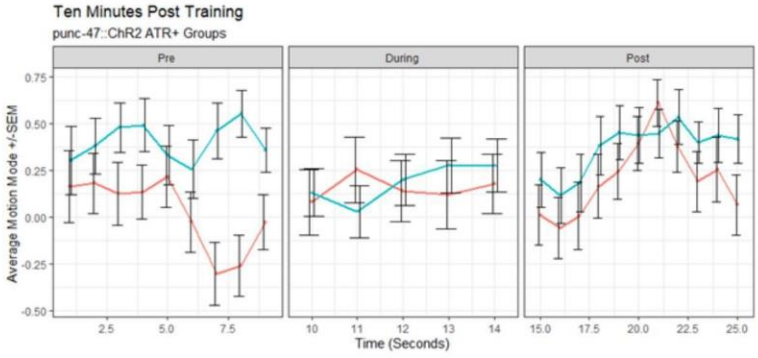

Ten Minutes Post Training
punc-47::ChR2 ATR+ Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)

Figure 11. Average motion mode measurement of *punc*-47::ChR2 locomotion pre-, during**,** and post-CS only presentation, one- and ten-minutes postconditioning. Line graphs depict variability in locomotion across the pre-, during-, and post- time segments in one-second bins (+1 is forward, 0 is paused, -1 is reversal). (A) The average motion mode of pooled ATR- trained and untrained *C. elegans* at one-minute postconditioning and (B) ten-minutes postconditioning. (C) Average motion mode for trained and untrained ATR+ *punc*-47::ChR2 animals one-minute postconditioning, and (D) ten-minutes postconditioning. Relative differences in locomotion show that the observed changes in locomotor behavior (i.e., time and distance ratios) are due to conditioning, and not an artifact of the cofactor rendering the animals immobile.

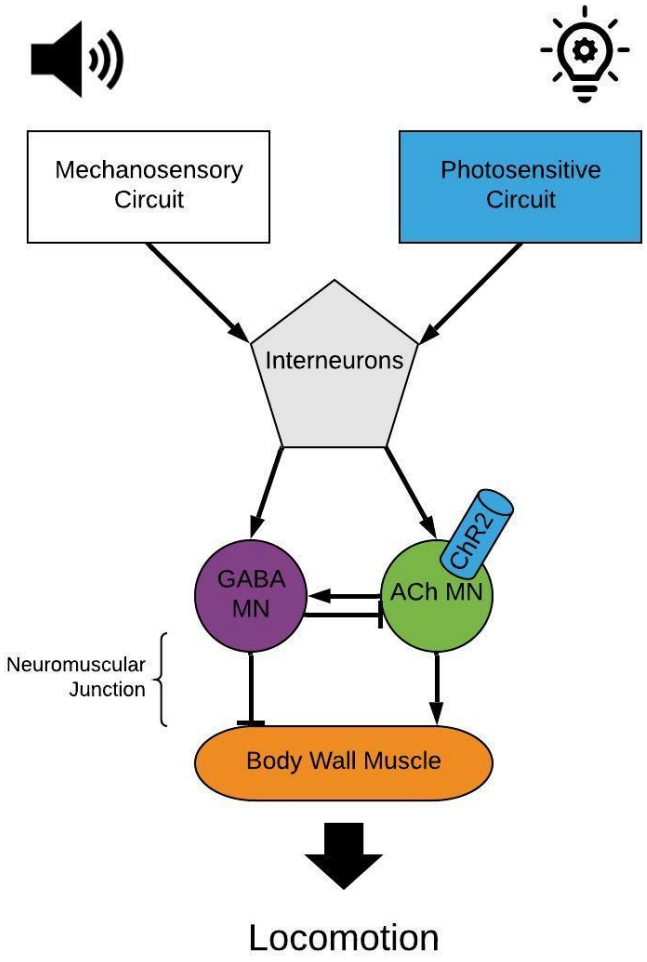


Figure 12. Schematic of the mechanosensory and phototaxis circuit, with the indicated expression profile of *punc-17*::ChR2. During vibration-light conditioning, blue light drives both the photosensitive circuit and channelrhodopsin (ChR2) overactivation of the cholinergic circuit, thus experimentally disrupting the sequential, top-down signaling process, but not disrupting integration of both mechanosensory and photosensitive circuits that underlie the association. Expression profile of *unc-17* in cholinergic motor neurons as previously reported by Eastman, Horvitz**,** and Jin, (1999). As before, the white box represents sensory neurons driving mechanosensation, Chalfie et al. (1985), Wicks and Rankin (1995). The blue box represents neurons that express the LITE-1 photoreceptor that drives photosensation, Edwards, et al. (2008) and Ward et al. (2008). Downstream interneurons (gray hexagon) and motor neurons (purple/green circles) controlling directionality of locomotive movement: Haspel et al. (2010).

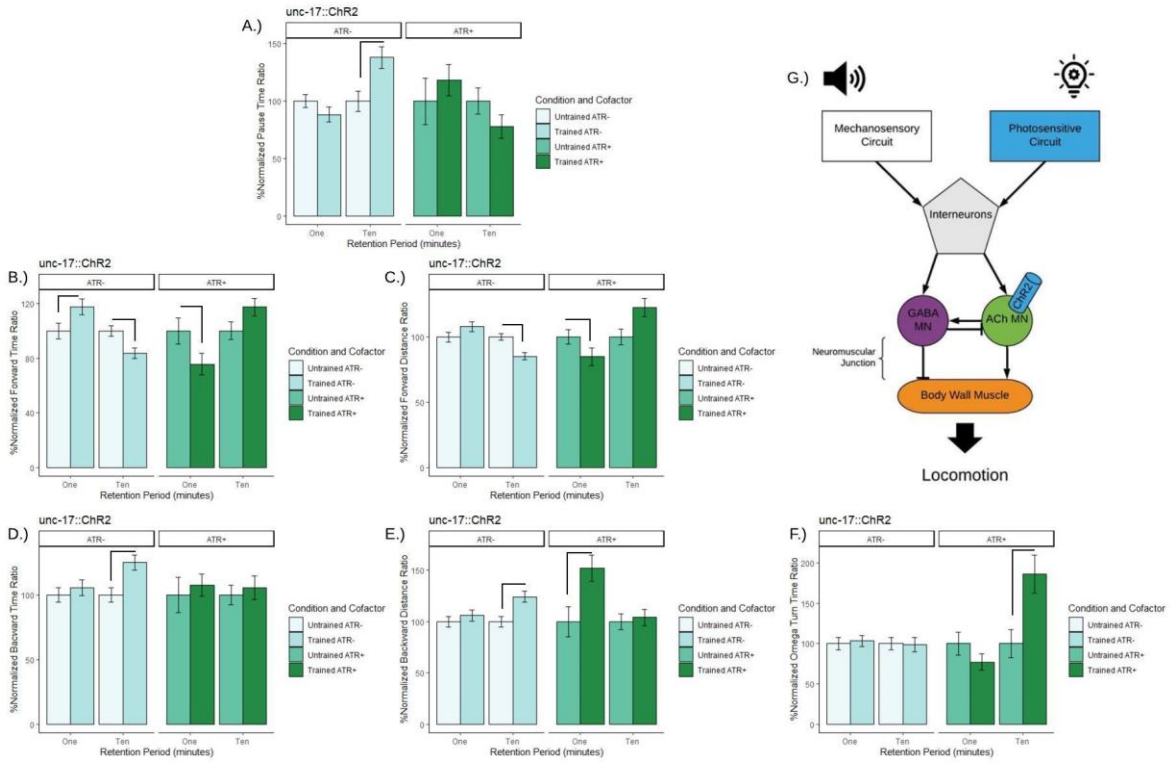
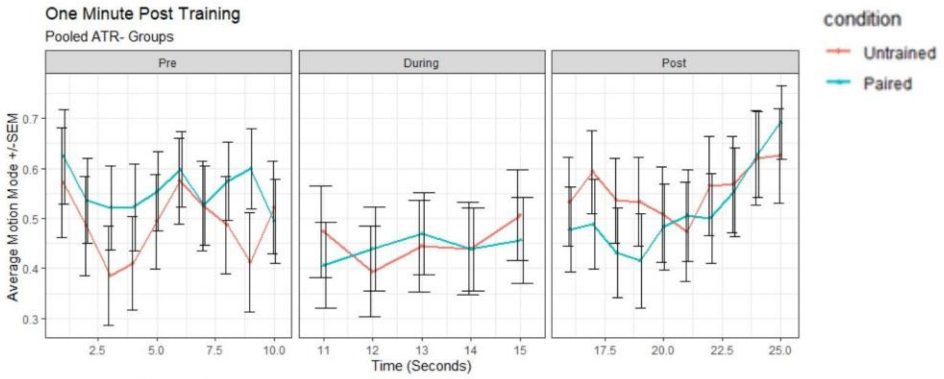
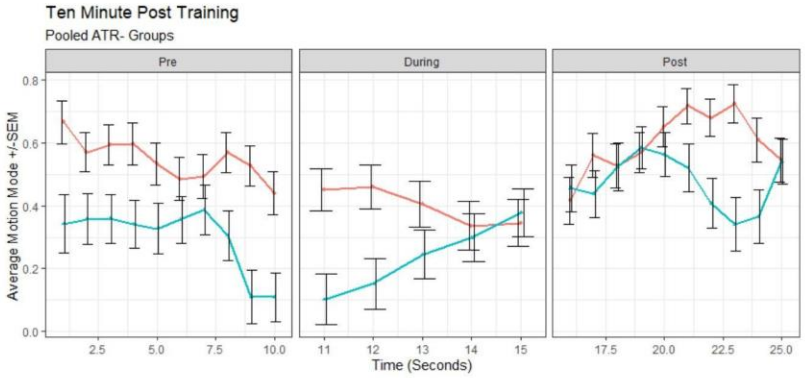


Figure 13. Vibration-light conditioning with *punc*-17::ChR2 *C. elegans*. All trained output were normalized to untrained output and presented as percentage points. Error bars are normalized +/-SEM. Two-way student's t-tests were used to assess differences between trained and untrained groups of the same cofactor level. (A, B, D, F) Time ratios are the average proportion of time animals of each group spent moving in each possible locomotor direction, pausing, moving forward or backward, or performing an omega turn. (C, E) Distance ratios are the average proportion of the total trajectory that each group of animals moved in either the forward or backward direction. (G) Illustrated schematic from fig 12. Trained ATR+ *punc*-17::ChR2 animals showed differential conditioned locomotor behavior at one-minute postconditioning, and were the only animals to show any change in omega turns, at ten-minutes postconditioning.

A.)


One Minute Post Training
Pooled ATR- Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)
condition
Untrained
Paired

B.)


Ten Minute Post Training
Pooled ATR- Groups
Pre
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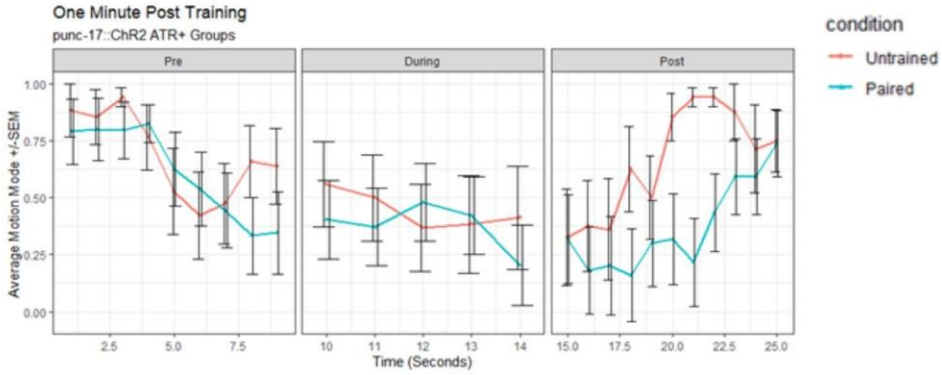
Average Motion Mode +/-SEM
Time (Seconds)

C.)


One Minute Post Training
punc-17::ChR2 ATR+ Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)
condition
Untrained
Paired

D.)

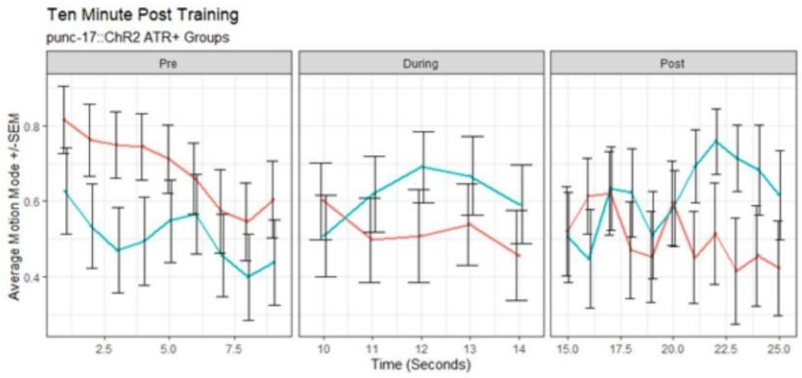

Ten Minute Post Training
punc-17::ChR2 ATR+ Groups
Pre
During
Post
Average Motion Mode +/-SEM
Time (Seconds)

Figure 14. Average motion mode of *punc*-17::ChR2 locomotion pre-, during- and post-CS presentation, one- and ten-minutes postconditioning. Line graphs depict variability in locomotion across the pre-, during-, and post- bins (+1 is forward, 0 is paused, -1 is reversal). (A) The average motion mode of trained and untrained pooled ATR- *C. elegans* at one-minute postconditioning and (B) ten-minutes postconditioning. (C) Average motion mode for trained and untrained *punc*-17::ChR2 ATR+ animals one-minute postconditioning, and (D) ten-minutes postconditioning. Relative differences in locomotion show that the observed changes in locomotor behavior (i.e., time and distance ratios) are due to conditioning, and not an artifact of the cofactor rendering the animals immobile.

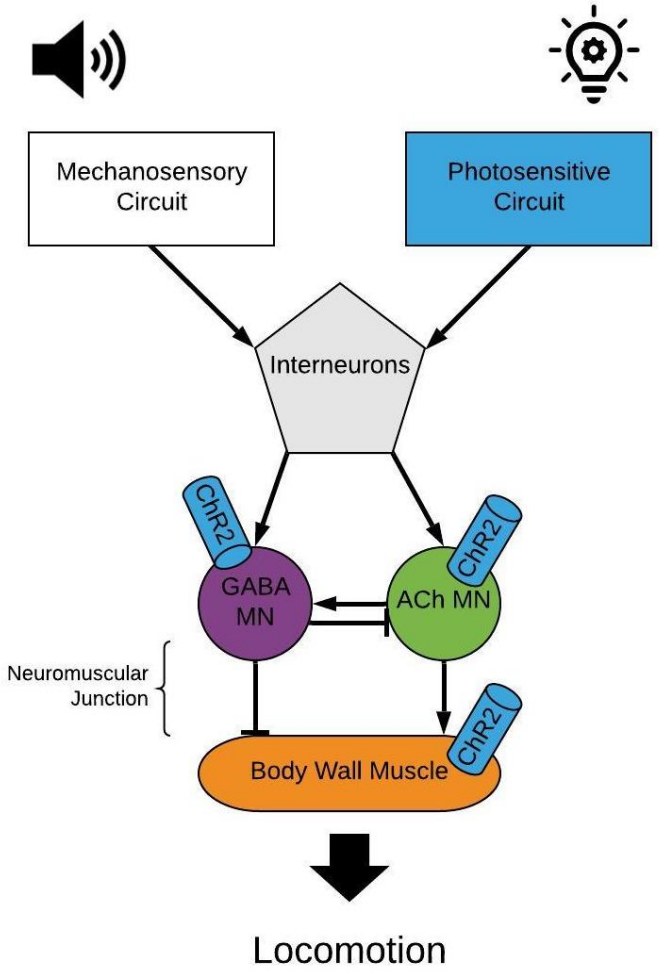


Figure 15. Proposed schematic of the mechanosensory and phototaxis circuit, with the indicated expression profile of *pmyo*-3::ChR2 (Nagel et al., 2005), *punc*-17::ChR2 (Eastman, Horvitz**,** and Jin, 1999) and *punc*-47::ChR2 (Eastman, Horvitz**,** and Jin, 1999; Schuske, Beg and Jorgensen, 2004). The white box represents sensory neurons driving mechanosensation, Chalfie et al. (1985), Wicks and Rankin (1995). Blue box represents both sensory and interneu**ro**ns that express the LITE-1 photoreceptor, Edwards, et al. (2008) and Ward et al. (2008). Downstream interneurons (grey hexagon) and motor neuron subcircuits (purple and green circles) that control the directionality of locomotive movement: Haspel et al. (2010).

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